Mesenchymal Stem Cells for the Treatment of Myocardial Infarction-Induced Ventricular Dysfunction.

Dr James David Richardson

MBBS (Hons) MRCP

Department of Medicine

Faculty of Health Sciences

The University of Adelaide, South Australia

&

Cardiovascular Research Centre

The Royal Adelaide Hospital, South Australia

&

Bone and Cancer Research Laboratories

Division of Haematology

Institute of Medical and Veterinary Science

A thesis submitted to the University of Adelaide

in candidature for the degree of Doctor of Philosophy

December 2013

Table of Contents

Declaration....vii
Acknowledgements....viii
Personal Bibliography....ix
Abbreviations....xii
Thesis Abstract....xiii

Chapter 1 - Optimisation of MSC....1

1. Abstract 3
2. Introduction....3
    2.1. Lessons from conventional MSC transplantation....5
    2.2. Preclinical experience....6
    2.3. Mechanisms of benefit: direct differentiation vs indirect repair....7
    2.4. Clinical application....9
3. Addressing limitations of conventional MSC transplantation....14
    3.1. Enrichment of primitive mesenchymal precursors....14
    3.2. Alternative sources of MSCs....18
4. In vitro modification of MSCs....20
    4.1. (1) Culture pre-conditioning....21
    4.2. (2) Paracrine factor augmentation....23
    4.3. (3) Activation of cytoprotective pathways....24
    4.4. (4) Directing MSC cardiopoiesis....26
5. Modification of the myocardial substrate....30
6. Optimization of cell delivery....31
7. Bionanotechnology....32
8. Future directions....34
9. Conclusion....37
    9.1. Acknowledgements....37
    9.3. References 38

2.Chapter 2- Rat Cardiac Imaging....47

2.1. ABSTRACT....49
2.2. INTRODUCTION....50

2.3. Animals, Material and Methods 51
2.3.1. Study protocol 51
2.3.2. CMR protocol 52
2.3.1. CMR image analysis 52
2.3.2. TTE protocol 54
2.3.3. Transoesophageal echocardiogram protocol 54
2.3.4. Data analysis 56
2.3.5. Statistical analysis 57
2.4. RESULTS 57
2.4.1. Correlation between modalities 57
2.4.2. Intra-observer agreement 58
2.4.3. Inter-observer agreement 58
2.5. DISCUSSION 63
2.5.1. Utility of 1.5T CMR 63
2.5.2. Reproducibility 64
2.5.3. Utility of TOE 65
2.5.4. Limitations 66
2.6. CONCLUSIONS 67
2.6.1. Acknowledgements 67
2.6.2. References 68

3.Chapter 3 – Prospective Isolation & Hypoxic Conditioning of MSC ...75

3.1. ABSTRACT 77
3.2. INTRODUCTION 78
3.3. METHODS 80
3.3.1. Study protocol 80
3.3.2. Prospective Isolation of Rat MSC 80
3.3.3. MSC immunophenotype 83
3.3.4. MSC differentiation capacity 83
3.3.5. Colony efficiency assay 84
3.3.6. Cardiac Magnetic Resonance 84
3.3.7. CMR image analysis 85
3.3.8. Infarct surgery and MSC transplantation 85
3.3.9. Histological analysis 86
3.3.10. Statistical analysis 86

3.4. RESULTS....87
3.4.1. Characterisation of MSC....87
3.4.2. Colony forming efficiency....87
3.4.3. Differentiation capacity....87
3.4.4. Global Left Ventricular Function....88
3.4.5. Regional left ventricular morphology and function....92
3.4.6. Myocardial Fibrosis....93
3.4.7. Arteriolar Density....94
3.5. DISCUSSION....95
3.5.1. Need for optimisation....95
3.5.2. Rationale for prospective isolation....95
3.5.3. Rationale for hypoxic conditioning....96
3.5.4. Combining the two optimisation strategies....97
3.5.5. Clinical relevance....98
3.5.6. Limitations 98
3.6. CONCLUSIONS....99
3.6.2.Reference 100

4.Chapter 4 - Timing of MSC Intervention 106

4.1. ABSTRACT 108
4.2. Introduction....109
4.3. Methods....110
4.3.1. Study protocol....110
4.3.2. Prospective Isolation of Rat MSC....111
4.3.3. Hypoxic conditioning....113
4.3.4. Green Florescent Protein-labelled MSC....113
4.3.5. Cardiac Magnetic Resonance Imaging....114
4.3.6. CMR image analysis....114
4.3.7. Infarct surgery and MSC transplantation....115
4.3.8. Histological analysis....116
4.3.9. Statistical analysis....116
4.4. Results 117
4.4.1. Characterisation of MSC....117
4.4.2. Global Left Ventricular Function....117
4.4.3. Regional left ventricular morphology and function....121

4.4.4. Arteriolar Density....126
4.4.5. Green Florescent Protein-labelled MSC....129
4.5. Discussion....129
4.5.2. Implications and Future Directions....134
4.6. Conclusion....135
4.6.1.References 136

5.Chapter 5 - Combination of Early and Late MSC Intervention 143

5.1. ABSTRACT....145
5.3. INTRODUCTION....146
5.4. MATERIALS and METHODS....147
5.4.1. Study protocol....147
5.4.2. Prospective Isolation of Rat MSC....149
5.4.3. Hypoxic conditioning....149
5.4.4. Cardiac Magnetic Resonance....150
5.4.5. CMR analysis....150
5.4.6. Infarct surgery and MSC transplantation....151
5.4.7. Histological analysis....151
5.4.8. Statistical analysis....152
5.5. RESULTS....152
5.5.1. Characterisation of MSC....152
5.5.2. Global Left Ventricular Function....153
5.5.3. Regional left ventricular morphology and function.....154
5.5.4. Myocardial Fibrosis....159
5.5.5. Arteriolar Density....159
5.5.6. Apoptosis 162
5.6. DISCUSSION....162
5.6.1. Limitations 165
5.6.2. Implications and Future Directions....165
5.7. Conclusion....167
5.7.1.References 168

6.Chapter - Summary 175

6.1. General Overview....176

6.2. Optimising MSC 177
6.3. Rat cardiac imaging 177
6.4. Pre-clinical model of MI 180
6.5. In vivo effects of optimised MSC 183
6.6.Timing of MSC intervention. 183
6.7. MSC Intervention at multiple time points 185
6.8.Future directions 185
6.9. Conclusion 186

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

James David Richardson

December 2013

Acknowledgements

My time as a PhD student has been an amazing journey. I am very grateful for the opportunities and experiences that this time has provided me with. Most of all, I have been blessed to have met so many outstanding people, whom I now count as lifelong friends, and to have been reminded of how extremely fortunate I am to have the unwavering love and support of my family and friends.

This undertaking required the collaberation of two departments: the Cardiovascular Research Centre, at the Royal Adelaide Hospital, led by Professor Stephen Worthley and the Bone and Cancer Research Laboratories, at the Institute of Medical and Veterinary Science, co-headed by Professors Andrew Zannettino and Stan Gronthos. I am indebted to you, Steve, Andrew and Stan, for having had the confidence in me to perform this research and to have allowed me great scope to develop my study ideas and plans, and to run with them. When obstacles appeared, your support was fundamental in helping me confront them. I value your friendship and mentorship greatly. Thank you Steve, for your remarkable optimism and positive encouragement. Thank you Andrew and Stan, for your dedication, passion, patience and attention to detail – I greatly enjoyed our time that we shared.

To my colleagues and co-authors; Mrs Sharon Paton, Mr Angelo Carbone, Dr Angela Bertaso, Mr Lachlan Frost, Dr Adam Nelson, Dr Dennis Wong, Dr Peter Psaltis, thank you for your friendship and support. The success of this work would not have been possible without you.

Personal Bibliography

Full-text publications arising directly from work conducted toward this thesis:

1. Optimization of the cardiovascular therapeutic properties of mesenchymal stromal/ stem cells-taking the next step. **Richardson JD**, Nelson AJ, Zannettino AC, Gronthos S, Worthley SG, Psaltis PJ. Stem Cell Rev. 2013 Jun;9(3):281-302. doi: 10.1007/s12015-012-9366-7.
2. Cardiac magnetic resonance, transthoracic and transoesophageal echocardiography: a comparison of in vivo assessment of ventricular function in rats. **Richardson JD**, Bertaso AG, Frost L, Psaltis PJ, Carbone A, Koschade B, Wong DT, Nelson AJ, Paton S, Williams K, Azarisman S, Worthley MI, Teo KS, Gronthos S, Zannettino AC, Worthley SG. Lab Anim. 2013 Oct; 47(4):291-300. doi: 10.1177/0023677213494373
3. Impact of timing and dose of mesenchymal stromal cell therapy in a preclinical model of acute myocardial infarction. **Richardson JD**, Bertaso AG, Psaltis PJ, Frost L, Carbone A, Paton S, Nelson AJ, Wong DT, Worthley MI, Gronthos S, Zannettino AC, Worthley SG. J Card Fail. 2013 May;19(5):342-53. doi: 10.1016/ j.cardfail.2013.03.011. PMID: 23663817 [PubMed - in process]
4. Incremental Benefits of Repeated Mesenchymal Stromal Cell Administration Compared to Solitary Intervention in a Preclinical Model of Myocardial Infarction. **JD Richardson**, PJ Psaltis, L Frost, A Carbone, S Paton, AG Bertaso, AJ Nelson, DT Wong, MI Worthley, S Gronthos, A Zannettino, SG Worthley. Cytotherapy 2013

Manuscript in preparation:

5. **JD Richardson**, S Paton, PJ Psaltis, A Carbone, L Frost, S Gronthos, ACW Zannettino, SG Worthley. Prospectively-Isolated Hypoxic-Conditioned Mesenchymal Stem Cells Have Superior in vitro Capacity and Significantly Attenuate Myocardial Infarction-Induced Ventricular Dysfunction.

Abstracts arising from the Thesis:

1. **Richardson JD**, Frost F, Bertaso AG, Carbone A, Paton S, Nelson AJ, Psaltis PJ, Wong DT, Worthley MI, Gronthos S, Zannettino ACW, Worthley SG. Immediate Mesenchymal Stem Cell Therapy Provides Greater Attenuation of Myocardial Injury Than Deferred Treatment in Rats After Acute Myocardial Infarction. Heart, Lung & Circulation 2012; S21: S1-S142 (**CSANZ YIA finalist**)

2. **Richardson JD**, Paton S, Frost F, Carbone A, Bertaso AG, Nelson AJ, Psaltis PJ, Wong DT, Worthley MI, Gronthos S, Zannettino ACW, Worthley SG. Prospectively Isolated, Hypoxic-Preconditioned Mesenchymal Stem Cells Significantly Attenuate Myocardial Infarction-Induced Ventricular Dysfunction In Rats. Heart, Lung & Circulation 2012; S21: S1-S142.

3. **Richardson JD**, Frost F, Bertaso AG, Carbone A, Paton S, Nelson AJ, Psaltis PJ, Wong DT, Worthley MI, Gronthos S, Zannettino ACW, Worthley SG. Sequential Mesenchymal Stem Cell Interventions Produce Greater Myocardial Repair Than Solitary Treatment in Rats After Acute Myocardial Infarction. Heart, Lung & Circulation 2012; S21: S1-S142.

4. **Richardson JD**, Bertaso AG, Koschade B, Wong DT, Williams K, Frost F, Carbone A, Paton S, Nelson AJ, Psaltis PJ, Worthley MI, Teo KS, Gronthos S, Zannettino ACW, Worthley SG. Cardiac Magnetic Resonance, Transthoracic and Transoesphageal Echocardiography: A Comparison of In Vivo Ventricular Function Assessment in Rats. Heart, Lung & Circulation 2012; S21: S143-S316.

5. **Richardson JD**, Bertaso AG, Wong DT, Frost F, Carbone A, Nelson AJ, Psaltis PJ, Paton S, Koschade B, Williams K, Worthley MI, Teo KS, Gronthos S, Zannettino ACW, Worthley SG. Assessment of Regional Myocardial Function in Rats using 1.5T Cardiac Magnetic Resonance Imaging. Heart, Lung & Circulation 2012; S21: S143-S316.

Presentations & Prizes arising from the Thesis:

- Cardiac Society (CSANZ) 2012 Oral Presentations:
    - "Immediate Mesenchymal Stem Cell Therapy Provides Greater Attenuation of Myocardial Injury Than Deferred Treatment in Rats After Acute Myocardial Infarction."
    - "Sequential Mesenchymal Stem Cell Interventions Produce Greater Myocardial Repair Than Solitary Treatment in Rats After Acute Myocardial Infarction"
- Cardiac Society (CSANZ) 2012 Poster Presentations:
    - "Prospectively Isolated, Hypoxic-Preconditioned Mesenchymal Stem Cells Significantly Attenuate Myocardial Infarction-Induced Ventricular Dysfunction In Rats".
    - "Cardiac Magnetic Resonance, Transthoracic and Transoesphageal Echocardiography: A Comparison of In Vivo Ventricular Function Assessment in Rats".
    - "Assessment of Regional Myocardial Function in Rats using 1.5T Cardiac Magnetic Resonance Imaging"
- Ralph Reader Young Investigator Award, Cardiac Society Australia and New Zealand 2012 (Runner-up)
- CSANZ ACC Travelling Fellowship 2011 (Top Ranked Candidate)
- Genesis Research Award 2012 (Winner)

Scholarships:

- International Postgraduate Research Scholarship, Uni. of Adelaide (2011).
- Australian Postgraduate Award, Uni. of Adelaide (2011).

Abbreviations

αMEM	Alpha modification of Eagle's medium	ESV	End-systolic volume
µg	Microgram	FACS	Fluorescence-activated cell sorting
µL	Microlitre	FCS	Foetal calf serum
µm	Micrometre	FITC	Fluorescein isothiocyanate
µM	Micromolar	FS	Fractional Shortening
ANOVA	Analysis of Variance	g	Gram
AUC	Area under the curve	GFP	Green fluorescent protein
BM	Bone marrow	HBSS	Hanks' balanced salt solution
BMMNC	Bone marrow mononuclear cells	HGF	Hepatocyte growth factor
CD	Cluster of differentiation	HR	Heart rate
cDNA	Complementary deoxyribonucleic acid	IGF	Insulin-like growth factor
CFU-F	Colony forming units-fibroblast	IHD	Ischaemic heart disease
CI	Confidence interval	IL	Interleukin
CMR	Cardiac magnetic resonance	PBS	Phosphate buffered saline
CXCL12	Stromal cell-derived factor 1 (SDF-1)	LV	Left ventricle (or left ventricular)
DMEM	Dulbecco's modification of Eagle's medium	MACS	Magnetic-activated cell sorting
DMSO	Dimethyl sulphoxide	mg	Milligram
DNA	Deoxyribonucleic acid	MI	Myocardial Infarction
EC	Endothelial cells	SD	Standard deviation
EDD	End-diastolic dimension	MPC	Mesenchymal precursor cells
EDTA	Ethylenediaminetetraacetic acid	MSC	Mesenchymal stromal/stem cells
EDV	End-diastolic volume	n	Sample number
EF	Ejection Fraction	TGF	Transforming growth factor
ELISA	Enzyme-linked immunosorbent assay	VEGF	Vascular endothelial growth factor
EPC	Endothelial progenitor cells	v/v	Volume per volume
ESD	End-systolic dimension	w/v	Weight per volume

Thesis Abstract

Despite current treatment options, cardiac failure after myocardial infarction (MI) is associated with significant morbidity and mortality so highlighting a compelling clinical need for novel therapeutic approaches. Based on promising pre-clinical data, stem cell therapy has been suggested as a possible therapeutic strategy. Early studies largely utilised autologous bone marrow cells with only modest benefits observed in clinical trials. Of the alternative candidate cell types evaluated, mesenchymal stromal/stem cells (MSCs) have shown promise, however their clinical application for mainstream cardiovascular use is currently hindered by several important limitations. Consequently, this has prompted intense efforts to advance the therapeutic properties of MSCs through cell optimisation strategies.

Allogeneic sources of MSC appear to hold several important advantages over autologous bone marrow/BM mononuclear cells (BMMNC); (1) MSC can be derived from young, healthy donors thereby enhancing the absolute yield and functional biology of MSCs; (2) The cell product could be prepared well ahead of time, so making very early MSC treatment feasible, e.g. after primary percutaneous intervention, when myocardium remains viable; (3) MSC could be optimised to potentially advance their therapeutic efficacy.

The studies described in this thesis utilised all of the above features to address the primary aims of:

1. Reviewing the literature and writing a review regarding the optimisation of the cardiovascular therapeutic properties of MSC.
2. Develop an allogeneic MSC population optimised by the novel combination of prospective-isolation enrichment and hypoxic preconditioning. Furthermore, evaluate the in vivo function of optimised MSC compared to conventional plastic-adherent isolation of MSC (PA-MSC).

3. Develop a reliable non-invasive assessment of rat ventricular function using 1.5T cardiac magnetic resonance and evaluate this modality against conventional methods (transthoracic echocardiography) and novel modalities in rats (transoesophageal echocardiography).
4. Explore the impact of the timing of MSC intervention and cell dose after MI, now that immediate cell intervention is feasible clinically and these factors have not previously been investigated.
5. Explore the potential benefits of immediate and deferred MSC treatment after MI, two very different time points – a novel concept.

An allogeneic source of MPCs was derived from donor rat bone marrow. In contrast to conventional plastic-adherent isolation of MSC, an enriched and optimised MSC population prepared by prospective isolation of immature MPCs (via a CD45 immunodepletion step) and hypoxic preconditioning was established. In cell-based experiments, optimised MSC were compared to same-donor plastic-adherence isolated MSC and demonstrated superior in-vitro differentiation and colony forming capacity than PA-MSC.

To evaluate the effects of MSC treatment after MI in rats, highly accurate and reproducible imaging techniques are required. Cardiac magnetic resonance (CMR) is widely regarded as the gold standard modality, however the use of standard 1.5T “clinical” MR scanners in rodents has only been achieved by a handful of investigators worldwide and none have used contemporary MR techniques. CMR was then evaluated against conventional imaging modalities (transthoracic echocardiography) and novel methods in rats (transoesophageal echocardiography).

Allogeneic MSC permits immediate treatment, previously impossible with autologous stem cells, therefore this potentially important variable (timing) was assessed. Myocardial infarction was induced by ligation of the left anterior descending artery in rats. Optimised

MSC were then injected into the myocardium either immediately after MI or one week later, at one of two cell doses. This study provided an innovative comparison of these clinically relevant time points and demonstrated value at both times. Furthermore, greater efficacy was observed with immediate treatment, which displayed high sensitivity to MSC dose, with benefits largely localised to the infarct territory. Deferred treatment, though less effective, was not dose dependant and primarily influenced non-infarct myocardium.

Given the disparate, yet beneficial effects, of immediate and deferred MSC intervention the benefit of combining MSC treatment at both time points was investigated. Again, this was undertaken in the rat model of MI, with CMR determination of ventricular function. This novel study showed clinically relevant improvements in LV function and confirmed the differential distribution of MSC repair according to timing of cell intervention.

In summary, the studies described in this thesis provide new evidence outlining the merits of prospective isolation and hypoxic preconditioning of MSC. Furthermore they demonstrate the reparative effects of these cells and provide novel insights into the significance of timing of MSC intervention on efficacy and mode/distribution of effect, which can be further augmented through treatment both time points after MI.

Chapter 1 - Optimisation of MSC

Optimization of the Cardiovascular Therapeutic Properties of Mesenchymal Stromal/Stem Cells – Taking the Next Step

James D Richardson[1,2] MBBS, Adam J Nelson[1] MBBS, Andrew CW Zannettino[2] PhD, Stan Gronthos[2] PhD, Stephen G Worthley[1,2] MBBS PhD, Peter J Psaltis[1,3] MBBS PhD

[1] Cardiovascular Research Centre, Royal Adelaide Hospital and Department of Medicine, University of Adelaide, South Australia, Australia.

[2] Department of Haematology, SA Pathology and Centre for Stem Cell Research, Robinson Institute, Discipline of Medicine, University of Adelaide, South Australia, Australia

[3] Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, USA

1. Abstract

Despite current treatment options, cardiac failure is associated with significant morbidity and mortality highlighting a compelling clinical need for novel therapeutic approaches. Based on promising pre-clinical data, stem cell therapy has been suggested as a possible therapeutic strategy. Of the candidate cell types evaluated, mesenchymal stromal/stem cells (MSCs) have been widely evaluated due to their ease of isolation and ex vivo expansion, potential allogeneic utility and capacity to promote neo-angiogenesis and endogenous cardiac repair. However, the clinical application of MSCs for mainstream cardiovascular use is currently hindered by several important limitations, including suboptimal retention and engraftment and restricted capacity for bona fide cardiomyocyte regeneration. Consequently, this has prompted intense efforts to advance the therapeutic properties of MSCs for cardiovascular disease. In this review, we consider the scope of benefit from traditional plastic adherence-isolated MSCs and the lessons learned from their conventional use in preclinical and clinical studies. Focus is then given to the evolving strategies aimed at optimizing MSC therapy, including discussion of cell-targeted techniques that encompass the preparation, pre-conditioning and manipulation of these cells ex vivo, methods to improve their delivery to the heart and innovative substrate-directed strategies to support their interaction with the host myocardium.

Key words: Cardiomyopathy; Ischemic heart disease; Limitations; Mesenchymal stem cells; Mesenchymal precursor cells; Myocardial infarction; Optimization; Paracrine; Pre-conditioning; Tissue engineering.

2. Introduction

Ischemic heart disease (IHD) remains a leading cause of morbidity and mortality worldwide. Emergency reperfusion treatment has significantly improved survival rates from its all too frequent complication - myocardial infarction (MI). However, survivors

are often left with significant left ventricular (LV) dysfunction which greatly impedes both quality of life and prognosis. Contemporary treatment of cardiac failure principally consists of pharmacological approaches in addition to selected use of device therapy. These measures deliver only a modest reduction in morbidity and mortality, with 5-year survival rates less than 50% frequently observed [1]. Owing to the lack of suitable donor organs, cardiac transplantation remains a solution reserved for a minority of patients who demonstrate the most severe forms of cardiac failure. Therefore a compelling clinical need exists for novel therapeutic approaches, of which stem cell treatment has emerged as one of the most promising.

Cardiac cell-based therapy was initially conceived as an innovative way to circumvent the apparent inability of the heart to replace lost cardiomyocytes after MI or other injury. Many potential candidate cells have been evaluated, including unfractionated bone marrow cells (BMCs) [2], bone marrow mononuclear cells (BM MNCs) [3], hematopoietic stem cells (HSCs) [4], endothelial progenitor cells or pro-angiogenic cells [5], skeletal myoblasts [6] and mesenchymal stromal/stem cells (MSCs). Of these, MSCs or MSC-like cells have been widely studied at both preclinical and clinical levels, owing to their ease of isolation, proclivity for ex vivo expansion, range of pro-angiogenic and cardiac supportive benefits and the potential for their allogeneic use due to their relatively immunoprivileged nature [7].

MSCs are rare, non-hematopoietic progenitor cells of mesodermal and neuroectodermal derivation. Following birth, these cells reside in a number of tissue niches characteristically in a perivascular distribution [8]. While they have been best characterized in the BM where they play a supportive role for cells of hematopoietic lineage, MSCs have also been isolated from the lungs [9], adipose [10], dental pulp and periodontal ligament tissue [11], peripheral circulation [12], umbilical cord blood [13] and placenta [14]. In the cardiovascular realm, MSCs have been assessed for their regenerative, or more precisely, reparative properties in a range of settings including acute MI [15-17], chronic ischemic cardiomyopathy [18] and non-ischemic cardiomyopathy

(NICM) [19]. There has also been interest in their utility for tissue valvular engineering [20] and the treatment of cardiac conduction disease [21]. However, as is the case for other adult-derived cell types, the potential application of MSCs to mainstream cardiovascular therapy is currently hindered by several important limitations. These include (1) suboptimal retention and engraftment after transplantation into the diseased heart, (2) inherently constrained potential for bona fide cardiomyocyte regeneration and (3) safety concerns relating to their delivery, most notably by the intracoronary arterial route [22]. In this review, we firstly consider the evidence from the conventional use of plastic adherence-isolated MSCs in preclinical and clinical studies of cardiovascular disease. Focus will then be given to different strategies that are evolving to optimize the potential of MSC therapy. These encompass cell-targeted techniques relating to their preparation, pre-conditioning and manipulation ex vivo, emerging methods to improve their delivery to the heart and innovative substrate-directed strategies to support their interaction with the host myocardium.

2.1. Lessons from conventional MSC transplantation

The methods by which MSCs are selected and cultured may have a profound impact on their disease modifying efficacy. The traditional method employed in the vast majority of cardiovascular studies has utilized MSCs isolated from density-separated MNCs via simple plastic adherence culture [23]. Despite its ease and popularity, this technique is compromised by suboptimal specificity, as the resulting MSC population may be contaminated by non-mesenchymal cells during early culture (e.g. monocytes, hematopoietic cells) and increasingly senescent mesenchymal cells following sequential passage [24]. Thus the product of this isolation process and subsequent ex vivo expansion may comprise a heterogeneous admixture of cells with a limited and unpredictable content of primitive MSCs that possess high clonogenicity and multipotency. Minimum criteria have therefore been established to ensure some degree of uniformity between different laboratories working in the field. MSC preparations must satisfy the fundamental requirements of displaying trilineage plasticity for bone, cartilage and fat under inductive culture conditions and exhibiting a characteristic immunophenotypic profile [25].

2.2. Preclinical experience

Mesenchymal stromal cells have been extensively evaluated in animal models of ischemic and non-ischemic cardiac disease, spanning a range of species from small rodents through to porcine and ovine models. In small animal studies of MI, MSC transplantation has resulted in reduced infarct size, diminution of cardiomyocyte apoptosis in peri-infarct tissue, enhanced neoangiogenesis and normalization of myocardial metabolo-energetics, ultimately improving indices of LV contractile function [26, 27]. Furthermore, recent studies also suggest that MSCs may be able to decrease the potential for re-entrant arrhythmias following acute MI [28]. Similarly, beneficial effects have also been observed in experimental models of inflammatory myocarditis and established NICM, including attenuation of inflammatory cell infiltration and collagen deposition and protection against cardiomyocyte oxidative stress and apoptosis [29-31].

In large animal studies, delivery of MSCs to the heart has involved clinically applicable methods such as intracoronary infusion [32] or percutaneous catheter-based transendocardial injection [16, 33]. In addition, there has been substantial interest in pre-labeling cells with direct or indirect strategies to enable their in vivo detection by non-invasive imaging modalities, such as magnetic resonance imaging (MRI) [34] or radionuclide techniques [35, 36]. These experiments have consistently revealed that irrespective of the delivery route employed, short-term retention of cells is suboptimal, amounting to less than 20% of the administered dose within one hour after transplantation [37]. Nevertheless, despite this difficulty, MSC therapy has translated into diverse benefits in the vast majority of experimental models. This has comprised improvements in regional and global systolic and diastolic function and reversal of LV remodeling after MI, as measured both by hemodynamic and imaging-based assessment [16]. In the settings of both MI and chronic ischemic cardiomyopathy, important evidence has also been presented for enhanced myocardial collateralization and coronary perfusion, which may precede benefits to contractile performance [18, 38, 39]. The scale of these functional

improvements, if translated into human clinical advances would certainly represent significant progress in the management of ischemic cardiac failure.

2.3. Mechanisms of benefit: direct differentiation vs indirect repair

Cardiovascular stem cell therapy was originally conceived to repopulate the damaged myocardium by transdifferentiation of donor stem cells into new functional cardiomyocytes or vascular cells [4]. As part of their multi-differentiation potential, MSCs can be induced to adopt cardiomyocyte properties under specific culture conditions and express transcription factors associated with fetal cardiomyocyte development [40, 41]. In vitro transdifferentiation has been achieved with both physical (e.g. mechanical strain [42], electrostimulation [43]) and chemical means. The latter encompass diverse strategies which have had variable levels of success, including DNA demethylation with 5-azacytidine [44, 45], histone acetylation with suberrylanilide hydroxamic acid [46], cytokine exposure [e.g. hepatocyte growth factor (HGF), bone morphogenic protein-2 (BMP-2), transforming growth factor-β1 (TGF-β1), exogenous Jagged1 protein) [47-50] and co-culture with neonatal or mature cardiomyocytes [51, 52] or extracts [53].

Evidence for cardiomyogenic differentiation in vivo requires stringent immunohistological and microscopic techniques to avoid confounding factors, such as tissue artefact or cellular fusion that may result in false positive results [54]. Incomplete proof of transdifferentiation has been reported on the basis of de novo expression of myocyte (e.g. troponin, desmin) or vascular markers (e.g. factor VIII, alpha-smooth muscle-actin) in studies transplanting xenogeneic BM MSCs in immunodeficient mice [26] and rats [55] and in larger animal studies of infarction [33, 56] and ischemia [18, 38], with either autologous or allogeneic cells. Recent reports have indicated that more efficient cardiomyocyte transformation may be achieved by non BM-derived MSCs, such as from amniotic membrane [57] or adipose [58].

The modest evidence for in vivo differentiation observed with conventional MSC preparations may partly be due to the heterogeneous nature of cells obtained through plastic adherence isolation and moreover the diminishing presence of immature, multipotent precursors after several passages of ex vivo expansion. In any case, owing to the underwhelming retention and engraftment of transplanted cells in the host myocardium, the majority of benefit from MSC transplantation cannot be attributed to differentiation and numerical replacement of lost cardiac cell mass. Increasingly, a paradigm has emerged in which MSCs (and indeed other cell types) are thought to provide cardiovascular reparative properties through indirect, paracrine effects. These are mediated by cell-to-cell interactions with constitutive cells and the secretion of a wide range of soluble growth factors and cytokines that influence nearby cells [59]. Relevant transcription and growth factors identified from both in vitro and in vivo experiments include stromal cell-derived factor-1 (SDF-1/CXCL12), HGF, insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), hypoxia inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), angiopoeitin-1 (Ang-1), monocyte chemoattractant protein-1 (MCP-1), interleukins-1 and 6, placental growth factor, plasminogen activator and tumour necrosis factor-alpha (TNF-α). The secretome of MSCs is known to be modulated by cell-specific factors (e.g. developmental status of the MSCs [24, 60]) and the local milieu in which they find themselves [61]. Paracrine interactions between transplanted cells and their host environment may impact on both mature and progenitor cell targets that are either resident in the myocardium or recruited there via the peripheral circulation [62]. These effects can be categorized as (1) trophic (anti-apoptotic; supportive of proliferation or differentiation of endogenous cells; pro-angiogenic), (2) immunomodulatory, (3) anti-fibrotic or (4) chemoattractant (Figure 1). The pro-angiogenic and cardioprotective properties of MSCs have been well demonstrated in vitro by co-culture or conditioned media experiments and have been corroborated in vivo [24, 62-65]. Exposure of cardiac cells to MSC-derived factors have resulted in reduced apoptosis and enhanced mitogenesis [24], metabolic protection [27] and preservation of intracellular calcium handling [66] and ion channel function [67]. Recently, it has been suggested that beyond releasing soluble cytokines, MSCs may also exert such actions by secreting small particles, such as phospholipid exosomes [68].

In addition to these trophic effects, the immune-modifying properties of MSCs may influence the local balance of pro- and anti-inflammatory mediators in the heart, ultimately limiting inflammatory cell infiltration after MI or non-ischemic myocarditis [69-71]. Similarly, their anti-proliferative actions on cardiac fibroblasts [72], and modulation of the production of extracellular matrix components and matrix-modifying enzymes [73] may also restore the balance of collagen deposition and turnover. In turn, this helps to attenuate myocardial scar formation and improve diastolic relaxation and LV geometry [74].

2.4. Clinical application

Significant biological differences are known to exist between MSCs isolated from different species, particularly between large mammals (including humans) and rodents [75]. Data from small animal studies can be difficult to interpret and may not satisfactorily represent either human disease or the biological potential of the stem cell. An example of this discordance relates to the important question of tumor risk after MSC transfer. Whereas some rodent experiments have reported alarming rates of chromosomal instability and neoplastic transformation [76, 77], these findings have not been reliably recapitulated during prolonged culture of human MSCs or long-term follow-up of MSC therapy in large animals. Despite rigorous debate within the scientific community, there has therefore been ongoing momentum to evaluate the safety and efficacy of stem cell treatments in the clinical setting.

The specific use of MSCs in human studies of cardiovascular disease has been relatively limited, lagging considerably behind the clinical experience with autologous BMCs or BM MNCs, which have been the subject of numerous trials since 2000 [3, 78, 79]. Although the practicality of using freshly isolated BM cells is suited to the acute time frame required to treat MI, the heterogeneous nature of unfractionated BM preparations, along with a lack of standardization in study design and methodology, has led to discrepant outcomes in cases of BMC therapy after MI. Meta-analyses have concluded modest benefits overall,

consisting of absolute improvement in LV EF in the order of 3% and reduced MI size, especially in patients with the most compromised cardiac function and those receiving the highest content of progenitor cells [80]. While the magnitude of this therapeutic effect is modest, it is comparable with conventional pharmacological treatment after MI, including the use of angiotensin converting enzyme (ACE) inhibitors [81]. Nevertheless, there has been increasing recognition that better results may be achievable with more selective progenitor/stem cell preparations, such as MSCs.

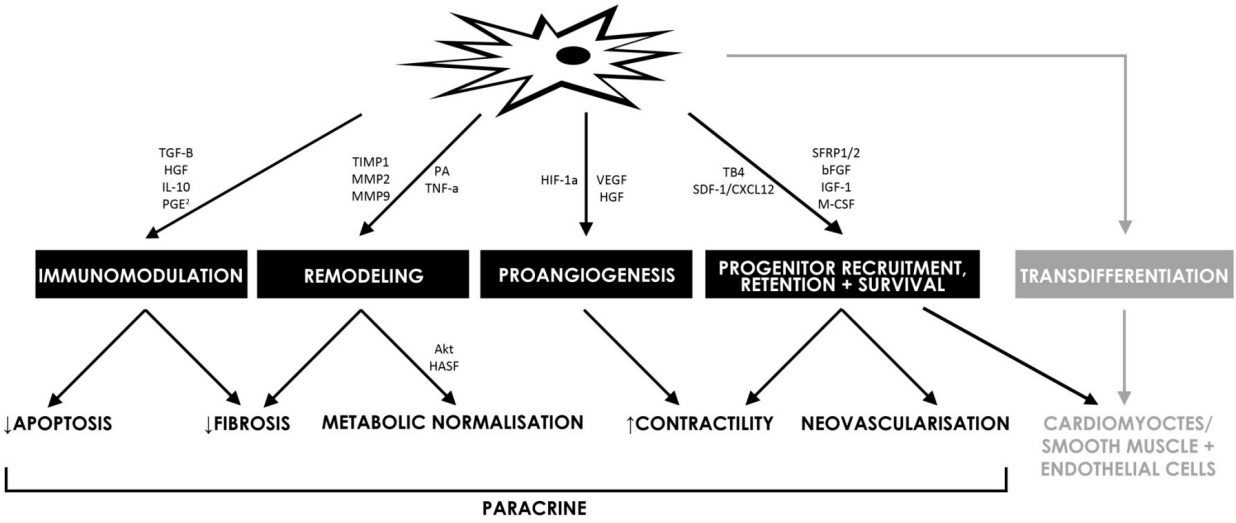

Figure 1. Proposed mechanisms of action of Mesenchymal Stromal Cells on cardiovascular repair. Despite some evidence for the cardiomyogenic differentiation potential of conventional MSCs, current consensus views these cells as mediating much of their reparative function through pleiotropic paracrine actions on various endogenous cellular and extracellular targets. Abbreviations: bFGF: basic fibroblast growth factor; CXCL12: chemokine (C-X-C motif) ligand 12; HASF: hypoxia regulated Akt mediated stem cell factor; HGF: hepatocyte growth factor; HIF-1a: hypoxia-inducible factor-1alpha; IGF-1: insulin-like growth factor-1; IL-10: interleukin-10; M-CSF: macrophage colony-stimulating factor; MMP2 (and MMP9): matrix metalloproteinase 2 (and 9); PGE2: prostaglandin E2; SDF-1: stromal-cell derived factor-1; SFRP-1: secreted frizzled-related protein 1; TB4: thymosin beta-4; TGF-B: transforming growth factor-beta; TIMP1: tissue inhibitor of metalloproteinase-1; TNF-α: tumor necrosis factor-alpha, VEGF: vascular endothelial growth factor.

To date, delivery of autologous MSC therapy for acute [15, 82, 83] or chronic MI [84, 85] has been mainly via the intracoronary route, either alone or in combination with endothelial progenitor cells. Although results have been encouraging in terms of therapeutic safety and benefit [improved ejection fraction (EF), myocardial perfusion, arrhythmia burden], these were small studies with follow-up of up to six months. More recently, intramyocardial catheter-based delivery of MSCs was reported to result in reversal of LV remodeling (reduction in end-diastolic volume) and improvement in infarct size and regional contractile function at one year in a small non-controlled series of patients with chronic MI [86]. Generally, the use of autologous MSCs provides several challenges in clinical practice, such as the time required to expand cells to adequate number and the inconsistent yield of functional, immature stem cells from sick, elderly patients with chronic comorbidities. Owing to the immunotolerant and immunomodifying properties of MSCs [87], it has been anticipated that these concerns can be circumvented by the use of allogeneic, 'off-the-shelf' MSC formulations. This approach could potentially allow rapid access to MSC therapy in the immediate aftermath of MI reperfusion, while the availability of a donor bank of cells prepared from healthy, young donors would ensure appropriate quality assurance and consistency in cell preparation.

This was exemplified by a randomized, double-blind, placebo-controlled study of 53 patients, in whom the allogeneic MSC product, Prochymal® (Osiris Therapeutics, Inc., Baltimore, Maryland), was evaluated after acute MI [17]. Notably, the expanded yield of MSCs isolated by plastic adherence from a single donor provided up to 5000 ready-to-use doses. Cells were delivered via peripheral intravenous route within 10 days of percutaneous intervention for MI. The allogeneic product was safely tolerated despite preclinical data highlighting the risk of pulmonary entrapment after systemic administration [88]. Six month benefit was reported for LV EF and reverse remodeling in the cohort of patients with anterior infarcts and moreover, there was evidence for a reduction in arrhythmic events. Further basic scientific and clinical investigation is still required to resolve some lingering uncertainties about the immunotolerance of allogeneic MSCs [89, 90], while cell therapy companies must also satisfy rigorous safety and regulatory standards to ensure compliance with good manufacturing practice (GMP).

Nevertheless, there are numerous ongoing trials currently utilizing plastic adherence-isolated MSCs from autologous and allogeneic sources in patients with acute MI and ischemic cardiomyopathy (www.clinicaltrials gov) (Table 1).

It is clear from both animal and human studies that a number of shortcomings exist in relation to conventional MSC strategies for cardiovascular therapy that center around the inherently modest cardiomyogenic plasticity of plastic adherence-isolated MSCs and their reduced reparative potential when derived autologously from older, diseased donors. These limitations are compounded by current inadequacies in the levels of cellular retention, engraftment and viability after their transfer to the host myocardium. Much of this relates to rapid clearance of injected stem cells from the myocardium in the initial hours following their transplantation [91]. Contraction of the myocardium causes leakage of cells at the injection site which is further accentuated by lymphatic and venous drainage. The hostile milieu of the ischemic and inflamed myocardium is thought to result in further attrition of the cells deposited within the myocardium and reduced functional capacity of those which engraft [92]. Considerable effort is now centered on countering such obstacles, by targeting key steps in the cell transplantation process. Optimization strategies can be broadly considered as those that aim to promote the survival and/or reparative/ regenerative properties of stem cells prior to their administration and those that modify the myocardial substrate to support the interaction of transplanted cells with their recipient tissue.

Table 1. Ongoing clinical trials using conventional plastic adherence isolated MSCs

Condition	Cell type / Dose	Delivery method	Primary Endpoint	Design	Patient number	Site / Company / Trial name	Trial ID number
Acute MI	Allogeneic MSCs	IV	Safety	Phase I, RDBPCT	48	US, Osiris Therapeutics, Multicenter	NCT00114452
Acute MI EF 20-45%	Allogeneic MSCs	IV	ESV	Phase II, RDBPCT	220	US, Osiris Therapeutics, Multicenter	NCT00877903
Acute MI	Autologous MSCs	IC	EF at 6 months (SPECT)	Phase II/III, RCT, open label	80	South Korea, FCB-Pharmicell Co Ltd. Multicenter	NCT01392105
STEMI EF 30-50%	Allogeneic MSCs	IV	MACE	Phase I/II, RDBPCT	20	India, Stempeutics Research Pvt Ltd. Multicenter	NCT00883727
Ischemic CM EF 15–45%	Autologous MSCs	TEn	EF (CMR)	Phase II, RDBPCT	60	Finland, Single Center	NCT00418418
Ischemic CM EF 20–50%	Autologous MSCs or BMCs	TEn	MACE & Safety	Phase I/II, RDBPCT	60	US, TAC-HFT trial, Single center	NCT00768066
Ischemic CM EF 15-50%	Autologous MSCs	TEp	MACE & Safety	Phase I/II, RDBPCT	45	US, Multicenter	NCT00587990
Ischemic CM EF <35%	Autologous MSCs	TEn	Safety	Phase I/II, NR, single arm, open label	10	France, Single center	NCT01076920
Ischemic CM EF 20-50%	Allogeneic or Autologous MSCs	TEn	MACE & Safety	Phase I/II, RCT, open label	30	US, POSEIDON-Pilot Study, Multicenter	NCT01087996
NICM	Allogeneic or Autologous MSCs	TEn	MACE & Safety	Phase I/II, RCT, open label	36	US, POSEIDON-DCM Study, Multicenter	NCT01392625
Ischemia / Angina	Autologous MSCs	IM	Perfusion (SPECT)	Phase I/II. NR, single arm, open label	40	Denmark, Single center	NCT00260338

3. Addressing limitations of conventional MSC transplantation

3.1. Enrichment of primitive mesenchymal precursors

As discussed above, the traditional method of plastic adherence isolation and expansion which was first described in the 1970s [23] suffers from a degree of non-specificity, yielding immature and mature mesenchymal subpopulations with diverse clonogenic, proliferative and differentiative potential. Consequently, the strategy of prospective immunoselection has been advocated as a means of isolating a more homogeneous, immature starting population of mesenchymal precursor cells (MPCs) prior to ex vivo culture [7] (Figure 2). This entails using specific monoclonal antibodies either individually

or in combination, which recognize and enrich for MPCs with the greatest clonogenicity and differentiative potential, whilst excluding contaminating cells of hematopoietic origin. Examples of such, include: stromal precursor antigen-1 (STRO-1) [93], CD49a/CD29 [94], CD106 [95], CD146 [96], low-affinity nerve growth factor receptor [97], platelet-derived growth factor receptor [98, 99], epidermal growth factor receptor [98], insulin-like growth factor receptor [98], non-tissue specific alkaline phosphatase (STRO-3) [100] and heat shock protein-90 (STRO-4) [101].

The STRO-1 antigen is widely regarded as a marker of the most immature MPC populations. The STRO-1 antibody recognizes a yet to be defined cell surface molecule that is expressed by 10-20% of human BM MNCs, comprising all colony forming units-fibroblast (CFU-F), Glycophorin-A+ nucleated red cells, and a small subset of CD19+ B-cells, but not hematopoietic stem/progenitor cells. Its enrichment in human BM MNC populations has been shown to confer several positive characteristics to the resulting mesenchymal progeny, including much lower contamination with CD14+ macrophages at early passage, higher replicative capacity and trilineage differentiation potential and increased levels of MSC-related mRNA transcripts [24]. Studies comparing same-donor plastic adherence MSCs with STRO-1 enriched human MPCs show that these prospectively isolated MPCs retain higher expression of the STRO-1 antigen during ex vivo expansion and this correlates with increased production of certain cardiovascular relevant cytokines, notably SDF-1α and HGF. This translates to STRO-1 MPCs conferring greater paracrine support to target cardiac muscle and endothelial cell populations [24] and considerable reparative benefit to LV function, as shown after xenogeneic transplantation in a rat model of MI [55].

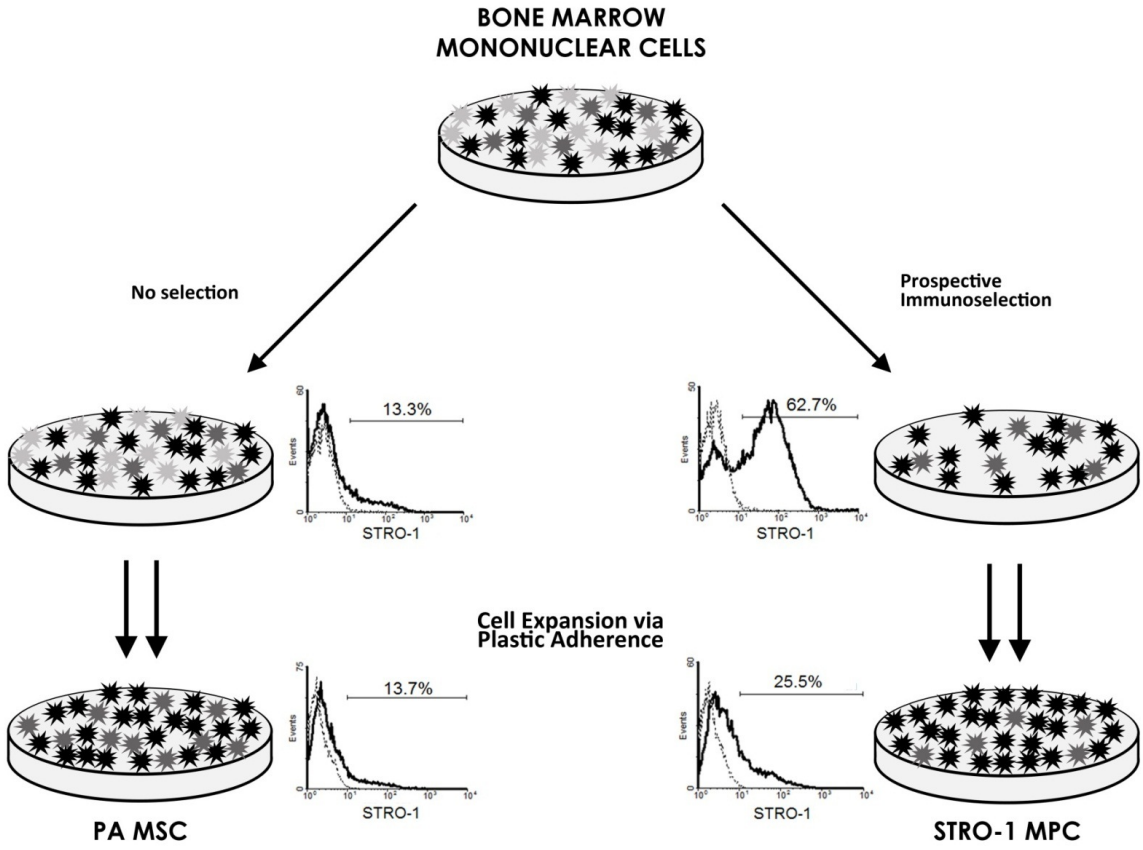

Figure 2. Approach for prospective isolation of Mesenchymal Precursor Cells. By comparison to conventional plastic adherence-based isolation of MSCs, prospective immunoselection (e.g. by magnetic or fluorescence activated cell sorting) has been shown to enrich for cells displaying greater "stemness" properties (shown as black cells) with less contamination of mature and non-mesenchymal cell types (gray cells). This applies at the time of initial mononuclear cell plating and also after serial passage. The monoclonal antibody for STRO-1 has been used for this purpose and its level of expression (shown as solid black line on representative flow cytometry histograms) correlates with important mesenchymal stem cell properties. PA MSC: plastic adherence-isolated mesenchymal stromal cells. STRO-1 MPC: STRO-1-isolated mesenchymal precursor cells

Unfortunately STRO-1-based immunoselection is limited by the instability of its pentameric IgM monoclonal antibody, hindering its implementation for GMP-grade MPC preparation. In addition, its relative specificity to human BM has prevented its use during large animal, preclinical evaluation. Alternative antibodies such as the IgG1 mAb, STRO-3 are therefore likely to be more widely applicable [100]. Immunoselection of human BM MNCs using STRO-3 has been shown to enrich for a similarly potent MPC population as STRO-1 [62] and allogeneic ovine STRO-3 MPCs have shown promising results in models of ischemic [74, 102] and nonischemic cardiac disease [19]. The latter study was also the first to administer these cells by catheter-based, transendocardial injection in the setting of nonischemic cardiomyopathy, using the NOGA XP® electromechanical mapping system (Biologics Delivery Systems Group, Cordis Corporation, Diamond Bar, CA, USA). Allogeneic human STRO-3 MPCs, produced and marketed as the "off-the-shelf" product Revascor™ (Mesoblast Ltd), have just undergone investigation in a completed phase II dose-escalation trial of heart failure, while another ongoing study is evaluating these cells in patients with recent MI.

Negative immunoselection, involving up-front depletion of cells expressing hematopoietic markers has also been used to prepare more homogeneous mesenchymal cell populations, using commercially available cell separation methodologies [13, 103]. This formed the basis of a recent study in which Lin-c-kit- MSCs were isolated from murine myocardium [104]. It has been claimed that single cell clonal expansion of negatively selected cells from different species results in potent, primitive cell populations, labeled as multipotent adult progenitor cells (MAPCs), with embryonic stem cell-like properties, including extensive proliferative capacity and totipotent differentiation into all three germ cell layers [105]. Despite early excitement surrounding this cell population, other laboratories have failed to reproduce these initial findings, leading to considerable controversy about their existence. More recent efforts to transplant them into small animal models of MI have shown favorable improvement in myocardial contractile function and infarct scar, without providing evidence for their in vivo differentiation into mature cardiac or vascular cells [106].

The cell medium in which MSCs are cultured and subsequently transplanted, can contain foreign proteins that may undermine their otherwise immunoprivileged characteristics. These proteins can provide an antigenic stimulus to the host immune system, triggering a local inflammatory or systemic humoral response and thus potentially exposing the MSCs to immune detection and clearance [107]. This may also result in immunological priming which could jeopardize repeat doses of cell therapy. Consequently, recent efforts in manufacturing MSC preparation for clinical use have increasingly centered around the use of serum-free growth and cryopreservation media (e.g. human platelet lysate) [108].

In summary, there is convincing evidence that a relatively simple up-front strategy of prospective immunoselection (positive or negative) can reliably provide a pure cell population, enriched for immature MPCs. This simple step provides cell populations with greater clonogenic and proliferative potential in vitro and potentially greater reparative capacity in vivo. Furthermore, the majority of the immunoselection processes described are feasible and readily transferable to clinical use.

3.2. Alternative sources of MSCs

Bone marrow has been the dominant source of MSCs used in studies of cardiac regeneration, however, a number of alternative tissue sources have also been investigated. These include adipose [10], umbilical cord blood [13], placental tissue [14] and the heart itself [109]. There are several proposed advantages to using adipose-derived MSCs, including the abundant supply of this tissue source. The peripheral distribution of fat allows easy access via liposuction procedures with large numbers of cells readily isolated. This potentially means that there is less need for culture-based cell expansion, which otherwise is known to diminish the "stemness" of mesenchymal cell populations. In a recent immunodeficient mouse model of MI, freshly derived human adipose cells, containing a heterogeneous mixture of mesenchymal (CD44+, CD105+), hematopoietic (CD34+, CD45+) and endothelial (CD31+) subpopulations, performed comparably with cultured adipose-derived MSCs in terms of achieving myocardial engraftment and improvement in LV function [110]. Cells with mesenchymal phenotype have also been

obtained from biopsies of adult human cardiac adipose tissue and have been shown to possess myocardial reparative properties, including pro-angiogenic paracrine actions [111]. At present, there are two ongoing clinical trials, sponsored by Cytori Therapeutics, Inc. (San Diego, CA), that are administering fresh adipose-derived cells to patients with MI (APOLLO trial, NCT00442806) and non-revascularizable IHD (PRECISE trial, NCT00426868) (Table 2). Although it appears that both fresh and cultured adipose cell products will be added to the cell therapy armamentarium for cardiovascular disease, it remains unclear if either will ultimately provide an advantage over traditional BM-derived cells.

Condition	Cell type / dose	Delivery method	Primary Endpoint	Design	Patient number	Site / Company / trial name	Trial ID number
Acute MI EF 30-50%	ADRCs	IC	MACE	Phase I, RDBPCT	48	Netherlands, Spain. APOLLO Trial. Cytori Therapeutics. Multicenter	NCT00442806
STEMI	Allogeneic WJ-MSCs	IC	EF & Perfusion (SPECT & PET)	Phase II, RDBPCT	160	China, Multicenter	NCT01291329
STEMI EF 30-50%	Allogeneic MPCs	TEn	Safety & feasibility	Phase Ib/IIa, RSBPCT, dose escalation	25	US, Angioblast Systems, Multicenter	NCT00555828
Cardiomyopathy EF <40%	Allogeneic MPCs	TEn	Safety & feasibility	Phase II, RSBCT	60	US, Angioblast Systems, Multicenter	NCT00721045
Cardiomyopathy with indication for LVAD	MPCs	TEp	Safety	Phase II, RDBPCT,	10	US. NHLBI & Angioblast Systems. Multicenter	NCT00927784
Childhood NICM EF 20-50%	Allogeneic UC-MSCs	Intramuscular	EF (Echo)	Phase I/II, RCT, open label	30	China, Single center	NCT01219452
Severe CAD	ADRCs	TEn	MACE	Phase I, RDBPCT	36	PRECISE Trial. Cytori Therapeutics. Multicenter.	NCT00426868

Table 2: Ongoing clinical trails using mesenchymal precursor cells.

Mesenchymal cell preparations from fetal and neonatal tissues, including placenta, umbilical cord blood [13], Wharton's jelly [112] and amniotic fluid [113] have also drawn interest, largely due to their primitive status. This has been postulated to confer greater cardiomyogenic plasticity and lower immunogenicity than observed with BM-derived MSCs, although this remains controversial [114]. Notably, comparative evaluation of amniotic fluid and BM-derived MSCs in a cryoinjury model has indicated that they may possess distinct differentiation capabilities for cardiomyocyte, endothelial and smooth

muscle cell lineages [113]. Recently, differences were also shown for other therapeutic effects (e.g. collagen deposition, cardiomyocyte apoptosis) following the transfer of different sources of human MSCs (BM, adipose, cord blood) in an immunodeficient mouse model of MI [115]. Currently, the clinical application of fetal or neonatal MSCs remains challenged by the difficult nature of isolating these cells and expanding them to sufficient number for therapeutic administration [116]. Therefore their use may need to be reserved for specific niche applications, such as in the field of heart valve tissue engineering [117].

Myocardial-derived MSCs have also shown promise [109], but require more invasive procedures for isolation (e.g. cardiac biopsy) and are relatively difficult to expand to high number. In addition, cardiosphere-derived cells [118] and c-kit+ cardiac progenitors [119] have been observed to express MSC phenotypic markers. Recently, the latter cells were found to have less multilineage mesenchymal plasticity compared to BM MSCs, but higher capacity for cardiomyogenic differentiation [119].

The age of the donor source is also an important consideration. With advancing age, there is considerable diminution of the absolute yield and functional biology of MSCs from aspirates of BM [120], negatively impacting on multilineage differentiation potential [121], gene expression profile, cell cycle [122] and ultimately myocardial reparative capacity [123, 124]. These insights have substantial clinical relevance given the hitherto reliance on autologous cell therapy from older comorbid patients. As mentioned earlier, allogeneic use of MSCs derived from young healthy donors might overcome this significant hurdle.

4. In vitro modification of MSCs

In vitro priming of MSCs by a wide variety of techniques has also been used to influence cell fate after in vivo administration. These may target any number of biological functions, including cell homing, migration, retention, engraftment, survival, proliferation, paracrine factor synthesis, cell-cell interaction and transdifferentiation.

4.1. (1) Culture pre-conditioning

During MI, blood flow to a portion of the myocardium is interrupted and accordingly cardiomyocytes are starved of oxygen. Oxygen levels have been observed to fall as low as 0.2% during infarction [125]. Shortly after, the combination of residual tissue hypoxia, reperfusion oxidative stress, cardiomyocyte death and inflammatory cell influx creates a hostile milieu that transplanted cells must withstand in order to engraft within the recipient myocardium. By virtue of their constitutive exposure to low oxygen tensions (~2%) within their native BM [126], one might surmise that MSCs should be quite well-placed to tolerate myocardial ischemia. However, there is scope to further augment their inherent resilience, by priming MSCs with different pre-conditioning factors.

Studies have assessed the in vitro and in vivo qualities of MSCs exposed to reduced levels of oxygen during cell culture. Hypoxia-preconditioned cells have been shown to produce increased quantities of pro-survival transcription and growth factors, including VEGF, HIF-1α, survivin and B-cell lymphoma-2 (Bcl-2) compared with their preparation under normoxia [127]. Sequential culture of MSCs under hypoxic followed by normoxic conditions may also enrich for those cells capable of thriving under hypoxia and this has translated into enhanced retention, survival and functional benefit after these cells are transplanted in models of MI [127] and diabetic cardiomyopathy [128]. In part, hypoxic pre-conditioning may confer its benefits to cell viability via induction and stabilization of intracellular HIF-1α, which undergoes nuclear translocation to bind to several important promoter regions. The downstream targets it may influence include the glucose-6-phosphate transporter, which serves to enhance the availability of glucose to the cell via enhanced gluconeogenesis. Alternatively, MSCs have been pre-conditioned by treatment with nitric oxide agents [129] or incubation with hydrogen peroxide or diazoxide [130] to increase their survival and paracrine capacity. The latter agent is known to stimulate opening of mitochondrial ATP-sensitive potassium channels (Mito-KATP) which is thought to impart cell protection during ischemic stress. Brief exposure of MSCs to diazoxide has been shown to improve cell viability by upregulation of various pro-survival

growth and signaling factors (e.g. VEGF, HGF, NF-κB, Akt pathway, micro-RNA-146a) [130, 131].

For several decades, heat shock proteins (HSPs) have been known to uphold a pivotal role in cell protection against a number of environmental stressors. Heat shock protein transcription can be induced in cultured cells by exposure to hyperthermia (e.g. by incubating for one hour at 42^{o}C). This has been applied for pre-conditioning of skeletal myoblasts to increase their myocardial engraftment in vivo [130], while MSCs have been genetically manipulated to over-express different HSPs prior to transplantation [133, 134]. While the precise mechanisms by which HSP augmentation subtends its benefits remain uncertain, evidence suggests that these proteins inhibit signaling pathway regulators of necrosis and apoptosis and ultimately enhance Akt activation. Furthermore, they may also promote the trophic properties of MSCs by increasing their secretion of different soluble factors, such as VEGF, FGF-2, and IGF-1 [133].

A diverse range of pharmacological drugs have been studied in conjunction with MSCs, either as adjunctive therapy at the time of cell delivery (e.g. statins [135, 136], sildenifil [137]) or as a means of cellular pre-conditioning. Examples of the latter have included the use of phosphodiesterase inhibitors [138], angiotensin pathway modulators [139] and neuropeptide Y [140]. Different mechanisms of benefit have been attributed to these agents, including the recent suggestion that angiotensin II blockade may augment transdifferentiation of human MSCs down the cardiomyocyte pathway [139].

The great appeal of all of these pre-conditioning strategies is their feasibility, simplicity and translatability to the clinical setting. However, the nature of MSC biology is exceedingly complex, even more so when cells are translocated from the controlled environment of tissue culture to the dynamic and unpredictable environment of a diseased heart. One of the great challenges to the field of optimizing cell therapy is to unravel the most crucial pathways implicated in cell survival and reparative function, so that pre-

conditioning strategies can be targeted more specifically to maximize their intended benefit.

4.2. (2) Paracrine factor augmentation

As detailed above, much of the cardiovascular reparative potential of MSCs seems to be mediated by release of a broad spectrum of soluble factors. This has prompted considerable efforts to enhance this paracrine capacity using either gene transfer methods or by pre-incubation strategies that induce cytokine or growth factor over-expression.

Vascular endothelial growth factor is an important regulator of MSC-induced vasculogenesis and angiogenesis [141] and may be upregulated in MSCs during hypoxia via upstream induction of HIF-1α. Pre-incubation of MSCs with TGF-β [142], SDF-1α [143] and lipopolysaccharides [144] has also been shown to increase cellular production of VEGF, with accompanying benefits to cell survival after transplantation and improved angiogenesis and myocardial recovery in rodent models of MI. Adenoviral transfection of rat MSCs with the human VEGF165 gene has also been reported as a successful "hybrid" strategy for combining cell transplantation with gene therapy to augment therapeutic angiogenesis after MI [145]. Insulin-like growth factor-1 also has pleiotropic activity, influencing cell growth, proliferation and survival predominantly by activating the Akt and mitogen-activated protein (MAP) kinase pathways. In one rat study of MI, pre-conditioning of MSCs with IGF-1 enhanced homing and engraftment of cells in the myocardium after systemic delivery and resulted in augmented functional benefit compared to transfer of conventional MSCs [146].

An important component of the paracrine benefit of MSCs is their ability to attract appropriate cells, including hematopoetic, endothelial or cardiac progenitor cells, to the site of myocardial injury. Stromal cell derived factor-1 and its G-protein transmembrane receptor, CXCR4, perform a pivotal chemotactic role to influence the recruitment of BMCs to the infarcted myocardium [147]. Immediately following MI, serum and myocardial

SDF-1 levels rise rapidly and peak at 48 and 72 hours respectively, before returning to baseline. This axis encourages progenitor cell homing to the damaged myocardium, including that of circulating, pro-angiogenic CD34+ cells that contribute to neovascularization. In addition, SDF-1 may also exert direct pro-angiogenic effects by inducing cellular expression of HIF-1α and VEGF [148].

In cardiovascular disease models, the SDF-1/CXCR4 axis has been manipulated in different ways to mediate beneficial effects on myocardial function and perfusion and to augment the reparative properties of transplanted cells, such as MSCs. In one rodent study of subacute MI, SDF-1α was injected directly into the myocardium as an adjunct to intravenous MSC injection, resulting in greater homing and engraftment of these exogenous cells to recipient heart, which could be reversed with the addition of a functional blocking antibody [149]. Pre-incubation of MSCs with SDF-1α has also been shown to confer pro-mitogenic and anti-apoptotic effects during in vitro exposure to hydrogen peroxide and after transplantation to infarcted myocardium [143]. In the latter case, this was associated with reduction in MI size and fibrosis and improvement in cardiac function, compared to the administration of unconditioned cells. Similar augmentation of MSC properties has also been achieved by stable overexpression of SDF-1α [150]. Following delivery to the heart, transduced cells were more resistant to apoptosis and achieved better engraftment than unmodified MSCs, resulting in decreased collagen deposition and expression of matrix metalloproteinases. Some of this effect was attributed to augmentation of the paracrine properties of MSCs, especially upregulation of their production of HGF. Other preclinical studies have utilized overexpression of HGF [151] or other factors (e.g. CCR1 [152]) to enhance the therapeutic promise of MSCs, while incremental gains have also been reported after performing dual transfection for VEGF and SDF-1 [153].

4.3. (3) Activation of cytoprotective pathways

The cytokine factors described above have complex interactions and function at multiple levels on different cellular and tissue substrates. Much of their ability to protect against

cell death is in part regulated through their actions on the Akt pathway. The Akt gene encodes a serine/threonine protein kinase, which when activated stimulates downstream signaling pathways (e.g. PI3K/mTOR) and ultimately downregulates BAD, a BCL-2 family protein and upregulates NFκB [154]. This leads to inhibition of apoptosis and transcription of pro-survival genes, respectively [155]. Activation of Akt gene expression is also known to stimulate angiogenesis and overcome cell cycle arrest [156]. Accordingly, manipulation of the Akt pathway in stem cells has been well studied as a means of optimizing survival both of the transplanted cells and the target host cardiac or vascular cells. In a series of highly cited basic and preclinical studies, Dzau and colleagues have demonstrated that not only are the reparative properties of MSCs predominantly paracrine-mediated, but that they can be significantly enhanced by genetic modification to overexpress Akt [27, 157, 158]. The benefits from using Akt-transfected MSCs compared to conventional MSCs were seen very early after in vivo transplantation to infarcted rat myocardium (≤72 hours). In particular, cardiac cell apoptosis was strongly attenuated, MSC engraftment was enhanced and there was upregulation of VEGF, HGF and IGF-1 within the myocardium. Impressively, some of these findings were also reproduced by administering only conditioned medium from these transfected cells, highlighting the paracrine basis for their cytoprotective and reparative properties. More recently, treatment of MI with Akt-modified MSCs has also been shown to restore normal cardiac metabolic function, with sparing of high energy phosphate content and normalization of myocardial pH and glucose metabolism [27]. Genomic analysis has identified that secreted frizzled related protein 2 (Sfrp2) is markedly upregulated in Akt-MSCs and this is a key mediator of the cytoprotective paracrine properties possessed by these cells [159].

Other transfection strategies used to confer cytoprotection to MSCs have focused on their over-expression of integrin-linked kinase (ILK) [160] and heme oxygenase-1 (HO-1) [161]. Heme oxygenase-1 enzymatically degrades heme to bilirubin, carbon monoxide and free iron and has anti-inflammatory, anti-oxidative, anti-apoptotic and pro-angiogenic actions. Over-expression of HO-1 in MSCs has been shown to enhance viability of donor cells and furthermore improve LV function in a porcine ischemia-reperfusion model [161].

Collectively, the studies described above demonstrate that there is great scope to improve the trophic capacity of MSCs, their resilience to stress and apoptosis and in turn their capacity to achieve cardiovascular repair. Moreover, they have helped uncover many of the critical molecules and signaling pathways that regulate stem cell function (survival, proliferation and migration). To date, the clinical translation of strategies for paracrine augmentation and enhanced cytoprotection remains largely untested. While genetic modification of MSCs and other cell types raises regulatory issues surrounding safety, early inroads into human evaluation have been made. In the ongoing placebo-controlled ENACT-AMI trial (clinicaltrials.gov NCT00936819), patients with large MI are being treated with pro-angiogenic circulating MNCs that have been transfected with human endothelial nitric oxide synthase [162]. Other pre-conditioning techniques should theoretically be more feasible in the clinical realm. Based on preclinical evidence in rodents [163], the MESAMI II trial aims to investigate melatonin pre-treated MSCs in patients with refractory chronic ischemic cardiomyopathy.

4.4. (4) Directing MSC cardiopoiesis

Efforts are also ongoing to enhance the capacity of MSCs to undergo cardiomyogenic differentiation so that they are capable of achieving some degree of active cardiomyocyte regeneration to accompany their paracrine reparative effects. As mentioned previously, early attempts to verify and then manipulate the cardiomyogenic potential of MSCs were focused on using the DNA methyltransferase inhibitor, 5-azacytidine [44]. Although this non-specific agent may facilitate modest cardiomyocyte transdifferentiation, its actions have been less reproducible with human primary MSCs than immortalized murine MSC lines [44]. Moreover, 5-azacytidine pre-conditioning may have genotoxic effects on cells which have implications for their safe transplantation in vivo. Treated cells also promptly enter cell cycle arrest, thereby losing the replicative potential that they require to achieve cardiomyocyte repopulation. Other strategies are therefore desirable that can reliably direct MSCs toward cardiomyocyte differentiation whilst maintaining their proliferative capacity.

During embryogenesis, stem cell commitment to the cardiac lineage is mediated by a series of signals promoting nuclear transcription of regulatory factors [homeobox gene Nkx2.5, myocyte enhancer factor-2 (MEF-2C) and GATA-4]. Embryonic stem cells (ESC) offer an ideal model to elucidate these mechanisms further. ESCs have extraordinary proliferative capacity, and therefore regenerative potential, but their clinical application has been impeded by neoplastic liability and ethical considerations. Cardiopoietic programming was devised as a method to modify these cells by exposing them to combinations of specific recombinant factors present in the embryonic milieu, with the aim to mitigate their tumorigenic risk but enhance their cardiomyocyte-specific regenerative potential [162]. Important insights have been provided into cardiac differentiation mechanisms, with members of the TNF-α, TGF-β and FGF families identified as crucial regulators of stem cell cardiopoiesis that in combination can reprogram ESCs to augment functional benefit to the myocardium without tumor risk [165].

Recently, this principle of directed cardiopoiesis was elegantly extended to human BM MSCs [166]. Screening of different patients with coronary artery disease identified rare individuals whose MSCs displayed a spontaneous capacity to improve myocardial contractile performance, along with high de novo expression of early and late cardiac transcription factors [e.g. Nkx-2.5, T-box transcription factor (TBX5), MESP1, MEFC2]. These cells were subjected to a recombinant cocktail consisting of TGF-β1, BMP4, activin A, retinoic acid, IGF-1, FGF-2, alpha-thrombin, and interleukin-6 which further consolidated their cardiac lineage pre-specification. In a nude mouse model of chronic MI, delivery of cardiopoietic MSCs, as compared to unmodified cells, achieved sustained functional and structural benefits without adverse sequelae (Figure 3). This was associated with higher retention of MSCs in the myocardium and greater evidence for their in vivo transformation to cardiomyocytes and their paracrine stimulation of endogenous c-kit+ cardiac stem cells.

This work has been further progressed to clinical investigation in Europe, where the C-cure trial has been undertaken with lineage-specified MSCs for cardiac repair in human patients

with chronic ischemic cardiomyopathy [167]. Cardiopoietic MSCs (0.6-1.2 x109) were transplanted transendocardially under electromechanical guidance into viable, dysfunctional LV myocardium in 21 patients. At median follow-up of just under a year, LV EF was increased to a greater extent in recipients of MSCs compared to non-placebo controls (absolute increase of 5.2% vs 1%), while there was also improved functional status (6 minute walk test results) and fewer arrhythmic episodes.

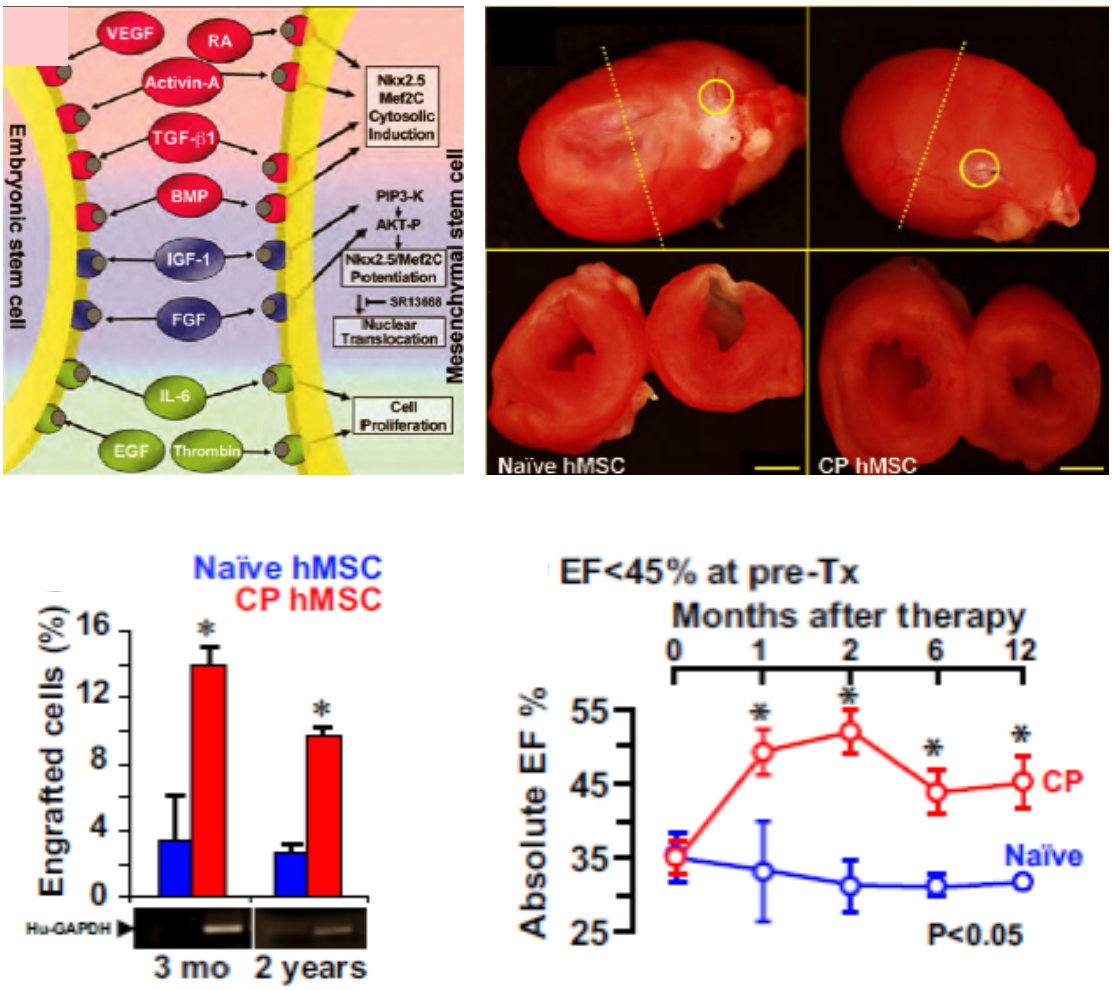

Figure 3. Guided cardiopoiesis of MSCs. (A) Example of human recombinant cardiogenic cocktail used with platelet lysate to potentiate nuclear translocation of homeobox transcription factor Nkx-2.5 and MEF2C (myocyte enhancer factor), while maintaining cell cycle progression during cardiopoietic induction of human bone marrow MSCs. (B) Examples of murine hearts showing remuscularization, reduced scar formation and diminished left ventricular remodeling downstream of left anterior descending artery ligation, three months after treatment with cardiopoietic MSCs (right) versus naive MSCs (left). (C) There is also evidence for greater short and long-term engraftment of cardiopoietic (CP) human MSCs along with (D) sustained benefit to left ventricular ejection fraction. Adapted from [164] (adapted with permission [166).

5. Modification of the myocardial substrate

Refinement of the selection of MSCs or their cardiac-specific functional properties has thus far provided the primary focus for investigators in their endeavor to optimize MSC therapy. However, modification of the myocardial substrate has also emerged as a potentially useful strategy to improve therapeutic outcomes. One of the best examples of this is the adjuvant use of specific pharmacological agents that are already in widespread clinical use and are known to have pleiotropic effects on the coronary vasculature and myocardium. Beyond their lipid lowering actions, HMG CoA reductase inhibitors ("statins") possess anti-inflammatory properties that may prime the myocardium to be more receptive to the engraftment of transplanted stem cells. In a porcine study of stem cell therapy for MI, concurrent treatment with atorvastatin resulted in reduction of inflammatory cytokine expression and apoptotic cell burden in the myocardium [168]. This translated into superior functional and perfusion recovery when compared to cell transfer alone. Lovastatin has also been shown to confer cytoprotection to MSCs exposed to hypoxia or serum-deprivation, via activation of the PI3K/Akt and ERK1/2 pathways [169].

Pre-conditioning of rats with hyperbaric oxygen treatment prior to permanent ligation MI was also shown to enhance the benefits of MSC therapy [170]. Notably, some of this effect may have been mediated by an increase in cell engraftment, as measured by detection of iron oxide-labeled cells with MRI and Prussian blue staining. Other mechanical strategies designed to improve myocardial perfusion are also likely to support the retention of cells in the heart, including conventional (e.g. percutaneous angioplasty, bypass grafting) and experimental (e.g. laser therapy) techniques for coronary revascularization. One group used low level laser irradiation to prime rat myocardium before induction of infarction and observed an increase in VEGF and GRP-78 (a heat shock protein) levels in the heart and a doubling of MSC retention two days after transplantation [171]. At the clinical level, percutaneous transmyocardial laser revascularization has previously been investigated in patients with refractory ischemia [172]. Although its effectiveness has ultimately proven to be disappointing, there has been

some interest in combining this technique with transendocardial cell delivery under the navigation of the NOGA® XP electromechanical mapping system [173]. Mid-term results from a small single-arm observational study have shown improvement in angina symptoms, hospitalization rates and LV EF [174], although rigorous controlled studies are clearly required if this combination approach is to be evaluated further.

6. Optimization of cell delivery

A crucial requirement of effective myocardial repair is that sufficient viable cells reach their target sites soon after administration and are retained there to enable their long-term survival, engraftment, proliferation and function. Cells can be administered to the heart (1) systemically by peripheral venous injection, (2) regionally, by coronary arterial or venous infusion or (3) locally, by direct transepicardial, transendocardial or intrapericardial implantation. Unfortunately, despite some differences in the effectiveness of these different delivery routes, rates of cell retention and engraftment currently remain disappointing, across all cell types, delivery methods and myocardial disease substrates [37].

Fluoroscopically-guided intracoronary infusion has been the commonest method used in clinical studies of cell therapy with the aim of distributing cells in the affected coronary artery territory [3, 175]. Its advantages include its low cost, minimally invasive nature, relatively short procedure time and immediate clinical feasibility during primary percutaneous coronary intervention for acute MI. Whilst myocardial retention rates are superior to intravenous delivery, direct wash-out of cells still limits first-pass retention and may result in undesirable entrapment of cells in non-cardiac organs. Furthermore, intracoronary delivery may be very challenging in the setting of totally occluded coronary arteries and may have potential risks associated with the aggregation of adherent or large-sized cells (e.g. MSCs) within the coronary microvasculature [22, 32, 176].

Delivery by intramyocardial injection, either by open transepicardial or percutaneous, catheter-based transendocardial approach, targets specific areas of myocardium more directly. While early injectate loss is still considerable, a direct injection approach appears to have advantages over systemic and intracoronary delivery relating to cardiac cell retention [177] non-cardiac cell entrapment [32] and overall therapeutic effect [178]. There is some potential risk of creating focal substrates for electrophysiological heterogeneity and arrhythmia, especially if cells are deposited at excessive density. However, this seems to be largely specific for skeletal myoblasts [6]. Although cell retention and distribution are similar between the transendocardial and transepicardial strategies [179], percutaneous catheter delivery is less invasive and therefore has broader clinical applicability [180].

7. Bionanotechnology

To date, stem cell therapy has largely consisted of administering suspensions of individual cells made up in medium. These preparations lack the physical characteristics needed to confine injectates within the target myocardium and prevent rapid efflux of the majority of cells to the surrounding capillaries and lymphatics. Cells that are retained in the myocardium are vulnerable to inflammatory clearance, ischemic death and apoptosis, as well as biomechanical disturbances in tissue architecture, homeostasis and matrix support that are prominent after infarction. The integration of cells into bioscaffolds is therefore appealing as a means to improve early retention after delivery and mitigate the obstacles that confound cell engraftment and survival.

Bioscaffolds are constructs with a three-dimensional architecture made from biocompatible and bioabsorbable materials that are ideally designed to reproduce the native structure of extracellular matrix. They often have "smart" surfaces that may be seeded with progenitor cells or growth factors. The most basic example of this has involved the injection of cells suspended in materials such as rapidly gelling fibrin glue [181], alginate [182] or matrigel [183], which may themselves possess vasculogenic properties. It has been reported that these cell-bioscaffold combinations result in superior engraftment and better therapeutic

outcomes in terms of myocardial vascularization, remodeling and function when compared to administering cells in conventional medium. A study in rats has also shown that cell retention can be substantially enhanced by simply applying fibrin glue over the epicardium where cells have been injected to restrict their backflow through myocardial puncture sites [184]. Beyond supporting cell engraftment, bioscaffolds also provide mechanical and functional support to the myocardium's extracellular matrix. This may be especially beneficial after MI to limit infarct expansion and coordinate collagen deposition in such a way as to minimize ventricular wall thinning and chamber dilatation.

Cells have also been incorporated into more sophisticated biomaterials, such as self-assembling peptides that form nanofibers after injection and exposure to myocardial levels of pH and osmolarity [185]. The tethering of cytokines to such structures may enable additional enhancement of cell survival and function and may accentuate the recruitment of endogenous progenitor cells to the treated myocardium [186]. Another notable development has been the production of biological composite scaffolds that are composed of cells suspended in a fibrin hydrogel and layered over a decellularized myocardial substrate [60]. By retaining most of its extracellular matrix intact, decellularized myocardial tissue provides a structural and biomechanical platform that imparts long-range signaling cues to the cells, while short-term cell retention is enhanced by the presence of fibrin. Importantly, these scaffolds can retain sutures and have enough pliability to be molded over the surface of the recipient heart. In a recent study, composites containing TGF-β pre-conditioned human STRO-3 MPCs were implanted into the infarct bed of nude rats after acute and chronic MI. This strategy greatly enhanced vascular network formation in the infarct territory by a combination of paracrine cytokine release and migration of MPCs into the ischemic myocardium and resulted in virtual restoration of normal contractile function [60]. Although developed outside of the cardiovascular context, another emerging technique is the microencapsulation of cells with semi-permeable capsules (e.g. alginate) that are embedded with contrast agents (e.g. bismuth sulphate, perfluorocarbon-hydrocarbons). These contrasts appear to provide a dual function of improving cell viability and function, while also rendering cells visible to different imaging modalities so that their fate can be tracked noninvasively in vivo [37,187].

8. Future directions

A wide variety of optimization strategies have been discussed for MSC-based therapies for cardiac regeneration, with particular focus on optimization of cell biology, modification of myocardial substrate or improvement of cell delivery (Figure 4). The immediate clinical applicability of these different techniques varies substantially. As mentioned earlier, prospective immunoselection of MPCs and cocktail-guided cardiopoiesis of MSCs are two examples that on the basis of solid preclinical evidence have been applied in clinical trials (Table 2). Alternative strategies such as the use of genotoxic differentiation agents (e.g. 5-azacytidine) are unlikely to be deemed safe for clinical use. While feasible, genetic engineering of cells faces considerable obstacles before satisfying stringent safety and regulatory requirements to allow human investigation.

Furthermore, it is increasingly apparent that there is no single "one-size-fits-all" answer to the clinical need that stem cell therapies aim to address. Cell-based interventions will ultimately need to be individually tailored to best suit patient-specific characteristics, including age, cardiovascular disease target, time of treatment relative to myocardial insult and the presence of comorbidities. For instance, the biological properties and optimization of cells used to treat early MI are likely to be very different from those that will be most helpful in chronic MI or in nonischemic cardiac diseases. In the case of the former, cells might be required to achieve preservation of LV function, through paracrine-mediated reduction of inflammatory cell infiltrates, modulation of cardiomyocyte protection and attenuation of extracellular matrix remodeling. Prospectively isolated MPCs with hypoxic pre-conditioning and/or Akt overexpression might adeptly serve this purpose. In contrast, cells delivered beyond the acute period of MI should be maximized in their cardiomyogenic regenerative potential (e.g. by directed cardiopoiesis), while in chronic IHD, VEGF-based augmentation of their vasculogenic properties may be most helpful.

Beyond the manipulation of cell biology, viability, retention, engraftment and differentiation, it is very likely that the most significant future direction of cell therapy will be to deviate away from the conventional approach of utilizing a solitary cell-based intervention. Investigators may be asking a great deal of one type of treatment to provide a significant and sustained improvement in cardiac function over a prolonged period. Owing to their intrinsic supportive properties, MSCs have already been recognized as an ideal candidate to use in combination with other progenitor cells to provide synergistic interaction and incremental therapeutic effect [188]. Moreover, pending future investigation, it is easy to imagine that cell therapy will be administered as a multi-step process, whereby specific optimized cell populations are delivered at precise intervals to satisfy time-dependent objectives for a given disease state. For example, one approach to the patient with acute MI might be to combine the early use of "preserver" cells with the later administration of "regenerator" cells. Such strategies may also draw on the current paradigm of paracrine effect to administer specialized cocktails of cell-derived soluble mediators or small molecules, either as adjuncts to cell transfer or as stand-alone treatment.

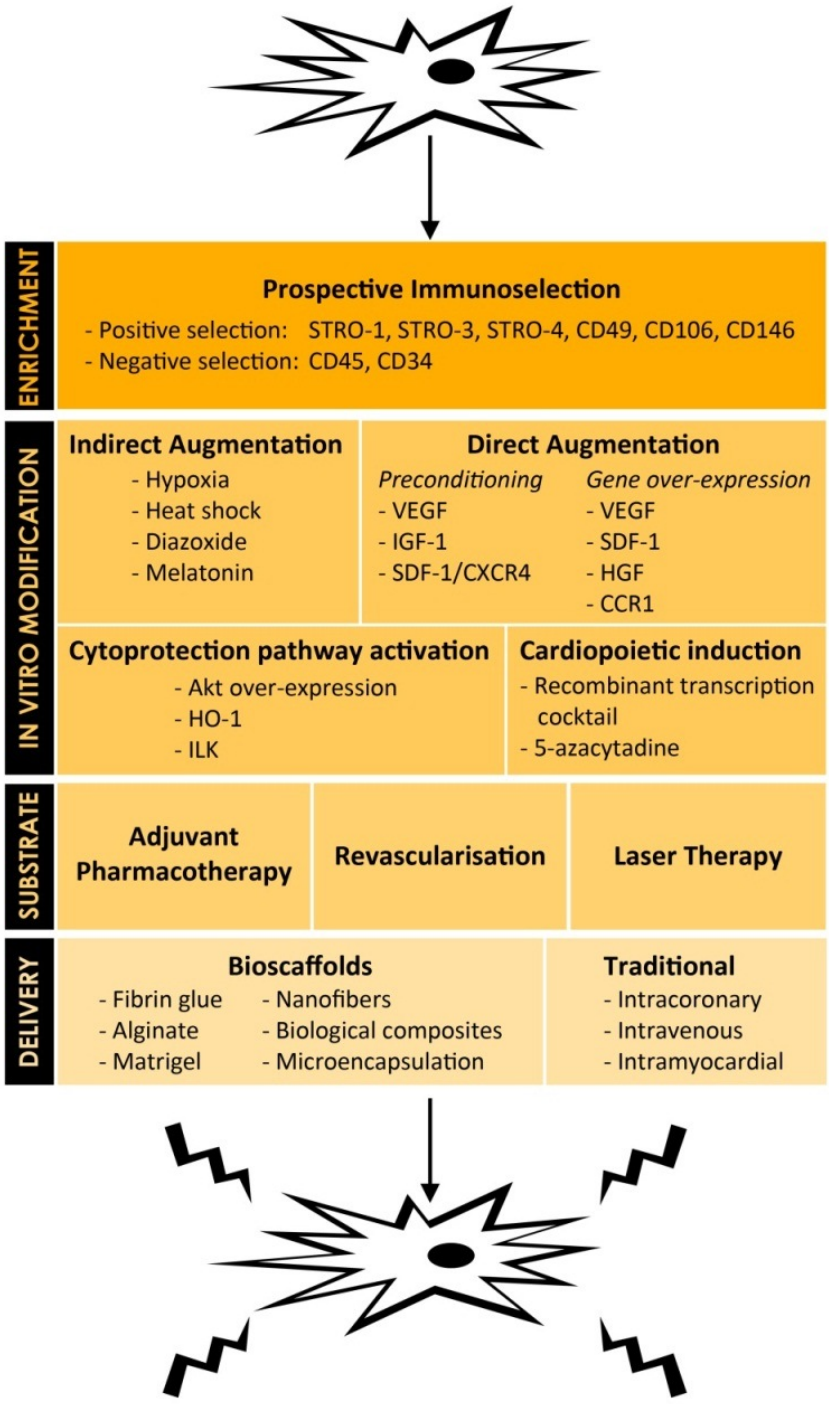

Figure 4. Overview of strategies studied to optimize outcomes of MSC-based cardiovascular therapy. Abbreviations: HO-1: Heme oxygenase-1, ILK: integrin-linked kinase (ILK); For other abbreviations see Figure 1.

9. Conclusion

While preclinical results have not yet translated into clinical benefits of equal magnitude, cell-based therapy still holds great promise for cardiac preservation, repair and regeneration. Many shortcomings of first generation progenitor cells, such as MSCs, have now been identified, including poor rates of engraftment and survival in vivo, deficient cardiomyogenic potential and compromised reparative effect when autologous cells are derived from older, comorbid patients. As outlined above, numerous diverse strategies have rapidly emerged from basic scientific innovation that target each of these limitations. However, considerable challenges await to progress these encouraging optimization techniques to the clinical realm.

9.1. Acknowledgements

Dr Richardson is supported by an International Postgraduate Research Scholarship, University of Adelaide and an Australian Postgraduate Award. Dr Psaltis has received funding from the National Health and Medical Research Council of Australia and the Royal Australasian College of Physicians.

9.3. References

1. Ho K, Anderson K, Kannel W, Grossman W, Levy D. Survival after the onset of congestive heart failure in Framingham Heart Study subjects. Circulation 1993;88:107-15.
2. Fuchs S, Baffour R, Zhou YF, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 2001;37:1726-32.
3. Lunde K, Solheim S, Aakhus S, et al. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 2006;355:1199-209.
4. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701-5.
5. Losordo DW, Henry TD, Davidson C, et al. Intramyocardial, Autologous CD34+ Cell Therapy for Refractory Angina. Circ Res 2011;109:428-36.
6. Menasche P, Hagege AA, Vilquin JT, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 2003;41:1078-83.
7. Psaltis PJ, Zannettino ACW, Worthley SG, Gronthos S. Concise Review: Mesenchymal Stromal Cells: Potential for Cardiovascular Repair. Stem Cells 2008;26:2201-10.
8. Psaltis PJ, Harbuzariu A, Delacroix S, Holroyd EW, Simari RD. Resident vascular progenitor cells - diverse origins, phenotype, and function. J Cardiovasc Transl Res 2011;4:161-76.
9. Sabatini F, Petecchia L, Tavian M, Jodon de Villeroche V, Rossi GA, Brouty-Boye D. Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab Invest 2005;85:962-71.
10. Zannettino ACW, Paton S, Arthur A, et al. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. Journal of Cellular Physiology 2008;214:413-21.
11. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 2003;18:696-704.
12. Roufosse CA, Direkze NC, Otto WR, Wright NA. Circulating mesenchymal stem cells. Int J Biochem Cell Biol 2004;36:585-97.
13. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669-75.
14. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, et al. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 2004;22:1338-45.
15. Chen SL, Fang WW, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92-5.
16. Amado LC, Saliaris AP, Schuleri KH, et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proceedings of the National Academy of Sciences of the United States of America 2005;102:11474-9.
17. Hare JM, Traverse JH, Henry TD, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol 2009;54:2277-86.
18. Quevedo HC, Hatzistergos KE, Oskouei BN, et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci U S A 2009;106:14022-7.
19. Psaltis PJ, Carbone A, Nelson AJ, et al. Reparative effects of allogeneic mesenchymal precursor cells delivered transendocardially in experimental nonischemic cardiomyopathy. JACC Cardiovasc Interv 2010;3:974-83.
20. Knight RL, Booth C, Wilcox HE, Fisher J, Ingham E. Tissue engineering of cardiac valves: re-seeding of acellular porcine aortic valve matrices with human mesenchymal progenitor cells. J Heart Valve Dis 2005;14:806-13.
21. Yokokawa M, Ohnishi S, Ishibashi-Ueda H, et al. Transplantation of mesenchymal stem cells improves atrioventricular conduction in a rat model of complete atrioventricular block. Cell Transplant 2008;17:1145-55.
22. Vulliet PR, Greeley M, Halloran SM, MacDonald KA, Kittleson MD. Intra-coronary arterial injection of mesenchymal stromal cells and microinfarction in dogs. The Lancet 2004;363:783-4.
23. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Proliferation 1970;3:393-403.
24. Psaltis PJ, Paton S, See F, et al. Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol 2010;223:530-40.
25. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.
26. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93-8.
27. Gnecchi M, He H, Melo LG, et al. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing Akt on cardiac metabolism after myocardial infarction. Stem Cells 2009;27:971-9.
28. Mills WR, Mal N, Kiedrowski MJ, et al. Stem cell therapy enhances electrical viability

in myocardial infarction. J Mol Cell Cardiol 2007;42:304-14.
29. Ohnishi S, Yanagawa B, Tanaka K, et al. Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J Mol Cell Cardiol 2007;42:88-97.
30. Nagaya N, Kangawa K, Itoh T, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112:1128-35.
31. Van Linthout S, Savvatis K, Miteva K, et al. Mesenchymal stem cells improve murine acute coxsackievirus B3-induced myocarditis. Eur Heart J. 2011;32(17): 2168-78.
32. Freyman T, Polin G, Osman H, et al. A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. European Heart Journal 2006;27:1114-22.
33. Hatzistergos KE, Quevedo H, Oskouei BN, et al. Bone Marrow Mesenchymal Stem Cells Stimulate Cardiac Stem Cell Proliferation and Differentiation / Novelty and Significance. Circulation Research 2010;107:913-22.
34. Kraitchman DL, Heldman AW, Atalar E, et al. In vivo magnetic resonance imaging of mesenchymal stem cells in myocardial infarction. Circulation 2003;107:2290-3.
35. Gyongyosi M, Blanco J, Marian T, et al. Serial noninvasive in vivo positron emission tomographic tracking of percutaneously intramyocardially injected autologous porcine mesenchymal stem cells modified for transgene reporter gene expression. Circ Cardiovasc Imaging 2008;1:94-103.
36. Perin EC, Tian M, Marini FC, 3rd, et al. Imaging long-term fate of intramyocardially implanted mesenchymal stem cells in a porcine myocardial infarction model. PLoS One 2011;6:e22949.
37. Psaltis PJ, Simari RD, Rodriguez-Porcel M. Emerging roles for integrated imaging modalities in cardiovascular cell-based therapeutics: a clinical perspective. Eur J Nucl Med Mol Imaging 2012;39:165-81.
38. Silva GV, Litovsky S, Assad JA, et al. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. Circulation 2005;111:150-6.
39. Schuleri KH, Amado LC, Boyle AJ, et al. Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. Am J Physiol Heart Circ Physiol 2008;294:H2002-11.
40. Arminan A, Gandia C, Bartual M, et al. Cardiac differentiation is driven by NKX2.5 and GATA4 nuclear translocation in tissue-specific mesenchymal stem cells. Stem Cells Dev 2009;18:907-18.
41. Gao LR, Zhang NK, Bai J, et al. The apelin-APJ pathway exists in cardiomyogenic cells derived from mesenchymal stem cells in vitro and in vivo. Cell Transplant 2010;19:949-58.
42. Ge D, Liu X, Li L, et al. Chemical and physical stimuli induce cardiomyocyte differentiation from stem cells. Biochem Biophys Res Commun 2009;381:317-21.
43. Genovese JA, Spadaccio C, Chachques E, et al. Cardiac pre-differentiation of human mesenchymal stem cells by electrostimulation. Front Biosci 2009;14:2996-3002.
44. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. The Journal of Clinical Investigation 1999;103:697-705.
45. Balana B, Nicoletti C, Zahanich I, et al. 5-Azacytidine induces changes in electrophysiological properties of human mesenchymal stem cells. Cell Res 2006;16:949-60.
46. Feng C, Zhu J, Zhao L, et al. Suberoylanilide hydroxamic acid promotes cardiomyocyte differentiation of rat mesenchymal stem cells. Exp Cell Res 2009;315:3044-51.
47. Yoon J, Min BG, Kim YH, Shim WJ, Ro YM, Lim DS. Differentiation, engraftment and functional effects of pre-treated mesenchymal stem cells in a rat myocardial infarct model. Acta Cardiol 2005;60:277-84.
48. Forte G, Minieri M, Cossa P, et al. Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. Stem Cells 2006;24:23-33.
49. Li H, Yu B, Zhang Y, Pan Z, Xu W. Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes. Biochem Biophys Res Commun 2006;341:320-5.
50. Herrmann JL, Abarbanell AM, Weil BR, et al. Postinfarct intramyocardial injection of mesenchymal stem cells pretreated with TGF-alpha improves acute myocardial function. Am J Physiol Regul Integr Comp Physiol 2010;299:R371-8.
51. Koninckx R, Hensen K, Daniels A, et al. Human bone marrow stem cells co-cultured with neonatal rat cardiomyocytes display limited cardiomyogenic plasticity. Cytotherapy 2009;11:778-92.
52. He XQ, Chen MS, Li SH, et al. Co-culture with cardiomyocytes enhanced the myogenic conversion of mesenchymal stromal cells in a dose-dependent manner. Mol Cell Biochem 2010;339:89-98.
53. Labovsky V, Hofer EL, Feldman L, et al. Cardiomyogenic differentiation of human bone marrow mesenchymal cells: Role of cardiac extract from neonatal rat cardiomyocytes. Differentiation 2010;79:93-101.
54. Nygren JM, Jovinge S, Breitbach M, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10:494-501.
55. Martens TP, See F, Schuster MD, et al. Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. Nat Clin Pract Cardiovasc Med 2006;3 Suppl 1:S18-22.
56. Shake JG, Gruber PJ, Baumgartner WA, et al. Mesenchymal stem cell implantation in a

swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 2002;73:1919-25.
57. Tsuji H, Miyoshi S, Ikegami Y, et al. Xenografted human amniotic membrane-derived mesenchymal stem cells are immunologically tolerated and transdifferentiated into cardiomyocytes. Circ Res 2010;106:1613-23.
58. Jumabay M, Zhang R, Yao Y, Goldhaber JI, Bostrom KI. Spontaneously beating cardiomyocytes derived from white mature adipocytes. Cardiovasc Res 2010;85:17-27.
59. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204-19.
60. Godier-Furnemont AF, Martens TP, Koeckert MS, et al. Composite scaffold provides a cell delivery platform for cardiovascular repair. Proc Natl Acad Sci U S A 2011;108:7974-9.
61. Thangarajah H, Vial IN, Chang E, et al. IFATS collection: Adipose stromal cells adopt a proangiogenic phenotype under the influence of hypoxia. Stem Cells 2009;27:266-74.
62. See F, Seki T, Psaltis PJ, et al. Therapeutic Effects of Human STRO-3-Selected Mesenchymal Precursor Cells and their Soluble Factors in Experimental Myocardial Ischemia. J Cell Mol Med 2010.
63. Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9:1195-201.
64. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004;94:678-85.
65. Xu M, Uemura R, Dai Y, Wang Y, Pasha Z, Ashraf M. In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function. J Mol Cell Cardiol 2007;42:441-8.
66. Rogers TB, Pati S, Gaa S, et al. Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes. J Mol Cell Cardiol 2011;50:346-56.
67. Benzhi C, Limei Z, Ning W, et al. Bone marrow mesenchymal stem cells upregulate transient outward potassium currents in postnatal rat ventricular myocytes. J Mol Cell Cardiol 2009;47:41-8.
68. Lai RC, Arslan F, Lee MM, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 2010;4:214-22.
69. Du YY, Zhou SH, Zhou T, et al. Immuno-inflammatory regulation effect of mesenchymal stem cell transplantation in a rat model of myocardial infarction. Cytotherapy 2008;10:469-78.
70. Lee RH, Pulin AA, Seo MJ, et al. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell 2009;5:54-63.
71. Ishikane S, Yamahara K, Sada M, et al. Allogeneic administration of fetal membrane-derived mesenchymal stem cells attenuates acute myocarditis in rats. J Mol Cell Cardiol 2010;49:753-61.
72. Li L, Zhang S, Zhang Y, Yu B, Xu Y, Guan Z. Paracrine action mediate the antifibrotic effect of transplanted mesenchymal stem cells in a rat model of global heart failure. Mol Biol Rep 2009;36:725-31.
73. Dixon JA, Gorman RC, Stroud RE, et al. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation 2009;120:S220-9.
74. Schneider C, Jaquet K, Geidel S, et al. Transplantation of Bone Marrow-Derived Stem Cells Improves Myocardial Diastolic Function: Strain Rate Imaging in a Model of Hibernating Myocardium. Journal of the American Society of Echocardiography 2009;22:1180-9.
75. Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells 2001;19:219-25.
76. Miura M, Miura Y, Padilla-Nash HM, et al. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells 2006;24:1095-103.
77. Jeong JO, Han JW, Kim JM, et al. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. Circ Res 2011;108:1340-7.
78. Schachinger V, Erbs S, Elsasser A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006;355:1210-21.
79. Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. The Lancet 2004;364:141-8.
80. Lipinski MJ, Biondi-Zoccai GGL, Abbate A, et al. Impact of Intracoronary Cell Therapy on Left Ventricular Function in the Setting of Acute Myocardial Infarction: A Collaborative Systematic Review and Meta-Analysis of Controlled Clinical Trials. Journal of the American College of Cardiology 2007;50:1761-7.
81. Pfeffer MA, Greaves SC, Arnold JMO, et al. Early Versus Delayed Angiotensin-Converting Enzyme Inhibition Therapy in Acute Myocardial Infarction : The Healing and Early Afterload Reducing Therapy Trial. Circulation 1997;95:2643-51.
82. Katritsis DG, Sotiropoulou PA, Karvouni E, et al. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. Catheter Cardiovasc Interv 2005;65:321-9.
83. Yang Z, Zhang F, Ma W, et al. A novel approach to transplanting bone marrow stem cells to repair human myocardial infarction:

delivery via a noninfarct-relative artery. Cardiovasc Ther 2010;28:380-5.
84. Chen S, Liu Z, Tian N, et al. Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery. J Invasive Cardiol 2006;18:552-6.
85. Katritsis DG, Sotiropoulou P, Giazitzoglou E, Karvouni E, Papamichail M. Electrophysiological effects of intracoronary transplantation of autologous mesenchymal and endothelial progenitor cells. Europace 2007;9:167-71.
86. Williams AR, Trachtenberg B, Velazquez DL, et al. Intramyocardial stem cell injection in patients with ischemic cardiomyopathy: functional recovery and reverse remodeling. Circ Res 2011;108:792-6.
87. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838-43.
88. Fischer UM, Harting MT, Jimenez F, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells Dev 2009;18:683-92.
89. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood 2006;108:2114-20.
90. Poncelet AJ, Vercruysse J, Saliez A, Gianello P. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. Transplantation 2007;83:783-90.
91. Robey TE, Saiget MK, Reinecke H, Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. J Mol Cell Cardiol 2008;45:567-81.
92. Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, Integration, and Differentiation of Cardiomyocyte Grafts : A Study in Normal and Injured Rat Hearts. Circulation 1999;100:193-202.
93. Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 1991;78:55-62.
94. Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 2001;28:174-81.
95. Simmons PJ, Masinovsky B, Longenecker BM, Berenson R, Torok-Storb B, Gallatin WM. Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. Blood 1992;80:388-95.
96. Sacchetti B, Funari A, Michienzi S, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 2007;131:324-36.
97. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 2002;30:783-91.
98. Gronthos S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 1995;85:929-40.
99. Schwab KE, Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Human Reproduction 2007;22:2903-11.
100. Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC. A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. Stem Cells Dev 2007;16:953-63.
101. Gronthos S, McCarty R, Mrozik K, et al. Heat shock protein-90 beta is expressed at the surface of multipotential mesenchymal precursor cells: generation of a novel monoclonal antibody, STRO-4, with specificity for mesenchymal precursor cells from human and ovine tissues. Stem Cells Dev 2009;18:1253-62.
102. Hamamoto H, Gorman JH, 3rd, Ryan LP, et al. Allogeneic mesenchymal precursor cell therapy to limit remodeling after myocardial infarction: the effect of cell dosage. Ann Thorac Surg 2009;87:794-801.
103. Tondreau T, Lagneaux L, Dejeneffe M, et al. Isolation of BM mesenchymal stem cells by plastic adhesion or negative selection: phenotype, proliferation kinetics and differentiation potential. Cytotherapy 2004;6:372-9.
104. Lushaj EB, Anstadt E, Haworth R, et al. Mesenchymal stromal cells are present in the heart and promote growth of adult stem cells in vitro. Cytotherapy 2011;13:400-6.
105. Jiang Y, Jahagirdar B, Reinhardt R, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41 - 9.
106. Pelacho B, Nakamura Y, Zhang J, et al. Multipotent adult progenitor cell transplantation increases vascularity and improves left ventricular function after myocardial infarction. J Tissue Eng Regen Med 2007;1:51-9.
107. Spees JL, Gregory CA, Singh H, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9:747-56.
108. Crespo-Diaz R, Behfar A, Butler GW, et al. Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. Cell Transplant 2011;20:797-811.
109. Hoogduijn MJ, Crop MJ, Peeters AM, et al. Donor-derived mesenchymal stem cells remain present and functional in the

transplanted human heart. Am J Transplant 2009;9:222-30.
110. Bai X, Yan Y, Song YH, et al. Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. Eur Heart J 2010;31:489-501.
111. Bayes-Genis A, Soler-Botija C, Farre J, et al. Human progenitor cells derived from cardiac adipose tissue ameliorate myocardial infarction in rodents. J Mol Cell Cardiol 2010;49:771-80.
112. Troyer DL, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 2008;26:591-9.
113. Iop L, Chiavegato A, Callegari A, et al. Different cardiovascular potential of adult- and fetal-type mesenchymal stem cells in a rat model of heart cryoinjury. Cell Transplant 2008;17:679-94.
114. Kadivar M, Khatami S, Mortazavi Y, Shokrgozar MA, Taghikhani M, Soleimani M. In vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells. Biochem Biophys Res Commun 2006;340:639-47.
115. Gaebel R, Furlani D, Sorg H, et al. Cell origin of human mesenchymal stem cells determines a different healing performance in cardiac regeneration. PLoS One. 2011;6(2):e15652.
116. Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 2004;22:625-34.
117. Schmidt D, Mol A, Breymann C, et al. Living autologous heart valves engineered from human prenatally harvested progenitors. Circulation 2006;114:I125-31.
118. Lee ST, White AJ, Matsushita S, et al. Intramyocardial injection of autologous cardiospheres or cardiosphere-derived cells preserves function and minimizes adverse ventricular remodeling in pigs with heart failure post-myocardial infarction. J Am Coll Cardiol 2011;57:455-65.
119. Gambini E, Pompilio G, Biondi A, et al. C-kit + cardiac progenitors exhibit mesenchymal markers and preferential cardiovascular commitment. Cardiovasc Res 2011;89:362-73.
120. Sethe S, Scutt A, Stolzing A. Aging of mesenchymal stem cells. Ageing Res Rev 2006;5:91-116.
121. Tokalov SV, Gruner S, Schindler S, Wolf G, Baumann M, Abolmaali N. Age-related changes in the frequency of mesenchymal stem cells in the bone marrow of rats. Stem Cells Dev 2007;16:439-46.
122. Hacia JG, Lee CC, Jimenez DF, et al. Age-related gene expression profiles of rhesus monkey bone marrow-derived mesenchymal stem cells. J Cell Biochem 2008;103:1198-210.
123. Zhang H, Fazel S, Tian H, et al. Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle myocardial cell therapy. Am J Physiol Heart Circ Physiol 2005;289:H2089-96.
124. Khan M, Mohsin S, Khan SN, Riazuddin S. Repair of senescent myocardium by mesenchymal stem cells is dependent on the age of donor mice. J Cell Mol Med 2011;15:1515-27.
125. Khan M, Kwiatkowski P, Rivera BK, Kuppusamy P. Oxygen and oxygenation in stem-cell therapy for myocardial infarction. Life Sciences 2010;87:269-74.
126. Kofoed H, Sjontoft E, Siemssen SO, Olesen HP. Bone marrow circulation after osteotomy. Blood flow, pO2, pCO2, and pressure studied in dogs. Acta Orthop Scand 1985;56:400-3.
127. Hu X, Yu SP, Fraser JL, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. The Journal of Thoracic and Cardiovascular Surgery 2008;135:799-808.
128. Li JH, Zhang N, Wang JA. Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy. J Endocrinol Invest 2008;31:103-10.
129. Rebelatto CK, Aguiar AM, Senegaglia AC, et al. Expression of cardiac function genes in adult stem cells is increased by treatment with nitric oxide agents. Biochem Biophys Res Commun 2009;378:456-61.
130. Afzal MR, Haider H, Idris NM, Jiang S, Ahmed RP, Ashraf M. Pre-conditioning promotes survival and angiomyogenic potential of mesenchymal stem cells in the infarcted heart via NF-kappaB signaling. Antioxid Redox Signal 2010;12:693-702.
131. Suzuki Y, Kim HW, Ashraf M, Haider H. Diazoxide potentiates mesenchymal stem cell survival via NF-kappaB-dependent miR-146a expression by targeting Fas. Am J Physiol Heart Circ Physiol 2010;299:H1077-82.
132. Suzuki K, Smolenski RT, Jayakumar J, Murtuza B, Brand NJ, Yacoub MH. Heat Shock Treatment Enhances Graft Cell Survival in Skeletal Myoblast Transplantation to the Heart. Circulation 2000;102:III-216-21.
133. Wang X, Zhao T, Huang W, et al. Hsp20-engineered mesenchymal stem cells are resistant to oxidative stress via enhanced activation of Akt and increased secretion of growth factors. Stem Cells 2009;27:3021-31.
134. Chang W, Song B-W, Lim S, et al. Mesenchymal Stem Cells Pretreated with Delivered Hph-1-Hsp70 Protein Are Protected from Hypoxia-Mediated Cell Death and Rescue Heart Functions from Myocardial Injury. Stem Cells 2009;27:2283-92.
135. Yang YJ, Qian HY, Huang J, et al. Combined therapy with simvastatin and bone marrow-derived mesenchymal stem cells increases benefits in infarcted swine hearts. Arterioscler Thromb Vasc Biol 2009;29:2076-82.
136. Yang Y, Mou Y, Hu SJ, Fu M. Beneficial effect of rosuvastatin on cardiac dysfunction is associated with alterations in calcium-regulatory proteins. Eur J Heart Fail 2009;11:6-13.
137. Lin YC, Leu S, Sun CK, et al. Early combined treatment with sildenafil and adipose-derived mesenchymal stem cells

preserves heart function in rat dilated cardiomyopathy. J Transl Med 2010;8:88.
138. Haider H, Lee YJ, Jiang S, Ahmed RP, Ryon M, Ashraf M. Phosphodiesterase inhibition with tadalafil provides longer and sustained protection of stem cells. Am J Physiol Heart Circ Physiol 2010;299:H1395-404.
139. Numasawa Y, Kimura T, Miyoshi S, et al. Treatment of Human Mesenchymal Stem Cells with Angiotensin Receptor Blocker Improved Efficiency of Cardiomyogenic Transdifferentiation and Improved Cardiac Function via Angiogenesis. Stem Cells 2011;29:1405-14.
140. Wang Y, Zhang D, Ashraf M, et al. Combining neuropeptide Y and mesenchymal stem cells reverses remodeling after myocardial infarction. Am J Physiol Heart Circ Physiol 2010;298:H275-86.
141. Kinnaird T, Stabile E, Burnett MS, et al. Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms. Circulation 2004;109:1543-9.
142. Herrmann JL, Wang Y, Abarbanell AM, Weil BR, Tan J, Meldrum DR. Pre-conditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated cardioprotection. Shock 2010;33:24-30.
143. Pasha Z, Wang Y, Sheikh R, Zhang D, Zhao T, Ashraf M. Pre-conditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. Cardiovasc Res 2008;77:134-42.
144. Yao Y, Zhang F, Wang L, et al. Lipopolysaccharide pre-conditioning enhances the efficacy of mesenchymal stem cells transplantation in a rat model of acute myocardial infarction. J Biomed Sci 2009;16:74.
145. Matsumoto R, Omura T, Yoshiyama M, et al. Vascular Endothelial Growth Factor–Expressing Mesenchymal Stem Cell Transplantation for the Treatment of Acute Myocardial Infarction. Arteriosclerosis, Thrombosis, and Vascular Biology 2005;25:1168-73.
146. Guo J, Lin G, Bao C, Hu Z, Chu H, Hu M. Insulin-like growth factor 1 improves the efficacy of mesenchymal stem cells transplantation in a rat model of myocardial infarction. J Biomed Sci 2008;15:89-97.
147. Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal Cell–Derived Factor-1α Plays a Critical Role in Stem Cell Recruitment to the Heart After Myocardial Infarction but Is Not Sufficient to Induce Homing in the Absence of Injury. Circulation 2004;110:3300-5.
148. Kijowski J, Baj-Krzyworzeka M, Majka M, et al. The SDF-1-CXCR4 axis stimulates VEGF secretion and activates integrins but does not affect proliferation and survival in lymphohematopoietic cells. Stem Cells 2001;19:453-66.
149. Zhuang Y, Chen X, Xu M, Zhang LY, Xiang F. Chemokine stromal cell-derived factor 1/CXCL12 increases homing of mesenchymal stem cells to injured myocardium and neovascularization following myocardial infarction. Chin Med J (Engl) 2009;122:183-7.
150. Tang J, Wang J, Guo L, et al. Mesenchymal stem cells modified with stromal cell-derived factor 1 alpha improve cardiac remodeling via paracrine activation of hepatocyte growth factor in a rat model of myocardial infarction. Mol Cells 2010;29:9-19.
151. Guo YH, He JG, Wu JL, et al. Hepatocyte growth factor and granulocyte colony-stimulating factor form a combined neovasculogenic therapy for ischemic cardiomyopathy. Cytotherapy 2008;10:857-67.
152. Huang J, Zhang Z, Guo J, et al. Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. Circ Res 2010;106:1753-62.
153. Tang J, Wang J, Zheng F, et al. Combination of chemokine and angiogenic factor genes and mesenchymal stem cells could enhance angiogenesis and improve cardiac function after acute myocardial infarction in rats. Molecular and Cellular Biochemistry 2010;339:107-18.
154. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. Cell 1997;88:435-7.
155. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905-27.
156. Somanath PR, Razorenova OV, Chen J, Byzova TV. Akt1 in endothelial cell and angiogenesis. Cell Cycle 2006;5:512-8.
157. Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:367-8.
158. Gnecchi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006;20:661-9.
159. Mirotsou M, Zhang Z, Deb A, et al. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci U S A 2007;104:1643-8.
160. Song SW, Chang W, Song BW, et al. Integrin-linked kinase is required in hypoxic mesenchymal stem cells for strengthening cell adhesion to ischemic myocardium. Stem Cells 2009;27:1358-65.
161. Jiang Y, Chen L, Tang Y, et al. HO-1 gene overexpression enhances the beneficial effects of superparamagnetic iron oxide labeled bone marrow stromal cells transplantation in swine hearts underwent ischemia/reperfusion: an MRI study. Basic Research in Cardiology 2010;105:431-42.
162. Taljaard M, Ward MR, Kutryk MJ, et al. Rationale and design of Enhanced Angiogenic Cell Therapy in Acute Myocardial Infarction (ENACT-AMI): the

first randomized placebo-controlled trial of enhanced progenitor cell therapy for acute myocardial infarction. Am Heart J 2010;159:354-60.
163. Mias C, Trouche E, Seguelas MH, et al. Ex vivo pretreatment with melatonin improves survival, proangiogenic/mitogenic activity, and efficiency of mesenchymal stem cells injected into ischemic kidney. Stem Cells 2008;26:1749-57.
164. Behfar A, Zingman LV, Hodgson DM, et al. Stem cell differentiation requires a paracrine pathway in the heart. FASEB J 2002;16:1558-66.
165. Behfar A, Perez-Terzic C, Faustino RS, et al. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med 2007;204:405-20.
166. Behfar A, Yamada S, Crespo-Diaz R, et al. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. J Am Coll Cardiol 2010;56:721-34.
167. Bartunek J, Wijns W, Dolatabadi D, et al. C-cure multicenter trial: Lineage specific bone marrow derived cardiopoietic mesenchymal stem cells for the treatment of ischaemic cardiomyopathy. J Am Coll Cardiol 2011;57:E200-.
168. Yang Y-J, Qian H-Y, Huang J, et al. Atorvastatin treatment improves survival and effects of implanted mesenchymal stem cells in post-infarct swine hearts. European Heart Journal 2008;29:1578-90.
169. Xu R, Chen J, Cong X, Hu S, Chen X. Lovastatin protects mesenchymal stem cells against hypoxia- and serum deprivation-induced apoptosis by activation of PI3K/Akt and ERK1/2. Journal of Cellular Biochemistry 2008;103:256-69.
170. Khan M, Meduru S, Mohan IK, et al. Hyperbaric oxygenation enhances transplanted cell graft and functional recovery in the infarct heart. J Mol Cell Cardiol 2009;47:275-87.
171. Zhang H, Hou JF, Shen Y, Wang W, Wei YJ, Hu S. Low level laser irradiation precondition to create friendly milieu of infarcted myocardium and enhance early survival of transplanted bone marrow cells. J Cell Mol Med 2010;14:1975-87.
172. Briones E, Lacalle JR, Marin I. Transmyocardial laser revascularization versus medical therapy for refractory angina. Cochrane Database Syst Rev 2009:CD003712.
173. Psaltis PJ, Worthley SG. Endoventricular electromechanical mapping-the diagnostic and therapeutic utility of the NOGA XP Cardiac Navigation System. J Cardiovasc Transl Res 2009;2:48-62.
174. Reyes G, Allen KB, Alvarez P, et al. Mid term results after bone marrow laser revascularization for treating refractory angina. BMC Cardiovasc Disord 2010;10:42.
175. Schachinger V, Erbs S, Elsasser A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006;355:1210-21.
176. Ly HQ, Hoshino K, Pomerantseva I, et al. In vivo myocardial distribution of multipotent progenitor cells following intracoronary delivery in a swine model of myocardial infarction. Eur Heart J 2009;30:2861-8
177. Hou D, Youssef EA, Brinton TJ, et al. Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials. Circulation 2005;112:I150-6.
178. Perin EC, Silva GV, Assad JA, et al. Comparison of intracoronary and transendocardial delivery of allogeneic mesenchymal cells in a canine model of acute myocardial infarction. J Mol Cell Cardiol 2008;44:486-95.
179. Mitchell AJ, Sabondjian E, Sykes J, et al. Comparison of initial cell retention and clearance kinetics after subendocardial or subepicardial injections of endothelial progenitor cells in a canine myocardial infarction model. J Nucl Med 2010;51:413-7.
180. Psaltis P, Zannettino A, Gronthos S, Worthley S. Intramyocardial Navigation and Mapping for Stem Cell Delivery. Journal of Cardiovascular Translational Research 2010;3:135-46.
181. Christman KL, Vardanian AJ, Fang Q, Sievers RE, Fok HH, Lee RJ. Injectable fibrin scaffold improves cell transplant survival, reduces infarct expansion, and induces neovasculature formation in ischemic myocardium. JACC 2004;44:654-60.
182. Leor J, Amsalem Y, Cohen S. Cells, scaffolds, and molecules for myocardial tissue engineering. Pharmacology & Therapeutics 2005;105:151-63.
183. Kofidis T, Lebl DR, Martinez EC, Hoyt G, Tanaka M, Robbins RC. Novel Injectable Bioartificial Tissue Facilitates Targeted, Less Invasive, Large-Scale Tissue Restoration on the Beating Heart After Myocardial Injury. Circulation 2005;112:I-173-7.
184. Terrovitis J, Lautamaki R, Bonios M, et al. Noninvasive quantification and optimization of acute cell retention by in vivo positron emission tomography after intramyocardial cardiac-derived stem cell delivery. J Am Coll Cardiol 2009;54:1619-26.
185. Davis ME, Motion JPM, Narmoneva DA, et al. Injectable Self-Assembling Peptide Nanofibers Create Intramyocardial Microenvironments for Endothelial Cells. Circulation 2005;111:442-50.
186. Segers VF, Tokunou T, Higgins LJ, MacGillivray C, Gannon J, Lee RT. Local delivery of protease-resistant stromal cell derived factor-1 for sc recruitment after myocardial infarction. Circulation 2007;116:1683-92.
187. Barnett BP, Ruiz-Cabello J, Hota P, et al. Fluorocapsules for improved function, immunoprotection, and visualization of cellular therapeutics with MR, US, and CT imaging. Radiology 2011;258:182-91.
188. Melero-Martin JM, De Obaldia ME, Kang SY, et al. Engineering robust and functional vascular networks in vivo. Circ Res 2008;103:194-202.

Statement of Authorship

Title of Paper	**Optimization of the cardiovascular therapeutic properties of mesenchymal stromal/stem cells-taking the next step.**
Publication Status	Published
Publication Details	Stem Cell Rev. 2013 Jun;9(3):281-302. doi: 10.1007/s12015-012-9366-7.

Author Contributions

Name of Principal Author (Candidate) Contribution to the Paper	**Dr James David Richardson** Primary contributor to the conception and design of the work; Drafted the work; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work. 18th December 2013
Name of Co-Author Contribution to the Paper	**Dr Adam J Nelson** Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work 18th December 2013
Name of Co-Author Contribution to the Paper	**Professor Andrew Zannettino** Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the

	version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013
Name of Co-Author	**Professor Stan Gronthos**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Professor Stephen G Worthley**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013
Name of Co-Author	**Dr Peter J Psaltis**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013

2. Chapter 2- Rat Cardiac Imaging

Cardiac Magnetic Resonance, Transthoracic and Transoesophageal Echocardiography; A Comparison of in vivo Assessment of Ventricular Function in Rats.

JD Richardson1,2, AG Bertaso1, L Frost1, PJ Psaltis1,3, A Carbone1, B Koschade1, DT Wong 1, AJ Nelson1, S Paton2, K Williams1, S Azarisman1, MI Worthley1, KS Teo1, S Gronthos2, ACW Zannettino2, SG Worthley1,2

1 Cardiovascular Research Centre, Royal Adelaide Hospital and Department of Medicine, University of Adelaide, South Australia, Australia.

2 Centre for Stem Cell Research, Robinson Institute, School of Medical Sciences, University of Adelaide, South Australia, Australia

3 Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, USA

2.1. ABSTRACT

In vivo assessment of ventricular function in rodents has largely been restricted to transthoracic echocardiography (TTE). However 1.5T cardiac magnetic resonance (CMR) and transoesophageal echocardiography (TOE) have emerged as possible alternatives. Yet, to date, no study has systematically assessed these three imaging modalities in determining ejection fraction (EF) in rats. Twenty rats underwent imaging 4 weeks after surgically induced myocardial infarction. CMR was performed on a 1.5T scanner, TTE was conducted using a 9.2MHz transducer and TOE was performed with a 10MHz intracardiac echo catheter. Correlation between the three techniques for EF determination and analysis reproducibility was assessed. Moderate-strong correlation was observed between the three modalities; the greatest between CMR and TOE (intraclass correlation coefficient (ICC) =0.89), followed by TOE and TTE (ICC=0.70) and CMR and TTE (ICC=0.63). Intra- and inter-observer variation were excellent with CMR (ICC=0.99 and 0.98 respectively), very good with TTE (0.90 and 0.89) and TOE (0.87 and 0.84). Each modality is a viable option for evaluating ventricular function in rats, however the high image quality and excellent reproducibility of CMR offers distinct advantages even at 1.5T with conventional coils and software.

Key Words: Cardiac Magnetic Resonance; Transthoracic echocardiography; Transeosophageal echocardiography; Left ventricular function; Intracardiac echocardiography.

\

2.2. INTRODUCTION

Small animal models are frequently used to evaluate cardiovascular disorders and response to therapeutic interventions. The primary end-point in many of these studies is the evaluation of ventricular function, with in vivo methods now largely superseding ex vivo assessment. There are specific challenges to imaging rodent hearts, namely their minute size (≤10mm left ventricles) and extreme heart rates (~300-600 beats/minute) [1]. Accordingly, adequate spatial and temporal resolution of candidate imaging modalities is essential.

Transthoracic echocardiography (TTE) has been the principal imaging modality employed owing to its widespread availability and excellent temporal, yet modest spatial resolution. In conjunction with high frequency echo transducers and skilled operators, a variety of images can be readily obtained. In practice, most TTE assessments of ventricular function rely on either 1-dimensional M-mode evaluation, or 2-D left ventricular (LV) short axis views of at one or more levels [2]. M-mode only evaluates two opposing myocardial walls, e.g. the anteroseptum and the posterior wall obtained from parasternal views, thus providing no information regarding the remaining myocardium. Calculation of LV ejection fraction (EF) from short axis views can provide a more representative evaluation of function as a 360 degree view of myocardium is obtained, however even this ignores important changes in geometric shape in diseased states, such as after myocardial infarction [3]. Additionally TTE can suffer from reduced reproducibility, as it is more operator dependant and ensuring that the views obtained are truly orthogonal to the LV long axis can be challenging.

Transoesophageal echocardiography (TOE) might offer an alternative echocardiographic strategy. Suitable transducer probes, of sufficiently small calibre and cost-effective nature, have recently become available in the form of intracardiac echocardiogram (ICE) catheters [4]. These devices are routinely used in many interventional cardiology centres for assistance with some structural or electrophysiology procedures. A typical ICE catheter is

8Fr (2.7mm) in diameter with a high frequency (10MHz) transducer and is amenable to repeated use. However there is a paucity of published evidence demonstrating the utility of rodent TOE.

Cardiac magnetic resonance (CMR), however, is the gold standard method of assessing LV function in humans and large animals. The high spatial and moderate temporal resolution of MRI provides highly reproducible imaging with minimal inter and intra-observer variation [5, 6]. Until recently, CMR of rodents has been most often performed using very expensive high-field MR scanners which are available only in a minority of research institutions. Accordingly investigators have explored the utility of standard low-field clinical MR scanners for this purpose with promising results [7]. However, whether CMR retains any advantage over TTE or TOE when performed on 1.5T scanners is unclear. To the best of our knowledge, no evaluation of 1.5T CMR, TTE and TOE has been undertaken in small animals. Therefore we sought to determine the utility of these three modalities, all directly relevant and available to many research groups, in a rat model of myocardial infarction.

2.3. Animals, Material and Methods

2.3.1. Study protocol

All procedures were performed in accordance with local animal research and ethics committee approval. Male Sprague-Dawley rats (n=20, weight 350-370g) participating in a separate study evaluating novel therapy after surgically induced myocardial infarction. All animals underwent imaging by all three modalities (CMR, followed by TTE and TOE) at day 28 post-MI. Anaesthesia was maintained using 1% isoflurane, which provides effective sedation but no significant effects on cardiac function [8]. Cine CMR sequences were acquired after 5-10 minutes of isoflurane and both echocardiography investigations were also performed after 5-10 minutes of a second period of anaesthesia (≥2 hours after CMR), ensuring comparable depth and duration of anaesthesia was provided in all animals.

2.3.2. CMR protocol

Magnetic resonance imaging was performed on a 1.5T MR system (Magnetom Sonata, Siemens, Germany), equipped with a dedicated cardiac software package. The thorax and right paw were shaved, cleaned with alcohol and adhesive electrocardiogram (ECG) electrodes attached thereby generating a vector ECG. Animals were placed supine recumbent in the isocentre of the magnet, with a 4-channel phased array carotid radiofrequency coil placed over the thorax, with 1% isoflurane administered via nose cone to maintain anaesthesia. Accordingly, all CMR images were free breathing, ECG-gated, acquisitions. Transverse and coronal localiser images were acquired followed by long (4-chamber and 2-chamber) and short axis pilot images, from which a true short axis stack was prescribed. Balanced steady-state free-precession (SSFP) cine images (gated to alternate R waves) were acquired. The stack comprised three contiguous LV slices (each 3mm thick, with no intersection gap) providing full coverage of the LV (Figure 1). The image matrix was 384 x 384, field of view 185 mm, repetition time 14.72ms, echo time 1.55ms, flip angle 90°, image resolution 0.5 x 0.5 mm and 8 phases per cardiac cycle were acquired. Three cardiac cycles were acquired at each level, with an imaging time of 52 seconds per short axis slice.

2.3.1. CMR image analysis

Left ventricular volumes and derived EF were measured off-line from cine images using commercially available software (QMass v7.2, Medis, Netherlands). Of the 3 cardiac cycles acquired at each level, the largest and smallest silhouettes corresponding to end-diastole and end-systole were identified and the endocardial border was manually traced. Papillary muscles were excluded from calculations. The end-diastolic (EDV) and end-systolic volumes (ESV) were then calculated using the true disk summation technique (i.e. sum of cavity volumes across all continuous slices), as previously described [9].

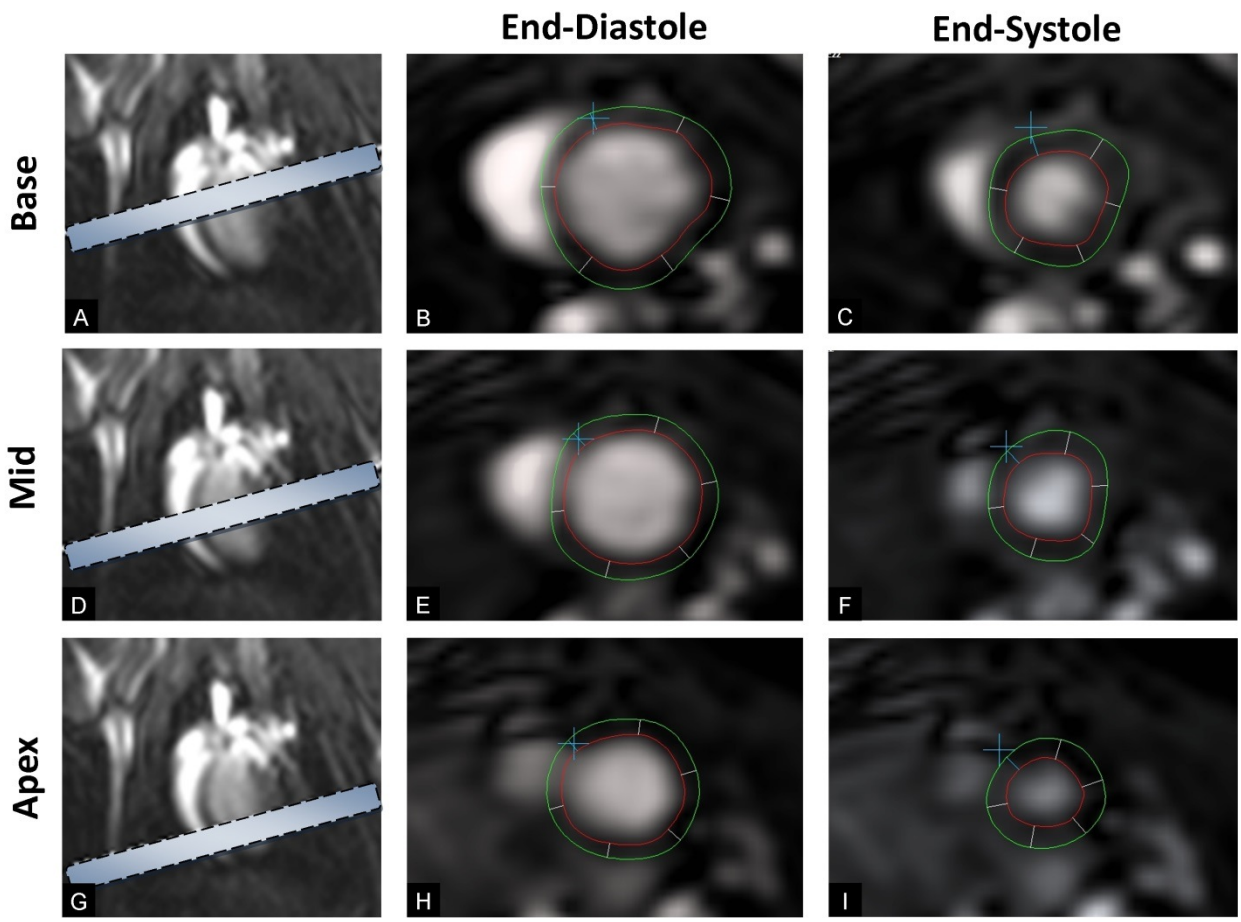

Figure 1: Cardiac magnetic resonance. Short axis stack comprising three short slices, each 3mm thick covering the base (a), mid-LV (d) and apex (g) of the heart. Representative examples of images at end-diastole (B,E,H) and end-systole (C,F,I) at each of the above levels, with endocardial and epicardial contours annotated.

2.3.2. TTE protocol

Transthoracic echocardiograms were performed on a clinical Siemens Acuson X300 ultrasound system (Siemens, Germany) in conjunction with a Siemens P9-4 (9.2MHz) transducer. Animals were placed in a supine recumbent position whilst maintained on 1% isoflurane. An LV short axis slice at the level of the papillary muscles was obtained and used to calculate EF. Additionally, an M-mode image was acquired by prescribing a cursor beam in the antero-inferior orientation of the above short axis slice (Figure 2). A minimum of 3 cardiac cycles were acquired and the largest and smallest silhouettes identified from these were analysed. Endocardial and epicardial borders were manually traced off-line (ProSolv Cardiovascular V4.0, FujiFilm, USA), with ejection fraction and fractional shortening (FS) calculated. We utilised the widely-used Teichholz formula (Volume = 7D3/2.4+D, where D=dimension) to calculate EF.

2.3.3. Transoesophageal echocardiogram protocol

Following TTE, rats were placed on an intubation rack by their front teeth, thereby lying in a vertical orientation (Figure 3) and 1% Isoflurane was delivered via nose cone. An 8Fr (2.7mm diameter) Biosense Webster AcuNav (10MHz) ICE catheter was used in combination with the Siemens Acuson ultrasound system used for TTE imaging. The ICE catheter was introduced into the oesophagus by directing the catheter posteriorly using minimal pressure. The catheter, steerable in the antero-posterior and right-left direction, readily identifies a 2 chamber view which illustrates the anterior and inferior walls from base to apex. As above, a minimum of 3 cardiac cycles were acquired and the largest and smallest silhouettes were analysed. Ejection fraction was calculated as for TTE using the same analysis software. In addition, FS was calculated using end-diastolic and end-systolic diameters, derived from calliper measurement of the mid LV just below the papillary muscle, using the same 2-chamber cine.

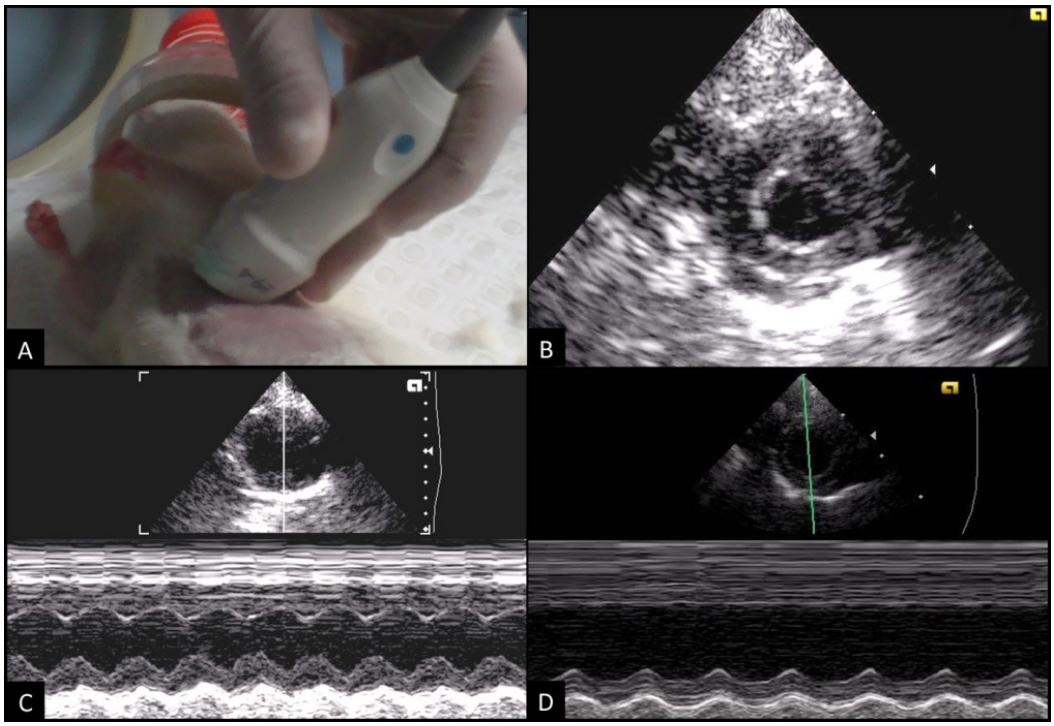

Figure 2: Transthoracic echocardiography. Photograph illustrating TTE set-up with 9.4MHz transducer over the shaved thorax (a). Representative 2-D short axis image (b) and M-mode acquisitions (through the anterior and inferior walls) o stem cell recipients (c) and controls (d) respectively

2.3.4. Data analysis

CMR studies were independently analysed by two CMR cardiologists (AGB, JDR). For the purpose of intraobserver assessment, the entire dataset was analysed on two separate occasions, with an interval of at least one month. An analogous format was followed for both TTE and TOE, with analysis performed by two experienced echocardiogram readers (BK, JDR). Image quality was quantitatively graded by two observers as good (=1), adequate (=2) or poor (=3) and mean scores for each 2-D technique were determined (40 scores per imaging modality). Criteria included image clarity, blood-myocardium border definition and absence of artefact.

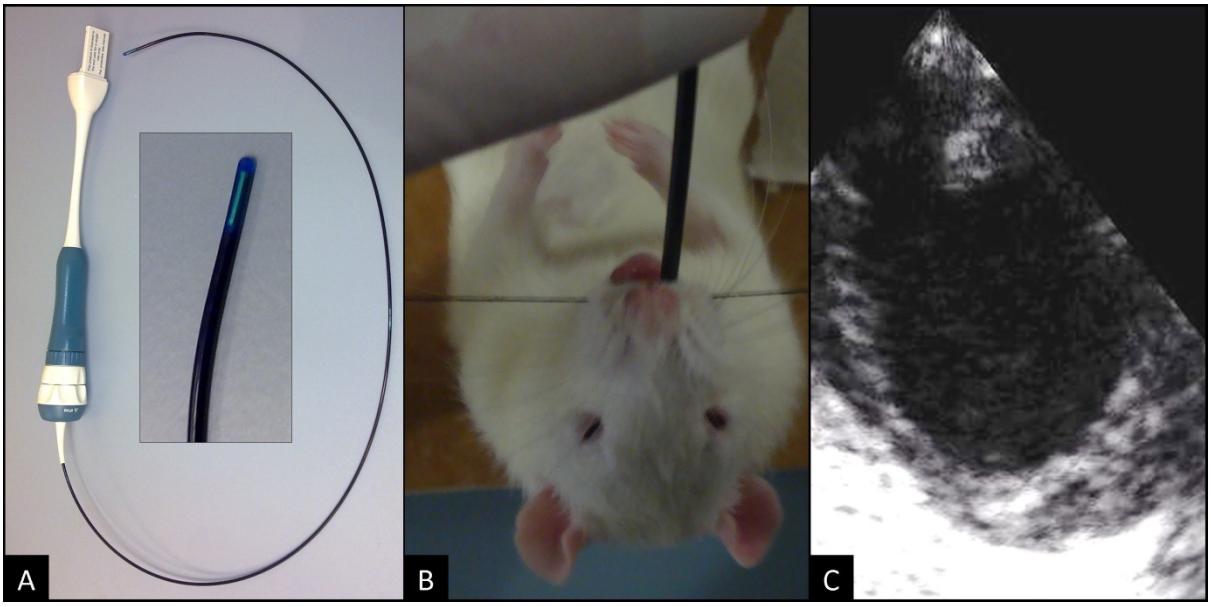

Figure 3: Transoesophageal echocardiography. Photograph of an 8Fr (2.7mm) ICE catheter, with an enlarged view of the transducer tip (a). Picture of a rat undergoing TOE, whilst suspended from an intubation rack (isoflurane nose-cone temporarily removed) (b) and representative TOE image (c).

2.3.5. Statistical analysis

Values are expressed as mean ± standard deviation. Groups were compared using paired t-tests (if normally distributed) or using Wilcoxon rank test for non-parametric data. Correlation between modalities, intra- and inter-observer agreement were assessed using Pearson or Spearman correlation (if normally distributed or not, respectively) and intra-class correlation coefficients (ICC). Bland-Altman analysis was conducted, with mean bias, standard deviation and 95% limits of agreement calculated. A p value < 0.05 was considered statistically significant. Statistical analysis was performed with SPSS 17.0 (SPSS Inc, Chicago, IL, USA).

2.4. RESULTS

Imaging with the three technologies was completed in all 20 animals without complication and with the full set of views obtained. The typical duration of the CMR investigation was 10 minutes, 5-7 minutes for TTE and 3-4 minutes for TOE. Mean heart rate was 300 beats per minute for each imaging assessment. Superior image quality was noted with CMR, which overall was graded as good (mean 1.16 ± 0.41, $p<0.0001$), whereas TTE and TOE were scored as adequate (2.16 ± 0.55 and 2.09 ± 0.53) respectively.

2.4.1. Correlation between modalities

The mean EF values determined by CMR and TOE were very similar (40.1 ±17.4% and 39.9 ±14.6% respectively, $p=0.96$), whereas there was a strong trend towards higher EF values when assessed by TTE (45.7 ±14.7%, $p=0.06$). Fractional shortening results, derived from TTE M-mode and TOE 2-chamber images, were also highly comparable (31.8 ±2.9% and 30.7 ±2.3% respectively, $p=0.90$). Conformity of EF measurements between each imaging modality was evaluated (Figure 4). Excellent correlation was noted between CMR and TOE ($r=0.89$, ICC=0.89, $p<0.0001$), whereas moderate-good agreement

was noted between TTE and TOE (r=0.75, ICC=0.70, p<0.0001) and CMR and TTE (r=0.67, ICC=0.63, p<0.001). Bland-Altman plots demonstrated a bias towards lower EF for both CMR (bias -5.86) and TOE (bias -5.78) when compared to TTE across the range of EF measurements (Fig.4). No significant bias was observed between CMR and TOE, with all measurements falling within 95% limits of agreement.

2.4.2. Intra-observer agreement

The absolute differences in EF (ΔEF) for each animal study between the first and second analysis were evaluated for each modality (Figure 5). For CMR the mean difference was 1.47 ±1.29%, but this was appreciably higher for TTE (ΔEF=4.85 ±3.78%, p<0.05) and TOE (ΔEF=5.84 ±4.41%, p<0.0001). Intra-observer agreement was excellent for CMR (r=0.97, ICC=0.99) and very good with TTE (r=0.88, ICC=0.90) and TOE (r=0.91, ICC=0.87). Reproducibility for fractional shortening was excellent for TTE-derived M-mode images (ICC =0.97) and very good for TOE (ICC=0.87). Bland-Altman analysis reiterated the high level of intra-observer agreement for all three modalities, particular CMR (Figure 6).

2.4.3. Inter-observer agreement

Mean differences in EF between two independent observers were lowest for CMR (ΔEF=2.6 ±1.5%) compared to TTE (ΔEF=4.6±5.4%, p<0.05) and TOE (ΔEF=6.3±5.7%, p<0.01) (Fig.5). Inter-observer agreement was excellent for CMR (r=0.97, ICC=0.98) and very good for both TTE (r=0.87, ICC=0.89) and TOE (r=0.87, ICC=0.84). Regarding FS, TTE-derived M-mode demonstrated excellent reproducibility (ICC=0.93), whereas TOE-derived fractional shortening was only moderate in agreement (ICC=0.71). Bland-Altman analysis (Figure 7) revealed a trend for one observer to systematically measure CMR EFs marginally higher (bias 1.71). For TTE, there was no overall significant bias, but differences were found between observers at either end of the EF spectrum. As with CMR, TOE analysis was systematically rated higher by one reader, but with greater variation (bias 4.32 ±6.74).

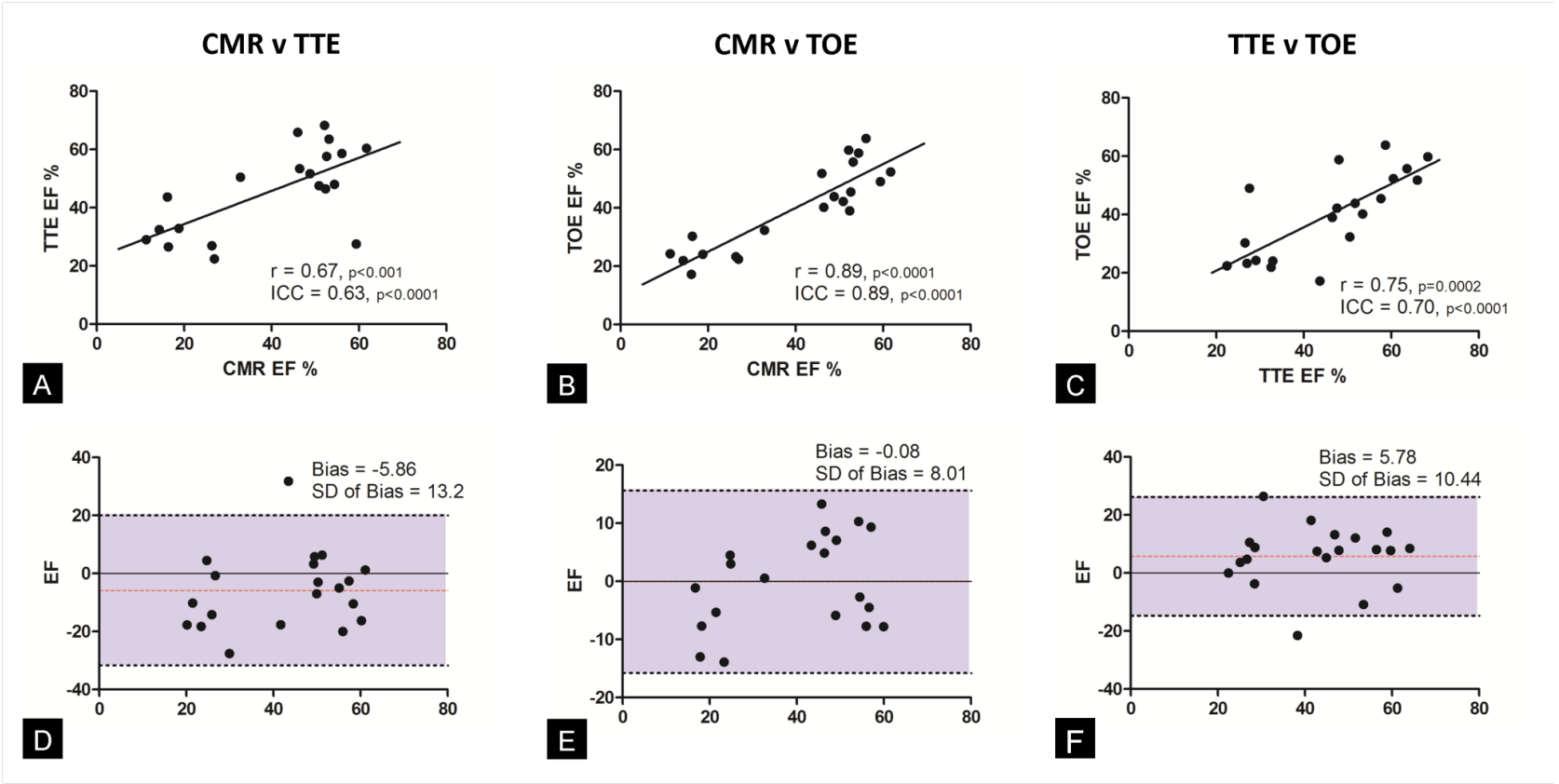

Figure 4: Correlation between imaging modalities. Linear correlation chart with regression line for each comparison pair (a,b,c), with correlation r value and intraclass correlation coefficient (ICC). Corresponding Bland-Altman plots (d,e,f) with mean bias, standard deviation and 95% limits of agreement displayed.

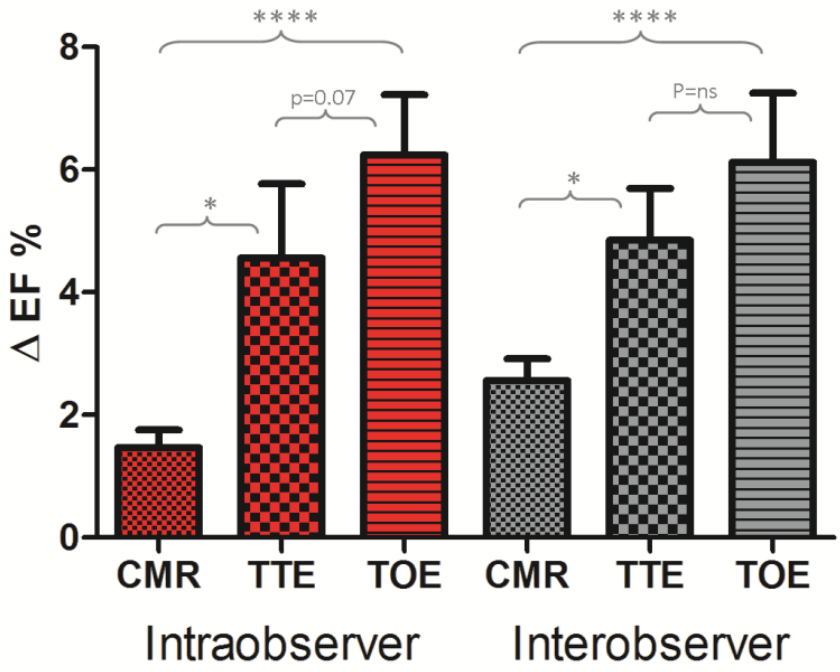

Figure 5: Mean absolute differences in paired EF measurements. Bar chart illustrating intraobserver data for each modality (red) and interobserver data (grey). Statistical significance of the differences between modalities displayed. *p<0.05, **** p<0.0001, ns = non-significant.

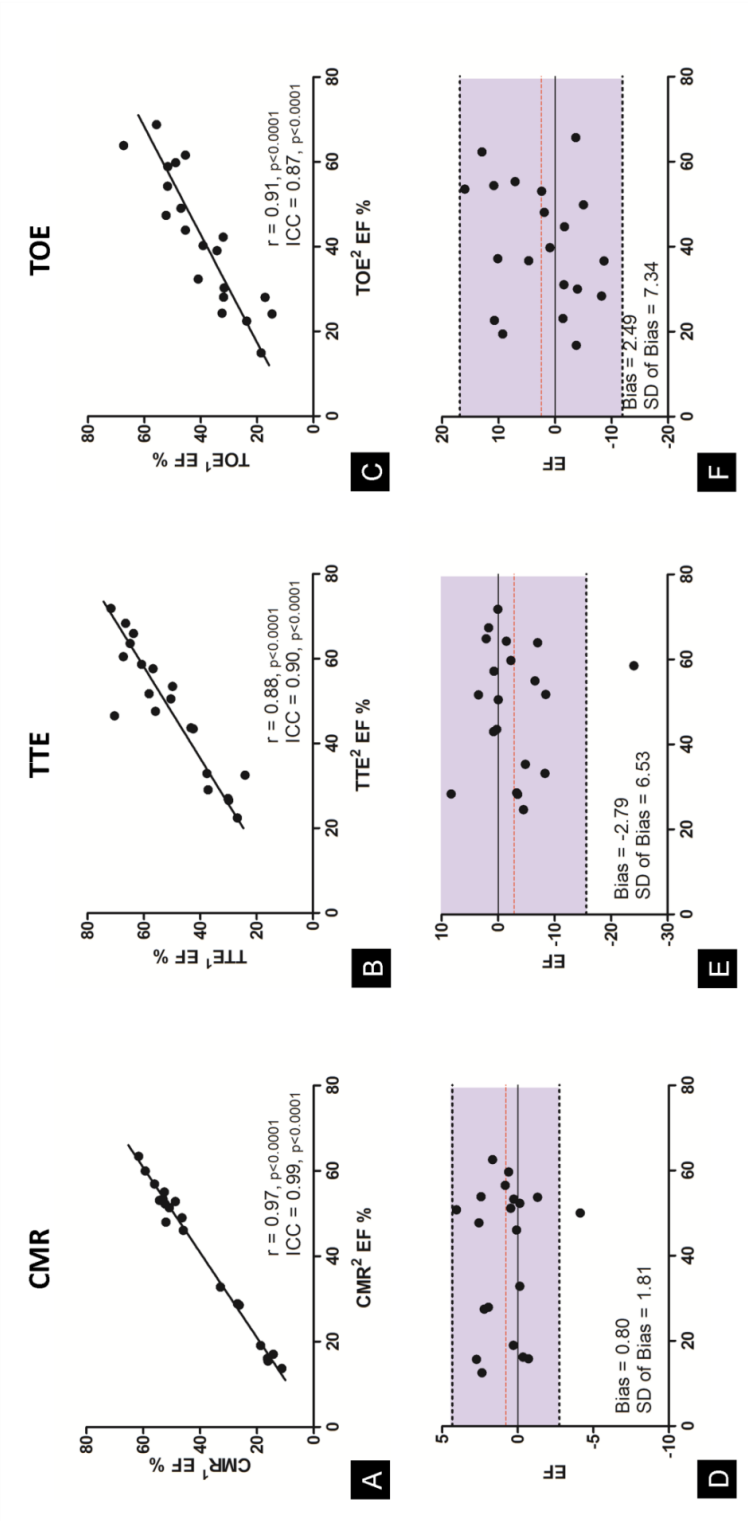

Figure 6: Intraobserver agreement. Linear correlation chart with regression line for each comparison pair (a,b,c), with correlation r value and intraclass correlation coefficient (ICC). Corresponding Bland-Altman plots (d,e,f) with mean bias, standard deviation and 95% limits of agreement displayed.

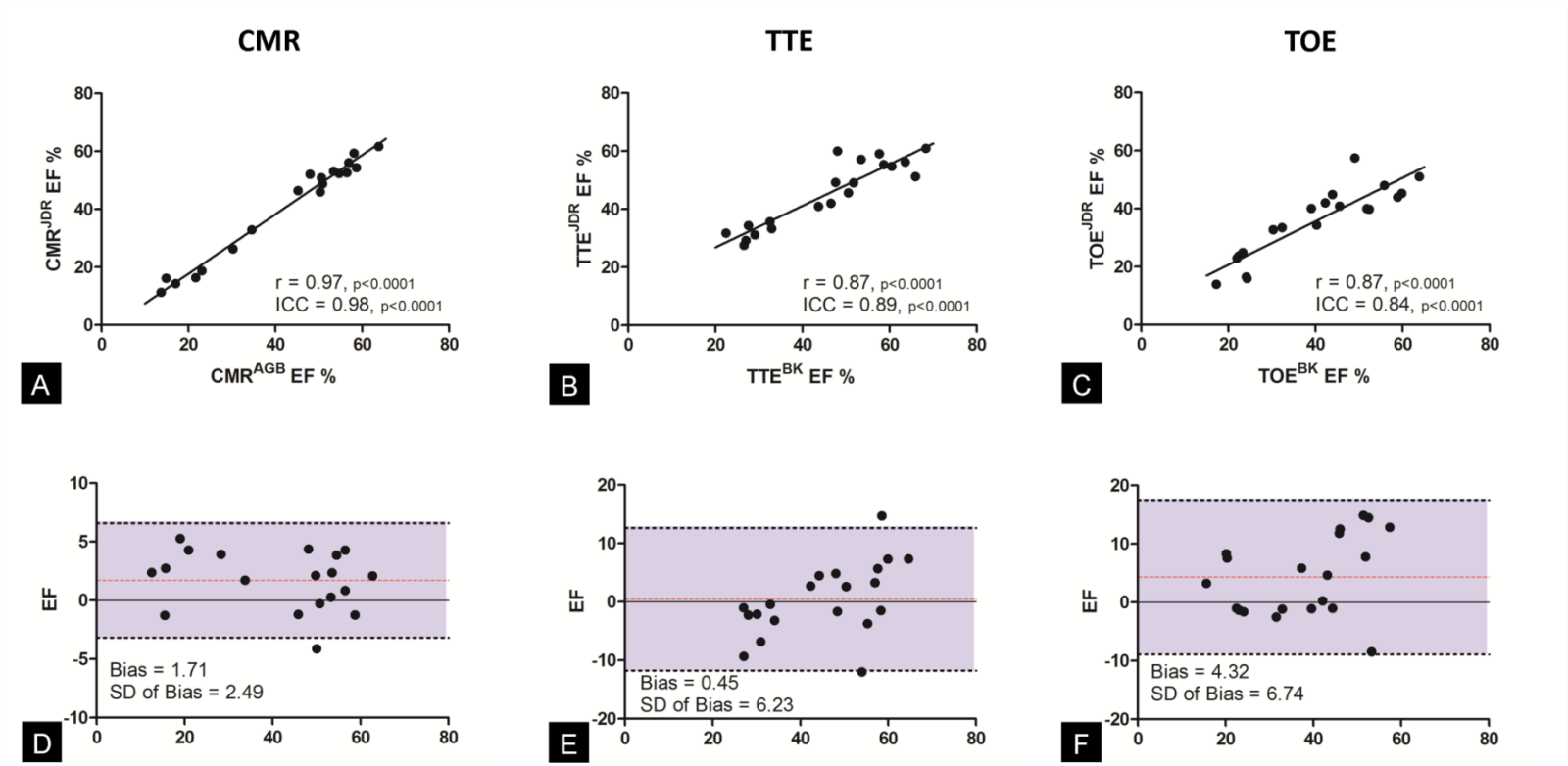

Figure 7: Interobserver variation. Linear correlation chart with regression line for each comparison pair (a,b,c), with correlation r value and intraclass correlation coefficient (ICC). Corresponding Bland-Altman plots (d,e,f) with mean bias, standard deviation and 95% limits of agreement displayed.

2.5. DISCUSSION

This study demonstrates: (1) The first systematic comparison of 1.5T CMR, TTE and TOE in rodents and suggests that 1.5T CMR is a feasible and highly reproducible method of evaluating LV EF in rats; (2) Support for TOE as a feasible and reproducible method to evaluate LV function; (3) CMR has greater reproducibility than the alternative modalities studied, with excellent intra and inter-observer agreement

2.5.1. Utility of 1.5T CMR

We believe this is only the second description of balanced SSFP cine imaging of rodents using 1.5T CMR [10]. This cine sequence has been widely adopted in clinical practice given the high signal-to-noise ratio (SNR) and excellent sub-endocardial contrast that it provides. In contrast, in the few other published studies using 1.5T CMR, investigators have used older generation spoiled gradient echo sequences, (e.g. FLASH) [5, 11-13]. CMR imaging of small animals had until recently been the preserve of research-dedicated high-field (≥4.7T) magnets in conjunction with customised surface coils. In order to accurately image such small anatomy, spatial resolution up to 10-fold higher than that used for humans is required, inevitably causing significant SNR losses [14]. Higher magnetic fields and small/customised coils can help boost signal to compensate for this obstacle, hence the use of this hardware in the few institutions where is it available. However, 1.5T CMR scanners are the principal MR technology used in clinical practice, consequently this equipment is more widely available to researchers. Accordingly, the utility of small animal imaging on 1.5T scanners is of significant interest to potential investigators. At present, reports have documented the utility of 1.5T CMR in evaluating cardiac structure/function for MI [7, 12, 15], myocarditis [16], transplant rejection [11, 13], stem cell tracking [10] and myocardial mass [17]. In only one study was an alternative imaging modality used to provide a comparison [7], which demonstrated a high correlation between single slice CMR and transthoracic M-mode FS (r=0.86). We have demonstrated that it is feasible to

conduct rat CMR on a 1.5T scanner using clinical software in combination with a conventional carotid coil. This provided image quality qualitatively rated as superior to both TTE and TOE, thereby enhancing the potential for accurate and reproducible data to be collected.

This study shows a moderate-strong correlation of CMR to both echocardiographic based techniques. Differences in EF measurements are expected, given the inherent differences in how the LV is interrogated by the different modalities. Transthoracic echocardiography usually depends upon a single measurement – either 1-D M-mode evaluating two opposing myocardial walls, or a short axis slice at a solitary LV level (usually mid LV/papillary muscle level). In comparison, CMR allows a complete assessment of the entire LV by means of contiguous LV slices with no gaps, as utilised in this study (Fig.1). Accordingly, as the entire LV is evaluated it is more likely to be a representative assessment of ventricular function. It was notable that in rodents with extremely poor LV function (EF<20%) as determined by CMR, TTE tended to provide higher EFs with none recorded as being below 20%. Reliance on a single echocardiography measure can therefore cause tighter grouping of measurements, so making it more difficult to discern smaller differences in function.

2.5.2. Reproducibility

For 1.5T CMR to be a feasible alternative, analysis of the images obtained must be highly reproducible. The findings from this study show this to be conclusively the case, with excellent intra- and inter-observer agreement observed for CMR. Rates of intra and inter-observer agreement were also high with both TTE and TOE, albeit lower than with CMR. The very low variability observed between repeated EF measurements by CMR has important implications for study sample sizes used in research projects. For experiments using EF as an end-point, the sample size will be determined by the expected effect of the therapy, but importantly, also the variability of EF measurement in a population. The implication of the improved reproducibility with CMR is that it enables the systematic detection of smaller changes in ventricular function and therefore potentially allows a

substantial reduction in sample size. This is of considerable scientific, ethical and economic value.

Reproducibility is crucial when serial imaging is performed to monitor function over time and CMR provides a number of advantages over echocardiographic based techniques in this respect. In addition to the breadth of LV coverage discussed above, CMR also allows LV short axis slices to be prescribed at clearly identifiable anatomical sites in a truly perpendicular manner. When the short axis stack of three slices (3mm slice thickness, no intersection gap) is prescribed the alignment is checked in two orthogonal (the 2 and 4-chamber) views, thereby ensuring truly perpendicular images are obtained. This reduces the impact of partial volume effects or undue influence of an obliquely-bisected myocardial wall.

Fractional shortening data provided by TTE M-mode had excellent intra and inter-observer reproducibility. One-dimensional data, however, is inherently limited with the provision of information about only two opposing walls, thereby underestimating the potential impact of remaining myocardium on ventricular function. This is particularly important with disease states associated with regional myocardial dysfunction such as ischaemia/ infarction. In contrast, CMR provides almost complete LV coverage in conjunction with excellent reproducibility.

2.5.3. Utility of TOE

Very few studies have described the use of TOE in small animals [18-20]. These have used intravascular ultrasound [18, 20] which has high frequency/resolution, yet is not readily steerable, thereby limiting the potential views that can be obtained. However, one study has utilised ICE to conduct TOE examinations and demonstrated that a complete study, including Doppler acquisitions, is possible in rats [19]. The vertical orientation of the long axis of the rat heart lends itself to TOE imaging and the ICE catheter readily identifies the 2 chamber view, with minimal radial manipulation of the probe. By directing the ICE

probe into the stomach, a transgastric short axis view can be obtained with more assertive manipulation. In our study, the first comparative study of ICE-derived TOE measures of EF, we used the 2-chamber image as our default view given the ease and reproducible nature of obtaining this view. This provides a 2-D assessment of the anterior and inferior walls allowing the calculation of EF, and also FS by manually measuring mid-LV cavity dimension. We observed TOE to have a greater correlation with CMR-derived EF measurements than with TTE. Accordingly, it is possible that the long axis view was more representative of the more extensive assessment provided by CMR, than the single mid-LV slice with TTE. Notably image quality was rated very similar to TTE but not as highly as CMR.

There are a number of scenarios where TOE (or CMR) may be advantageous to TTE imaging. Following thoracic surgery, wound margins may be raised, large intrathoracic collections or residual air may be present [21], therefore limiting optimal image views with TTE, which can be circumvented by transoesophageal imaging. It is feasible to perform TOE intraoperatively to guide procedures and for researchers investigating posterior cardiac structures (e.g. atria and pulmonary veins) TOE would also be particularly suitable. We used the same ICE catheter, itself previously used once clinically, for all 20 rat examinations. As ICE catheters are increasingly used in many interventional/ electrophysiological cardiology centres, availability should not be limiting. Accordingly, for institutions which do not have easy access to expensive high frequency TTE transducers, ICE TOE is an economically and scientifically sound alternative.

2.5.4. Limitations

Cardiac MR imaging of rodents takes 1.5T MRI to the edge of its technological capabilities. Optimal spatial and temporal resolution, both readily achievable with high field scanners, is not possible on clinical scanners and compromises in these factors are inevitable. The spatial resolution of the CMR sequence used in this study is marginally lower than other investigators have used, but we considered this a better balance with higher temporal resolution, which allowed a minimum of 8 phases per cardiac cycle to be

acquired. We found that by optimising other aspects, particularly ECG-trace quality, use of 3mm thick cine slices, signal averaging and contemporary balanced-SSFP software (the latter three enhancing SNR), that high quality images could be reproducibly obtained. This study evaluated 3-dimensional CMR against TTE and TOE, which will in part explain the favourable results for CMR. Bi-dimensional techniques, such as the area-length method, can provide increased accuracy in EF determination compared to the techniques used in this study. We also acknowledge that relative low frequency echo transducers were utilised (9.2MHz), largely as this was the highest frequency transducer available to our unit, which is likely to be the case for many research groups. However, for those facilities with access to high-frequency research-dedicated transducers, superior echo image quality should be obtained which might enhance the accuracy and reproducibility of the echo data.

2.6. CONCLUSIONS

This is the first study to systematically evaluate 1.5T CMR, TTE and TOE in rodents. It shows 1.5T CMR to be feasible, productive of high quality images with extremely high reproducibility. Moderate-strong correlation was observed between all three modalities, identifying all technologies as potential options for researchers evaluating ventricular function in rats. The availability, associated costs and institutional expertise with these imaging modalities will determine the equipment used, but these data suggest that CMR offers distinct advantages even at 1.5T with conventional coils and software.

2.6.1. Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

2.6.2. References

1. Litwin S, Katz S, Morgan J, Douglas P. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. Circulation. 1994 January 1, 1994;89(1):345-54.
2. Ram R, Mickelsen DM, Theodoropoulos C, Blaxall BC. New approaches in small animal echocardiography: imaging the sounds of silence. American Journal of Physiology - Heart and Circulatory Physiology. 2011 November 1, 2011;301(5):H1765-H80.
3. Dawson D, Lygate CA, Saunders J, Schneider JE, Ye X, Hulbert K, et al. Quantitative 3-Dimensional Echocardiography for Accurate and Rapid Cardiac Phenotype Characterization in Mice. Circulation. 2004 September 21, 2004;110(12):1632-7.
4. Allan JJ, Smith RS, DeJong SC, McKay CR, Kerber RE. Intracardiac echocardiographic imaging of the left ventricle from the right ventricle: quantitative experimental evaluation. J Am Soc Echocardiogr. 1998 Oct;11(10):921-8.
5. Jones JR, Mata JF, Yang Z, French BA, Oshinski JN. Left ventricular remodeling subsequent to reperfused myocardial infarction: evaluation of a rat model using cardiac magnetic resonance imaging. J Cardiovasc Magn Reson. 2002;4(3):317-26.
6. Schneider JE, Cassidy PJ, Lygate C, Tyler DJ, Wiesmann F, Grieve SM, et al. Fast, high-resolution in vivo cine magnetic resonance imaging in normal and failing mouse hearts on a vertical 11.7 T system. Journal of Magnetic Resonance Imaging. 2003;18(6):691-701.
7. Voelkl JG, Haubner BJ, Kremser C, Mayr A, Klug G, Loizides A, et al. Cardiac imaging using clinical 1.5 t MRI scanners in a murine ischemia/reperfusion model. J Biomed Biotechnol. 2011;2011:185683.
8. Kober F, Iltis I, Cozzone PJ, Bernard M. Cine-MRI assessment of cardiac function in mice anesthetized with ketamine/xylazine and isoflurane. MAGMA. 2004 Dec;17(3-6): 157-61.
9. Teo KS, Carbone A, Piantadosi C, Chew DP, Hammett CJ, Brown MA, et al. Cardiac MRI assessment of left and right ventricular parameters in healthy Australian normal volunteers. Heart Lung Circ. 2008 Aug;17(4): 313-7.
10. Campan M, Lionetti V, Aquaro GD, Forini F, Matteucci M, Vannucci L, et al. Ferritin as a reporter gene for in vivo tracking of stem cells by 1.5-T cardiac MRI in a rat model of myocardial infarction. Am J Physiol Heart Circ Physiol. 2011 Jun;300(6):H2238-50.
11. Penno E, Johnsson C, Johansson L, Ahlstrom H. Macrophage uptake of ultra-small iron oxide particles for magnetic resonance imaging in experimental acute cardiac transplant rejection. Acta Radiol. 2006 Apr; 47(3):264-71.
12. Franco F, Thomas GD, Giroir B, Bryant D, Bullock MC, Chwialkowski MC, et al. Magnetic resonance imaging and invasive evaluation of development of heart failure in transgenic mice with myocardial expression of tumor necrosis factor-alpha. Circulation. 1999 Jan 26;99(3):448-54.
13. Johansson L, Johnsson C, Penno E, Bjornerud A, Ahlstrom H. Acute cardiac transplant rejection: detection and grading with MR imaging with a blood pool contrast agent--experimental study in the rat. Radiology. 2002 Oct;225(1):97-103.
14. Gilson WD, Kraitchman DL. Cardiac magnetic resonance imaging in small rodents using clinical 1.5 T and 3.0 T scanners. Methods. 2007 Sep;43(1):35-45.
15. Arai T, Kofidis T, Bulte JW, de Bruin J, Venook RD, Berry GJ, et al. Dual in vivo magnetic resonance evaluation of magnetically labeled mouse embryonic stem cells and cardiac function at 1.5 t. Magn Reson Med. 2006 Jan;55(1):203-9.
16. Kromen W, Korkusuz H, Korkusuz Y, Esters P, Bauer RW, Huebner F, et al. Correlation of left ventricular wall thickness, heart mass, serological parameters and late gadolinium enhancement in cardiovascular magnetic resonance imaging of myocardial inflammation in an experimental animal model of autoimmune myocarditis. Int J Cardiovasc Imaging. 2012 Feb 12.
17. Franco F, Dubois SK, Peshock RM, Shohet RV. Magnetic resonance imaging accurately estimates LV mass in a transgenic mouse model of cardiac hypertrophy. Am J Physiol. 1998 Feb;274(2 Pt 2):H679-83.
18. Ramani R, Mathier M, Dawson J, McTiernan CF, Feldman AM. Assessment of infarct size and myocardial function in mice using transesophageal echocardiography. J Am Soc Echocardiogr. 2004 Jun;17(6):649-53.
19. Gao Z, Li J, Kehoe V, Davidson WR, Jr., Sinoway L, Pu M. An initial application of transesophageal Doppler echocardiography in experimental small animal models. J Am Soc Echocardiogr. 2005 Jun;18(6):626-31.
20. Schwarz ER, Pollick C, Meehan WP, Kloner RA. Evaluation of cardiac structures and function in small experimental animals: Transthoracic, transesophageal, and intraventricular echocardiography to assess contractile function in rat heart. Basic Research in Cardiology. 1998;93(6):477-86.
21. Kanno S, Lerner DL, Schuessler RB, Betsuyaku T, Yamada KA, Saffitz JE, et al. Echocardiographic evaluation of ventricular remodeling in a mouse model of myocardial infarction. J Am Soc Echocardiogr. 2002 Jun; 15(6):601-9.

Statement of Authorship

Title of Paper	**Cardiac magnetic resonance, transthoracic and transoesophageal echocardiography: a comparison of in vivo assessment of ventricular function in rats.**
Publication Status	Published
Publication Details	Lab Anim. 2013 Oct;47(4):291-300.

Author Contributions

Name of Principal Author (Candidate)	**Dr James David Richardson**
Contribution to the Paper	Primary contributor to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work; Drafted the work ; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Angela Bertaso**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Lachlan Frost**

Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Peter J Psaltis**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Angelo Carbone**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Ben Koschade**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and

	revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Dennis T Wong**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Adam J Nelson**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mrs Sharon Paton**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and

	revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mrs Kerry Williams**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Azarisman Shah**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Karen Teo**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval

	of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**A/Prof Matthew Worthley**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Professor Andrew Zannettino**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013
Name of Co-Author	**Professor Stan Gronthos**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013

Name of Co-Author	**Professor Stephen G Worthley**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013

3. Chapter 3 – Prospective Isolation & Hypoxic Conditioning of MSC

Prospectively-Isolated Hypoxic-Conditioned Mesenchymal Stem Cells Have Superior in vitro Capacity and Significantly Attenuate Myocardial Infarction-Induced Ventricular Dysfunction.

James D Richardson[1,2], Sharon Paton[2], Angelo Carbone[1], Lachlan Frost[1], Peter J Psaltis[1,3], Stan Gronthos[2#], Andrew CW Zannettino[2#], Stephen G Worthley[1,2#]

[1] Cardiovascular Research Centre, Royal Adelaide Hospital and Department of Medicine, University of Adelaide, South Australia, Australia.

[2] Centre for Stem Cell Research, Robinson Institute, School of Medical Sciences, University of Adelaide, South Australia, Australia

[3] Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, USA

[#] Joint senior authors

3.1. ABSTRACT

Introduction: Optimisation of mesenchymal stem/stromal cells (MSC) prior to transplantation might advance their therapeutic efficacy in regenerating myocardium after Infarction (MI). Previous experiments have largely examined the impact of one optimisation strategy only. We assessed the effect of combining two strategies – prospective immunoselection and hypoxic preconditioning - against conventional plastic-adherent (PA) MSC on in vitro efficacy. We then evaluated the utility of the optimised MSC in a pre-clinical model of MI.

Methods: The target cell population was prospectively isolated from bone marrow of donor Sprague-Dawley rats using magnetic activated cell sorting CD45-depletion. MSCs were subjected to hypoxic incubation (5% O_2) during primary culture. Quantitative assessment of colony forming efficiency and in vitro differentiation potential of optimised-MSC and PA-MSC was undertaken. In vivo efficacy of optimised-MSC was evaluated in Sprague-Dawley rats, with MSCs delivered by transepicardial injection at repeat thoracotomy one week after MI (n=20). Controls received cryopreservant solution (n=15). Left ventricular dimensions and ejection fraction (EF) were assessed by cardiac magnetic resonance immediately before MI and also at 1, 2 and 4 weeks post-MI.

Results: Optimised MSC demonstrated greater adipogenic (12.4±2.3% vs 2.1±0.3%, $p<0.01$) and osteogenic differentiation capacity (3.24±0.5mM vs 0.68±0.3mM, $p<0.01$) than PA-MSC. Chondrogenic differentiation was equivalent (Scintillation count/DNA= 31968±1537 vs 33178±923, $p=0.61$). In controls, MI reduced EF rom 58.5±1.2% to 22.1±2.0% at 1 week, with no subsequent improvement by week 4 (19.5±1.5%). In the MSC treatment group, EF also fell from 57.8±1.2% to 25.1±2.2%, but significantly improved to 38.8±1.4% by week 4 ($p<0.001$). Accordingly, optimised-MSC increased EF by 19.3±3.1% ($p<0.001$) at 4 weeks and was accompanied by improvements in LV volumes, mass, wall thickness, systolic wall thickening, fibrosis and arteriolar density.

Conclusion: Allogeneic MSC, optimised by prospective isolation and hypoxic preconditioning, exhibit superior in vitro differentiation capacity and attenuate LV dysfunction in rats following MI. These optimisation strategies, when used in combination, offer significant potential to improve therapeutic efficacy of stem cell therapy.

Key Words: Mesenchymal stem cell; Repair; Myocardial infarction; Cardiac magnetic resonance; Prospective isolation; Hypoxic conditioning; Immunoselection; Optimisation.

3.2. INTRODUCTION

There is a compelling need for novel therapies to treat left ventricular dysfunction after acute myocardial infarction (MI). Treatment with mesenchymal stem/stromal cells (MSC) has demonstrated significant promise in pre-clinical experiments, but only modest benefits were observed with autologous bone marrow cells in clinical trials [1]. Allogeneic sources of MSC appear to hold several important advantages over autologous BM/BM mononuclear cells (BMMNC); (1) MSC can be derived from young, healthy donors which is important as with advancing age there is considerable diminution of the absolute yield and functional biology of MSCs from BM aspirates [2], negatively impacting on multilineage differentiation potential [3], gene expression profile and myocardial reparative capacity [4]; (2) The cell product is prepared well ahead of time, so making very early MSC treatment feasible, e.g. after primary percutaneous intervention, when myocardium remains viable; (3) MSC could be optimised to potentially advance their therapeutic efficacy, e.g. through superior isolation techniques, pre-conditioning, paracrine augmentation, activation of cytoprotective pathways and directing cardiopoeisis [5]. Of the cell optimisation strategies currently evaluated prospective isolation and hypoxic conditioning strategies have individually been shown to enhance their in vitro [6] and in vivo efficacy [7, 8] and could be combined and readily applied to clinical use.

The traditional isolation method employed in the majority of cardiovascular studies has utilised MSCs isolated from density-separated mononuclear cells via simple plastic adherence culture (PA-MSC) [9]. Despite its ease and popularity, this technique is compromised by suboptimal specificity, as the resulting MSC population may be contaminated by non-mesenchymal cells during early culture (e.g. monocytes, hematopoietic cells) and increasingly senescent mesenchymal cells following sequential passage [6]. Thus the product of this isolation process and subsequent *ex vivo* expansion may comprise a heterogeneous admixture of cells with a limited and unpredictable content of primitive MSCs that possess high clonogenicity and multipotency. Consequently, the strategy of prospective immunoselection has been advocated as a means of isolating a more homogeneous, immature starting population of mesenchymal precursor cells prior to *ex vivo* culture [10]. Previously our group has demonstrated that human MSC procured by prospective isolation with positive selection monoclonal antibodies (e.g. STRO-1, STRO-3) have biological advantages over conventionally isolated MSC, in terms of purity, "stemness" and cardiovascular paracrine capacity ([6, 11]).

Studies have assessed the *in vitro* and *in vivo* qualities of MSCs exposed to reduced levels of oxygen during cell culture. Hypoxic-conditioned cells have been shown to produce increased quantities of pro-survival transcription and growth factors, including vascular endothelial growth factor, hypoxia inducible factor-1α, survivin and B-cell lymphoma-2 compared with their preparation under normoxia [7]. Sequential culture of MSCs under hypoxic followed by normoxic conditions may also enrich for those cells capable of thriving under hypoxia and this has translated into enhanced retention, survival and functional benefit after these cells are transplanted in models of MI [7].

Previous studies have focused on a single optimisation strategy, but we evaluated whether MSC optimised by the combination of prospective isolation and hypoxic conditioning have; (1) greater in vitro differentiation capacity than PA-MSC, (2) have in vivo reparative capability in a rat model of acute MI.

3.3. METHODS

3.3.1. Study protocol

This study was approved by the Animal Ethics Committees of the Central Adelaide Local Health Network (No 104/10) and the University of Adelaide (No. M-2010-105), South Australia. Animal handling was carried out in accordance with the guidelines outlined in the "Position of the American Heart Association on Research Animal Use".

Male Sprague-Dawley rats (n=35, baseline weight 350-370g) were used in the experimental protocol. Animals were randomised at baseline to one of two investigation arms: Optimised MSC group or controls (Figure 1). Baseline evaluation of ejection fraction (EF) was undertaken using cardiac magnetic resonance (CMR), followed by thoracotomy and permanent surgical ligation of the left anterior descending artery. At repeat thoracotomy one week later, MSC were delivered transepicardially whereas control animals received injections of ProFreeze^{TM} (PF) cryopreservation vehicle. Reassessment of EF with CMR was conducted at 1, 2 and 4 weeks after myocardial infarction (MI). Euthanasia was performed at week 4 after final imaging for tissue harvesting and histological evaluation.

3.3.2. Prospective Isolation of Rat MSC

3.3.2.1.(i) Bone marrow and compact bone isolation

Six donor male Sprague Dawley rats were euthanized (by CO_2 inhalation) and the femora and tibiae excised under sterile conditions in a laminar flow hood. The epiphyses were removed and bones flushed with Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 20% (v/v) fetal calf serum (FCS) (SAFC Biosciences,Lenexa, Kansas, USA), 1% Penicilllin/Streptomycin Solution (w/v) (Sigma-Aldrich, Saint Louis, MO, USA) and 50i.u/ml Deoxyribonuclease I from Bovine Pancreas (DNAse I) (Sigma-Aldrich, Saint Louis, MO, USA). The resultant cell suspension was centrifuged, supernatant removed and the cell pellet resuspended for density gradient

separation. The long bones, now BM deplete, were crushed into small fragments using sterile scissors and washed with Dulbeccos Phosphate Buffered Saline (PBS, Sigma-Aldrich Saint Louis, MO, USA) and 2% FCS. Digestion of the bone fragments, using Collagenase Type I 3mg/ml (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.2% DNAse I solution in PBS, was performed on a shaking platform for 45 min at 37°C. The supernatant was collected and the cell suspension strained through a 70 µm nylon cell strainer (BD Falcon, FranklinLakes, NJ, USA) and centrifuged at 305 *g*.

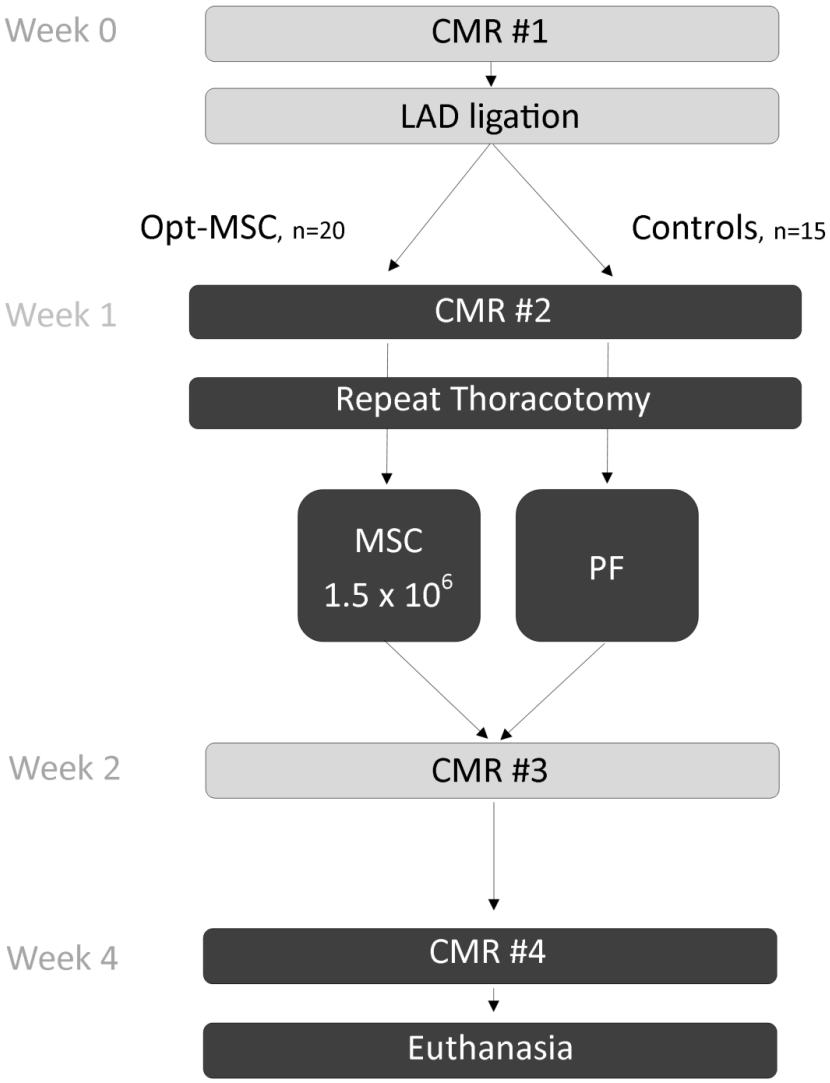

Figure 1: Flow chart of experimental protocol.

3.3.2.2.(ii) Density gradient separation of compact bone (CB) derived cells

Rat compact bone-derived cells were underlayed with 3ml Lymphoprep™ (Axis Shield PoC, Rodeløkka, Oslo, Norway) and centrifuged at 305g for 30 min with no brake. The mononuclear layer was separated and resuspended in HBSS/10% FCS before repeat centrifugation. The CB-derived mononuclear cell (CB-MNC) count was then determined using White Cell Fluid (2% Acetic acid in deionised water, tinged with methyl violet) using a haemocytometer.

3.3.2.3.(iii) Depletion of contaminating CD45 positive BMMNC

Magnetic activated cell sorting (MACS) was used to deplete contaminating CD45 positive haematopoietic cells from the CB-derived MNC population. Briefly, CB-MNC were pelleted, blocked for 20 min at 4^{o}C in blocking buffer (5% Normal Human Serum, 10% BSA, 5% FCS in HBSS), prior to incubation with purified Anti-Rat CD45 10ug/ml (BD Biosciences, San Diego, CA, USA) for 45 min at 4^{o}C. After repeated washes in HBSS 10%FCS, secondary incubation was performed with goat anti-mouse IgG-Biotin 10ug/ml (Southern Biotec, Birmingham, AL, USA) for 30 min at 4^{o}C, followed by two washes in MACS Buffer (5mM EDTA, 1% BSA, 0.01%NaN_3 in PBS) and incubation with Anti-Biotin Microbeads (MiltenyiBiotec, BegischGladbach, Germany) for 15 min at 4^{o}C. The cell suspension was then passed through a MACS MS Separation Column (MiltenyiBiotec, BegischGladbach, Germany). The CD45-negative fraction was collected as the target cell population harbouring the MSC, to establish primary cultures.

3.3.2.4. Hypoxic conditioning

CD45-negative cells were initially seeded in plastic culture flasks at $3x10^4/cm^2$ in alpha Modification of Eagles's Medium (Sigma-Aldrich, Saint Louis, MO, USA, supplemented with 20% (v/v) FCS, 2mM L-Glutamine (SAFC, Lenexa, Kansas, USA), 1mM sodium pyruvate (Sigma-Aldrich, Saint Louis, MO, USA), 100uM L-ascorbate-2-phosphate (WAKO Pure Chemical Industries, Ltd, Amagasaki, Hyogo-Ken, Japan) and penicillin (50i.u./ml)/streptomycin sulphate (50ug/ml) (Sigma-Aldrich, Saint Louis, MO, USA), in a

humidified hypoxic chamber in the presence of 5% O_2, and 10% CO_2 (COY Laboratory Products, Inc., Grass Lake, Michigan, USA). After reaching 80% confluence, cells were detached by Trypsin/EDTA treatment (Gibco-Life technologies, Grand Island, NY, USA) and subcultured in αMEM, supplemented with 10% (v/v) FCS, 2mM L-Glutamine, 1mM sodium pyruvate, 100uM L-ascorbate-2-phosphate and penicillin /streptomycin under normoxicconditions (21% O_2, 5% CO). At passage 4 (P4), MSC were cryopreserved in ProFreeze™ -CDM NAO, (LONZA BioWhittaker Walkersville, MD, USA) with a 7.5% DMSO (ChemSupply, Gillman, Adelaide, South Australia) at a mean concentration of $1.5x10^6$/100µl.

3.3.3. MSC immunophenotype

Cell surface antigen expression of passage 4 Opt-MSC and PA-MSC was assessed using flow cytometric analysis. Both MSC populations were incubated on ice for 45min against a panel of anti-rat primary antibodies directed at common cell markers including CD44, CD45. After washing to remove unbound primary antibody, cells were incubated for 30 minutes on ice with a fluorochrome-conjugated secondary antibody at a 1:50 dilution. Flow cytometric analysis was performed using an Epics®-XL-MCL flow cytometer (Beckman Coulter, USA) and EXPO32 software (Co name etc). For each sample, 10,000 events were analysed and positive fluorescence was defined as the level of fluorescence >99% of the corresponding isotype-matched control antibody.

3.3.4. MSC differentiation capacity

Adipogenic, chondrogenic and osteogenic differentiation capacity of Opt-MSC and PA-MSC, using inductive culture media was assessed. Passage 4 cells were seeded at 8 x 10^3/ well in standard media until reaching 90% confluence. Thereafter for adipogenic induction, media comprising α-MEM supplemented with FCS, ascorbic acid, indomethacin and dexamethasone was used. For osteogenesis the same media, with indomethacin omitted, was applied, For chondrogenesis, α-MEM supplemented with FCS, L-glutamine, Sodium pyruvate, Penicillin-Streptomycin, HEPES and Ascorbic acid was used. Culture medium

was replaced twice weekly until day 14 for adipogenic and day 28 for chondrogenic and osteogenic assays. Samples were fixed in 10% formalin and histological staining with oil red O or alizarin red for adipocytes and osteoblasts respectively. A GAG DNA assay was utilised for the chondrocyte analysis as previously described.

3.3.5. Colony efficiency assay

Colony efficiency was assayed by incubating primary cells of either: (i) unselected non-hypoxic conditioned cells; (ii) selected-only cells; (iii) selected and hypoxic conditioned cells in α-MEM-20, at densities of $1x10^3$ to $1x10^5/cm^2$, in triplicate, in six-well plates. On day 14 of culture, plates were washed twice with warm PBS, stained with 0.1% (w/v) toluidine blue (in 1% PFA w/v in PBS) for at least 1 h and finally rinsed in tap water. Aggregates of 50 cells or more were scored as colony forming units-fibroblast (CFU-F) under an Olympus SZ-PT dissecting light microscope (Olympus Optical Co Ltd, Japan).

3.3.6. Cardiac Magnetic Resonance

Imaging was performed on a 1.5T MR system (Magnetom Sonata, Siemens, Germany), equipped with a dedicated cardiac software package. The thorax and right paw were shaved, cleaned with alcohol and adhesive electrocardiogram (ECG) electrodes attached thereby generating a vector ECG. Animals were placed supine recumbent in the isocentre of the magnet, with a 4-channel phased array carotid radiofrequency coil placed over the thorax, with 1% isoflurane administered via nose cone to maintain anaesthesia. Accordingly, all CMR images were free breathing, ECG-gated acquisitions. Transverse and coronal localiser images were acquired followed by long (4-chamber and 2-chamber) and short axis pilot images, from which a true short axis stack was prescribed. Balanced steady-state free-precession cine images (gated to alternate R waves) were acquired. The stack comprised three contiguous LV slices (each 3mm thick, with no intersection gap) providing full coverage of the LV. The image matrix was 384 x 384, field of view 185 mm, repetition time 14.72ms, echo time 1.55ms, flip angle 90°, image resolution 0.5 x 0.5 mm,

8 phases per cardiac cycle were acquired with an imaging time of 52 seconds per short axis slice [12].

3.3.7. CMR image analysis

Left ventricular mass, volumes and derived EF were measured off-line from cine images using commercially available software (QMass v7.2, Medis, Netherlands). The end-diastolic and end-systolic cine frames were identified for each slice and the endocardial and epicardial borders were manually traced. The end-diastolic (EDV) and end-systolic volumes (ESV) were calculated using the true disk summation technique (i.e. sum of cavity volumes across all continuous slices), and EF calculated, as previously described [13]. Regional segmental function was analysed in each of 16 segments – 6 basal, 6 mid and 4 apical. Using the same software, a reference point was placed at the right ventricular insertion point in the anterior septum for each LV slice to assign myocardial segmentation. Left ventricular end-diastolic wall thickness and systolic wall thickening (SWT) measurements were automatically calculated.

3.3.8. Infarct surgery and MSC transplantation

Anaesthesia was induced using a closed chamber filled with 3% isoflurane and 2L/min O_2. Animals were intubated with a 16-gauge cannula and connected to a small animal ventilator (Harvard Apparatus, Holliston, MA, USA) and mechanically ventilated (tidal volume 3.6 mL, ventilatory rate 60 breaths/min) with 1-1.5% isoflurane and 2L/min O_2. A left anterior thoracotomy was performed, the pericardium excised and the left anterior descending artery permanently ligated as previously described [13]. Infarction was confirmed by the development of pallor and hypo-/akinesis of the anteroapical myocardium. The muscle and skin layers were closed, Isoflurane was discontinued and animals were extubated once spontaneous ventilation resumed. Noracillin (0.15mg/kg i.m.) was given prophylactically, while post-operative analgesia was achieved with a single dose of Ketamine (75mg/kg i.p.) and Carprofen (5mg/kg s/c) repeated over two days.

One week later, all animals underwent CMR and repeat thoracotomy using identical anaesthesia and medication as described above. Animals received transepicardial injections of MSC (mean $1.5x10^6$ cells) or PF (control) in four separate injection sites in the anterior myocardial wall border zone at a total volume of 100μL via a 30-gauge needle (i.e. 25μL per injection). MSCs were thawed from individual ampoules and underwent cell counts and cell viability (by trypan blue exclusion) immediately prior to injection.

3.3.9. Histological analysis

Euthanasia was performed at day 28 post-MI under deep anaesthesia (Metedomidine 0.5mg/kg i.p. and Ketamine 75mg/kg i.p.). Hearts were excised, drenched in sterile saline and immersed in a mixture of PBS/10% formalin. Myocardium below the occlusion site was later sectioned into three transverse slices providing a basal, mid and apical LV section. After paraffin embedding, replicate 6μm sections were cut and stained by haematoxylin and eosin, Masson's trichrome and immunohistochemistry. Whole slides were photographed (x40 magnification) with the NanoZoomer Digital Photography system (Hamamatsu Photonics, Japan) and complete image sets exported for blinded analysis using Image Pro Plus software (v5.1, Media Cybernetics, USA). Cardiac fibrosis was quantified using Masson's trichrome specimens and myocardial vascular density was quantified using staining for α-smooth muscle actin (αSMA - Clone 1A4, IgG_{2a} Imgenex, USA), as previously described [14]. The number of αSMA positive vessels (10 - 100μm diameter) was counted in ten random high power fields (x20 magnification), in the infarct zone, border zone and remote LV myocardium respectively [13].

3.3.10. Statistical analysis

All analyses were performed blind to study group and by two independent observers for CMR parameters. Numerical data are expressed as mean ± standard deviation. In view of repeated measures of the same subjects at different time points, a linear mixed effects model was adopted to compare the effectiveness of therapy between groups. A p-value

<0.05 was considered statistically significant. Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3.4. RESULTS

3.4.1. Characterisation of MSC

The immunophenotype of P4 Opt-MSC and PA-MSC, evaluated with flow cytometric analysis of cell surface markers as previously described [6], was found to be identical. Both rat MSC populations exhibited very high expression of CD105, CD90, CD73 and CD44, but negligible expression of CD45 and CD34 and minor expression of CD106 consistent with international guidelines [15]

3.4.2. Colony forming efficiency

The immunoselected cell population (derived by CD45 immunodepletion) demonstrated increased capacity to establish colony forming units-fibroblast (CFU-F) compared to unselected PA cells. The frequency of clonogenic CFU-F in selected cells was 101.5±0.5 compared to 4.0±0.1 for PA-cells ($p<0.01$). Hypoxic conditioning further increased CFU-F by almost half, with immunoselected and hypoxic conditioned cells exhibiting a CFU-F frequency of 145.5±4.5 compared to 101.5±0.5 for selected cells incubated at 21% O_2 ($p<0.05$).

3.4.3. Differentiation capacity

The relative capacity of Opt-MSC and PA-MSC to differentiate into adipocytes, chondroblasts and osteoblasts was assessed. Both MSC groups demonstrated trlineage differentiation, but the magnitude (and speed) of adipogensis and osteogeneis was substantially greater in Opt-MSC. For adipogenesis, the number of adipocytes as a proportion of cells was higher than PA-MSC (12.4±2.3% vs 2.1±0.3%, $p<0.01$) and for osteogenesis calcium concentration was higher (3.24±0.5mM vs 0.68±0.3mM, $p<0.01$).

However chondrogenic differentiation did not differ significantly (Scintillation count/DNA conc = 31968±1537 vs 33178±923, p=0.61).

3.4.4. Global Left Ventricular Function

There were no significant differences in LV volumes or EF between the investigation groups at baseline and changes in LVEF over time are illustrated in Table 1. Following LAD ligation, EF declined significantly by week 1 in both groups, to 22.1±1.9% in controls and 24.1±2.2% in the MSC group (p<0.0001 versus baseline EF for each group, p=not significant between control and MSC groups). By week 2 (one week after MSC transplantation), mean EF significantly improved to 40.2±3.1% (p<0.001 vs controls) whereas EF remained stable in the no-cell recipients (EF= 19.2±1.9%). By week 4, the MSC treatment group maintained significantly higher LV function than controls (final EF: control group 19.5±21.5%; MSC group 38.9±2.6%, p<0.001). The mean reduction in EF between baseline and week 4 in the control group was -38.8 ±1.4% which was significantly attenuated by MSC treatment (-18.9±2.6%). Accordingly the absolute difference in mean EF was 19.9±3.1%, (p<0.001, Figure 2). Ventricular dilatation was substantially assuaged by MSC intervention compared to no-cell control. Mean EDV in MSC recipients was 111±14μL compared to 389±74μL in controls (p<0.0001). Mean ESV was 164±23μL in the MSC group compared to 402±71μL in controls (p<0.001).

CMR Scan	Controls EF (%)	MSC EF (%)	Δ EF	Significance
Week 0	58.5 ±1.2	57.8 ±1.2	0.7±1.8	$p = 0.87$
Week 1	22.1 ±1.9	24.1 ±2.2	2.0±3.0	$p = 0.62$
Week 2	19.2 ±1.9	40.2 ±3.1	21.0±4.6	$p < 0.001$
Week 4	19.5 ±1.5	38.9 ±2.6	19.3±3.1	$p < 0.001$

Table 1: Change in ejection fraction over time according to experimental group.

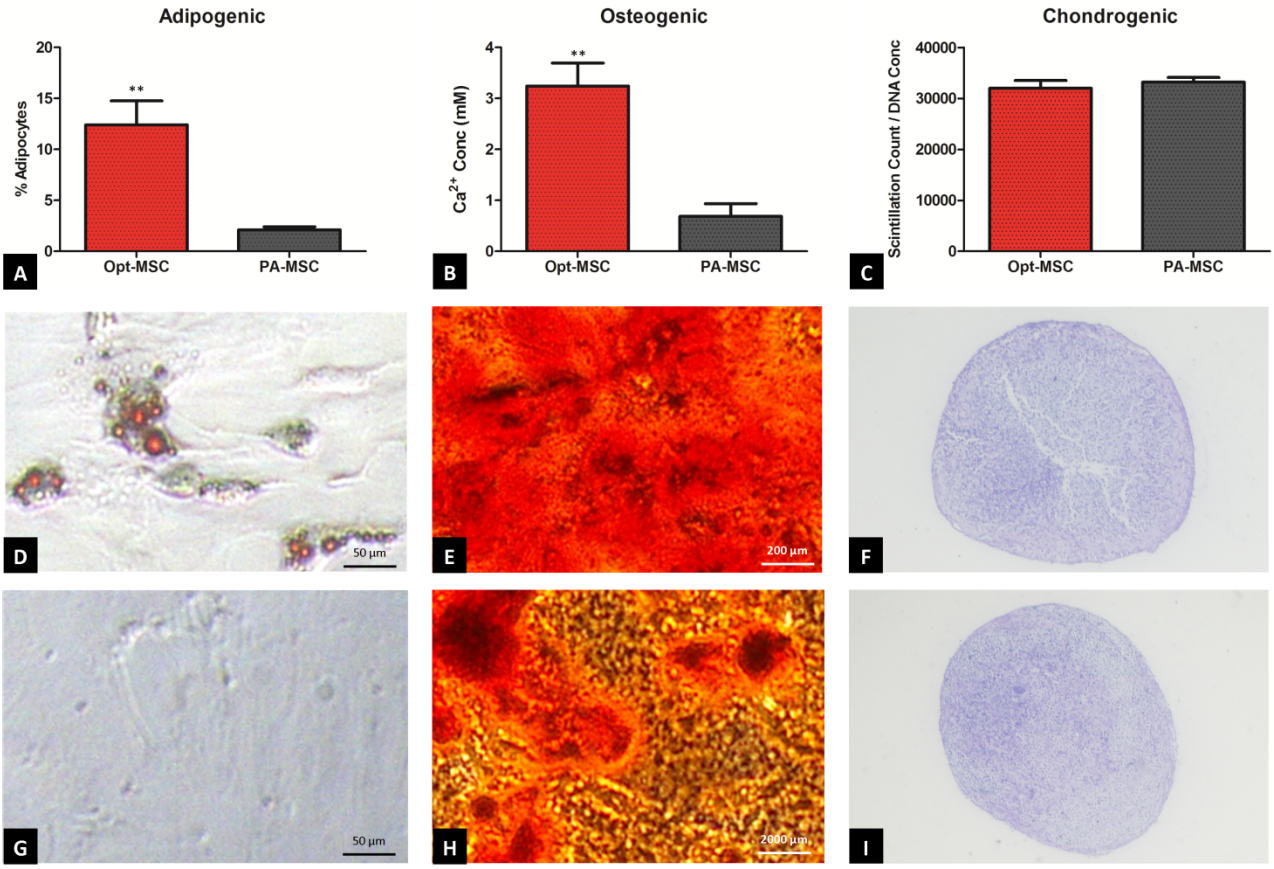

Figure 2: Adipogenic, Osteogenic and Chondrogenic differentiation capacity.

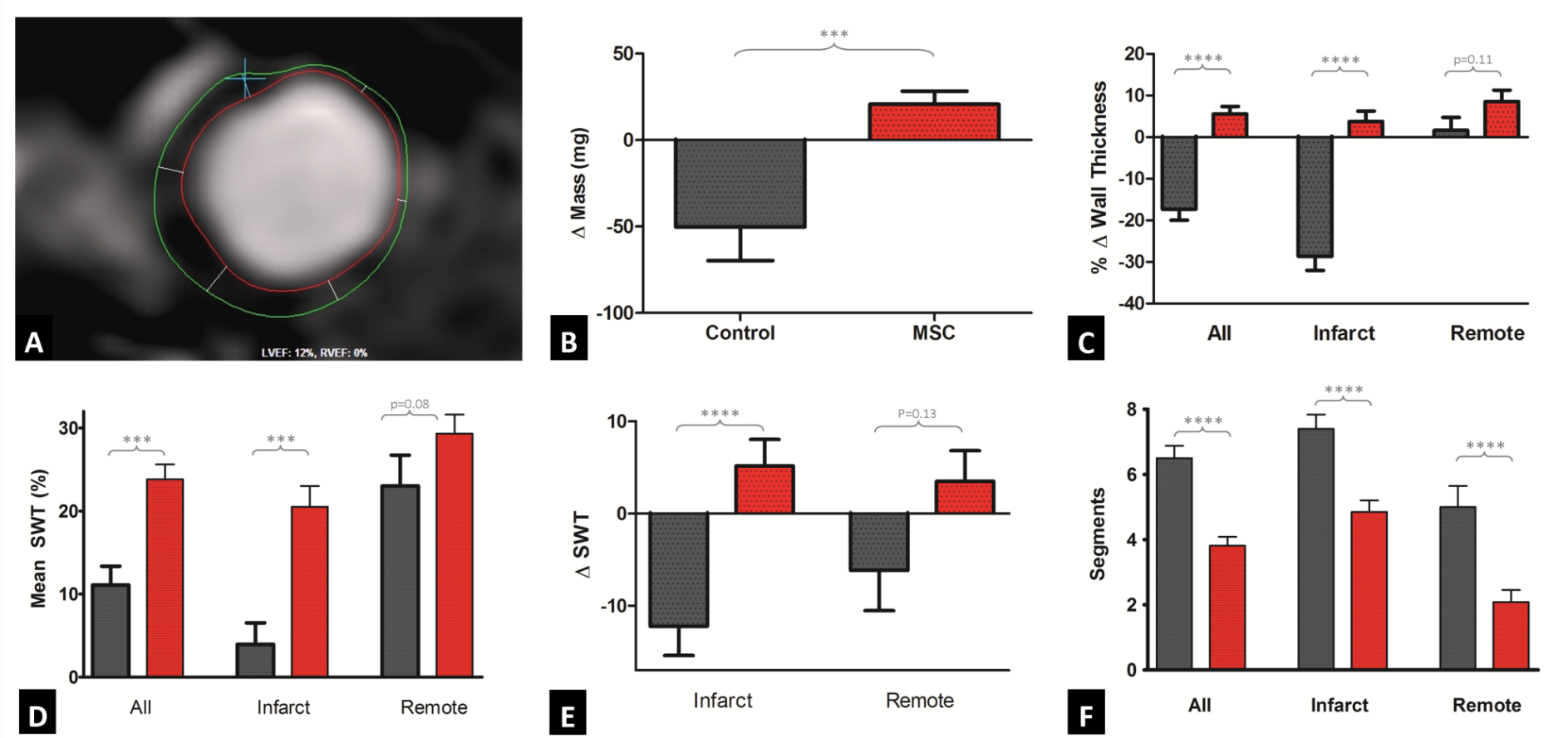

Figure 3: Wall thickness and systolic wall thickening. (A) Representative CMR example demonstrating typical contours in an infarcted rat heart. (B) Change in ventricular myocardial mass, (C) % change in wall thickness, (D) Mean systolic wall thickening, (E) change in systolic wall thickening, (F) Number of infarcted segments.

3.4.5. Regional left ventricular morphology and function

Global myocardial mass, derived from CMR imaging, was significantly reduced in control animals measured four weeks after MI (Δ -50.2 ±19.5mg). In contrast, myocardial mass was preserved in MSC recipients (Δ 20.7 ±7.6mg, $p<0.001$, Figure 3_B). Analysis of CMR-derived segmental LV wall thickness demonstrated preservation of wall thickness in the myocardial segments supplied by the LAD and in all segments overall in the MSC group compared with controls ($p<0.001$, Figure 3_C). In remote (non-infarct) segments, there was a trend to greater change in wall thickness in MSC recipients.

Overall mean SWT was significantly higher in MSC recipients at end-study compared to controls (Figure 3_D). This effect was most apparent in the myocardial segments supplied by the LAD (SWT: controls 3.9±2.6%, MSC cohort 20.5±2.5%, $p<0.001$). Systolic wall thickening in remote LV myocardium displayed a strong trend favouring MSC treatment. Similar findings were noted when considering the change in SWT from baseline to endpoint (Figure 3_E). The number of myocardial segments that had final SWT <50% of baseline was also determined, as this is a validated surrogate marker for infarcted myocardium [16]. The MSC group had fewer infarcted segments compared to controls (Figure 3_F). The number of dysfunctional segments was lowest in animals receiving MSC in the infarct territory, remote LV myocardium and in all segments overall (3.8 ±0.3 vs 6.5±0.4, $p<0.0001$).

3.4.6. Myocardial Fibrosis

Extensive myocardial fibrosis was documented in the control cohort with 28.6 ± 3.3% of the LV free wall staining positive for Masson's trichrome (Figure 4). In contrast, fibrosis was significantly reduced in the MSC group by almost half (14.8±2.4%, $p<0.01$).

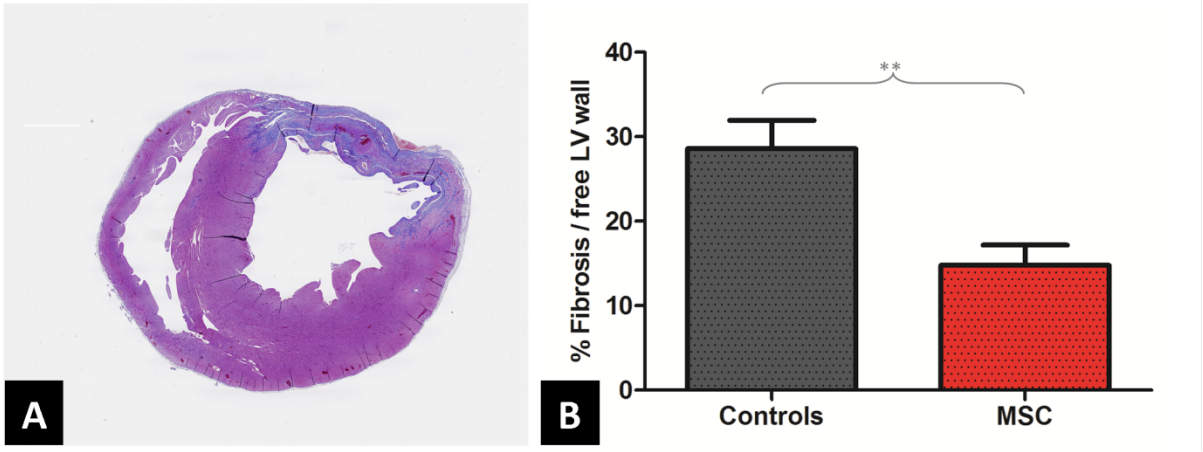

Figure 4: Myocardial Fibrosis. (A) Representative example of myocardial fibrosis staining in infarcted myocardium. (B) % Fibrosis of total LV myocardium.

3.4.7. Arteriolar Density

Arteriolar density was assessed histologically by staining for αSMA$^+$ vessels in the infarct zone (IZ), border zone (BZ) and remote LV myocardium (Figure 5). In the IZ, the MSC group displayed greater arteriolar density than control animals (4.5±0.7 /0.2mm^2 vs 0.9±0.4 /0.2mm^2, p<0.0001). Arteriolar density was also higher MSC recipients in the BZ (19.8±1.9 vs 8.6±1.2, p<0.0001) and remote LV (15.4±1.4 vs 8.9±0.9, p<0.001).

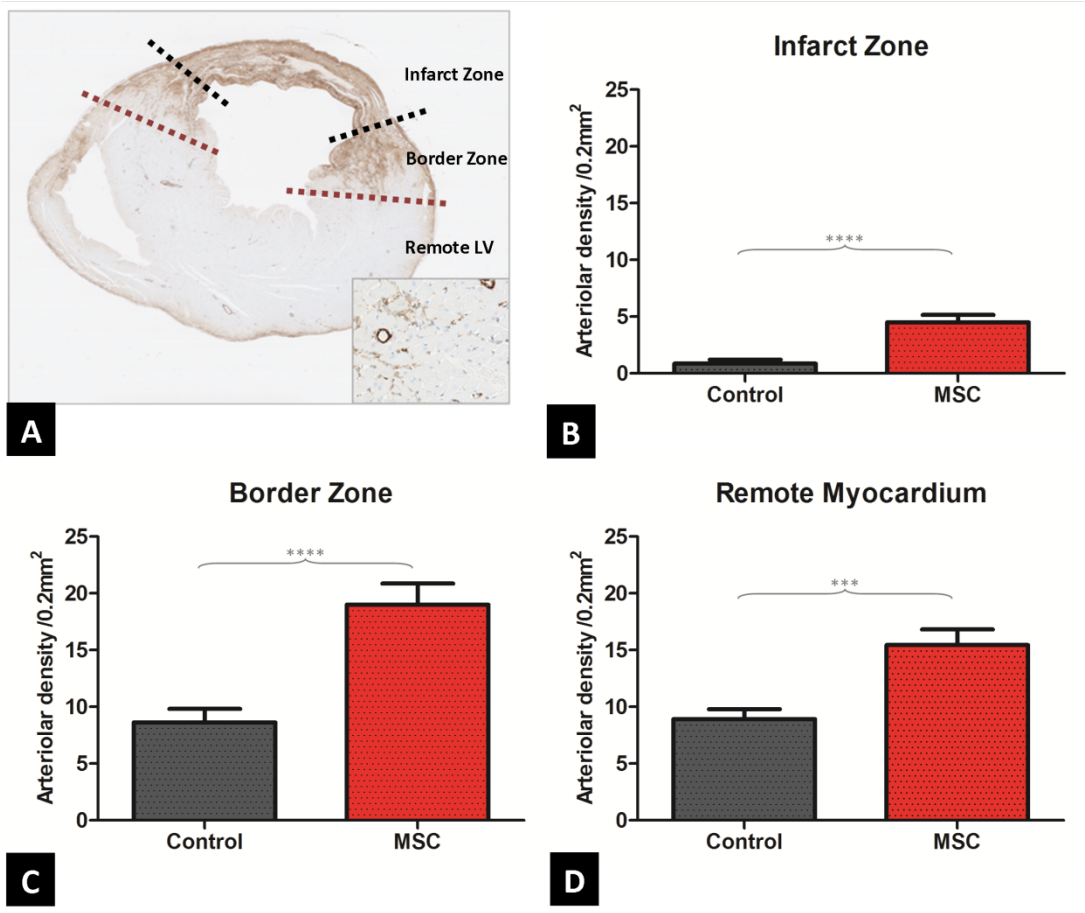

Figure 5: Arteriolar Density. (A) Representative example displaying typical staining patterns and delineation of LV myocardium into infarct zone, border zone of infarction and remote (non-infarcted) myocardium. (B) Arteriolar density in the infarct zone, border zone (C) and Remote myocardium (D).

3.5. DISCUSSION

The principal findings of this study are that: (1) Prospectively isolated MSC enriched via an initial immunodepletion step have superior in-vitro differentiation and colony forming capacity than PA-MSC; (2) Hypoxic conditioning during primary culture incrementally enhances these in vitro parameters; (3) MSC optimised by the combination of prospective isolation and hypoxic conditioning have significant reparative capacity in-vivo.

3.5.1. Need for optimisation

A paradigm has emerged in which MSCs are thought to provide cardiovascular reparative properties through indirect, paracrine effects, such as: (1) trophic (anti-apoptotic; supportive of proliferation or differentiation of endogenous cells; pro-angiogenic), (2) immunomodulatory, (3) anti-fibrotic or (4) chemoattractant. However, the potential application of MSCs to mainstream cardiovascular therapy is currently hindered by several important limitations, including suboptimal retention and engraftment after transplantation into the diseased heart [5]. Therefore, strategies to enhance their paracrine effects in an effort to improve retention, engraftment and efficacy are actively been explored. These include cell-targeted techniques relating to their preparation and pre-conditioning via in vitro modification of MSCs, augmentation of paracrine factors, activation of cytoprotective pathways and directing MSC cardiopoiesis [5].

3.5.2. Rationale for prospective isolation

The traditional method of plastic adherence isolation and expansion which was first described in the 1970s [9] suffers from a degree of non-specificity, yielding immature and mature mesenchymal subpopulations with diverse clonogenic, proliferative and differentiative potential. As few as one-third of PA-MSC are pluripotent [17] and only 30% of in vitro derived MSC demonstrate trilineage differentiation potential, with the remainder only displaying bi-lineage or uni-lineage potential [18]. Therefore enriching for primitive mesenchymal precursors could increase the number of pluripotent cells and therefore

overall differentitation capacity of a given MSC population. In this study we employed a negative immunoselection, involving up-front depletion of cells expressing hematopoietic markers (CD45 in this case) to prepare a more homogeneous mesenchymal cell population. Despite sharing an identical immunophentypic profile with PA-MSC at passage 4, significant differences in differentiation capacity and colony forming efficiency were observed. A near 6-fold increase in adipogenesis and an almost 5-fold increase in osteogenesis were observed with prospectively isolated, hypoxic conditioned MSC compared to same donor PA-MSC. However, no significant difference in chondrogenic differentiation capacity was observed. This data suggests that the optimisation strategies employed in this study identify an MSC population with enhanced adipogenic and osteogenic differentiation capacity. These lineages are known to share the upregulated expression of 235 genes, whereas only 3 and 10 genes are shared between chondro/ osteogenic and chondro/adipogenic differentiation respectively [19].

We utilised an immunodepletion step for enrichment, however a more conventional strategy of prospective immunoselection entails using specific monoclonal antibodies to positively select and enrich for MPCs with the greatest clonogenicity and differentiative potential. Examples include: stromal precursor antigen-1 (STRO-1) [20], CD49a/CD29 [21], CD106 [22], CD146 [23], low-affinity nerve growth factor receptor [24], platelet-derived growth factor receptor [25, 26], epidermal growth factor receptor [25], insulin-like growth factor receptor [25], non-tissue specific alkaline phosphatase (STRO-3) [27] and heat shock protein-90 (STRO-4) [28]. Allogeneic human STRO-3 MPCs, produced and marketed as the "off-the-shelf" product Revascor™ (Mesoblast Ltd), are under evaluation in patients with heart failure [NCT00721045] and recent MI [Allogeneic Mesenchymal Precursor Cell Infusion in MyoCardial Infarction (AMICI) study NCT01781390].

3.5.3. Rationale for hypoxic conditioning

Initial retention of transplanted MSCs is extremely poor, with as many as 99% of MSC dead within 3-4 days of cell delivery [29]. This is perhaps unsurprising given the hazardous myocardial microenvironment in to which they are delivered, including the extremely low

oxygen tension encountered. However MSC in the bone marrow are routinely subjected to low oxygen levels, potentially as low as 1-2% [30], suggesting MSC should be an ideal candidate cell for regenerative purposes. However, conventional approaches to cell culture involve culture and expansion in atmospheric oxygen (21%), which is at odds with the physiological conditions experienced *in vivo*. Hypoxia-preconditioned MSC have been shown to produce increased quantities of pro-survival transcription and growth factors, including vascular endothelial growth factor, hypoxia induced factor-1α, surviving, B-cell lymphoma-2 and *akt* upregulation compared with their preparation under normoxia [7]. Increased cell proliferation is also observed, though the mechanism explaining this in unresolved. Interestingly, the differentiation potential of MSC expanded under hypoxic conditions does not appear to be adversely affected [31]. Previous studies have shown mixed effects of hypoxic conditioning on osteogenic differentiation, varying with the degree, timing and duration of hypoxia [32]. Few studies have evaluated chondrogenic differentiation and have shown minimal impact of hypoxia, whereas several studies have demonstrated positive impact of hypoxia on adipogenic differentiation [32]. Our study showed even brief exposure to hypoxia during primary culture conferred significant improvements in both osteogenic and adipogenic differentiation, but no significant difference on chondrogenic differentiation. Recruitment and homing of MSC to ischaemic tissue may also be enhanced, with previous studies demonstrating increased SDF-1 production and CXCR4 expression [33]. Therefore, hypoxic pre-conditioning appears to convey multiple desirable effects including enhanced: cell survival, proliferation, differentiation capacity, paracrine augmentation and recruitment and homing.

3.5.4. Combining the two optimisation strategies

In this study we combined the two optimisation strategies to assess whether hypoxic pre conditioning adds incremental benefits to a prospective isolation technique and to evaluate the *in vivo* impact of these MSC in an infarct model of MI. The *in vitro* data shows significant improvements in differentiation capacity and colony forming efficiency compared to PA-MSC. The in vivo data shows that optimised-MSC increased EF by 19.3±3.1% ($p<0.001$) at 4 weeks compared to controls. This primary finding was

accompanied by improvements in LV volumes, mass, wall thickness, systolic wall thickening, myocardial fibrosis and arteriolar density. This degree of improvement in ventricular function is substantially higher than that frequently observed with PA-MSC in individual experiments [34, 35] or in meta-analysis of pre-clinical studies [36], where improvements in the order of 10% have been observed. The findings are comparable to outcomes noted in several other studies with optimised MSC, for example via overexpression of akt [37], SDF-1 [38] or VEGF [39] in rodents.

3.5.5. Clinical relevance

Many of the alternative methods to optimise stem cells referred to above target one particular facet – e.g. overexpression of a single (albeit important) gene/chemokine/growth factor, sometimes relying on retroviral manipulation of genes. It is clear that the proposed benefits of MSC therapy are multifactorial and complex, therefore targeting one aspect of MSC function may not be the ultimate solution. In comparison, both strategies employed in this study - prospective immunoselection and hypoxic conditioning – have a broader influence on the wide repertoire of MSC functions considered relevant. Furthermore, both strategies are relatively straightforward to perform and lack the potential controversy that accompanies gene modification, making these strategies immediately relevant and readily transferable to clinical practice.

3.5.6. Limitations

We opted to use a permanent ligation model of LAD occlusion, as it provides large infarcts with relatively high reproducibility of infarct size (as shown in the control group here, though this remains operator-dependant). Although ischaemia-reperfusion injury would more closely reflect the myocardial pathology encountered after patients with MI receive reperfusion treatment, this model is prone to causing infarcts of smaller and more variable size, potentially limiting the scope of therapeutic effect and complicating sample size and study power calculations.

3.6. CONCLUSIONS

Allogeneic MSC, optimised by prospective isolation and hypoxic preconditioning, exhibit superior in vitro differentiation capacity and colony forming efficiency compared to conventional plastic adherent MSC. Furthermore, they significantly attenuate LV dysfunction in rats following MI, with improvements in LV volumes, mass, wall thickness, systolic wall thickening, myocardial fibrosis and arteriolar density. These optimisation strategies, when used in combination, offer significant potential to improve therapeutic efficacy of stem cell therapy.

3.6.6.1. Acknowledgements

We thank Mr Tom Sullivan (University of Adelaide) for support with statistics. JDR is supported by an International Postgraduate Research Scholarship at the University of Adelaide, Australia. PJP has received funding from the National Health and Medical Research Council of Australia and the Royal Australasian College of Physicians. DW is supported by the National Health and Medical Research Council and Australian National Heart Foundation postgraduate scholarship.

3.6.2.Reference

1. Lipinski MJ, Biondi-Zoccai GGL, Abbate A, Khianey R, Sheiban I, Bartunek J, Vanderheyden M, Kim H-S, Kang H-J, Strauer BE, Vetrovec GW. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: A collaborative systematic review and meta-analysis of controlled clinical trials. *Journal of the American College of Cardiology*. 2007;50:1761-1767
2. Hacia JG, Lee CC, Jimenez DF, Karaman MW, Ho VV, Siegmund KD, Tarantal AF. Age-related gene expression profiles of rhesus monkey bone marrow-derived mesenchymal stem cells. *J Cell Biochem*. 2008;103:1198-1210
3. Zhang H, Fazel S, Tian H, Mickle DA, Weisel RD, Fujii T, Li RK. Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle myocardial cell therapy. *Am J Physiol Heart Circ Physiol*. 2005;289:H2089-2096
4. Khan M, Mohsin S, Khan SN, Riazuddin S. Repair of senescent myocardium by mesenchymal stem cells is dependent on the age of donor mice. *J Cell Mol Med*. 2011;15:1515-1527
5. Richardson J, Nelson A, Zannettino AW, Gronthos S, Worthley S, Psaltis P. Optimization of the cardiovascular therapeutic properties of mesenchymal stromal/stem cells–taking the next step. *Stem Cell Reviews and Reports*. 2012:1-22
6. Psaltis PJ, Paton S, See F, Arthur A, Martin S, Itescu S, Worthley SG, Gronthos S, Zannettino AC. Enrichment for stro-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. *J Cell Physiol*. 2010;223:530-540
7. Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang J-A, Wei L. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *The Journal of Thoracic and Cardiovascular Surgery*. 2008;135:799-808
8. Martens TP, See F, Schuster MD, Sondermeijer HP, Hefti MM, Zannettino A, Gronthos S, Seki T, Itescu S. Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. *Nat Clin Pract Cardiovasc Med*. 2006;3 Suppl 1:S18-22
9. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Proliferation*. 1970;3:393-403
10. Psaltis PJ, Zannettino ACW, Worthley SG, Gronthos S. Concise review: Mesenchymal stromal cells: Potential for cardiovascular repair. *STEM CELLS*. 2008;26:2201-2210
11. See F, Seki T, Psaltis PJ, Sondermeijer HP, Gronthos S, Zannettino AC, Govaert KM, Schuster MD, Kurlansky PA, Kelly DJ, Krum H, Itescu S. Therapeutic effects of human stro-3-selected mesenchymal precursor cells and their soluble factors in experimental myocardial ischemia. *J Cell Mol Med*. 2010
12. Richardson JD, Bertaso AG, Frost L, Psaltis PJ, Carbone A, Koschade B, Wong DT, Nelson AJ, Paton S, Williams K, Azarisman S, Worthley MI, Teo KS, Gronthos S, Zannettino AC, Worthley SG. Cardiac magnetic resonance, transthoracic and transoesophageal echocardiography: A comparison of in vivo assessment of ventricular function in rats. *Lab Anim*. 2013
13. Richardson JD, Bertaso AG, Psaltis PJ, Frost L, Carbone A, Paton S, Nelson AJ, Wong DT, Worthley MI, Gronthos S, Zannettino AC, Worthley SG. Impact of timing and dose of mesenchymal stromal cell therapy in a preclinical model of acute myocardial infarction. *J Card Fail*. 2013;19:342-353
14. Psaltis PJ, Carbone A, Nelson AJ, Lau DH, Jantzen T, Manavis J, Williams K, Itescu S, Sanders P, Gronthos S, Zannettino AC, Worthley SG. Reparative effects of allogeneic mesenchymal precursor cells delivered transendocardially in experimental nonischemic cardiomyopathy. *JACC Cardiovasc Interv*. 2010;3:974-983
15. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy*. 2006;8:315-317
16. Nowosielski M, Schocke M, Mayr A, Pedarnig K, Klug G, Kohler A, Bartel T, Muller S, Trieb T, Pachinger O, Metzler B. Comparison of wall thickening and ejection fraction by cardiovascular magnetic resonance and echocardiography in acute myocardial infarction. *Journal of Cardiovascular Magnetic Resonance*. 2009;11:22
17. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143 - 147
18. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci*. 2000;113 (Pt 7):1161-1166
19. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: Characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med*. 2004;8:301-316
20. Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone

marrow by a novel monoclonal antibody, stro-1. *Blood*. 1991;78:55-62
21. Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. *Bone*. 2001;28:174-181
22. Simmons PJ, Masinovsky B, Longenecker BM, Berenson R, Torok-Storb B, Gallatin WM. Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. *Blood*. 1992;80:388-395
23. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell*. 2007;131:324-336
24. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol*. 2002;30:783-791
25. Gronthos S, Simmons PJ. The growth factor requirements of stro-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. *Blood*. 1995;85:929-940
26. Schwab KE, Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. *Human Reproduction*. 2007;22:2903-2911
27. Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC. A novel monoclonal antibody (stro-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. *Stem Cells Dev*. 2007;16:953-963
28. Gronthos S, McCarty R, Mrozik K, Fitter S, Paton S, Menicanin D, Itescu S, Bartold PM, Xian C, Zannettino AC. Heat shock protein-90 beta is expressed at the surface of multipotential mesenchymal precursor cells: Generation of a novel monoclonal antibody, stro-4, with specificity for mesenchymal precursor cells from human and ovine tissues. *Stem Cells Dev*. 2009;18:1253-1262
29. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93-98
30. Cipolleschi MG, Dello Sbarba P, Olivotto M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood*. 1993;82:2031-2037
31. Grayson WL, Zhao F, Bunnell B, Ma T. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun*. 2007;358:948-953
32. Das R, Jahr H, van Osch GJ, Farrell E. The role of hypoxia in bone marrow-derived mesenchymal stem cells: Considerations for regenerative medicine approaches. *Tissue Eng Part B Rev*. 2010;16:159-168
33. Wang Y, Deng Y, Zhou GQ. Sdf-1alpha/cxcr4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. *Brain Res*. 2008;1195:104-112
34. Dai W, Hale SL, Martin BJ, Kuang J-Q, Dow JS, Wold LE, Kloner RA. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium. *Circulation*. 2005;112:214-223
35. Hou M, Yang K-m, Zhang H, Zhu W-Q, Duan F-j, Wang H, Song Y-h, Wei Y-j, Hu S-s. Transplantation of mesenchymal stem cells from human bone marrow improves damaged heart function in rats. *International Journal of Cardiology*. 2007;115:220-228
36. van der Spoel TIG, Jansen of Lorkeers SJ, Agostoni P, van Belle E, Gyöngyösi M, Sluijter JPG, Cramer MJ, Doevendans PA, Chamuleau SAJ. Human relevance of pre-clinical studies in stem cell therapy: Systematic review and meta-analysis of large animal models of ischaemic heart disease. *Cardiovascular Research*. 2011;91:649-658
37. Gnecchi M, He H, Melo LG, Noiseaux N, Morello F, de Boer RA, Zhang L, Pratt RE, Dzau VJ, Ingwall JS. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing akt on cardiac metabolism after myocardial infarction. *Stem Cells*. 2009;27:971-979
38. Dong F, Harvey J, Finan A, Weber K, Agarwal U, Penn MS. Myocardial cxcr4 expression is required for mesenchymal stem cell mediated repair following acute myocardial infarction / clinical perspective. *Circulation*. 2012;126:314-324
39. Kim SH, Moon H-H, Kim HA, Hwang K-C, Lee M, Choi D. Hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cells prevent myocardial ischemic injury. *Mol Ther*. 2011;19:741-750

Statement of Authorship

Title of Paper	**Prospectively-Isolated Hypoxic-Conditioned Mesenchymal Stem Cells Have Superior in vitro Capacity and Significantly Attenuate Myocardial Infarction-Induced Ventricular Dysfunction.**
Publication Status	Publication Style

Author Contributions

Name of Principal Author (Candidate)	**Dr James David Richardson**
Contribution to the Paper	Primary contributor to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work; Drafted the work ; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mrs Sharon Paton**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Angelo Carbone**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and

	revising it critically; Provided final approval of the version to be published; agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Lachlan Frost**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Peter J Psaltis**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Professor Andrew Zannettino**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the

	version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013
Name of Co-Author	**Professor Stan Gronthos**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Professor Stephen G Worthley**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013

4. Chapter 4 - Timing of MSC Intervention

Impact of Timing and Dose of Mesenchymal Stromal Cell Therapy in a Preclinical Model of Acute Myocardial Infarction

James D Richardson[1,2], Angela G Bertaso[1], Peter J Psaltis[1,3], Lachlan Frost[1], Angelo Carbone[1], Sharon Paton[2], Adam J Nelson[1], Dennis TL Wong[1], Matthew I Worthley[1], Stan Gronthos[2]#, Andrew CW Zannettino[2]#, Stephen G Worthley[1,2]#

1 Cardiovascular Research Centre, Royal Adelaide Hospital and Department of Medicine, University of Adelaide, South Australia, Australia.

2 Centre for Stem Cell Research, Robinson Institute, School of Medical Sciences, University of Adelaide, South Australia, Australia

3 Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, USA

joint senior authors

4.1. ABSTRACT

Introduction: Although mesenchymal stem/stromal cells (MSC) have shown therapeutic promise after myocardial infarction (MI), the impact of cell dose and timing of intervention remains uncertain. Here, we compared immediate and deferred administration of two doses of MSC in a rat model of MI.

Methods: Sprague-Dawley rats were used. Allogeneic, prospectively isolated MSC (dose: 'low' 1x106 or 'high' 2x106 cells) were delivered by transepicardial injection, immediately after MI ('Early-low', 'Early-high'), or one week later ('Late-low', 'Late-high'). Controls received cryopreservant solution alone. Left ventricular dimensions and ejection fraction (EF) were assessed by cardiac magnetic resonance.

Results: All four MSC-treatment cohorts demonstrated higher EF than control animals four weeks after MI ($p<0.01$ to $p<0.0001$), with function most preserved in the early-high group (absolute reduction in EF from baseline: control 39.1±1.7%, early-low 26.5±3.2%, early-high 7.9±2.6%, late-low 19.6±3.5%, late-high 17.9±4.0%). Cell treatment also attenuated left ventricular dilatation and fibrosis and augmented left ventricular mass, systolic wall thickening (SWT) and microvascular density. Although early intervention selectively increased SWT and vascular density in the infarct territory, delayed treatment caused greater benefit in remote (non-infarct) myocardium. All outcomes demonstrated dose-dependence for early MSC treatment, but not for later cell administration.

Conclusion: The nature and magnitude of benefit from MSC after acute MI is strongly influenced by timing of cell delivery, with dose-dependence most evident for early intervention. These novel insights have potential implications for cell therapy after MI in human patients.

Key Words: Mesenchymal stem cells; Timing; Repair: Myocardial Infarction; Cardiac magnetic resonance; Prospective isolation; Hypoxic conditioning; Optimisation.

4.2. Introduction

There is a compelling need for novel therapies for the treatment of left ventricular dysfunction after acute myocardial infarction (MI). Treatment with mesenchymal stromal cells (MSC) has demonstrated significant promise in pre-clinical experiments, but only modest benefits were observed with cell therapy in clinical trials [1]. Potential explanations include the type of stem cells used, an absence of optimisation strategies and importantly, the dose of cells delivered and the timing of intervention after MI.

Pre-clinical studies have utilised allogeneic plastic-adherent MSC (exhibiting characteristic immunophenotype and trilineage plasticity) whereas clinical trials have largely been limited to the use of autologous bone marrow (BM) cells comprising diverse cell populations. MSC have a relatively immunoprivileged phenotype that permits their allogeneic use (2), therefore offering the prospect of cell optimisation and immediate off-the-shelf therapy. Of the optimisation strategies currently evaluated (3] prospective isolation and hypoxic conditioning approaches have been shown to enhance their in vitro [4] and in vivo efficacy [5, 6] and could be readily applied to clinical use.

The optimal timing for cell intervention is uncertain, as this question remains largely untested. Early intervention might offer greater scope for preservation of function, however at this early stage the myocardium is inflamed, potentially limiting cell retention and engraftment [7]. In contrast, deferred therapy may afford greater opportunity for cells to engraft and mediate regenerative benefits, but by this time adverse remodelling and scar formation is underway [8, 9]. Pre-clinical research has commonly delivered cells either immediately or approximately one week after MI, with each interval proving effective [10]. A recent meta-analysis demonstrated a trend to greater efficacy in the deferred

treatment of large animals [10], although cell therapy 2-3 weeks post MI has been shown to be ineffective in humans [11]. A direct comparison of these two discrete time points is largely untested [12] and the mechanisms underlying MSC treatment at these differing times remains uncertain.

Furthermore, the optimal dose to be delivered is undefined with previous studies using doses in the order of 106 plastic adherent MSCs in rodent models [13]. The relationship between dose and efficacy is non-linear, where there is evidence of a plateau beyond which higher doses are ineffective and potentially detrimental [14]. However the optimal dose, with selected optimised cells at these differing but clinically relevant time points, has not been addressed. Therefore we performed a direct comparison of allogeneic rat MSC, optimised by prospective immunoselection and hypoxic conditioning, and transplanted either immediately or one week after MI, at low or high dose, to identify the optimal strategy.

4.3. Methods

4.3.1. Study protocol

This study was approved by the Animal Ethics Committees of the Central Adelaide Local Health Network (No. 104/10) and the University of Adelaide (No. M-2010-105), South Australia. Animal handling was carried out in accordance with the guidelines outlined in the "Position of the American Heart Association on Research Animal Use" (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD).

Male Sprague-Dawley rats (n=60, baseline weight 350-370g) were used in the experimental protocol. Animals were randomised at baseline to one of five investigation arms: 'Early-low dose' (E1), 'early-high' dose (E2), 'late-low dose' (L1), 'late-high dose' (L2) or controls (Figure 1). Baseline evaluation of ejection fraction (EF) was

undertaken using cardiac magnetic resonance (CMR), followed by thoracotomy and permanent surgical ligation of the left anterior descending artery. Allogeneic MSC or control injections (of ProFreezeTM (PF) cryopreservation vehicle) were then delivered transepicardially immediately after MI or at repeat thoracotomy one week later according to investigation arm. Reassessment of EF with CMR was conducted at 1, 2 and 4 weeks after MI. Euthanasia was performed at week 4 after final imaging for tissue harvesting and histological evaluation.

4.3.2. Prospective Isolation of Rat MSC

Six donor male Sprague Dawley rats were euthanized (by CO2 inhalation) and the femora and tibiae excised, the epiphyses removed and bones flushed. The resultant cell suspension was centrifuged, supernatant removed and the cell pellet resuspended for density gradient separation. The long bones, now BM deplete, were crushed into small fragments using sterile scissors and washed. Digestion of the bone fragments, using Collagenase Type I and 0.2% DNAse solution in PBS, was performed on a shaking platform for 45 min at 37°C. The supernatant containing compact bone (CB) cells was collected and processed for mononuclear cells (MNC) by centrifugation and strained. Magnetic activated cell sorting (MACS) was used to deplete any contaminating CD45-positive haematopoietic cells from the CB-derived MNC population. Briefly, CB-MNC were pelleted and blocked, prior to incubation with purified Anti-Rat CD45 10ug/ml (BD Biosciences, San Diego, CA, USA). After repeated washes in HBSS 10%FCS, secondary incubation was performed with goat anti-mouse IgG-Biotin 10ug/ml (Southern Biotec, Birmingham, AL, USA) followed by two washes in MACS Buffer (5mM EDTA, 1% BSA, 0.01%NaN3 in PBS) and incubation with Anti-Biotin Microbeads (MiltenyiBiotec, BegischGladbach, Germany). The cell suspension was then passed through a MACS MS Separation Column (MiltenyiBiotec, BegischGladbach, Germany). The CD45-negative fraction was collected as the target cell population harbouring the MSC, to establish primary cultures.

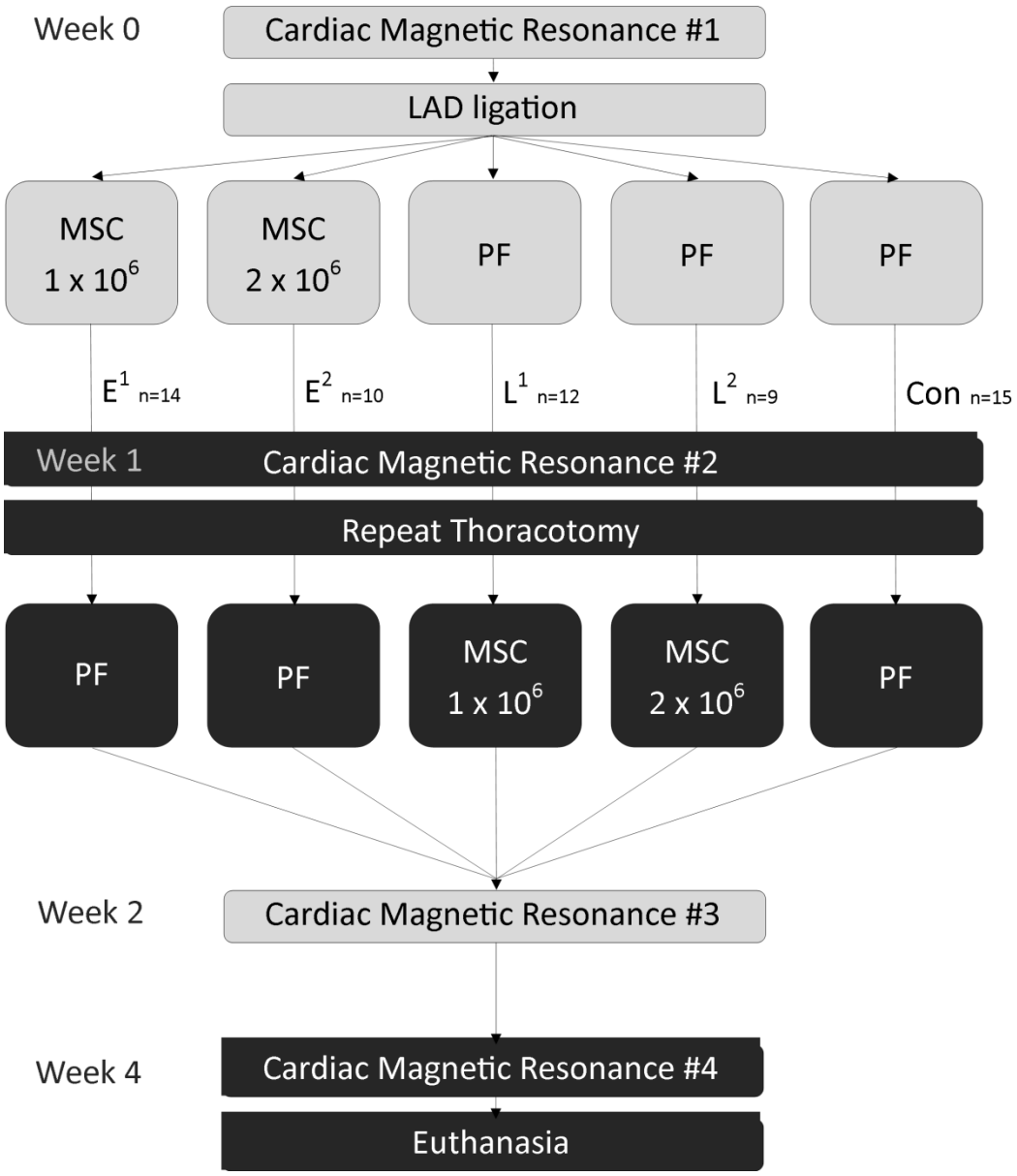

Fig.1. Study protocol: Flowchart illustrating the imaging and MSC interventions delivered over the 4 week study duration.

4.3.3. Hypoxic conditioning

CD45-negative cells were initially seeded in plastic culture flasks at 3x104/cm2 in alpha Modification of Eagle's Medium (supplemented with 20% (v/v) FCS, 2mM L-Glutamine, 1mM sodium pyruvate, 100μM L-ascorbate-2-phosphate and penicillin/streptomycin sulphate) in a humidified hypoxic chamber in the presence of 5% O2, and 10% CO2. After reaching 80% confluence, cells were detached by Trypsin/EDTA treatment and subcultured in αMEM, supplemented with 10% (v/v) FCS, 2mM L-Glutamine, 1mM sodium pyruvate, 100μM L-ascorbate-2-phosphate and penicillin /streptomycin under normoxic conditions (21% O2, 5% CO2). Hypoxic conditioning was undertaken only during primary culture and all subsequent cell culture was performed under normoxic conditions. At passage 4 (P4), MSC were cryopreserved in ProFreezeTM (LONZA BioWhittaker Walkersville, MD, USA) with 7.5% Dimethyl Sulfoxide at a concentration of 1 or $2x10^6$/100μl. The MSC doses used in this study were determined on the basis of previous human clinical trials which delivered approximately 1-$2x10^8$ cells to a heart consisting of $1.4x10^{10}$ myocardial cells (i.e.1-2% of total cells) [15]. Therefore, as rat hearts are comprised of an estimated $9x10^7$ cells [15], an equivalent dose was determined to be 1-$2x10^6$ allogeneic MSC.

4.3.4. Green Florescent Protein-labelled MSC

An additional 7 rats were injected with Green Florescent Protein (GFP)-labelled MSC to gain insights into the distribution of MSC according to timing of cell delivery. MSC were eGFP labelled by retroviral transduction with the eGFP reporter vector pRUFiG2 . Briefly, pRUFiG2 and packaging constructs (pGP and pVSVG) were introduced into HEK-293T cells using Lipofectamine 2000 (Invitrogen). Viral supernatant was collected 48 hrs post transfection, 0.45μm filtered (low protein binding;Thermo Scientific) then added to rat MSCs plated at 2.5 x104/cm2 in 10cm dishes. Transduced eGFP+ cells were isolated by flourescence activated cell sorting and expanded. Animals underwent permanent LAD ligation as described in detail below and received GFP-MSC either immediately (early n=4) or one week after MI (late n=3). One week after GFP-MSC transplantation, rats were euthanized (as per the main cohort below) and anti-GFP immunohistochemistry staining performed on three left ventricular (LV) sections per animal corresponding to base, mid

and apex. Namely, paraffin-embedded sections were de-waxed, quenched in endogenous peroxidase, washed and blocked with 5% normal goat serum and 3% horse serum. Primary antibody (rabbit α-GFP, 2 mg/mL, Invitrogen) and normal rabbit IgG were applied overnight, washed and secondary antibody (Goat α-Rabbit Biotin) applied. Sections were washed, incubated in Streptavidin (in the dark), washed and DAB peroxidise colour substrate solution applied. NanoZoomer photomicrographs were exported to Image Pro Plus software (v5.1, Media Cybernetics, USA) and the number of GFP positive cells/high power field were quantified in the infarct zone, border zone and remote LV myocardium.

4.3.5. Cardiac Magnetic Resonance Imaging

Cardiac magnetic resonance (CMR) was performed on rats at baseline prior to MI surgery and also at 1, 2 and 4 weeks post-MI using a 1.5T MR system (Magnetom Sonata, Siemens Medical Solutions, Erlangen, Germany), equipped with a dedicated cardiac software package. General anaesthesia was induced and maintained with inhalation of 1% isoflurane by nose cone. All CMR images were obtained as free breathing, electrocardiogram-gated, acquisitions utilising a carotid radiofrequency receiver coil. Transverse and coronal localiser images were acquired followed by long (4-chamber and 2-chamber) and short axis pilot images, from which a true short axis stack was prescribed. Steady-state free precession cine images (gated to alternate R waves) were acquired. The stack comprised three contiguous LV slices (each 3mm thick, with no intersection gap) providing almost complete coverage of the LV. The image matrix was 384 x 384, field of view 185 mm, repetition time 14.72ms, echo time 1.55ms, flip angle 90°, and 20 heart phases were acquired (8 per cardiac cycle).

4.3.6. CMR image analysis

Left ventricular mass, volumes and derived EF were measured off-line from cine images using commercially available software (QMass v7.2, Medis, Netherlands). The end-diastolic and end-systolic cine frames were identified for each slice and the endocardial and epicardial borders were manually traced. The end-diastolic (EDV) and end-systolic

volumes (ESV) were calculated using the true disk summation technique (i.e. sum of cavity volumes across all continuous slices), and EF calculated, as previously described [16]. Regional segmental function was analysed in each of 16 segments – 6 basal, 6 mid and 4 apical [17]. Using the same software, a reference point was placed at the right ventricular insertion point in the anterior septum for each LV slice to assign myocardial segmentation. Left ventricular end-diastolic myocardial wall thickness and systolic wall thickening (SWT) measurements were automatically calculated.

4.3.7. Infarct surgery and MSC transplantation

Anaesthesia was induced using 3% isoflurane, animals intubated and connected to a small animal ventilator (Harvard Apparatus, Holliston, MA, USA). Animals were mechanically ventilated (tidal volume 3.6mL, ventilatory rate 60 breaths/min) with 1-1.5% isoflurane and 2L/min O2. A left anterior thoracotomy was performed, the pericardium excised and the left anterior descending artery was permanently ligated, as previously described [13]. Infarction was confirmed by the development of pallor and hypo-/akinesis of the anteroapical myocardium. Rats were randomised to receive either transepicardial injections of MSC or ProFreeze (PF) in four separate injection sites in the anterior myocardial wall border zone at a total volume of 100μL via a 30-gauge needle. In the immediate-MSC treatment groups, MSC were delivered in doses of 1x106 (‘early-low’) or 2x106 (‘early-high’). Animals randomised to deferred therapy (‘late-low’ or ‘late-high’) or controls received PF control injections at this juncture. The muscle and skin layers were closed, Isoflurane was discontinued and animals were extubated once spontaneous ventilation resumed. Noracillin (0.15mg/kg i.m.) was given prophylactically, while post-operative analgesia was achieved with a single dose of Ketamine (75mg/kg i.p.) and Carprofen (5mg/kg s/c) repeated over two days.

One week later, all animals underwent CMR and repeat thoracotomy using identical anaesthesia and medication as described above. Once again, all animals received transepicardial injections of MSC or PF into the infarct border zone, depending on initial randomisation. Those in the deferred therapy groups (‘late-low’ and ‘late-high’) received

1x106 or 2x106 MSC respectively, whereas the control and early treatment groups were given PF injections. On all occasions, MSCs were thawed from individual ampoules and underwent cell counts and cell viability (by trypan blue exclusion) immediately prior to injection and were only used if they exhibited >70% viability (release criteria).

.

4.3.8. Histological analysis

Euthanasia was performed at day 28 post-MI under deep anaesthesia (Metedomidine 0.5mg/kg ip and Ketamine 75mg/kg ip). Hearts were excised, drenched in sterile saline and immersed in a mixture of PBS/10% formalin. Myocardium below the occlusion site was later sectioned into three transverse slices providing a basal, mid and apical LV section. After paraffin embedding, replicate 6μm sections were cut and stained by haematoxylin and eosin, Masson's trichrome and immunohistochemistry. Whole slides were photographed (x40 magnification) with the NanoZoomer Digital Photography system (Hamamatsu Photonics, Hamamtsu, Japan) and complete image sets exported for blinded analysis using Image Pro Plus software (v5.1, Media Cybernetics, USA). Cardiac fibrosis was quantified using Masson's trichrome specimens and myocardial vascular density was quantified using staining for α-smooth muscle actin (αSMA - Clone 1A4, IgG2a Imgenex, San Diego CA), as previously described [16]. The number of αSMA positive vessels (10-100μm diameter) was counted in ten random high power fields (x20 magnification), in the infarct zone, border zone and remote LV myocardium respectively.

4.3.9. Statistical analysis

All analyses were performed blind to study group and by two independent observers for CMR parameters. Numerical data are expressed as mean ± standard deviation. In view of repeated measures of the same subjects at different time points, a linear mixed effects model was adopted to compare the effectiveness of therapy between groups. A p-value <0.05 was considered statistically significant. Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

4.4. Results

4.4.1. Characterisation of MSC

The immunophenotype of P4 MSC was evaluated via flow cytometric analysis of cell surface markers as previously described [4]. Rat MSC exhibited very high expression (defined as ≥95% relative to isotype control) of CD105, CD73 and CD44, but negligible expression (defined as ≤2% relative to isotype control) of CD45, CD34 or CD106 consistent with international guidelines [18] (data not shown). Multilineage differentiation capacity was confirmed with adipogenic, osteogenic and chondrogenic formation under standard inductive conditions [4] (data not shown).

4.4.2. Global Left Ventricular Function

In general, animals tolerated administration of MSCs after LAD ligation, well. Deaths occurred intraoperatively or immediately post operation in relation to LAD ligation and were similarly distributed between the different MSC treatment groups. Importantly, no late deaths were observed that could be attributable to arrhythmia induced by MSC therapy. Figure 2 depicts the temporal trends and absolute changes in LV EF over time for each treatment group. There were no significant differences in LV volumes or EF between the five investigation groups at baseline (Table 1). In the three cohorts receiving control injections immediately after LAD ligation, LV EF declined significantly by week 1 after MI, to 22.1±1.9% in controls, 26.1±3.4% in the L1 group and 24.0±2.5% in the L2 cohort ($p<0.0001$ versus baseline EF for each group, with no significant difference between the control and late MSC groups) (Figure 2A). In contrast, recipients of early MSC intervention exhibited an early reduction in EF after infarction was significantly attenuated (week 1 EF: 31.7±3.1% for E1 $p<0.05$ versus control group and 45.3±3.2% for E2 $p<0.0001$ versus control). At week 2 (one week after late MSC delivery), mean EFs significantly improved from their week 1 measurements to 38.2±3.9% ($p<0.05$) and 42.6±5.2% ($p<0.01$) in the L1 and L2 groups respectively. Over the same interval, EFs remained stable in the no-cell (control) recipients and in both early MSC groups. By week

4, all MSC treatment groups maintained significantly higher LV function than controls (Table 1). Notably, in each experimental group, EF was most responsive to change in the first week after intervention, plateauing thereafter for the remainder of follow-up (Figure 2A).

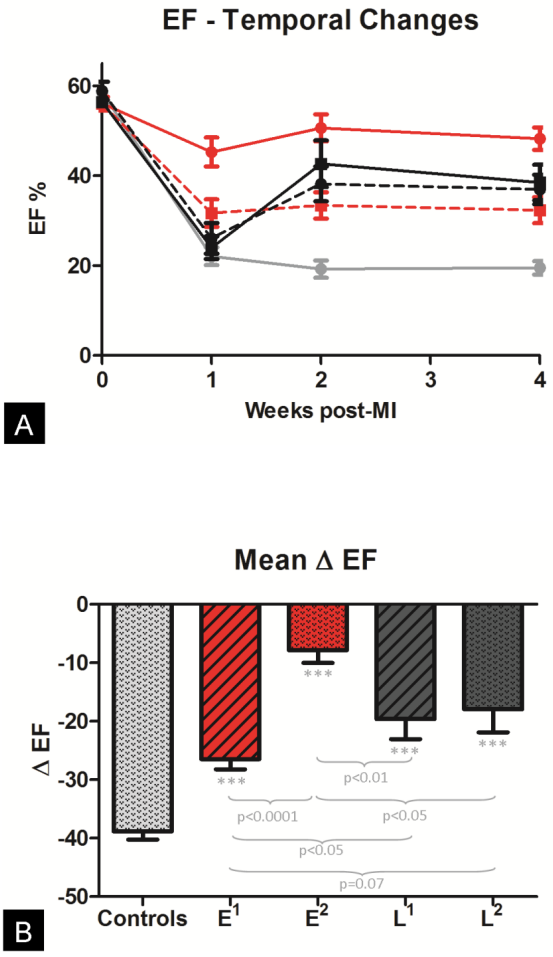

Fig. 2. Ejection fraction: (A) Temporal change in EF of the experimental groups from baseline to 1, 2 and 4 weeks post MI. (B) Absolute mean change in EF between baseline and week 4. ***p<0.001 from controls

Absolute changes in EF over four week follow-up are summarised for all experimental groups in table 1. Although each MSC treatment group experienced attenuation of the deterioration in EF associated with MI in controls, myocardial function was most preserved in early-high dose (E2) cohort, whereas the early-low dose (E1) strategy was the least effective, while both late interventions demonstrated similar intermediate levels of attenuation of LV dysfunction (Figure 2b). Representative end-diastolic and end-systolic CMR images are illustrated in Figure 3.

Experimental Group	Ejection Fraction (%)		
	Baseline	Week 4	ΔEF
E^1	58.8 ±1.5	32.3 ±2.9	26.5±3.2**
E^2	56.1 ±1.5	48.2 ±2.5	7.9±2.6****
L^1	56.5 ±2.0	36.9 ±3.3	19.6±3.5****
L^2	56.4 ±1.0	38.5 ±4.0	17.9±4.0****
Control	58.6 ±1.2	19.5 ±1.5	39.1±1.7

Data presented as mean ± SD. ** p<0.01, **** p<0.0001 to controls.

Table 1: Change in ejection fraction over time.

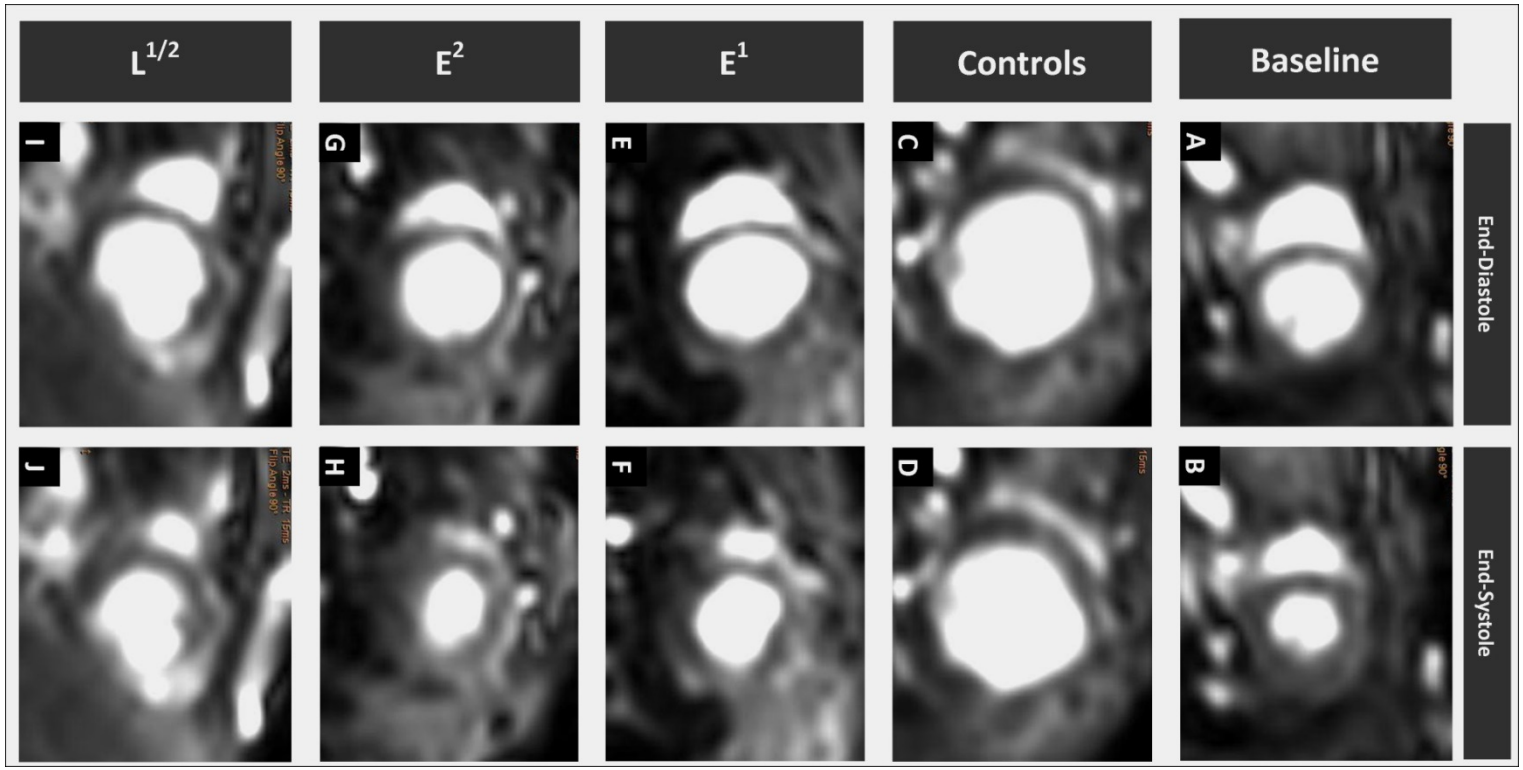

Fig. 3. Cardiac magnetic resonance: Representative examples of the mid LV short axis slice at end-diastole and end-systole. Images illustrate (A, B) normal baseline/day zero findings; (C, D) Control animal at day 28, showing anterolateral akinesia, wall thinning and substantial increase in EDV and ESV; (E, F) Rat in the E1 cohort, with less extensive akinesia, preservation of wall thickness and only moderate increase in EDV/ESV; (G, H) Animal in the E2 group, with mild hypokinesia, preserved wall thickness and only mild increase in EDV and ESV; (I, J) Rat representative of the late groups demonstrating more extensive regional wall motion abnormalities in the infarct territory, associated with wall thinning, but hyper-contractile remote myocardium, and moderate increase in ventricular volumes.

in LV EDV and ESV mirrored the EF findings, with ventricular dilatation at both points in the cardiac cycle substantially assuaged by all four MSC approaches compared to no-cell control ($p<0.05$, to 0.01 versus control, Figure 4a, b). In particular, the early-high dose group had the greatest impact on LV volume change, almost preventing post-MI LV remodelling altogether ($p<0.05$ to 0.01 for comparisons to other MSC groups for ΔEDV and ΔESV). Attenuation of LV dilatation was achieved to a similar extent in the E1, L1 and L2 cohorts.

Global myocardial mass, derived from CMR imaging, was significantly reduced in control animals measured four weeks after MI (Δ Mass = -50.2±19.5 mg $p<0.001$ versus baseline). In contrast, myocardial mass was preserved in all four MSC treatment groups ($p<0.05$ to 0.01 versus control group), without clear superiority for any of the individual timing/ dosing strategies (Figure 4c).

4.4.3. Regional left ventricular morphology and function

Analysis of CMR-derived segmental LV wall thickness (Figure 5a-c) demonstrated preservation of wall thickness in the myocardial segments supplied by the LAD, and globally, in all MSC treatment groups compared with controls (all $p<0.001$). Notably, thickness in the infarcted region was dose-responsive for both early and late intervention. In contrast, only late MSC delivery significantly increased wall thickness in remote LV myocardial segments ($p<0.05$), although this was not dose-dependent.

Overall, systolic wall thickening (SWT) was significantly higher in MSC recipients at end-study compared to controls ($p<0.001$). This effect was most apparent in the myocardial segments supplied by the LAD (SWT: 3.9±2.6% control group, 18.7±2.2% E1, 20.0±4.2%

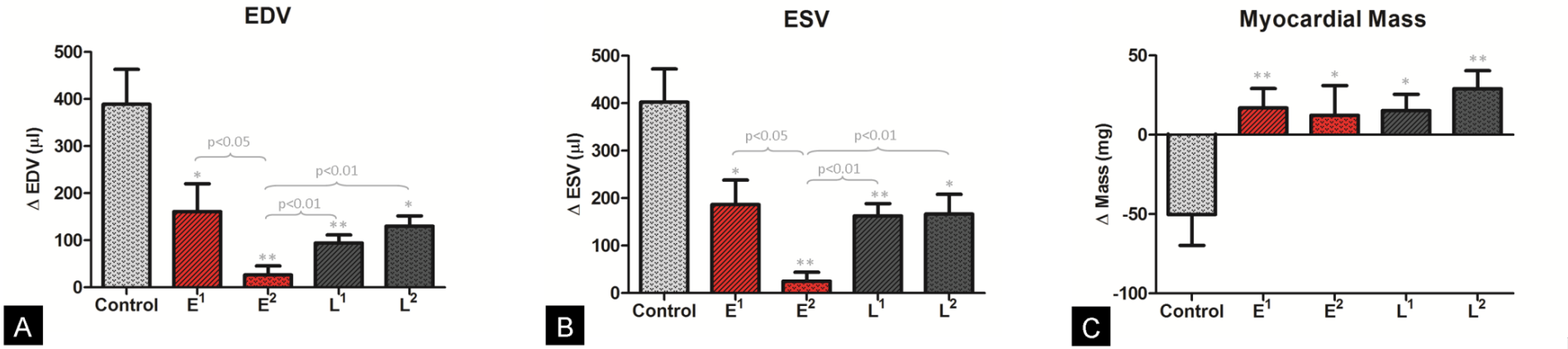

Fig. 4. Left ventricular volumes and mass: (A) Absolute mean change in end-diastolic volume between baseline and week 4 for each experimental group. (B) Same for end-systolic volumes. (C) Mean change in myocardial mass from baseline to week 4. * $p<0.05$, ** $p<0.01$ from controls.

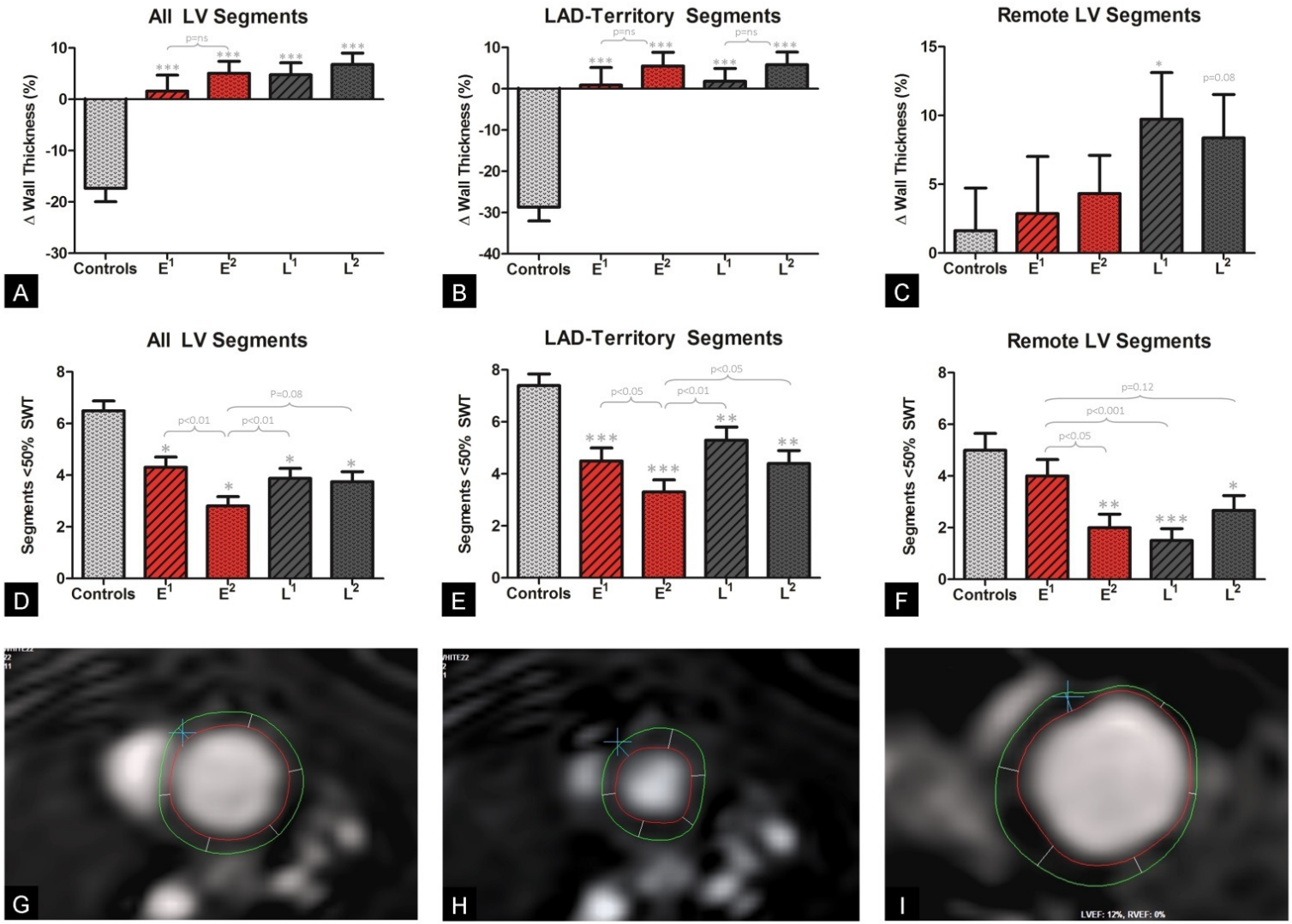

Fig. 5. Wall thickness and systolic wall thickening: Mean change in wall thickness per experimental group in (A) all segments, (B) segments supplied by the LAD and (C) remote LV myocardium. Number of segments with systolic wall thickening (SWT) <50% of baseline SWT (indicating dysfunctional/infarcted segments) in (D) all segments, (E) segments supplied by the LAD and (F) remote LV myocardium. Representative examples of LV contours and segmentation in the mid-LV slice at end-diastole (G) and end-systole (H) in a recipient of E2 MSC showing well preserved wall thickness and wall thickening. (I) End-diastolic equivalent in a control animal showing marked thinning in the infarct territory and significant ventricular dilatation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ from controls.

E2, 19.2±3.2% L1 and 22.3±4.0% L2, $p<0.001$). Although the SWT in the remote LV myocardium was not significantly different, the E2 and late intervention groups did show the trends to increased SWT. The number of myocardial segments that had final SWT <50% of baseline, a validated surrogate CMR parameter for infarcted myocardium [19], was determined. Each MSC group had fewer dysfunctional segments compared to controls ($p<0.0001$) (Figure 5d). The least number of infarcted segments was seen in the E2 group (2.8±0.4) compared to E1 (4.3±0.4, $p<0.01$), L1 (3.9±0.4, $p<0.01$) and L2 (3.8±0.4, $p=0.08$) and control (6.5±0.4, $p<0.0001$). Notably, early MSC intervention had a greater relative impact in reducing dysfunctional segments in the LAD territory, with 3.9±0.3 infarcted segments compared to 4.9±0.4 segments in animals receiving late MSC intervention ($p<0.05$, Figure 5e). In contrast late MSC intervention had a greater impact on remote LV myocardial segments with 2.1±0.4 dysfunctional segments compared to 3.0±0.4 segments in animals receiving early MSC intervention ($p<0.05$, Figure 5f).

4.4.3.1. Myocardial Fibrosis

Extensive myocardial fibrosis was documented in the control cohort with 28.6 ± 3.3% of the LV free wall staining positive for Masson's trichrome (Figure 6). Myocardial fibrosis was significantly reduced in the E2 group (7.6±1.6%, $p<0.001$), L1 (14.5±2.7%, $p<0.01$) and L2 cohort (15.1±4.6%, $p<0.05$). The E1 group demonstrated a trend to reduction in fibrosis (20.2±4.2%, $p=0.12$ versus control). Histologically, we observed no examples of bone or cartilage formation within the myocardium after MSC delivery.

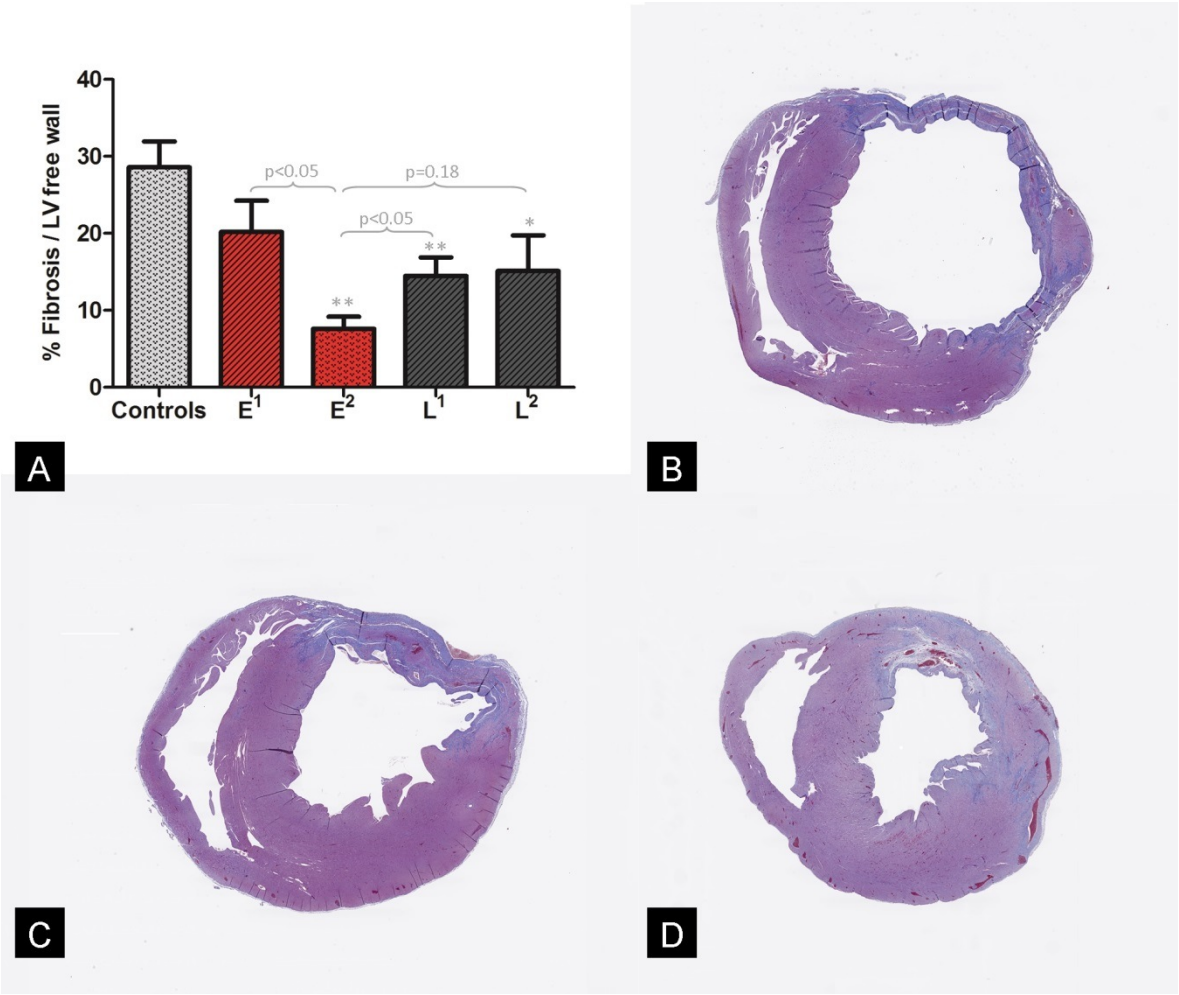

Fig. 6. Myocardial fibrosis: (A) Proportion of the LV free wall occupied by fibrotic tissue in each experimental group. Representative Masson trichrome stained histopathology slides of animals receiving: (B) control injections (note the marked ventricular dilatation and wall thinning in addition to extensive myocardial fibrosis (blue staining); (C) L2 MSC treatment (intermediate dilatation, wall thinning and fibrosis); (D) E2 MSC therapy (preserved volume, wall thickness and less fibrosis). * $p<0.05$, ** $p<0.01$ from controls.

4.4.4. Arteriolar Density

Arteriolar density was assessed histologically by staining for αSMA+ vessels in the infarct zone (IZ), border zone (BZ) and remote LV myocardium. In the IZ, all four MSC treatment groups displayed greater arteriolar density than control animals (Figure 7a). The E2 group had the highest density of arterioles present in the IZ (11.4±1.9 /0.2mm2), almost twice that measured in the E1 (6.2±1.2 /0.2mm2, $p<0.05$) and L2 groups (5.8±0.6 /0.2mm2, $p<0.05$) and more than three-fold that observed in L1 recipients (3.2±1.0 /0.2mm2, $p<0.01$).

Similarly arteriolar density was also augmented in each MSC group in the BZ of infarction, when compared with controls ($p<0.001$, Figure 7b). No significant differences were noted between E1, L1 and L2 groups, but E2 demonstrated higher BZ vascularity than the E1 and L2 cohorts.

Arteriolar density in the LV myocardium remote to the IZ and BZ was not significantly higher in the E1 group (9.7±1.2 /0.2mm2, $p=0.60$), and only marginally higher in the E2 cohort (11.7±0.8 /0.2mm2, $p<0.05$) compared with controls (8.9±0.9 /0.2mm2, Figure 7c). Conversely, substantially higher density was observed in the groups where MSC was delivered late; L1 (16.1±2.4 /0.2mm2, $p<0.001$) and L2 (14.8±1.4 /0.2mm2, $p<0.001$).

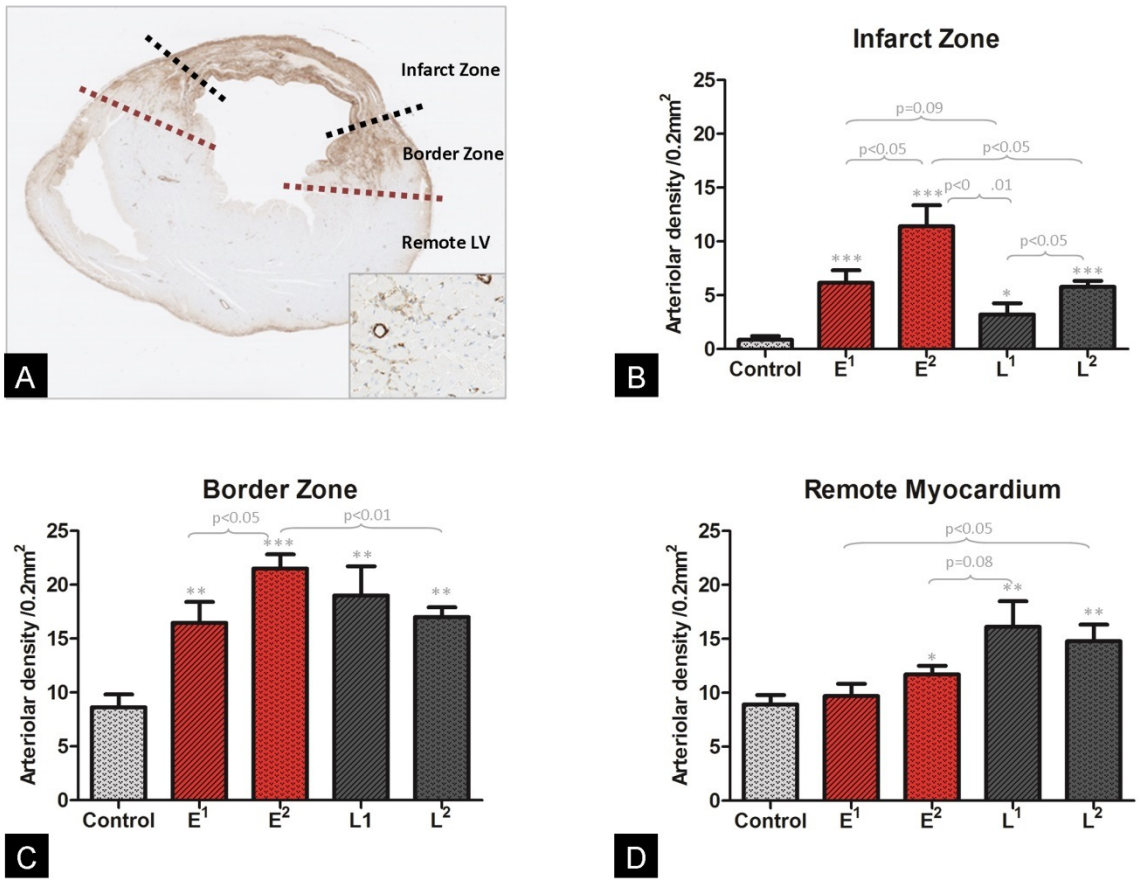

Fig. 7. Arteriolar density: (A) Representative example of αSMA stained slide, illustrating the infarct zone, border zone and remote LV myocardium. Insert shows examples of αSMA vessels. Graphs demonstrating arteriolar density in the (B) infarct zone (note the impact of early intervention); (C) border zone of infarction; (D) remote LV myocardium (note impact of late intervention). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ from controls.

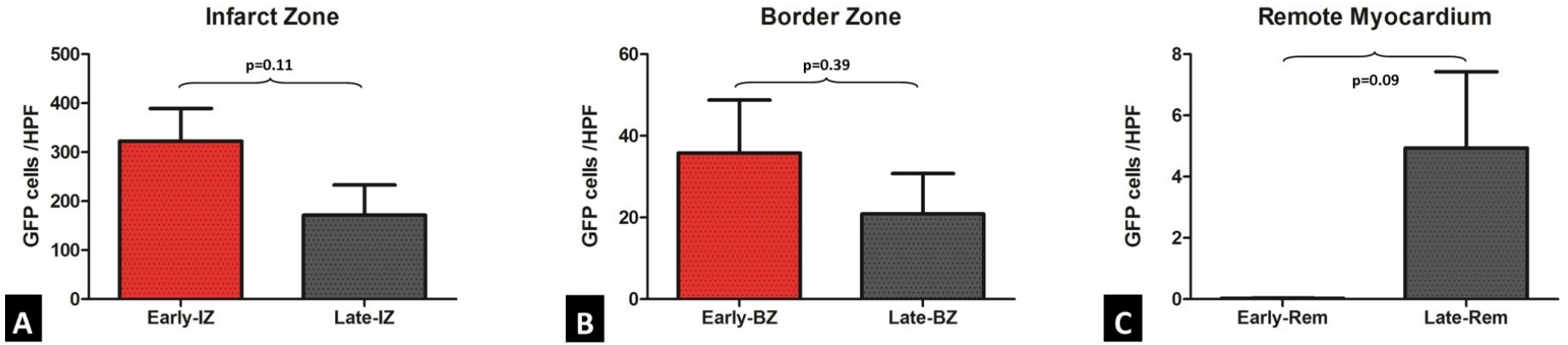

Fig. 8. Distribution of MSC according to timing of cell transplantation: (A) Quantification of retention of GFP positive cells in the infarct zone, (B) border zone of infarction and (C) remote left ventricular myocardium.

4.4.5. Green Florescent Protein-labelled MSC

Detection of GFP-labelled MSC was used to determine and compare cell retention in the different myocardial territories between the therapeutic groups. In the infarct zone (Figure 8), there was a trend toward greater cell retention in recipients of early MSC compared to late delivery (323±67 v 171±62 GFP cells/HPF, p=0.11). In the border zone of infarction, there was no statistically significant difference in retention between the two timing strategies (early: 35.8±13.0 v late: 20.9±9.9, p=0.39). In contrast, in remote LV myocardium, we observed a strong trend toward greater cell retention in the late MSC group (4.9±2.5 v 0.1±0.1 GFP cells/HPF, p=0.09).

4.5. Discussion

This study systematically assessed the efficacy of MSC treatment at varying dose, in the clinically relevant immediate and sub-acute period after MI in rats. The majority of previous experimental studies have used one of these time points for stem cell intervention [10, 20, 21]. In the immediate period during/after MI the myocardium represents a hazardous environment consisting of hypoxic, metabolically disordered tissue undergoing necrosis and apoptosis [9, 22]. Accordingly, myocardium is likely to be less receptive to stem cell retention and engraftment at this juncture, potentially limiting MSC efficacy [7]. However at this time myocardial function potentially remains salvageable, as adverse remodelling has not commenced and myocardial scar has not formed, therefore an early/ immediate intervention offers the greatest scope for preservation of function [8]. In contrast, MSC intervention deferred until one week after MI encounters a less hazardous myocardial microenvironment that is likely to be more receptive to cellular retention and engraftment [7, 12]. However, by this time the infarction is complete, remodelling and scar formation has commenced and the potential for benefit of stem cell treatment may be reduced.

One meta-analysis has attempted to clarify the relative importance of different variables of cell therapy, including timing of administration, on recovery of ventricular function in large animal models of MI [10]. Cell therapy was found to be moderately beneficial (mean 7.5% increase in EF) when given at either early (≤24 hours) or delayed (≥day 7) intervals after MI. Although neither time-frame was found to be definitively superior, there were trends toward better results when cell dosing was deferred beyond day seven. However, it is important to emphasise that the individual studies included in this meta-analysis were very heterogeneous in terms of such key parameters as cell type used, cell dose, infarct model and territory of infarction. One previous study has directly evaluated the timing of plastic adherence-isolated MSC in a rat model of MI, comparing administration immediately after MI and at one and two weeks after coronary ligation [12]. MSC engraftment was higher in the one week group, and EF (measured by echocardiography) and infarct size both showed non-significant trends toward better outcome with delayed intervention.

In contrast to conventional plastic adherence-isolated MSC that have been used in the vast majority of prior studies [23, 24], our study used CD45-depleted compact bone derived allogeneic rat MSC which were hypoxic conditioned. Previously our group has demonstrated that human MSC isolated by prospective isolation using monoclonal antibodies (e.g. STRO-1, STRO-3) directed against cell surface proteins expressed by MSC possess superior biological properties over conventionally isolated MSC, in terms of purity, "stemness" and cardiovascular paracrine capacity [4, 25]. Prospectively selected human and ovine MSC have been used to promising effect in small and large animal studies of MI [6, 14] and non-ischemic cardiomyopathy [16]. In light of the paucity of anti-rat antibodies to well defined MSC-markers [18], we opted to use a strategy of negative selection to exclude contaminating hematopoietic (CD45+) cells when initiating MSC cultures. In addition, hypoxic pre-conditioning was also applied in order to selectively cultivate MSC with the capacity to grow in suboptimal growth conditions, such as those found in the post-MI setting [26]. In the present study, we have shown that prospectively

isolated MSC are highly effective at mitigating myocardial injury and deleterious LV remodelling post-MI when compared to a control (no-cell) injectate solution.

Therapeutic benefits were most striking when the higher cell dose was administered immediately after coronary ligation, consisting of greater preservation of LV EF, almost complete attenuation of ventricular dilation and marked reduction of myocardial fibrosis. In contrast, the least effective of the four dosing strategies was the early-low dose cohort, whereas an intermediate impact was observed with both of the late intervention groups. In each case, improvement following cell administration was apparent within the first week of treatment with maintenance of the effect thereafter. The dichotomous findings seen in the early intervention groups suggest that in the immediate aftermath of infarction, the myocardium is dose-sensitive to the actions of exogenous cells. In particular, our data indicate that there is a higher threshold of cell number required to overcome the challenges that acute ischemia imparts upon the retention, survival and function of newly transferred MSC to elicit a reparative effect. The substantial incremental benefit in preservation of LV EF observed in the E2 group over the apparent ceiling of repair in the late intervention groups probably reflects the greater scope for myocardial salvage when treatment is initiated at the earliest time-point possible after MI. The similarity in outcomes from delayed low-dose (L1) and high-dose (L2) treatment demonstrates that in the subacute setting post-MI there is a lower dose threshold for effectiveness of MSC but a distinct lack of dose-responsiveness, at least within the range of doses evaluated in this study.

The substantial impact of the E2 strategy is comparable to outcomes noted in several other studies with MSC (particularly when delivered early after MI at high dose) when optimised, for example via overexpression of akt [27], SDF-1 [28] or VEGF [29] in rodents. More modest improvements have been noted using plastic-adherent MSC, particularly if delivery was deferred until at least one week after MI [13, 30].

It is now more widely believed that indirect paracrine mechanisms of action largely account for the benefits of MSC therapy, rather than bona fide transdifferentiation into

functional cardiomyocytes [2, 31]. Indirect paracrine effects are mediated by cell-to-cell interactions with endogenous cells (comprising mature cardiomyocytes and vascular cells, endogenous progenitor cells and inflammatory cells) and the secretion of a wide range of soluble growth factors and cytokines that influence cellular and extracellular targets [31]. The secretome of MSC is known to be modulated by cell-specific factors (e.g. developmental status of the MSC [4]) and the local milieu in which they find themselves [32]. Accordingly, as the myocardial microenvironments differ greatly in the immediate and sub-acute stages of MI, it follows that the reparative actions and mechanisms of MSC may also vary depending on the timing of intervention. This is supported by the current study's findings. Although early MSC treatment had a greater relative impact in the infarct territory in terms of preserving wall thickness and systolic thickening, enhancing microvascularity, abrogating fibrosis and reducing the number of infarcted segments, the same parameters were selectively influenced by late MSC intervention in remote LV segments. One possible explanation for these intriguing observations is that immediately after MI, the infarct territory is still viable and the predominant site of upregulated homing signals, so that injected cells are more likely to be retained within this region for sufficient periods to confer an early, perhaps transient, paracrine influence. However, the lack of blood flow in the culprit vessel presumably also results in high cell attrition limiting the effectiveness of low dose therapy (recapitulated here in the E1 group) and the number of cells that may ultimately survive to migrate to remote myocardium at later time-points. Furthermore, at this early juncture, remote segments are not yet compromised and therefore there are fewer homing signals originating from remote areas competing for the attention of stem cells. By comparison, after one week, the infarcted rat myocardium has already undergone much cardiomyocyte death and completed the early wave of acute inflammatory infiltration, with collagen deposition well underway [22]. As such, there is less scope for repair of the infarcted region and fewer inflammatory homing cues. However, as the ventricular chamber continues to remodel there is likely to be increasing strain on remote (non-infarcted) myocardium possibly accompanied by upregulation of remote homing signals. Experiments evaluating SDF-1/CXCL12 have shown earliest SDF-1 upregulation to occur in the direct infarct zone by 24 hours, with subsequent upregulation in the border zone by 48-72 hours and peaking at 96 hours [33]. However,

timing of SDF-1 expression in the remote LV myocardium was not assessed. We hypothesize that these factors, perhaps in conjunction with lower cell attrition after delayed delivery, may enable more cells to reach remote LV segments to promote repair there. The GFP-MSC data presented appear to support this hypothesis, with trends to higher retention in the infarct zone of early MSC recipients and in remote LV myocardium in late recipients. At this stage these mechanisms are highly speculative and will need careful evaluation in future studies by tracking the survival and distribution of cells in myocardium after different dosing/timing protocols, preferably with high resolution, non-invasive imaging modalities [34].

4.5.5.1. Limitations

A permanent ligation method of LAD occlusion was used to instigate infarction, a method frequently employed in rodent models of MI as this provides large infarcts with relatively high reproducibility of infarct size (as shown in the control group here, though this remains operator dependant). It could be argued that ischemia-reperfusion models might more closely reflect the myocardial pathology encountered in clinical practice in patients after reperfusion treatment. However, a limitation of ischemia-reperfusion models is that smaller MIs are produced and often with greater inherent variability in the magnitude of the infarct, which has implications for sample size and study power. We used a relatively narrow window of dosing and future studies may wish to explore broader dose ranges, especially at the early dose-dependent time point to see if effects can be augmented further, and to see if dose-responsiveness does actually occur at higher dose range with late treatment. This study primarily compares differing MSC strategies according to timing and dose, using P4 MSC. This passage number enables sufficient ex vivo culture to obtain adequate numbers of cells for therapeutic application and has been widely utilized in other pre-clinical and clinical studies [16, 24, 35]. However, the optimal passage number for MSC remains an important topic of ongoing investigation, as biological functions and by extension therapeutic efficacy, may vary with ex vivo expansion through serial passage [36]. Finally, the current study evaluated myocardial endpoints four weeks after MI and cell administration, as consistent with previous small animal studies [9, 21]. As future studies investigate the effect of cell dose and timing in large animal or clinical settings,

longer follow-up will be warranted to determine whether differential effects like those observed here, are maintained over time.

4.5.2. Implications and Future Directions

This study provides insights, albeit in a surrogate small animal model, that may be useful for refocusing clinical researchers in terms of the proposed timing and dose range of cell therapy following MI. To date, the vast majority of clinical studies have utilized unfractionated BM (MNC) cell therapy after acute MI delivered by coronary infusion, with highly variable results and at best, modest benefit [1, 37]. The widespread adoption of primary angioplasty/stenting for acute MI together with the allogeneic properties of MSC [38], suggest that it is clinically feasible for MSC to be administered as an "off-the-shelf" product immediately after coronary reperfusion [24]. Indeed, an early dosing strategy would negate the need for additional (potentially invasive and expensive) procedures, making this approach more attractive to patients and health commissioners alike.

Clearly, the current study's results need to be interpreted in the context of intramyocardial injection (as delivery route) of BM MSC (as cell type), both of which have been heavily under-represented until now in published clinical studies of cell therapy for acute MI. However, our revelation of discrepant dose-responsiveness with early and late MSC administration is highly novel and underscores the complexities associated with selecting the optimal combination of cell type, dose and timing interval in the early MI setting. In particular, this sheds new light on the risks inherent in comparing the outcomes of different stem cell studies (both preclinical and clinical) and in trying to reach a generic summary of the effectiveness of cell therapy, as attempted by recent meta-analyses.

The next generation of MSC-based studies should adopt optimisation strategies to advance the biological and therapeutic properties of these cells, through the use of techniques such as gene engineering, exposure to preconditioning or adjuvant agents and combination with bioscaffolds [3]. In the case of early cell therapy, strategies targeted to maximise initial cell

survival (e.g. through hypoxic conditioning or akt overexpression [27, 29]) and augment paracrine anti-apoptotic, anti-inflammatory and angiogenic actions are likely to be most beneficial. Conversely, for delayed therapy, MSC may be selectively manipulated to enhance their capacity to remodel extracellular matrix and fibrosis and/or promote cardiomyocyte replenishment [3]. Some of these strategies, including the prospective immunoselection and hypoxic conditioning used in our study, are immediately relevant and transferable to clinical practice. Finally, the finding that early and late intervention differentially effect different regions of the LV, also raises the prospect that additive or even synergistic repair may be achievable with repeated cell dosing.

4.6. Conclusion

Allogeneic MSC, enriched by prospective isolation and hypoxic conditioning, have substantial pleiotropic reparative capacity for MI. Although cell therapy is effective both when given immediately or deferred, early, high dose intervention is most potent. In addition to influencing the magnitude of benefit, timing of cell delivery is also an important determinant of dose-responsiveness and the regional distribution of myocardial repair.

4.6.6.1. Acknowledgements

We thank Mr Tom Sullivan (University of Adelaide) for support with statistics and Dr Stephen Fitter, (University of Adelaide) for assistance with the GFP retroviral transduction. JDR is supported by an International Postgraduate Research Scholarship at the University of Adelaide, Australia. PJP has received funding from the National Health and Medical Research Council of Australia and the Royal Australasian College of Physicians. DW is supported by the National Health and Medical Research Council and Australian National Heart Foundation postgraduate scholarship.

4.6.1. References

1. Lipinski MJ, Biondi-Zoccai GGL, Abbate A, Khianey R, Sheiban I, Bartunek J, et al. Impact of Intracoronary Cell Therapy on Left Ventricular Function in the Setting of Acute Myocardial Infarction: A Collaborative Systematic Review and Meta-Analysis of Controlled Clinical Trials. Journal of the American College of Cardiology. 2007;50(18):1761-7.
2. Psaltis PJ, Zannettino ACW, Worthley SG, Gronthos S. Concise Review: Mesenchymal Stromal Cells: Potential for Cardiovascular Repair. Stem Cells. 2008;26(9):2201-10.
3. Richardson J, Nelson A, Zannettino A, Gronthos S, Worthley S, Psaltis P. Optimization of the Cardiovascular Therapeutic Properties of Mesenchymal Stromal/Stem Cells–Taking the Next Step. Stem Cell Reviews and Reports.1-22.
4. Psaltis PJ, Paton S, See F, Arthur A, Martin S, Itescu S, et al. Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol. 2010 May;223(2):530-40.
5. Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang J-A, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. The Journal of Thoracic and Cardiovascular Surgery. 2008;135(4): 799-808.
6. Martens TP, See F, Schuster MD, Sondermeijer HP, Hefti MM, Zannettino A, et al. Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. Nat Clin Pract Cardiovasc Med. 2006 Mar;3 Suppl 1:S18-22.
7. Li R-K, Mickle DAG, Weisel RD, Rao V, Jia Z-Q. Optimal time for cardiomyocyte transplantation to maximize myocardial function after left ventricular injury. The Annals of Thoracic Surgery. 2001;72(6): 1957-63.
8. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation. 1990 April 1, 1990;81(4):1161-72.
9. Virag JI, Murry CE. Myofibroblast and Endothelial Cell Proliferation during Murine Myocardial Infarct Repair. The American Journal of Pathology. 2003;163(6):2433-40.
10. van der Spoel TIG, Jansen of Lorkeers SJ, Agostoni P, van Belle E, Gyöngyösi M, Sluijter JPG, et al. Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. Cardiovascular Research. 2011 September 1, 2011;91(4):649-58.
11. Traverse Jh HTDESG, et al. Effect of intracoronary delivery of autologous bone marrow mononuclear cells 2 to 3 weeks following acute myocardial infarction on left ventricular function: The latetime randomized trial. JAMA: The Journal of the American Medical Association. 2011;306(19):2110-9.
12. Hu X, Wang J, Chen J, Luo R, He A, Xie X, et al. Optimal temporal delivery of bone marrow mesenchymal stem cells in rats with myocardial infarction. European Journal of Cardio-Thoracic Surgery. 2007 March 1, 2007;31(3):438-43.
13. Dai W, Hale SL, Martin BJ, Kuang J-Q, Dow JS, Wold LE, et al. Allogeneic Mesenchymal Stem Cell Transplantation in Postinfarcted Rat Myocardium. Circulation. 2005 July 12, 2005;112(2):214-23.
14. Dixon JA, Gorman RC, Stroud RE, Bouges S, Hirotsugu H, Gorman JH, 3rd, et al. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation. 2009 Sep 15;120(11 Suppl):S220-9.
15. Black Schaffer B, Grinstead CE, 2nd, Braunstein JN. Endocardial Fibroelastosis of Large Mammals. Circ Res. 1965 Apr; 16:383-90.
16. Psaltis PJ, Carbone A, Nelson AJ, Lau DH, Jantzen T, Manavis J, et al. Reparative effects of allogeneic mesenchymal precursor cells delivered transendocardially in experimental nonischemic cardiomyopathy. JACC Cardiovasc Interv. 2010 Sep;3(9):974-83.
17. Segmentation AHAWGoM, Imaging: RfC, Cerqueira MD, Weissman NJ, Dilsizian V, Jacobs AK, et al. Standardized Myocardial Segmentation and Nomenclature for Tomographic Imaging of the Heart. Circulation. 2002 January 29, 2002;105(4): 539-42.
18. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
19. Nowosielski M, Schocke M, Mayr A, Pedarnig K, Klug G, Kohler A, et al. Comparison of wall thickening and ejection fraction by cardiovascular magnetic resonance and echocardiography in acute myocardial infarction. Journal of Cardiovascular Magnetic Resonance. 2009;11(1):22.
20. Armiñán A, Gandía C, García-Verdugo JM, Lledó E, Trigueros C, Ruiz-Saurí A, et al. Mesenchymal Stem Cells Provide Better Results Than Hematopoietic Precursors for the Treatment of Myocardial Infarction. Journal of the American College of Cardiology. 2010;55(20):2244-53.
21. Tang J, Wang J, Yang J, Kong X, Zheng F, Guo L, et al. Mesenchymal stem cells over-expressing SDF-1 promote angiogenesis and improve heart function in experimental myocardial infarction in rats. European Journal of Cardio-Thoracic Surgery. 2009 October 1, 2009;36(4):644-50.
22. Ertl G, Frantz S. Healing after myocardial infarction. Cardiovascular Research. 2005 April 1, 2005;66(1):22-32.
23. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, et al. Multilineage

potential of adult human mesenchymal stem cells. Science. 1999;284:143 - 7.
24. Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol. 2009 Dec 8;54(24):2277-86.
25. See F, Seki T, Psaltis PJ, Sondermeijer HP, Gronthos S, Zannettino AC, et al. Therapeutic Effects of Human STRO-3-Selected Mesenchymal Precursor Cells and their Soluble Factors in Experimental Myocardial Ischemia. J Cell Mol Med. 2010 Dec 14.
26. Das R, Jahr H, van Osch GJ, Farrell E. The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Eng Part B Rev. 2010 Apr;16(2):159-68.
27. Gnecchi M, He H, Melo LG, Noiseaux N, Morello F, de Boer RA, et al. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing Akt on cardiac metabolism after myocardial infarction. Stem Cells. 2009 Apr;27(4):971-9.
28. Dong F, Harvey J, Finan A, Weber K, Agarwal U, Penn MS. Myocardial CXCR4 Expression Is Required for Mesenchymal Stem Cell Mediated Repair Following Acute Myocardial Infarction / Clinical Perspective. Circulation. 2012 July 17, 2012;126(3): 314-24.
29. Kim SH, Moon H-H, Kim HA, Hwang K-C, Lee M, Choi D. Hypoxia-inducible Vascular Endothelial Growth Factor-engineered Mesenchymal Stem Cells Prevent Myocardial Ischemic Injury. Mol Ther. 2011;19(4): 741-50.
30. Hou M, Yang K-m, Zhang H, Zhu W-Q, Duan F-j, Wang H, et al. Transplantation of mesenchymal stem cells from human bone marrow improves damaged heart function in rats. International Journal of Cardiology. 2007;115(2):220-8.
31. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008 Nov 21;103(11):1204-19.
32. Thangarajah H, Vial IN, Chang E, El-Ftesi S, Januszyk M, Chang EI, et al. IFATS collection: Adipose stromal cells adopt a proangiogenic phenotype under the influence of hypoxia. Stem Cells. 2009 Jan;27(1): 266-74.
33. Zhang M, Mal N, Kiedrowski M, Chacko M, Askari AT, Popovic ZB, et al. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. FASEB J. 2007 Oct; 21(12):3197-207.
34. Psaltis PJ, Simari RD, Rodriguez-Porcel M. Emerging roles for integrated imaging modalities in cardiovascular cell-based therapeutics: a clinical perspective. Eur J Nucl Med Mol Imaging. 2011 Sep 8.
35. Huang J, Zhang Z, Guo J, Ni A, Deb A, Zhang L, et al. Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. Circ Res. 2010 Jun 11;106(11):1753-62.
36. Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B. Aging of mesenchymal stem cell in vitro. BMC Cell Biol. 2006;7:14.
37. Schächinger V, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, Hölschermann H, et al. Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. European Heart Journal. 2006 December 1, 2006;27(23): 2775-83.
38. Aggarwal S, Pittenger M. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005;105:1815 - 22.

Statement of Authorship

Title of Paper	**Impact of timing and dose of mesenchymal stromal cell therapy in a preclinical model of acute myocardial infarction.**
Publication Status	Published
Publication Details	J Card Fail. 2013 May;19(5):342-53. doi: 10.1016/j.cardfail.2013.03.011. PMID: 23663817

Author Contributions

Name of Principal Author (Candidate)	**Dr James David Richardson**
Contribution to the Paper	Primary contributor to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work; Drafted the work ; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Angela Bertaso**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Peter J Psaltis**

Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Lachlan Frost**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Angelo Carbone**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Dennis T Wong**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and

	revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Adam J Nelson**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mrs Sharon Paton**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**A/Prof Matthew Worthley**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval

	of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Professor Andrew Zannettino**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013
Name of Co-Author	**Professor Stan Gronthos**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Professor Stephen G Worthley**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work
	18th December 2013

5. Chapter 5 - Combination of Early and Late MSC Intervention

Incremental Benefits of Repeated Mesenchymal Stromal Cell Administration Compared to Solitary Intervention after Myocardial Infarction

James D Richardson[1,2], Peter J Psaltis[1,3], Lachlan Frost[1], Sharon Paton[2], Angelo Carbone[1], Angela G Bertaso[1], Adam J Nelson[1], Dennis TL Wong[1], Matthew I Worthley[1], Stan Gronthos[2#], Andrew CW Zannettino[2#], Stephen G Worthley[1,2#]

[1] Cardiovascular Research Centre, Royal Adelaide Hospital and Department of Medicine, University of Adelaide, South Australia, Australia.

[2] Centre for Stem Cell Research, Robinson Institute, School of Medical Sciences, University of Adelaide, South Australia, Australia

[3] Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, USA

[#]Joint senior authors

5.1. ABSTRACT

Introduction: Traditionally, stem cell therapy for myocardial infarction (MI) has been administered as a single treatment in the acute or subacute period after MI. These time intervals coincide with marked differences in post-infarct myocardial environment, raising the prospect that repeat cell dosing could provide incremental benefit above solitary intervention. This was evaluated here with the use of mesenchymal stromal cells (MSC).

Methods: Three groups of rats were studied. Single and dual therapy groups received allogeneic, prospectively isolated MSC ($1x10^6$ cells) by transepicardial injection immediately after MI, with additional dosing one week later in the dual treatment cohort. Control animals received cryopreservant solution only. Left ventricular dimensions and ejection fraction (EF) were assessed by cardiac magnetic resonance immediately before MI and also at 1, 2 and 4 weeks post-MI.

Results: Immediate MSC treatment attenuated early myocardial damage with EF of 35.3±3.1% (dual group, n=12) and 35.2±2.2% (single group, n=15) at 1 week post-MI, compared to 22.1±1.9% in controls (n=17, $p<0.01$). In animals receiving a second dose of MSC, EF increased to 40.7±3.1% by week 4, which was significantly higher than in the single intervention group (EF 35.9±1.8%, $p<0.05$). Dual MSC treatment was also associated with greater myocardial mass and arteriolar density, with trends toward reduced myocardial fibrosis. These incremental benefits were especially observed in remote (non-infarct) segments of left ventricular myocardium.

Conclusion: Repeated stem cell intervention in both the acute and sub-acute period after MI provides additional improvement in ventricular function beyond solitary cell dosing, largely due to beneficial changes remote to the area of infarction.

Key Words: Cardiac magnetic resonance; Hypoxic conditioning; Mesenchymal stem cells; Multiple intervention; Myocardial infarction; Optimisation. Prospective isolation; Repair: Timing.

5.3. INTRODUCTION

There is a compelling need for novel therapies for the treatment of left ventricular dysfunction after acute myocardial infarction (MI). Therapy with mesenchymal stem/ stromal cells (MSC) has shown significant promise in pre-clinical experiments, however, only modest benefits have been observed in clinical trials [1]. MSC have a relatively immunoprivileged phenotype that permits their allogeneic use, therefore offering the prospect of cell optimisation and immediate off-the-shelf treatment, previously unachievable with autologous cell therapy. Of the cell optimisation strategies currently evaluated for MSC [2], prospective isolation and hypoxic pre-conditioning have been shown to enhance their *in vitro* [3, 4] and *in vivo* capacity for repair [5, 6] and could be readily applied to clinical use.

Almost all studies evaluating stem cell treatment have utilised a solitary dose intervention, often applied either acutely or deferred to the sub-acute period, 1-2 weeks after MI [7]. These two time points represent greatly differing myocardial environments; early after MI the myocardium is highly inflamed and consists of hypoxic, metabolically disordered tissue undergoing a mixture of necrosis and apoptosis that may be particularly noxious to transplanted stem cells [8, 9]. In contrast, one week later the infarcted myocardium has already undergone considerable cardiomyocyte death and completed the early wave of acute inflammatory infiltration, with commencement of collagen deposition and remote myocardial remodeling [10]. Thus far, the therapeutic potential of MSC post-MI has largely been attributed to their paracrine properties [3, 11, 12] and their secretome is known to be modulated by the local milieu in which they find themselves [13]. Recently, we reported that timing of MSC intervention in a rat model of MI influenced their reparative effects, such that immediate cell administration had a greater relative impact on

the infarct territory, whereas deferral of treatment for one week caused more beneficial change in remote (non-infarct) left ventricular myocardium [14]. Accordingly, as mechanisms appear to differ, we hypothesised that repeated cell dosing delivered at both these times could provide incremental benefit to ventricular function compared to a single early intervention. In this study, we evaluated whether a second/late delivery of MSC (optimised by prospective isolation and hypoxic conditioning), in animals already treated with MSC immediately post-MI, would enhance myocardial repair.

5.4. MATERIALS and METHODS

5.4.1. Study protocol

This study was approved by the Animal Ethics Committees of the Central Adelaide Local Health Network (No. 104/10) and the University of Adelaide (No. M-2010-105), South Australia.Animal handling was carried out in accordance with the guidelines outlined in the "Position of the American Heart Association on Research Animal Use" [15].

Male Sprague-Dawley rats (n=44, baseline weight 350-370g) were used in the experimental protocol. Animals were randomised at baseline to one of three investigation arms: Single group, dual treatment group or controls (Figure 1). Baseline evaluation of ejection fraction (EF) was undertaken using cardiac magnetic resonance (CMR), followed by thoracotomy and permanent surgical ligation of the left anterior descending artery. Allogeneic MSC were delivered transepicardially immediately after MI whereas control animals received injections of ProFreeze^{TM} (PF) cryopreservation vehicle. At repeat thoracotomy one week later, a second MSC dose was transplanted in the combination group, whereas the solitary and control groups received PF injections. Reassessment of EF with CMR was conducted at 1, 2 and 4 weeks after MI. Euthanasia was performed at week 4 after final imaging for tissue harvesting and histological evaluation.

Six donor male Sprague Dawley rats were euthanized (by CO_2 inhalation) and the femora and tibiae excised, the epiphyses removed and bones flushed. The resultant cell suspension was centrifuged, supernatant removed and the cell pellet resuspended for density gradient separation. The long bones, now BM deplete, were crushed into small fragments using sterile scissors and washed. Digestion of the bone fragments, using Collagenase Type I and 0.2% DNAse solution in PBS, was performed on a shaking platform for 45 min at 37°C. The supernatant containing compact bone (CB) cells was collected and processed for mononuclear cells (MNC) by centrifugation using Lymphoprep™ (Axis Shield PoC, Rodeløkka, Oslo, Norway) as previously described [3].

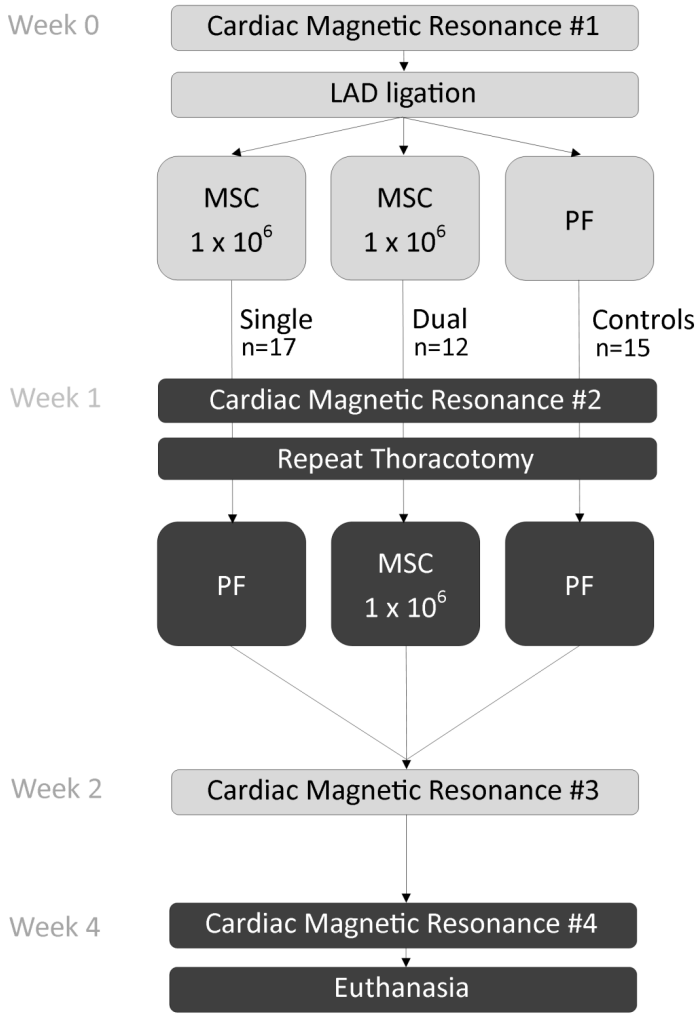

Figure 1. Study protocol: Flowchart illustrating the imaging and MSC interventions delivered over the 4 week study duration.

5.4.2. Prospective Isolation of Rat MSC

Magnetic activated cell sorting (MACS) was used to deplete any contaminating CD45-positive haematopoietic cells from the CB-derived MNC population. Briefly, CB-MNC were pelleted and blocked, prior to incubation with purified Anti-Rat CD45 10ug/ml (BD Biosciences, San Diego, CA, USA). After repeated washes in HBSS 10%FCS, secondary incubation was performed with goat anti-mouse IgG-Biotin 10ug/ml (Southern Biotec, Birmingham, AL, USA) followed by two washes in MACS Buffer (5mM EDTA, 1% BSA, 0.01%NaN_3 in PBS) and incubation with Anti-Biotin Microbeads (MiltenyiBiotec, BegischGladbach, Germany). The cell suspension was then passed through a MACS MS Separation Column (MiltenyiBiotec, BegischGladbach, Germany). The CD45-negative fraction was collected as the target cell population harbouring the MSC, to establish primary cultures.

5.4.3. Hypoxic conditioning

CD45-negative cells were initially seeded in plastic culture flasks at $3x10^4/cm^2$ in alpha Modification of Eagle's Medium (supplemented with 20% (v/v) FCS, 2mM L-Glutamine, 1mM sodium pyruvate, 100µM L-ascorbate-2-phosphate and penicillin/streptomycin sulphate) in a humidified hypoxic chamber in the presence of 5% O_2, and 10% CO_2. After reaching 80% confluence, cells were detached by Trypsin/EDTA treatment and subcultured in αMEM, supplemented with 10% (v/v) FCS, 2mM L-Glutamine, 1mM sodium pyruvate, 100µM L-ascorbate-2-phosphate and penicillin /streptomycin under normoxic conditions (21% O_2, 5% CO). Hypoxic conditioning was undertaken only during primary culture and all subsequent cell culture was performed under normoxic conditions. At passage 4 (P4), MSC were cryopreserved in ProFreeze™(LONZA BioWhittaker Walkersville, MD, USA) with 7.5% Dimethyl Sulfoxide at a concentration of $1x10^6$/100µl [14].

5.4.4. Cardiac Magnetic Resonance

Imaging was performed on a 1.5T MR system (Magnetom Sonata, Siemens, Germany), equipped with a dedicated cardiac software package. The thorax and right paw were shaved, cleaned with alcohol and adhesive electrocardiogram (ECG) electrodes attached thereby generating a vector ECG. Animals were placed supine recumbent in the isocentre of the magnet, with a 4-channel phased array carotid radiofrequency coil placed over the thorax, with 1% isoflurane administered via nose cone to maintain anaesthesia. Accordingly, all CMR images were free breathing, ECG-gated acquisitions. Transverse and coronal localiser images were acquired followed by long (4-chamber and 2-chamber) and short axis pilot images, from which a true short axis stack was prescribed. Balanced steady-state free-precession cine images (gated to alternate R waves) were acquired. The stack comprised three contiguous LV slices (each 3mm thick, with no intersection gap) providing full coverage of the LV. The image matrix was 384 x 384, field of view 185 mm, repetition time 14.72ms, echo time 1.55ms, flip angle 90°, image resolution 0.5 x 0.5 mm, 8 phases per cardiac cycle were acquired with an imaging time of 52 seconds per short axis slice [14].

5.4.5. CMR analysis

Left ventricular mass, volumes and derived EF were measured off-line from cine images using commercially available software (QMass v7.2, Medis, Netherlands). The end-diastolic and end-systolic cine frames were identified for each slice and the endocardial and epicardial borders were manually traced. The end-diastolic (EDV) and end-systolic volumes (ESV) were calculated using the true disk summation technique (i.e. sum of cavity volumes across all continuous slices), and EF calculated, as previously described [16]. Regional segmental function was analysed in each of 16 segments – 6 basal, 6 mid and 4 apical [17]. Using the same software, a reference point was placed at the right ventricular insertion point in the anterior septum for each LV slice to assign myocardial segmentation. Left ventricular end-diastolic myocardial wall thickness and systolic wall thickening (SWT) measurements were automatically calculated.

5.4.6. Infarct surgery and MSC transplantation

Anaesthesia was induced using 3% isoflurane, animals intubated and connected to a small animal ventilator (Harvard Apparatus, Holliston, MA, USA). Animals were mechanically ventilated (tidal volume 3.6mL, ventilatory rate 60 breaths/min) with 1-1.5% isoflurane and 2L/min O_2. A left anterior thoracotomy was performed, the pericardium excised and the left anterior descending artery (LAD) was permanently ligated, as previously described [18]. Infarction was confirmed by the development of pallor and hypo-/akinesis of the anteroapical myocardium. Rats were randomised to receive either transepicardial injections of MSC ($1x10^6$ cells) or PF (control) in four injection sites in the anterior myocardial wall border zone at a total volume of 100µL via a 30-gauge needle (i.e. 25µL per injection site). The muscle and skin layers were closed, Isoflurane was discontinued and animals were extubated once spontaneous ventilation resumed. Noracillin (0.15mg/kg i.m.) was given prophylactically, while post-operative analgesia was achieved with a single dose of Ketamine (75mg/kg i.p.) and Carprofen (5mg/kg s/c) repeated over two days.

One week later, all animals underwent CMR and repeat thoracotomy using identical anaesthesia and medication as described above. Animals in the combination group received a second repeat MSC intervention ($1x10^6$ MSC) while control and solitary cohort animals received control injections of PF. On all occasions, MSC were thawed from individual ampoules and underwent cell counts and cell viability (by trypan blue exclusion) immediately prior to injection and were only used if they exhibited >70% viability.

5.4.7. Histological analysis

Euthanasia was performed at day 28 post-MI under deep anaesthesia (Metedomidine 0.5mg/kg i.p. and Ketamine 75mg/kg i.p.). Hearts were excised, drenched in sterile saline and immersed in a mixture of PBS/10% formalin. Myocardium below the occlusion site was later sectioned into three transverse slices providing a basal, mid and apical LV section. After paraffin embedding, replicate 6µm sections were cut and stained by haematoxylin and eosin, Masson's trichrome and immunohistochemistry. Whole slides

were photographed (x40 magnification) with the NanoZoomer Digital Photography system (Hamamatsu Photonics, Hamamtsu, Japan) and complete image sets exported for blinded analysis using Image Pro Plus software (v5.1, Media Cybernetics, USA). Apoptosis was assessed by activated Caspase-3 immunohistochemistry [19], with the number of cells displaying caspase-3 activity per high powered field evaluated. Cardiac fibrosis was quantified using Masson's trichrome specimens and myocardial vascular density was quantified using staining for α-smooth muscle actin (αSMA - Clone 1A4, IgG_{2a}Imgenex, San Diego CA), as previously described [20]. The number of αSMA positive vessels (10 - 100μm diameter) was counted in ten random high power fields (x20 magnification), in the infarct zone, border zone and remote LV myocardium respectively [14].

5.4.8. Statistical analysis

All analyses were performed blind to study group and by two independent observers for CMR parameters. Numerical data are expressed as mean ± standard deviation. In view of repeated measures of the same subjects at different time points, a linear mixed effects model was adopted to compare the effectiveness of therapy between groups. A p-value <0.05 was considered statistically significant. Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

5.5. RESULTS

5.5.1. Characterisation of MSC

The immunophenotype of P4 MSC was evaluated via flow cytometric analysis of cell surface markers as previously described [3]. Rat MSC exhibited very high expression of CD105, CD73 and CD44, but negligible expression of CD45, CD34 or CD106 consistent with international guidelines [21] (data not shown). Multilineage differentiation capacity

was confirmed with adipogenic, osteogenic and chondrogenic formation under standard inductive conditions [22] (data not shown).

5.5.2. Global Left Ventricular Function

There were no significant differences in LV volumes or EF between the three investigation groups at baseline. Figure 2 depicts the temporal trends and absolute changes in LV EF over time for each group. In the control group, LV EF declined significantly by week 1 after MI to 22.1±1.9% ($p<0.0001$ versus baseline EF Figure 2A). In contrast, both active treatment groups (that received early MSC intervention) had significant attenuation of the early reduction in EF after infarction (week 1 EF: 35.2±3.4% single group and 35.4±2.9% for dual group, $p<0.05$ versus controls). At week 2 (one week after repeat MSC delivery), mean EF significantly improved in the dual therapy group to 41.2±3.4% ($p<0.05$). Over the same interval, EF remained stable in the no-cell (control) recipients and in the solitary MSC intervention group. By week 4, both MSC treatment groups maintained significantly higher LV function than controls, but the dual treatment cohort demonstrated superior ventricular function overall (final EF: 19.5±1.5% control group; 35.9±2.9% single, 40.7±3.3% dual ($p<0.001$ vs control; $p<0.05$ vs single). Absolute changes in EF between week 1 and 4 of follow-up, to identify the impact of a second MSC intervention, are summarised in Figure 2B. Whereas the change in EF in the single cohort was negligible over this period, the dual group demonstrated an additional 4.6±2.1% absolute improvement in EF ($p<0.05$).

Ventricular dilatation, in terms of LV EDV and ESV, was substantially attenuated by both MSC dosing strategies compared to no-cell control ($p<0.05$ to 0.01 versus control, Figure 3). However, no significant differences were detected between solitary and repeated therapy cohorts. Global myocardial mass was significantly reduced in control animals measured four weeks after MI (Δ Mass = -50.2±19.5mg, $p<0.001$ versus baseline). In contrast, myocardial mass was preserved in both MSC treatment groups ($p<0.05$ to 0.001 versus control group), with the highest mass noted in recipients of dual intervention ($p<0.05$, Figure 4a).

5.5.3. Regional left ventricular morphology and function.

Analysis of CMR-derived segmental LV wall thickness (Figure 4B-D) demonstrated greater wall thickness in the myocardial segments supplied by the LAD, and globally, in both MSC treatment groups compared with controls ($p<0.001$). While there were no significant differences between the MSC intervention groups in these territories, there was a trend to higher wall thickness in remote LV myocardial segments in the dual therapy cohort compared to controls ($p=0.13$).

Overall SWT was significantly higher in MSC recipients at end-study compared to controls ($p<0.001$). This effect was most apparent in the myocardial segments supplied by the LAD (SWT: 3.9±2.6% control group, 18.7±2.2% single, 16.8±2.0% dual, $p<0.001$ versus controls). Systolic wall thickening in remote LV myocardium was higher in the repeat dosing group than the solitary cohort (29.2±2.5%vs 21.7±2.6%, $p<0.05$). Similar outcomes were noted for the change in SWT (ΔSWT), with equal attenuation in the infarct territory but only the dual therapy group demonstrating improvements in the remote LV myocardium.

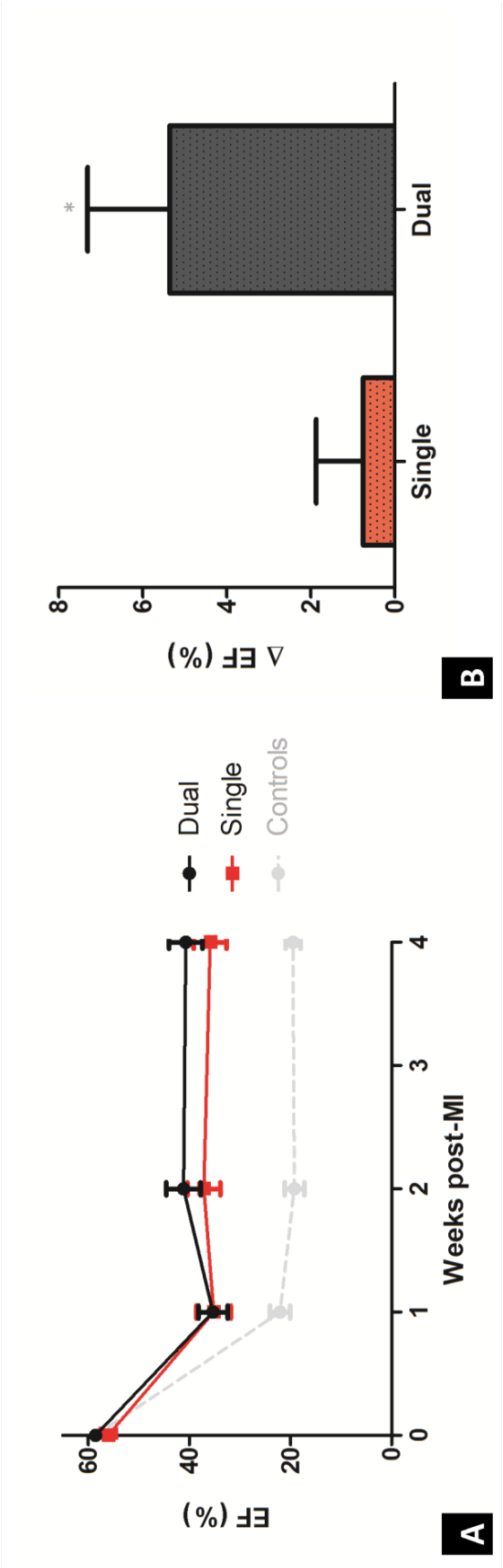

Figure 2. Ejection fraction: Temporal change in EF of the experimental groups from baseline to 1, 2 and 4 weeks post MI **(A)**. Absolute mean change in EF between week 1 and week 4 **(B)**.*$p<0.05$, ***$p<0.001$ (of controls versus dual and single groups).

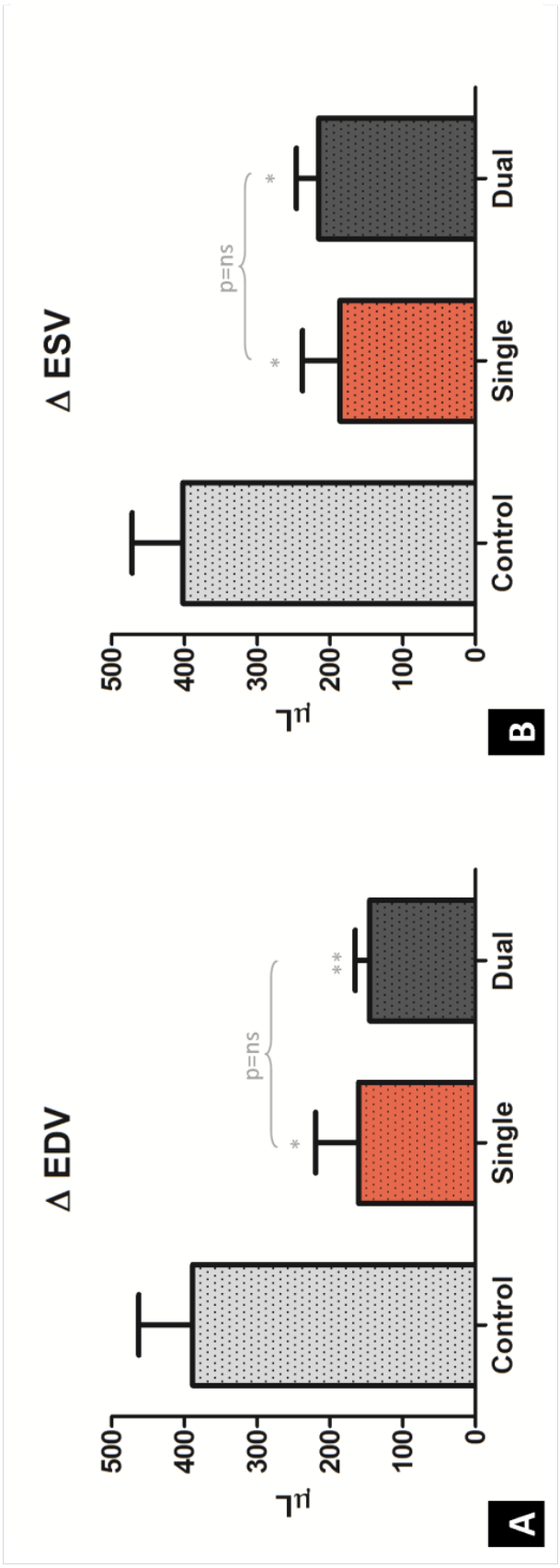

Figure 3. Left ventricular volumes: Mean changes in **(A)** end-diastolic and **(B)** end-systolic volumes between baseline and week 4 for each experimental group. * $p<0.05$, ** $p<0.01$ versus controls.

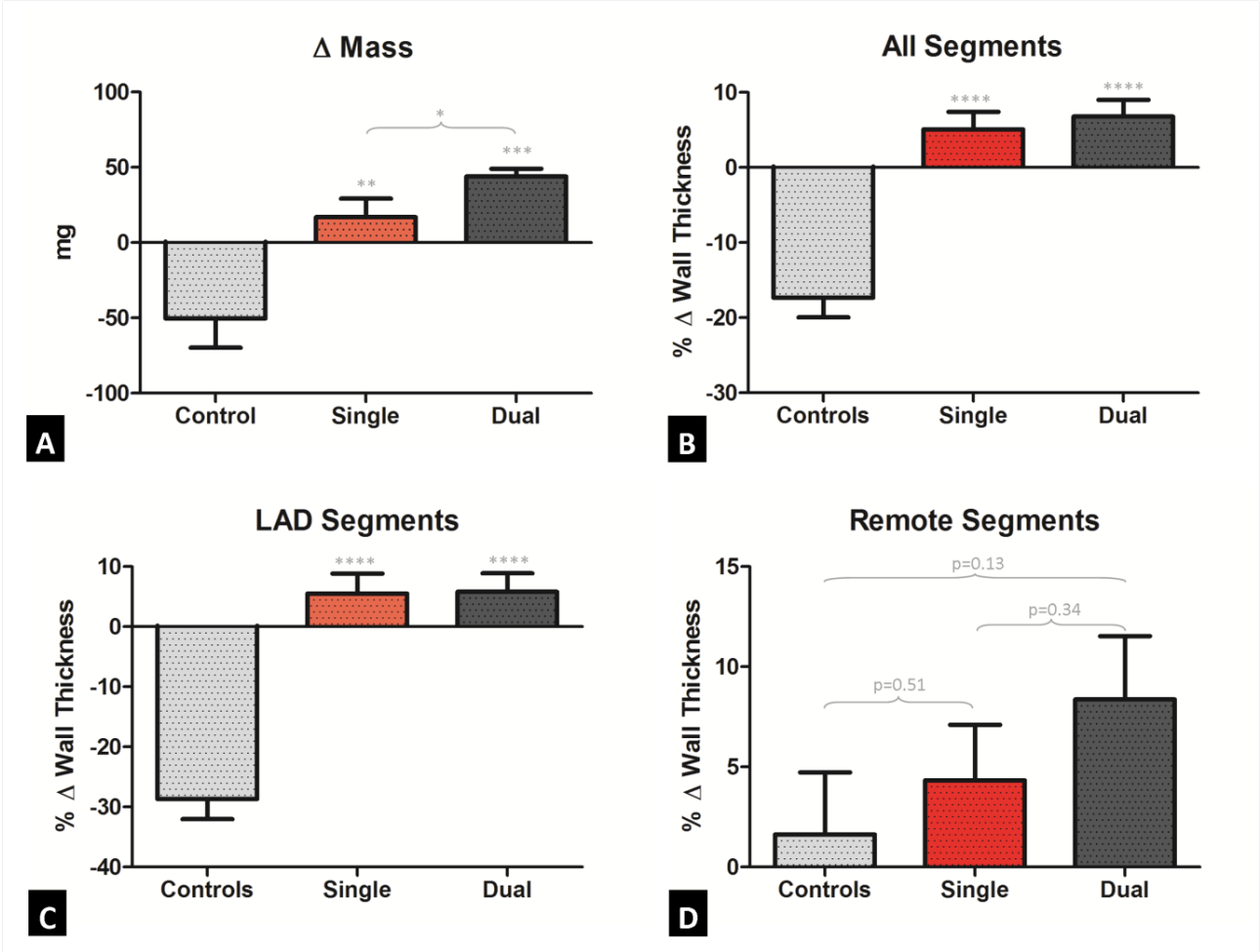

Figure 4. Myocardial mass and wall thickness: Mean changes in left ventricular myocardial mass from baseline to final study (A). Mean change in wall thickness in all segments (B), segments supplied by the left anterior descending artery (LAD) (C) and remote LV myocardium (D). ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus controls.

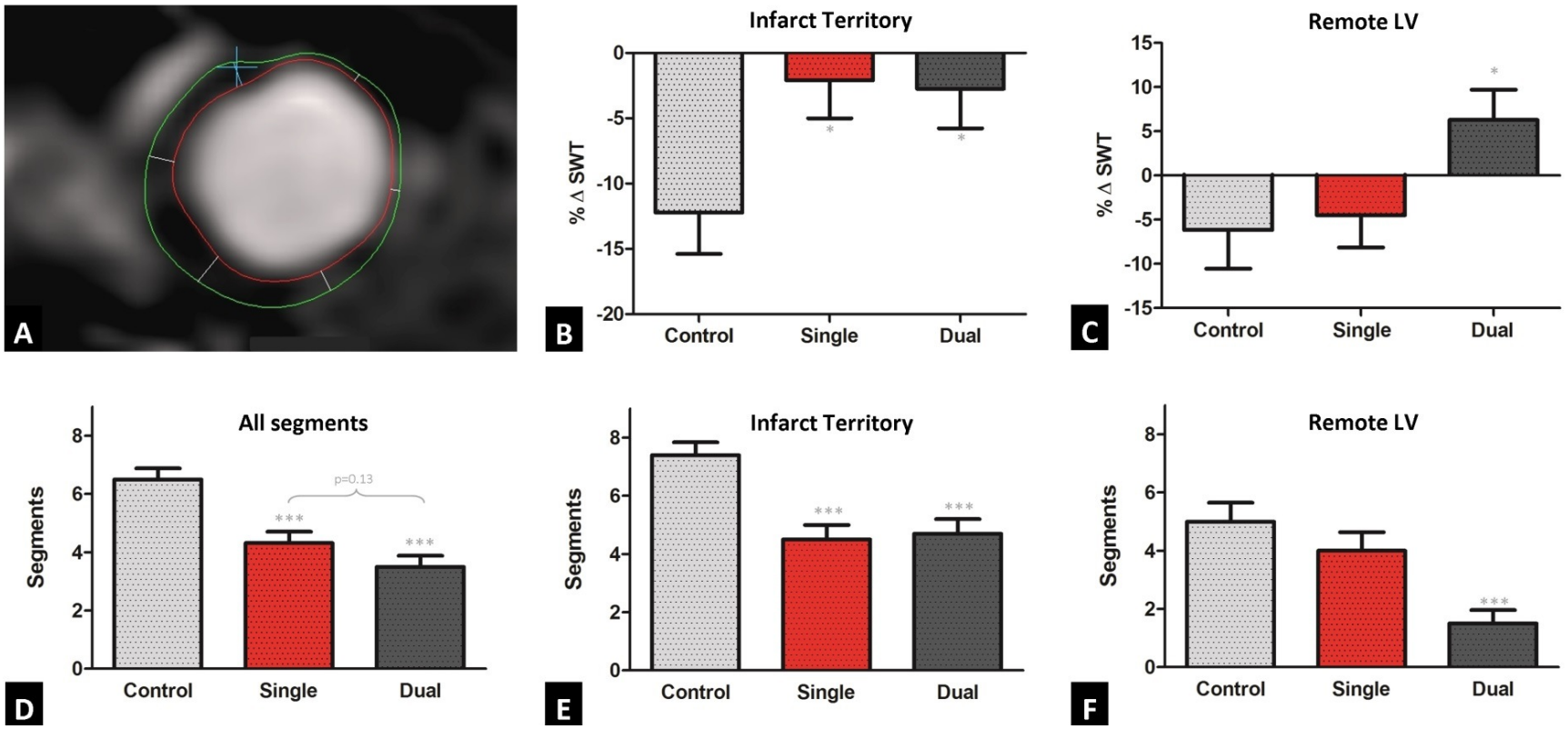

Figure 5. Systolic wall thickening and infarcted segments: Representative cardiac magnetic resonance images at end-diastole and end-systole in each experimental group – note the dilated LV dimensions and thinned myocardium in controls**(A)**. Mean change in systolic wall thickening (SWT) in the segments supplied by the LAD **(B)** and remote (non-infarct) LV myocardial segments **(C)**. Number of segments with SWT <50% of baseline (indicating dysfunctional/infarcted segments) in all segments **(D)**, in the infarct territory **(E)** and remote LV myocardium **(F)**. * $p<0.05$, *** $p<0.001$ versus controls.

The number of myocardial segments that had final SWT <50% of baseline, a validated surrogate CMR parameter for infarcted myocardium [23] was determined. Each MSC group had fewer dysfunctional/"infarcted" segments overall compared to controls (6.5±0.4 segments, $p<0.001$), with a trend to fewer affected segments in the dual dosing group (dual 3.5±0.4 vs single 4.3±0.4 segments, $p=0.13$). Notably, both MSC interventions had a similar impact in reducing dysfunctional segments in the LAD territory (Figure 5D), whereas in remote LV myocardium, significantly fewer dysfunctional segments were observed in the dual compared to solitary treatment cohort (1.5±0.5 vs, 4.0±0.6 segments respectively $p<0.01$, Figure 5E).

5.5.4. Myocardial Fibrosis

Extensive myocardial fibrosis was documented in the control cohort with 28.6 ±3.3% of the LV free wall staining positive for Masson's trichrome (Figure 6). Myocardial fibrosis was significantly reduced in the dual therapy animals (14.3±2.3%, $p<0.001$), with only a trend to a reduction in the solitary dose group compared to controls (20.2±4.1%, $p=0.12$). Comparison of the MSC groups, indicated a trend to less fibrosis in the dual dose group ($p=0.19$).

5.5.5. Arteriolar Density

Arteriolar density was assessed histologically by staining for αSMA^+ vessels in the infarct zone (IZ), border zone (BZ) and remote LV myocardium (Figure 7). In the IZ, both MSC treatment groups displayed greater arteriolar density than control animals, with a trend to higher density in the dual therapy group (dual 10.6±3.1 /0.2mm^2, single 6.2±1.2 /0.2mm^2, controls 0.9±0.4 /0.2mm^2; $p<0.001$ for MSC groups versus control and $p=0.24$ for dual vs single cohort). Similarly, arteriolar density was also equally augmented in each MSC group in the BZ of infarction, when compared with controls ($p<0.01$).

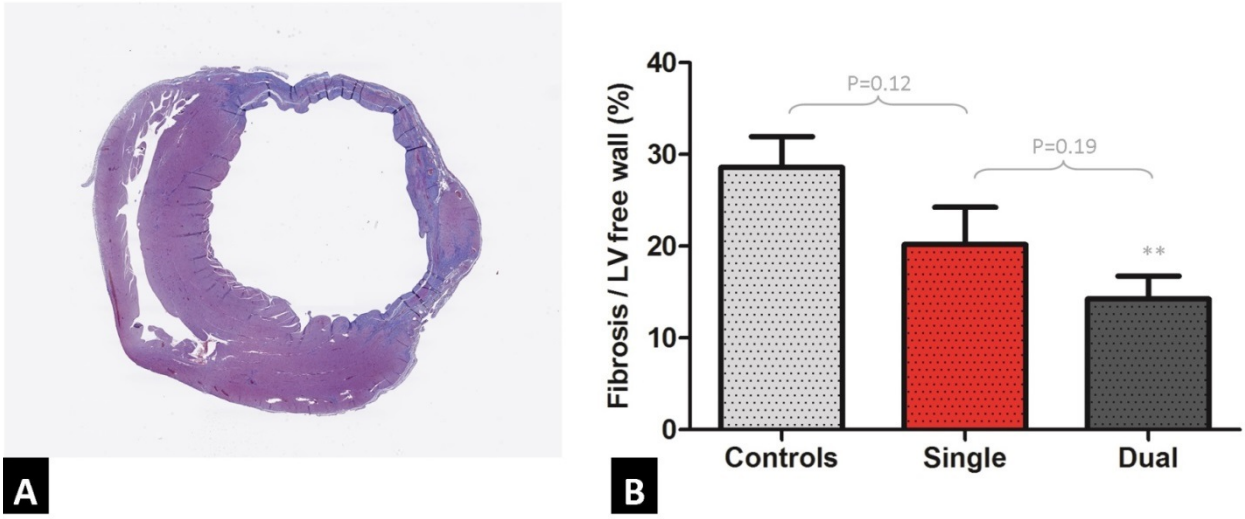

Figure 6. Myocardial fibrosis: Bar chart illustrating the myocardial fibrosis as a proportion of the LV free wall in the three experimental groups **(A)**. Representative Masson's trichrome staining of the heart at end-study in a control animal **(B)** - note the marked ventricular dilatation and wall thinning in addition to extensive myocardial fibrosis (blue staining). Representative examples from single **(C)** and dual MSC treatment groups **(D)** also shown. ** $p<0.01$ versus controls.

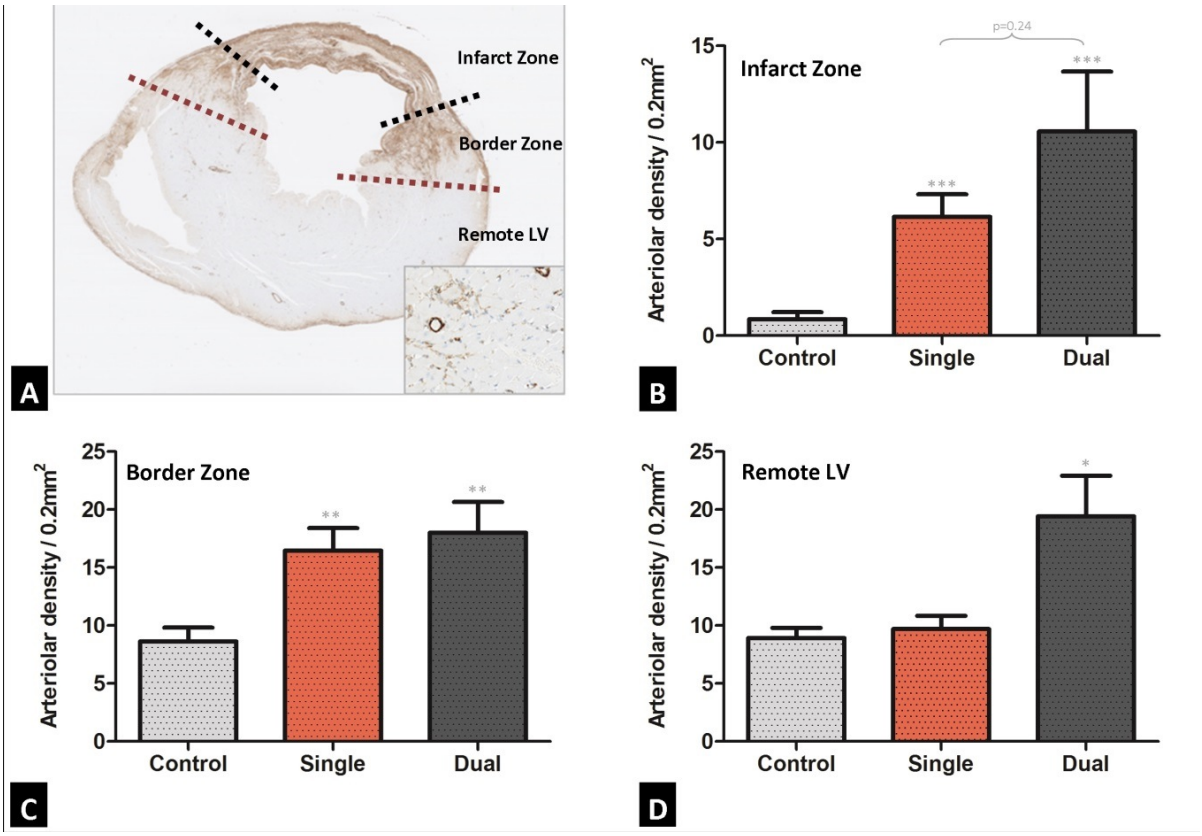

Figure 7. Arteriolar density: Representative example of alpha-smooth muscle actin (αSMA) staining, with the infarct zone, border zone and remote LV myocardium demarcated (A). Insert shows examples of αSMA^{+} vessels. Graphs summarising mean arteriolar density in the infarct zones (B), border zones of infarction (C), and remote LV myocardium (D) for each group.*$p<0.05$, ** $p<0.01$, *** $p<0.001$ versus controls.

Although arteriolar density in the LV myocardium remote to the IZ and BZ was not significantly higher in the solitary dose group compared to controls (single 9.7±1.2 / 0.2mm^2, controls 8.9±0.9 /0.2mm^2,p=0.60), it was substantially increased after repeated MSC delivery (dual 19.4±3.5 /0.2mm^2, p<0.01).

5.5.6. Apoptosis

Caspase-3 activity in the BZ was significantly reduced in the single and dual MSC treatment groups compared to controls, though no significant difference was observed between the two MSC groups (dual 142.6±24.8 cells/0.2mm^2, single 165.6±37.4 /0.2mm^2, controls 285.9±33.4 /0.2mm^2; p<0.05 for MSC groups versus control and p=0.60 for dual vs single cohort). In remote LV myocardium, there was a trend to reduced apoptosis in the dual treatment group compared to control and single MSC groups (dual 6.7 cells ±1.6 / 0.2mm^2, single 13.0±2.6 /0.2mm^2, controls 15.1±4.6 /0.2mm^2; p=0.07 dual versus single and p=0.11 dual versus control).

5.6. DISCUSSION

The principal findings of this study are: (i) Allogeneic MSC, enriched by prospective isolation and hypoxic pre-conditioning, have substantial pleiotropic reparative capacity for MI; (ii) A combination of interventions in the acute and sub-acute period after MI provides greater augmentation of ventricular function than solitary intervention; (iii) These additional improvements appear to be largely related to changes in the remote (non-infarct) LV myocardium.

This study systematically assessed optimised MSC delivered in the clinically relevant immediate and sub-acute period after MI in rats. We demonstrate that a second delivery of MSC, in animals already treated with MSC immediately post-MI, further enhances

ventricular function. Much of the reparative benefit of stem cell treatment was attained through the initial early intervention (absolute improvement in EF of 16.4% compared to control animals), consequently reducing the potential scope of repeat intervention. Though the magnitude of benefit obtained with repeat dosing was relatively moderate, with a further absolute increase in EF of 4.6%, this incremental effect is clinically relevant, especially when put in the context of benefits achievable with conventional post-MI pharmacotherapy. Furthermore, meta-analyses of human studies evaluating single therapy with autologous bone marrow mononuclear cells after MI have also demonstrated a mean improvement in EF of approximately 3% [1, 24].

One of the major limitations of current strategies of stem cell treatment for MI is the poor retention and engraftment of donor cells, therefore limiting their therapeutic efficacy. Cell optimisation strategies have been developed to improve the survival of transplanted cells by directly (e.g. *akt* overexpression [25]) or indirectly (e.g. hypoxic conditioning [4]) enhancing the expression of pro-survival factors [2]. Our study used rat allogeneic MSC, enriched by a prospective CD45 depletion step to exclude contaminating hematopoietic cells and hypoxic pre-conditioning, in contrast to conventional plastic adherence-isolated MSC that have been used in the vast majority of pre-clinical experiments [26-29)]. Previous studies have established that immunoselected MSC have enhanced stem cell purity and cardiovascular paracrine capacity when compared to conventionally isolated MSC [3, 5].

Repeated cell administration is another strategy that may increase cell retention and engraftment. Yet up until now it has been virtually un-investigated, as prior studies have delivered cells at only a solitary time-point after MI. The safety of repeat allogeneic MSC dosing, 14 days apart, has previously been demonstrated in healthy pigs, when delivered by the transendocardial route [30]. Our study evaluated repeat treatment in an infarct model at two clinically relevant time-points and demonstrates enhanced efficacy of repeat intervention over solitary therapy. In addition to the primary endpoint of ventricular function, we also identified a number of other important findings. The combination of

early and late MSC intervention provided greater global myocardial mass, superior SWT, fewer dysfunctional segments in remote LV myocardium and higher arteriolar density in the infarct zone and remote segments. There were also trends to reduced myocardial fibrosis and fewer infarcted segments overall compared to solitary MSC intervention. Given that the prevailing paradigm in the MSC field is one of paracrine related repair [3, 20, 31, 32], we focused on the impact of MSCs on arteriolar density, apoptosis and fibrosis according to single or dual treatment.

Interestingly, in the infarct territory myocardium, wall thickness, mean SWT, change in SWT and number of infarcted segments did not differ significantly between the solitary or repeated MSC groups. All of the above parameters were significantly improved compared to controls, and the equivalent findings may reflect the identical early MSC intervention that was common to both active treatment groups. When comparing early versus late MSC intervention, we have previously shown that early therapy has a greater relative impact in the *infarct* territory in terms of augmenting these functional and histological characteristics [14]. Furthermore, the same parameters were selectively influenced by late MSC intervention in *remote* LV segments, and this finding appears to be recapitulated in the present study, where the addition of a second MSC dose at one week post-MI resulted in beneficial changes in remote myocardium in a variety of parameters, including wall thickness and systolic thickening and microvascularity. One possible explanation for these interesting observations is that one week after MI, the infarcted rat myocardium has already undergone significant cardiomyocyte death and completed the early wave of acute inflammatory infiltration, with collagen deposition well underway [33]. As such, there is less scope for repair of the infarcted region and declining inflammatory homing cues. However, as the ventricular chamber continues to remodel, there may be increasing strain on remote (non-infarcted) myocardium possibly accompanied by upregulation of remote homing signals. Expression of cardiac relevant cytokines such as SDF-1/CXCL12 appear to vary over time in differing anatomical locations, first commencing in the infarct zone and spreading to the border zone before peaking at 96 hours post-MI [34]. We hypothesise that these factors, perhaps in conjunction with lower cell attrition after delayed delivery, may enable more cells to reach remote LV segments to promote repair there. Regional

differences in matrix metalloproteinases (MMPs) have been demonstrated in an ovine model evaluating mesenchymal precursor cells treatment 8 weeks after MI [31]. MMP-1, -2, and -7 levels in the remote myocardium were increased in the MI controls, whereas levels were decreased (therefore limiting collagen deposition) in the cell treatment groups. In the borderzone of infarction MMP-7 and -9 levels were reduced by cell treatment, most effectively at lower doses. These enzymes play a fundamental role in collagen turnover in the extracellular matrix and therefore influence LV remodelling in the infarct territory, border zone and remote myocardium.

5.6.1. Limitations

We opted to use a permanent ligation model of LAD occlusion, as it provides large infarcts with relatively high reproducibility of infarct size (as shown in the control group here, though this remains operator-dependant). Although ischaemia-reperfusion injury would more closely reflect the myocardial pathology encountered after patients with MI receive reperfusion treatment, this model is prone to causing infarcts of smaller and more variable size, potentially limiting the scope of therapeutic effect and complicating sample size and study power calculations.

5.6.2. Implications and Future Directions

This study reveals important insights around the timing and combination of stem cell interventions, which have not been available from previous studies that have relied on a single intervention delivered at various time points after infarction. In the clinical BOOST trial, a transient benefit was initially observed with unfractionated BM (MNC) cell therapy [35] that was later caught up by the placebo arm [36], raising the prospect that repeat intervention might enhance and prolong efficacy. On a rudimentary level, repeat dosing at any two intervals would provide a doubling of the cell dose delivered and therefore increased opportunity for cell retention, engraftment and therefore efficacy. However, the timing of cell intervention appears important [37] and therefore if targeted at specific critical time points and tailored to the underlying pathology, efficacy might be enhanced.

We investigated the immediate and sub-acute period after MI as the myocardium differs significantly at these times and they are clinically relevant intervals in the modern era of primary angioplasty/stenting for MI. We have shown in this study, and through comparisons of immediate versus deferred therapy [14], that MSC appear to affect contractile function in different regions of the LV, depending on timing of administration; early intervention has a greater relative impact on the infarct territory whereas late treatment has a greater influence on remote myocardium, raising the potential for additive or even synergistic benefit when the two are combined. In the same study, fluorescent labelled MSC were found to locate to the infarct and border zone to a greater extent when delivered early after MI, whereas deferred therapy was associated with greater numbers of transplanted MSC in remote LV myocardium, potentially explaining these findings [14]. Immediate cell treatment is now feasible as the allogeneic properties of MSC [38], allow their administration as an "off-the-shelf" product immediately after coronary reperfusion [29]. Patients with residual ventricular dysfunction could then be identified and offered repeat cell intervention in the sub-acute period. This could be via repeat intracoronary delivery, or targeted more specifically via transendocardial injection, which is increasingly being utilised in the post-MI setting [39]. Although in the current study we used the same MSC population for both interventions, specific strategies to optimise MSC could be tailored for different time-points of delivery [2]. For example, MSC used for early therapy could be modified to enhance their effects on cardiomyocyte survival [40], cardiac and vascular progenitor cell recruitment, and attenuation of inflammatory cell infiltration. Allogeneic cells to be transplanted in the sub-acute period after MI might benefit from techniques targeted at augmenting their anti-fibrotic, pro-angiogenic and cardiomyogenic potential [41]. The use allogeneic MSC for both interventions avoids the limitations of autologous cells, including the reduced cell function associated with advanced age and comorbidity of the donor commonly seen in patients with MI [2]. Contemporary clinical trials utilising allogeneic MSC are currently undergoing evaluation, with promising findings ([29], POSEIDON [42] and C-CURE trials [43]) and others still recruiting whose findings are eagerly awaited (e.g. Allogeneic Mesenchymal Precursor Cell Infusion in MyoCardial Infarction (AMICI) study NCT01781390).

5.7. Conclusion

Allogeneic MSC, enriched by prospective isolation and hypoxic conditioning, have substantial pleiotropic reparative capacity for MI. Although cell therapy is effective both when given as a single intervention or as dual therapy, an incremental advantage is achievable with repeat MSC intervention. These additional improvements appear to be largely related to changes in the remote (non-infarct) LV myocardium.

5.7.7.1. Acknowledgements

JDR is supported by an International Postgraduate Research Scholarship at the University of Adelaide, Australia. PJP has received funding from the National Health and Medical Research Council of Australia and the Royal Australasian College of Physicians. DW is supported by the National Health and Medical Research Council and Australian National Heart Foundation postgraduate scholarship.

1.

5.7.1.References

1, Lipinski MJ, Biondi-Zoccai GGL, Abbate A, Khianey R, Sheiban I, Bartunek J, et al. Impact of Intracoronary Cell Therapy on Left Ventricular Function in the Setting of Acute Myocardial Infarction: A Collaborative Systematic Review and Meta-Analysis of Controlled Clinical Trials. Journal of the American College of Cardiology. 2007;50(18):1761-7.

2. Richardson JD, Nelson AJ, Zannettino AC, Gronthos S, Worthley SG, Psaltis PJ. Optimization of the cardiovascular therapeutic properties of mesenchymal stromal/stem cells-taking the next step. Stem Cell Rev. 2013 Jun;9(3):281-302.

3. Psaltis PJ, Paton S, See F, Arthur A, Martin S, Itescu S, et al. Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol. 2010 May;223(2):530-40.

4. Das R, Jahr H, van Osch GJ, Farrell E. The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Eng Part B Rev. 2010 Apr;16(2):159-68.

5. See F, Seki T, Psaltis PJ, Sondermeijer HP, Gronthos S, Zannettino ACW, et al. Therapeutic Effects of Human STRO-3-Selected Mesenchymal Precursor Cells and their Soluble Factors in Experimental Myocardial Ischemia. Journal of Cellular and Molecular Medicine. 2010:no-no.

6. Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang J-A, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. The Journal of Thoracic and Cardiovascular Surgery. 2008;135(4): 799-808.

7. van der Spoel TIG, Jansen of Lorkeers SJ, Agostoni P, van Belle E, Gyöngyösi M, Sluijter JPG, et al. Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. Cardiovascular Research. 2011 September 1, 2011;91(4):649-58.

8. Virag JI, Murry CE. Myofibroblast and Endothelial Cell Proliferation during Murine Myocardial Infarct Repair. The American Journal of Pathology. 2003;163(6):2433-40.

9. Hawkins H. K EML, Zhu J. Y, Youker K. A, Berens K, Doré M, Smith C. W. . Acute inflammatory reaction after myocardial ischemic injury and reperfusion. Development and use of a neutrophil-specific antibody. Am J Pathol. 1996;148(6):1957–69.

10. Thangarajah H, Vial IN, Chang E, El-Ftesi S, Januszyk M, Chang EI, et al. IFATS collection: Adipose stromal cells adopt a proangiogenic phenotype under the influence of hypoxia. Stem Cells. 2009 Jan;27(1): 266-74.

11. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004 Mar 19;94(5):678-85.

12. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008 Nov 21;103(11):1204-19.

13. Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci U S A. 2009 Aug 18;106(33):14022-7.

14. Richardson JD, Bertaso AG, Psaltis PJ, Frost L, Carbone A, Paton S, et al. Impact of timing and dose of mesenchymal stromal cell therapy in a preclinical model of acute myocardial infarction. J Card Fail. 2013 May; 19(5):342-53.

15. Position of the American Heart Association on the use of research animals. A statement for health professionals from a task force appointed by the Board of Directors of the American Heart Association. Circulation Research. 1985 August 1, 1985;57(2):330-1.

16. Richardson JD, Bertaso AG, Frost L, Psaltis PJ, Carbone A, Koschade B, et al. Cardiac magnetic resonance, transthoracic and transoesophageal echocardiography: a comparison of in vivo assessment of ventricular function in rats. Lab Anim. 2013 Jul 8 [Epub ahead of print].

17. Segmentation AHAWGoM, Imaging: RfC, Cerqueira MD, Weissman NJ, Dilsizian V, Jacobs AK, et al. Standardized Myocardial Segmentation and Nomenclature for Tomographic Imaging of the Heart. Circulation. 2002 January 29, 2002;105(4): 539-42.

18. Dai W, Hale SL, Martin BJ, Kuang J-Q, Dow JS, Wold LE, et al. Allogeneic Mesenchymal Stem Cell Transplantation in Postinfarcted Rat Myocardium. Circulation. 2005 July 12, 2005;112(2):214-23.

19. Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS, Blomme EA. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. J Pathol. 2003 Feb;199(2):221-8.

20. Psaltis PJ, Carbone A, Nelson AJ, Lau DH, Jantzen T, Manavis J, et al. Reparative effects of allogeneic mesenchymal precursor cells delivered transendocardially in experimental nonischemic cardiomyopathy. JACC Cardiovasc Interv. 2010 Sep;3(9):974-83.

21. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.

22. Gronthos S, Zannettino ACW, Hay SJ, Shi S, Graves SE, Kortesidis A, et al. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. Journal of Cell Science. 2003 May 1, 2003;116(9):1827-35.
23. Nowosielski M, Schocke M, Mayr A, Pedarnig K, Klug G, Kohler A, et al. Comparison of wall thickening and ejection fraction by cardiovascular magnetic resonance and echocardiography in acute myocardial infarction. Journal of Cardiovascular Magnetic Resonance. 2009;11(1):22.
24. Abdel-Latif A, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, et al. Adult Bone Marrow-Derived Cells for Cardiac Repair: A Systematic Review and Meta-analysis. Arch Intern Med. 2007 May 28, 2007;167(10):989-97.
25. Gnecchi M, He H, Melo LG, Noiseaux N, Morello F, de Boer RA, et al. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing Akt on cardiac metabolism after myocardial infarction. Stem Cells. 2009 Apr;27(4):971-9.
26. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The Development of Fibroblastic Colonies in Monolayer Cultures of Guinea-pig Bone Marrow and Spleen Cells. Cell Proliferation. 1970;3(4):393-403.
27. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143 - 7.
28. Schuleri KH, Amado LC, Boyle AJ, Centola M, Saliaris AP, Gutman MR, et al. Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. Am J Physiol Heart Circ Physiol. 2008 May; 294(5):H2002-11.
29. Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol. 2009 Dec 8;54(24):2277-86.
30. Poh K-K, Sperry E, Young RG, Freyman T, Barringhaus KG, Thompson CA. Repeated direct endomyocardial transplantation of allogeneic mesenchymal stem cells: Safety of a high dose, "off-the-shelf", cellular cardiomyoplasty strategy. International Journal of Cardiology. 2007;117(3):360-4.
31. Dixon JA, Gorman RC, Stroud RE, Bouges S, Hirotsugu H, Gorman JH, 3rd, et al. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation. 2009 Sep 15;120(11 Suppl):S220-9.
32. Houtgraaf JH, de Jong R, Kazemi K, de Groot D, van der Spoel TI, Arslan F, et al. Intracoronary Infusion of Allogeneic Mesenchymal Precursor Cells Directly Following Experimental Acute Myocardial Infarction Reduces Infarct Size, Abrogates Adverse Remodeling and Improves Cardiac Function. Circ Res. 2013 May 8.
33. Ertl G, Frantz S. Healing after myocardial infarction. Cardiovascular Research. 2005 April 1, 2005;66(1):22-32.
34. Zhang M, Mal N, Kiedrowski M, Chacko M, Askari AT, Popovic ZB, et al. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. FASEB J. 2007 Oct; 21(12):3197-207.
35. Wollert KC, Meyer GP, Lotz J, Ringes Lichtenberg S, Lippolt P, Breidenbach C, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. The Lancet. 2004;364(9429):141-8.
36. Meyer GP, Wollert KC, Lotz J, Steffens J, Lippolt P, Fichtner S, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. Circulation. 2006 Mar 14;113(10):1287-94.
37. Traverse Jh HTDESG, et al. Effect of intracoronary delivery of autologous bone marrow mononuclear cells 2 to 3 weeks following acute myocardial infarction on left ventricular function: The latetime randomized trial. JAMA: The Journal of the American Medical Association. 2011;306(19):2110-9.
38. Aggarwal S, Pittenger M. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005;105:1815 - 22.
39. Psaltis PJ, Worthley SG. Endoventricular electromechanical mapping-the diagnostic and therapeutic utility of the NOGA XP Cardiac Navigation System. J Cardiovasc Transl Res. 2009 Mar;2(1):48-62.
40. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005 Apr;11(4):367-8.
41. Behfar A, Yamada S, Crespo-Diaz R, Nesbitt JJ, Rowe LA, Perez-Terzic C, et al. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. J Am Coll Cardiol. 2010 Aug 24;56(9):721-34.
42. Hare JM, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, et al. Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. JAMA. 2012 Dec 12;308(22):2369-79.
43. Bartunek J, Behfar A, Dolatabadi D, Vanderheyden M, Ostojic M, Dens J, et al. Cardiopoietic Stem Cell Therapy in Heart Failure: The C-CURE (Cardiopoietic stem Cell therapy in heart failURE) Multicenter Randomized Trial With Lineage-Specified Biologics. J Am Coll Cardiol. 2013 Jun 11;61(23):2329-38.

Statement of Authorship

Title of Paper	I**ncremental Benefits of Repeated Mesenchymal Stromal Cell Administration Compared to Solitary Intervention in a Preclinical Model of Myocardial Infarction.**
Publication Status	Published
Publication Details	Cytotherapy. 2013 Oct 8. doi:pii: S1465-3249(13)

Author Contributions

Name of Principal Author (Candidate)	**Dr James David Richardson**
Contribution to the Paper	Primary contributor to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work; Drafted the work ; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Angela Bertaso**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Peter J Psaltis**

Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Lachlan Frost**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mr Angelo Carbone**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Dennis T Wong**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and

	revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Dr Adam J Nelson**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**Mrs Sharon Paton**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.
	18th December 2013
Name of Co-Author	**A/Prof Matthew Worthley**
Contribution to the Paper	Provided a substantial contribution to the acquisition, analysis, and interpretation of data for the work; Helped draft the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.

18th December 2013

Name of Co-Author	**Professor Andrew Zannettino**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work

18th December 2013

Name of Co-Author	**Professor Stan Gronthos**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work.

18th December 2013

Name of Co-Author	**Professor Stephen G Worthley**
Contribution to the Paper	Provided a contribution to the design of the work; Helped drafting the work and revising it critically; Provided final approval of the version to be published; Agreement to be accountable for all aspects of the work

18th December 2013

6. Chapter - Summary

6.1. General Overview

Ischemic heart disease remains a leading cause of morbidity and mortality worldwide. Emergency reperfusion treatment has significantly improved survival rates from its all too frequent complication - myocardial infarction. However, survivors are often left with significant left ventricular dysfunction which greatly impedes both quality of life and prognosis. Contemporaneous treatment of cardiac failure principally consists of pharmacological approaches in addition to selected use of device therapy. These measures deliver only a modest reduction in morbidity and mortality, with 5-year survival rates less than 50% frequently observed. Owing to the lack of suitable donor organs, cardiac transplantation remains a solution reserved for a minority of patients who demonstrate the most severe forms of cardiac failure. Therefore a compelling clinical need exists for novel therapeutic approaches, of which stem cell treatment has emerged as one of the most promising.

Many potential candidate cells have been evaluated, including unfractionated bone marrow cells, BM MNCs, hematopoietic stem cells, endothelial progenitor cells, skeletal myoblasts and mesenchymal stromal/stem cells. Of these, MSCs have been widely studied at both preclinical and clinical levels, owing to their ease of isolation, proclivity for ex vivo expansion, range of pro-angiogenic and cardiac supportive benefits and the potential for their allogeneic use due to their relatively immunoprivileged nature. Increasingly, a paradigm has emerged in which MSCs (and indeed other cell types) are thought to provide cardiovascular reparative properties through indirect, paracrine effects. These are mediated by cell-to-cell interactions with constitutive cells and the secretion of a wide range of soluble growth factors and cytokines that influence nearby cells. These effects can be categorized as (1) trophic (anti-apoptotic; supportive of proliferation or differentiation of endogenous cells; pro-angiogenic), (2) immunomodulatory, (3) anti-fibrotic or (4) chemoattractant. Many of these beneficial effects not only support efforts to repair injured myocardium, but also offer *preventative* functions that might limit the extent of injury sustained after MI. Therefore it follows that the greatest impact of MSC treatment

might be when myocardium retains its greatest viability, which is early after the onset of infarction. The allogeneic utility of MSC now makes intervention at this time point possible and accordingly a scientifically important subject to investigate. Furthermore, the use of allogeneic MSC also opens the door to optimisation of stem cells (derived from healthy, young donors) to enhance their therapeutic efficacy and help resolve the limitations that hinder the clinical application of stem cells. The studies in this thesis have investigated the above subjects, by using allogeneic MSC, optimised by two different strategies and investigated the impact of the two clinically relevant time points, either as solitary or dual treatment.

6.2. Optimising MSC

The various strategies to optimise stem cell treatment were outlined in the review paper in chapter 1. Of these approaches, many remain a distant prospect in particular those requiring genetic manipulation, whereas others could be more readily transferred to the clinic. The studies presented in this thesis used two of these techniques – prospective isolation and hypoxic preconditioning – and combined the two strategies in a novel attempt to further augment MSC optimisation. In a later chapter, the in vivo function of these optimised MSC were compared against same donor non-optimised plastic adherent cells. That study demonstrated greater differentiation capacity and colony forming efficiency with the optimised MSC. Adipogenic and osteogenic differentiation were almost 6- and 5-fold higher respectively than conventional PA-MSC, whereas chondrogenic differentiation was equivalent. Colony forming efficiency was over 20-fold higher after prospective isolation, and increased by a further 50% after hypoxic preconditioning, providing an overall 36-fold improvement of optimised over plastic adherent cells.

6.3. Rat cardiac imaging

The primary endpoint in evaluating the reparative effects of stem cell treatment after MI is usually the assessment of LV function. In adults and large animals the gold standard imaging modality is CMR, however in small animal models the use of CMR has largely

been restricted to expensive high field scanners of scarce availability. Our research unit has a substantial experience with CMR and investigated whether LV function could be reliably assessed with conventional 1.5T scanners. Rat hearts are approximately 1cm in length and beat at approx. 300bpm providing a significant challenge to the spatial and temporal resolution capabilities of 1.5T MRI. Indeed, only a handful of reports have been published worldwide regarding this technology.

Our study, presented in chapter 2, reports the unique comparison of 1.5T CMR against conventional transthoracic echocardiography and the novel assessment of rat LV function with transoesophageal echocardiography. In addition to been the first study to systematically compare 1.5T CMR, TTE and TOE in rodents, it demonstrated: (1) that 1.5T CMR is a feasible and highly reproducible method of evaluating LV EF in rats; (2) Support for TOE as a feasible and reproducible method to evaluate LV function; (3) CMR has greater reproducibility than the alternative modalities studied, with excellent intra and inter-observer agreement.

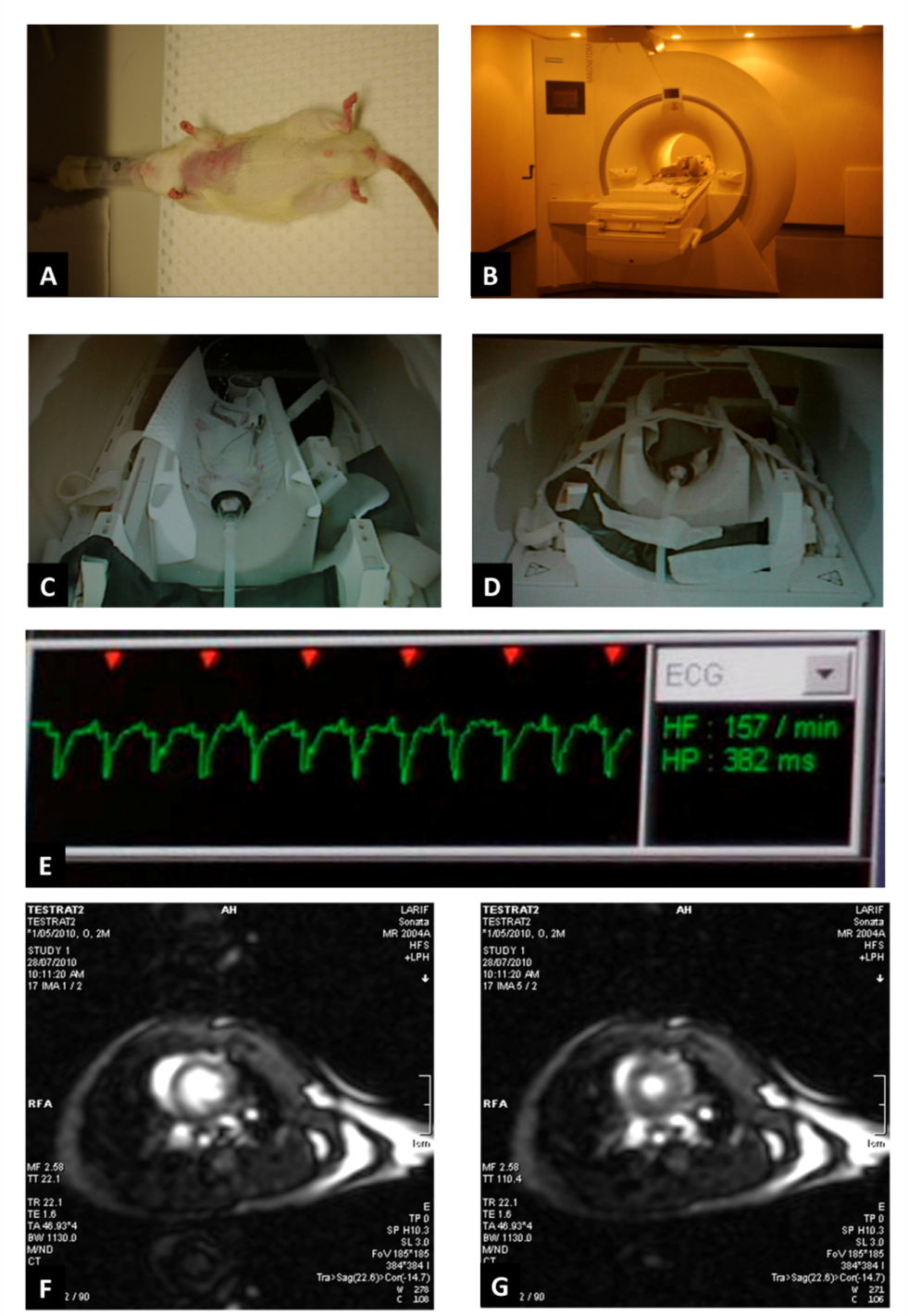

Figure 6.1: Rat Cardiac Magnetic Resonance Imaging. (A) Animals are anaesthetised with Isoflurane. (B) the 1.5T CMR clinical scanner with (C) ECG leads attached to chest and (D) Carotid receiver coil in pace of the anterior chest of the rats. (E) ECG gated acquisitins timed every other R wave. (F) representative end diastolic and (G) end systolic images, from which contours can be drawn in specialist software packages to calculate ejection fraction.

6.4. Pre-clinical model of MI

In common with the majority of rodent models of myocardial infarction, we used ligation of the left anterior descending artery to create infarction. This surgery was performed under general anaesthetic with Isoflurane under sterile conditions. MSC were then delivered by transepicardial injection into the border zone of infarction. ProFreeze media, the cryopreservant in which MSC were stored and delivered, was used for control injections. The intubation procedure is outlined in figure 6.2. The LAD ligation model employed in these experiments (illustrated in figure 6.3) are associated with significant mortality of rat subjects. We observed a mortality rate in the order of 30%, consistent with previously published studies. Animals were randomised to a experimental group prior to this operation so mitigating the potential for survival bias. Furthermore the experiments were not powered to detect a difference in mortality between experimental groups, as this was not a end point under evaluation.

One potential limitation of stem cell implantation is the potential for increased arrhythmogenesis. This concern primarily relates to the findings in subjects after transplantation of skeletal myoblasts. In that scenario the myoblasts developed functionally contractile tissue, however these cells were electrically inert. Accordingly this provides the substrate for re-entrant ventricular tachyarrhythmias that were observed in a significant proportion of patients. Fortunately, adverse effects on heart rhythm have not been recapitulated in other studies examining other stem cells, including mesenchymal stem cells/precursor cells. In the studies detailed in this thesis, we sought to investigate the arrhythmic potential following MSC transplantation. We performed electrophysiology testing on atrial and ventricular tissue retrieved following sacrifice using a micro electrode array system. We aimed to measure conduction velocities, conduction heterogeneity and total activation time. However, we were unable to reproducibly ascertain these measurements, such that no reliable comparisons between controls and the variety of MSC strategies employed in the thesis could be made. Future studies will need to address this important issue.

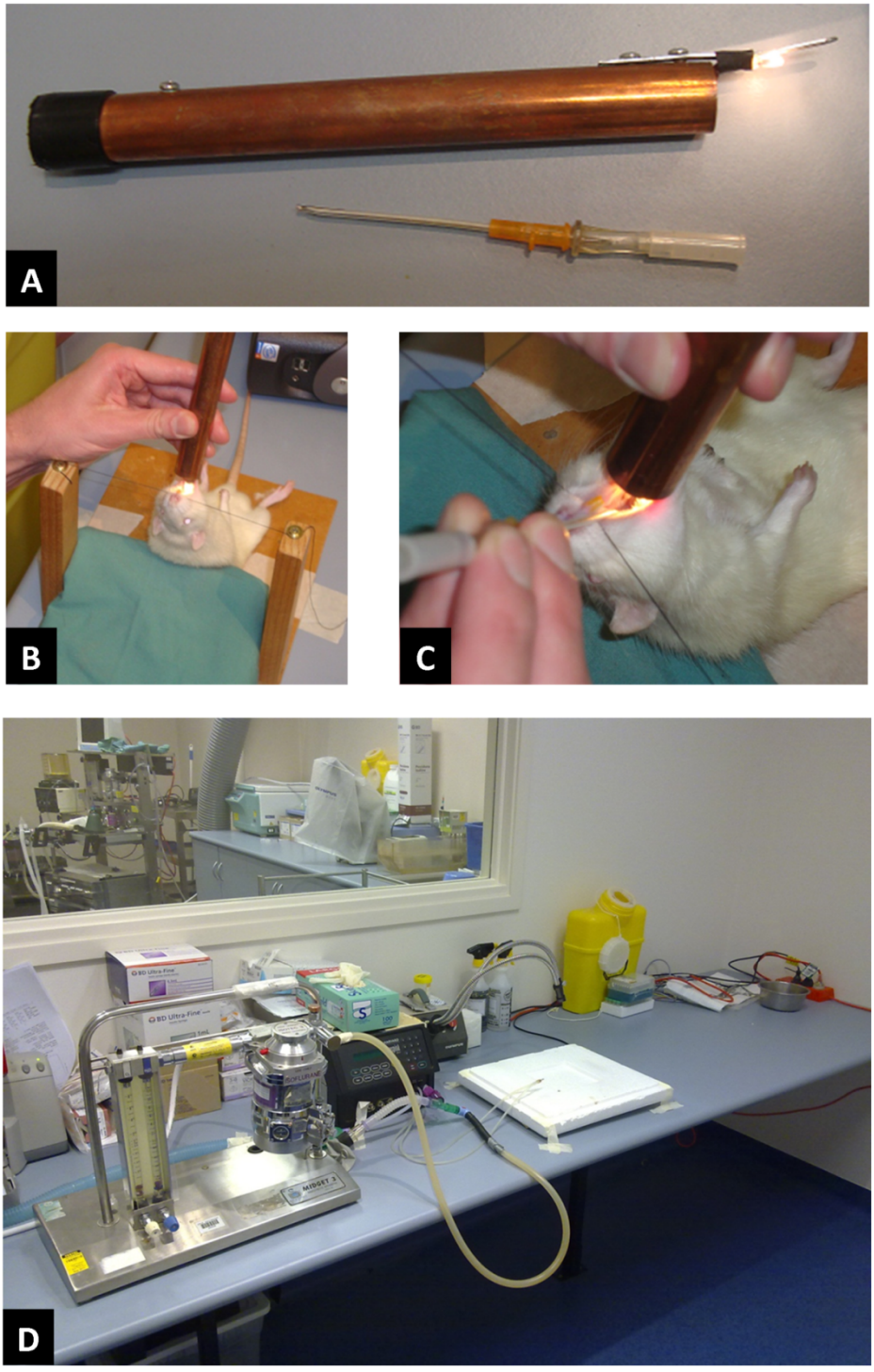

Figure 6.2: Rat Intubation and Ventilation. (A) A medical venous cannula is used as a novel endotracheal tube for ventilation and a purpose built laryngoscope are shown.. **(B)** Animals are suspended by their front teeth from a purpose built rack and **(C)** the cannula inserted through the vocal cords under direct vision. **(D)**Specialist rodent ventilator.

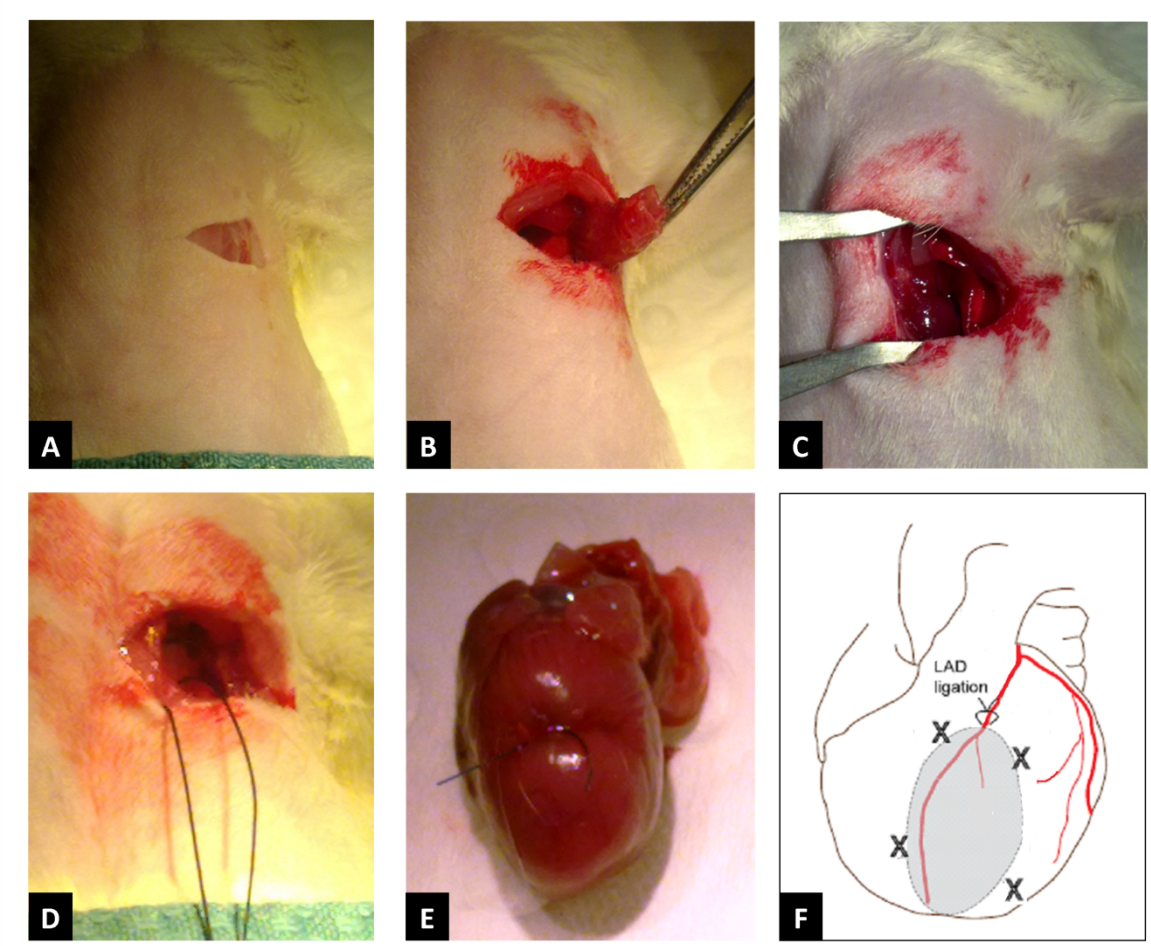

Figure 6.3: Thoracotomy, ligation and MSC injection. (A) Skin incision over the left 5th rib space. (B) Dissection down the ribs, with excision of the 5th rib, to allow adequate visualisation. (C) Retractors in situ providing visualisation of the rat heart. (D) Suture introduced from the lateral border through to the septum, just beneath the left atrium (so ligating the left anterior descending artery). (E) Excised heart example showing ligature in situ. (F) Schematic displaying the four injection sites for the MSC/Placebo.

6.5. In vivo effects of optimised MSC

Chapter 3 investigated the impact of MSC optimisation. The in vitro effects were discussed in section 6.2, demonstrating significant improvements in adipogenic and osteogenic capacity. This experimental study then evaluated whether these effects would be translated into significant in vivo effects. This was the case, with profound effects on left ventricular ejection fraction in MSC treated animals. In controls, MI reduced EF rom 58.5±1.2% to 22.1±2.0% at 1 week, with no subsequent improvement by week 4 (19.5±1.5%). In the MSC treatment group, EF also fell from 57.8±1.2% to 25.1±2.2%, but significantly improved to 38.8±1.4% by week 4 ($p<0.001$). Accordingly, optimised-MSC increased EF by 19.3±3.1% ($p<0.001$) at 4 weeks and was accompanied by improvements in LV volumes, mass, wall thickness, systolic wall thickening, fibrosis and arteriolar density. This study provided the rationale for investigating the optimal timing of MSC intervention and also whether stem cell transplantation at multiple pathophysiologically-relevant time points impacts upon MSC efficacy and mechanisms of action.

6.6.Timing of MSC intervention.

This study reveals important insights around the timing and combination of stem cell interventions, which have not been available from previous studies that have relied on a single intervention delivered at various time points after infarction. In the clinical BOOST trial, a transient benefit was initially observed with unfractionated BM (MNC) cell therapy that was later caught up by the placebo arm, raising the prospect that repeat intervention might enhance and prolong efficacy. On a rudimentary level, repeat dosing at any two intervals would provide a doubling of the cell dose delivered and therefore increased opportunity for cell retention, engraftment and therefore efficacy. However, the timing of cell intervention appears important and therefore if targeted at specific critical time points and tailored to the underlying pathology, efficacy might be enhanced. We investigated the immediate and sub-acute period after MI as the myocardium differs significantly at these

times and they are clinically relevant intervals in the modern era of primary angioplasty/ stenting for MI. We have shown in this study, and through comparisons of immediate versus deferred therapy in chapter 4, that MSC appear to affect contractile function in different regions of the LV, depending on timing of administration; early intervention has a greater relative impact on the infarct territory whereas late treatment has a greater influence on remote myocardium, raising the potential for additive or even synergistic benefit when the two are combined. In the same study, fluorescent labelled MSC were found to locate to the infarct and border zone to a greater extent when delivered early after MI, whereas deferred therapy was associated with greater numbers of transplanted MSC in remote LV myocardium, potentially explaining these findings.

Immediate cell treatment is now feasible as the allogeneic properties of MSC, allow their administration as an "off-the-shelf" product immediately after coronary reperfusion. Patients with residual ventricular dysfunction could then be identified and offered repeat cell intervention in the sub-acute period. This could be via repeat intracoronary delivery, or targeted more specifically via transendocardial injection, which is increasingly being utilised in the post-MI setting.

Although in our study we used the same MSC population for both interventions, specific strategies to optimise MSC could be tailored for different time-points of delivery. For example, MSC used for early therapy could be modified to enhance their effects on cardiomyocyte survival [40], cardiac and vascular progenitor cell recruitment, and attenuation of inflammatory cell infiltration. Allogeneic cells to be transplanted in the sub-acute period after MI might benefit from techniques targeted at augmenting their anti-fibrotic, pro-angiogenic and cardiomyogenic potential [41]. The use allogeneic MSC for both interventions avoids the limitations of autologous cells, including the reduced cell function associated with advanced age and comorbidity of the donor commonly seen in patients with MI [2].

6.7. MSC Intervention at multiple time points

The study in chapter 5 systematically assessed optimised MSC delivered in the clinically relevant immediate and sub-acute period after MI in rats. We demonstrate that a second delivery of MSC, in animals already treated with MSC immediately post-MI, further enhances ventricular function. Much of the reparative benefit of stem cell treatment was attained through the initial early intervention (absolute improvement in EF of 16.4% compared to control animals), consequently reducing the potential scope of repeat intervention. Though the magnitude of benefit obtained with repeat dosing was relatively moderate, with a further absolute increase in EF of 4.6%, this incremental effect is clinically relevant, especially when put in the context of benefits achievable with conventional post-MI pharmacotherapy. Furthermore, meta-analyses of human studies evaluating single therapy with autologous bone marrow mononuclear cells after MI have also demonstrated a mean improvement in EF of approximately 3%.

6.8.Future directions

A wide variety of optimization strategies have been discussed for MSC-based therapies for cardiac repair, with particular focus on optimization of cell biology, modification of myocardial substrate or improvement of cell delivery. The immediate clinical applicability of these different techniques varies substantially. Prospective immunoselection of MPCs and cocktail-guided cardiopoiesis of MSCs are two examples that on the basis of solid preclinical evidence have been applied in clinical trials. Alternative strategies such as the use of genotoxic differentiation agents (e.g. 5-azacytidine) are unlikely to be deemed safe for clinical use. While feasible, genetic engineering of cells faces considerable obstacles before satisfying stringent safety and regulatory requirements to allow human investigation.

Furthermore, it is increasingly apparent that there is no single "one-size-fits-all" answer to the clinical need that stem cell therapies aim to address. Cell-based interventions will ultimately need to be individually tailored to best suit patient-specific characteristics, including age, cardiovascular disease target, time of treatment relative to myocardial insult and the presence of comorbidities. For instance, the biological properties and optimization of cells used to treat early MI are likely to be very different from those that will be most helpful in chronic MI or in non-ischemic cardiac diseases. In the case of the former, cells might be required to achieve preservation of LV function, through paracrine-mediated reduction of inflammatory cell infiltrates, modulation of cardiomyocyte protection and attenuation of extracellular matrix remodeling. Prospectively isolated MPCs with hypoxic preconditioning and/or Akt overexpression might adeptly serve this purpose. In contrast, cells delivered beyond the acute period of MI could be maximized in their cardiomyogenic regenerative potential (e.g. by directed cardiopoiesis), while in chronic IHD, VEGF-based augmentation of their vasculogenic properties may be most helpful.

The widespread adoption of primary angioplasty/stenting for acute MI together with the allogeneic properties of MSC, suggest that it is clinically feasible for MSC to be administered as an "off-the-shelf" product immediately after coronary reperfusion. Indeed, an early dosing strategy would negate the need for additional (potentially invasive and expensive) procedures, making this approach more attractive to patients and health commissioners alike. Repeat cell therapy, as above, could then be targeted to those with impaired ventricular function.

6.9. Conclusion

In conclusion, the findings presented in this thesis provide new evidence highlighting the value of cell optimisation techniques comprising prospective isolation and hypoxic preconditioning and demonstrate the reparative properties when used early, late or at both clinically relevant time points after myocardial infarction.