The Cardiovascular Reparative Properties of Bone Marrow Mesenchymal Precursor Cells

Peter James Psaltis

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

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Abstract

Despite seminal advances in its management, the burden of cardiomyopathy remains substantial, providing the stimulus for intense research into new therapeutic options, including the use of stem cells. Of the various adult-derived cell types that have been investigated, mesenchymal stromal/stem cells (MSC) are notable for their lack of immunogenicity and their capacity to mediate vascular and myocardial repair. While numerous studies have evaluated the use of plastic adherence-isolated, *ex vivo* expanded MSC for the treatment of ischaemic heart disease, little attention has been given to the use of MSC in the context of nonischaemic heart disease.

The studies described in this thesis address the issue of optimising the efficacy of mesenchymal cell therapy, by using selective, antibody-based isolation of immature, mesenchymal precursor cells (MPC). Furthermore, they evaluate the reparative benefits of immunoselected MPC in a newly described, preclinical model of nonischaemic cardiomyopathy (NICM), using NOGA® XP navigation technology to perform targeted, intramyocardial cell delivery.

In cell-based experiments, human bone marrow MPC, prepared by prospective immunoselection using the STRO-1 monoclonal antibody, were compared to donor-matched, plastic adherence-isolated MSC (PA-MSC). The *ex vivo* cultured progeny of STRO-1-enriched cells were found to possess a more immature and “stem-like” phenotype than conventional PA-MSC, also displaying greater expression of cardiovascular-relevant cytokines and stronger cardiovascular paracrine activity. Similar benefits were observed for human MPC that were isolated by using an alternative antibody, STRO-3. Unlike STRO-1, this marker was not human-species specific, enabling its use for selective preparation of ovine bone marrow MPC, as well.

Preclinical NICM was reproducibly induced in Merino sheep, by titrated dosing of the anthracycline drug, doxorubicin, via the coronary arteries. Using a combination of cardiac magnetic resonance imaging, echocardiography and histology, this large animal model was found to share many of the features of clinical NICM, including the increased presence of myocardial fibrosis. Electromechanical characterisation of this model, using the NOGA® XP Cardiac Navigation System, demonstrated for the first time, the system's potential to identify myocardial segments with increased fibrosis in NICM.

This information was used to facilitate intramyocardial transplantation of allogeneic, STRO-3-selected, ovine MPC in the anthracycline model of cardiomyopathy. Cell therapy was found to attenuate left ventricular remodelling and prevent the deterioration of cardiac function that was observed in placebo-treated animals. The MPC-induced benefits were accompanied by reduction of cardiac fibrosis and enhancement of cardiomyocyte proliferation and myocardial neovascularisation, which were most likely mediated by paracrine mechanisms.

In summary, the studies described in this thesis provide new evidence confirming the merits of selective isolation of bone marrow MPC and demonstrate that the pleiotropic, reparative effects of these cells may extend their therapeutic utility to nonischaemic cardiac disease.

Declaration

I declare that this thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Peter James Psaltis. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

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

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Peter James Psaltis
25th March 2010

In dedication to the three little wonders in my life,

my children:

Jimmy, Lela & Labrini Psaltis

“There is no failure except in no longer trying. There is no defeat except from within, no insurmountable barrier except our own inherent weakness of purpose.”

Elbert Hubbard (1856-1915), American writer and philosopher

Table of Contents

Explanation Commentary of Revisions to Thesis

I thank both Professor Henry Krum and Professor Stephen Hunyor for their positive comments and helpful suggestions, submitted to the University of Adelaide as examiners of my thesis. This document outlines in detail the amendments made to my thesis in response to their feedback.

Revisions made in response to Professor Henry Krum

Professor Krum made minor recommendations for change to the Introduction, Methods, Chapter 5 and Chapter 7.

Chapter 1 Introduction

1. Section 1.6.2.3. Endothelial Progenitor Cells (page 14) has been slightly modified to respond to the reviewer's question about the variation of endothelial progenitor cell number in different disease states:

The numbers of circulating progenitor cells have also been shown to vary, often diminishing in the presence of cardiovascular risk factors, non-cardiac diseases and ageing and increasing in response to specific pharmacologic agents, such as HMG-CoA reductase inhibitors [78, 79].

2. With respect to the reviewer, we do not believe that the differences between iscaemic and nonischaemic heart failure were at all overstated in the introduction section of this thesis. Rather, the text describes the heterogeneous nature of pathological substrate in nonischaemic cardiomyopathy and important, published differences that may exist in the

regulation of homing and inflammatory signals in the myocardium and circulation in this disease context. As discussed in the text of this thesis, these factors may have implications for the selection of both cell type and delivery route of cell therapy in nonischaemic heart disease (Please refer to section 1.11.4 page 51).

Chapter 2 Methods

3. With regards to the question of sample size calculation, the establishment and description of the doxorubicin model of cardiomyopathy and its characterisation by electromechanical mapping (Chapters 5 and 6), required using relatively high numbers of animals to assess a variety of end-points, beyond simple left ventricular ejection fraction. As such, we did not believe that it was appropriate to limit the size of these validation studies to the minimum sample number required to measure the intended reduction of ejection fraction (n=8 animals for a 10% decline in ejection fraction, with 6% variance and 80% statistical power).

In chapter 7 (section 7.2), I have added an explanatory paragraph explaining the rationale for study group numbers:

Study numbers were based on observing a 5% absolute reduction in mean global ejection fraction in the placebo group after intramyocardial intervention, aiming for statistical power of 80% with P value < 0.05 and allowing for 4% variance. Allowance was made for two animals per group to not survive the entire study duration.

Chapter 5 Establishment of Preclinical Model of Doxorubicin Cardiomyopathy

4. In our experience which has been supported by other investigator groups, the accuracy of transthoracic echocardiography is quite limited in sheep, due to the shape of the chest wall and the unreliability of image quality. This has been described in some detail in the discussion section of chapter 5 (page 137). Therefore, with respect to the reviewer, we do not believe that the correlation between echocardiography and magnetic resonance imaging in individual animals would provide essential or indeed additive information to the question of establishing and characeterising this preclinical cardiomyopathy model.

With regard to the CMR assessment of fibrosis, this was described in section 5.3.3.4 pages 131-132.

Delayed enhancement of gadolinium was present on CMR in 10/21 surviving sheep (47.6%), involving 35% of all LV segments examined. In the majority of cases this was located either midmurally (Figure 5.13A) or subepicardially (Figure 5.13D), often corresponding with macroscopic foci of myocardial pallor at necropsy (Figure 5.13B,C,E) or microscopic evidence of fibrosis (Fibrosis 5.13F). Transmural or subendocardial enhancement, suggestive of infarction was not observed.

In addition, I have added the following sentence in the following paragraph confirming that myocardial segments with delayed enhancement contained a higher burden of histological fibrosis:

Notably, the percentage area of fibrosis was significantly higher in those LV segments showing late gadolinium uptake on CMR than those without delayed enhancement (9.0±2.8% versus 6.9±2.4%, P<0.001).

Chapter 7 Allogeneic MPC Therapy in Noniscaemic Cardiomyopathy

5. With respect to the reviewer, it appears as though he may have misunderstood the statistical analysis used in Chapter 7 to compare the effects of cell therapy and placebo intervention. In the original analysis, measurements at different time-points were compared within each group (MPC and placebo) by one-way repeated measures ANOVA and at final follow-up between groups by unpaired Student's t-test. As such, absolute values, rather than percent changes (as stated by the reviewer), had been used for this purpose.

However, we do agree with the reviewer that two-way ANOVA is the preferable test for intergroup comparisons and have repeated the analyses accordingly. Please refer to Section 7.2 page 158

Inter-group comparisons involved Mann-Whitney test, unpaired Student's t-test, one-way ANOVA or two-way ANOVA, as appropriate. A two-tailed P value of <0.05 was considered statistically significant.

The applicable results and figures have now been modified to reflect the amended analysis. This has included merging the previous Figures 7.4 and 7.5 into a new montage (Figure 7.4). Notably, this different approach to statistical analysis did not alter the main findings of this chapter.

Revisions made in response to Professor Stephen Hunyor

Professor Hunyor made some specific suggestions regarding the thesis prologue and lay-out:

1. As per his recommendations, an additional page has been provided in the prologue addressing financial disclosures. As stated, I have no financial conflicts of interest to disclose.

2. The table of contents has been shortened.

3. After review of the hard copies of the thesis and discussion with all of my supervisors, we believe that the formatting of figures, tables and legends should remain as they had been originally presented.

4. An opening section has been included at the start the Introduction (Section 1.1), stating the central hypotheses of the thesis. This is in addition to the objectives and aims of the thesis which remain at the end of the introduction section.

Responses to his other recommendations are found below:

5. Suitability of the pre-clinical model of NICM and the issue of fibrosis

With respect to the reviewer, we strongly disagree with his conclusions that "only 10/21 sheep had significant fibrosis". The reviewer has made this inference on the grounds that this was the number of sheep that demonstrated delayed enhancement on cardiac magnetic resonance. While this is so, CMR-based assessment of fibrosis is imperfect and only detects significant foci of replacement fibrosis, rather than interstitial and perivascular fibrosis that are more prevalent in clinical noniscaemic cardiomyopathy and indeed in our model of preclinical disease. In contrast to the reviewer's conclusion, all sheep in our model had increased fibrosis detected by the gold standard of histological analysis. This is stated in the text and is a key difference to previous models of pacing-induced cardiomyopathy that have inconsistently recapitulated the fibrosis burden reported in clinical disease.

Section 5.3.4 Page 132

By comparison to untreated control hearts, all sheep with doxorubicin-induced NICM had an increase in total LV fibrosis burden, as identified by Masson's trichrome staining (Figure 5.14).

The prevalence of delayed enhancement on CMR that we have reported in our model (approximately 50%) is actually at the higher limit of that observed clinically. Therefore, we stand by our original statements that this model of cardiomyopathy is accompanied by increased fibrosis burden and that this is a consistent finding, especially when assessed by the gold standard of histomorphometry.

Section 5.4.3 Page 138

The extent and nature of LV fibrosis, as determined both by delayed enhancement CMR and histomorphometry, was consistent with that observed in clinical studies of NICM from different aetiologies, including idiopathic disease [317, 318, 417].

6. Inclusion of a Limitations Section in Chapter 8

In accordance with the reviewer's recommendations, a limitations section has been incorporated into Chapter 8. Please note that this is in addition to the discussion of limitations that remains in each Results chapter, as per the original submission.

The addition of this section addresses the reviewer's suggestions relating to:

- Suitability of this preclinical model of NICM to the broader clinical context
- Load-dependence of LV functional characteristics
- The implications of the vasculotoxic and Purkinje system effects of doxorubicin
- The potential limitations of the NOGA/Myostar approach to cell delivery

8.6 Limitations (page 180-1).

As discussed previously, an important limitation of the imaging modalities used to quantify LV systolic function in Chapters 5-7 (CMR, echocardiography, electromechanical mapping), is that they all provide load-dependent measurements of contractility, which can be confounded by variations in cardiac pre-load and after-load, especially in the setting of severe heart failure. Lack of access to conductance catheters

prevented the acquisition of LV pressure-volume loops to provide more robust, load-independent analysis of ventricular function.

Although the anthracycline model of ovine cardiomyopathy recapitulates important characteristics of clinical NICM, including haemodynamic, structural and electrical LV remodelling and increased fibrosis burden, it is not representative of the full spectrum of human nonischaemic disease, which comprises numerous aetiologies and pathological substrates, with different natural courses and variable prognosis. Moreover, the intracoronary dosing regimen does not replicate the systemic administration of anthracyclines in clinical chemotherapy, and its invasive, time- and cost-intensive nature are all important factors when assessing the model's overall utility.

Some of the pathogenic changes shown to be induced by intracoronary doxorubicin (e.g. fibrosis, Purkinje fibre injury, small vessel damage) may be especially severe in this model, resulting in impairment to myocardial conduction, synchrony and perfusion, that are less prominent with other aetiologies of NICM. Anthracycline toxicity may also extend to resident cardiac and circulating progenitor cell populations, potentially impairing the recruitment and function of these endogenous cells in response to exogenous cell transplantation. This may have unique implications for the relative effectiveness of different cell types and delivery strategies in toxic NICM, as distinct from other types of cardiomyopathy.

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8.7.2 ***Cell delivery*** (page 183-4)

Despite the rationale for administering cell therapy by intramyocardial injection in NICM, the wider implementation of the NOGA® XP/MyoStar™ system is limited by its invasive, time-intensive nature, high cost and demands on operator expertise and training. These factors require special consideration when comparing NOGA® XP-guided therapy to the more accessible and widely used strategy of intracoronary injection. Plans are currently in place for future studies to compare the safety and efficacy of NOGA® XP-guided delivery with intracoronary MPC infusion, in the ovine doxorubicin model.

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We believe that the "off-target" effects of doxorubicin, including bone marrow suppression and troponin-T elevation were satisfactorily described in the original results and discussion sections of chapter 5 and thus do not require further discussion.

Section 5.4.2 Page 136

Despite its intracoronary administration, doxorubicin still resulted in bone marrow suppression, including transient but severe neutropaenia. However, systemic malaise was typically mild and surviving sheep recovered quickly from surgeries, maintaining their oral intake and weight throughout the duration of the study protocol.

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Although small elevations in troponin-T occurred with increasing frequency after repeat doses, there was minimal histopathological or CMR evidence for transmural or

subendocardial MI, suggesting that the underlying process was progressive, doxorubicin-induced myocarditis.

Acknowledgements

On reflection of my time as a PhD student, I am filled with a mixture of emotions. In making the transition from clinical Cardiology practice to basic scientific and preclinical research, I found myself confronted with numerous challenges: technical, emotional and mental. At the end of this period of my life, I am very grateful for the opportunities and experiences that my PhD has provided me with. Most of all, I have been blessed to have met, and interacted with, such a diverse group of people, whom I now count as friends, and to have been reminded, once again, of how extremely fortunate I am to have the unwavering love and support of my family.

This undertaking required the coming together of two different 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 Associate 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 together and the countless discussions, anecdotes and laughs that we shared.

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From the CRC, thank you first and foremost to my great friend, Angelo Carbone, who stood next to me during countless sheep surgeries, maintaining constant conversation and making sure that

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Finally, I want to pay great tribute to my beautiful wife, Maria, and our precious children – Jimmy, Lela and Labrini. I have greatly regretted not being able to devote more time or energy to our family unit over the duration of my PhD. Maria, your remarkable strength and understanding have enabled me to "get the job done", whilst you have selflessly and lovingly been the rock of our home. I am so blessed to have you by my side and to have provided me with three wonderful children, who fill our lives with so much warmth, laughter and happiness. As much as anything, I owe my ability to complete this undertaking to the incredible support and faith you have all provided me. Jimmy, Lela and Labrini – Daddy loves you with all his heart, thanks for being such great kids. This thesis is dedicated to you.

Financial Disclosures

I, Peter James Psaltis, declare that I have no financial conflicts of interest to disclose in performing and submitting the research described in this thesis.

With regard to my supervisors:
Professor Stephen Worthley has no financial conflicts of interest relating to this thesis.

Associate Professors Andrew Zannettino and Stan Gronthos report that they have received consultancy fees from Angioblast Systems, Inc.

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The National Heart Foundation of Australia, Overseas Travel Grant, to present the abstract entitled "STRO-1-immunoselection enhances the biological properties and cardiovascular paracrine effects of bone marrow mesenchymal stromal cells" at the International Society for Stem Cell Research (ISSCR) 7th Annual Meeting, Barcelona, Spain, July 2009.

The European Society of Cardiology, Council on Basic Cardiovascular Science Travel Award, to present the abstracts entitled "Nonischemic Cardiomyopathy is Associated with Purkinje Fiber Injury and Electrical Remodeling: Implications for Sudden Cardiac Death" and "STRO-1 immunoselection enhances the biological properties and cardiovascular paracrine effects of bone marrow mesenchymal stromal cells" at the European Society of Cardiology Congress, Barcelona, Spain, August 2009.

Awards

Hugh Gilmore Prize for the Best Oral Presentation by a Full-time Researcher, **The Royal Adelaide Hospital,** for the abstract entitled "Human STRO-1^{Bright} mesenchymal stem cells exert paracrine effects on cardiomyocytes and endothelial cells", Adelaide, October 2007.

Best Poster Prize, The University of Adelaide Research Expo, for the abstract entitled "Mesenchymal stromal cells immunoselected by the STRO-1 antibody exert paracrine effects on cardiomyocytes and endothelial cells", Adelaide, July 2008.

The Ross Wishart Memorial Award for the Best Oral Presentation by a Young Investigator, Australian Society of Medical Research (ASMR), for the abstract entitled "Cardiac repair with intramyocardial injection of allogeneic mesenchymal precursor cells for experimental nonischaemic cardiomyopathy", Adelaide, June 2009.

Best Mini-oral Presentation - Runner-up Award, International Society for Heart Research, Australasian Section, 33rd Annual Scientific Meeting, for the abstract entitled "Electromechanical characterisation of fibrosis in an ovine model of nonischaemic cardiomyopathy", Sydney, August 2009.

Nimmo Prize for the Best Oral Presentation by a Full-time Researcher, The Royal Adelaide Hospital, for the abstract entitled "Pleiotropic reparative effects of allogeneic mesenchymal precursor cells delivered transendocardially in nonischaemic cardiomyopathy", Adelaide, August 2009.

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8. **Psaltis PJ**, Zannettino ACW, Gronthos S, Worthley SG. Intramyocardial navigation and mapping for stem cell delivery. *Journal of Cardiovascular Translational Research* 2010;3(2):135-46.
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10. See F, Seki T, **Psaltis PJ**, Sondermeijer HP, Gronthos S, Zannettino ACW, Govaert K, Kurlansky PA, Kelly DJ, Krum H, Itescu S. Therapeutic potential of human mesenchymal precursor cells in acute myocardial ischemia: Cell biology and mechanisms of action. *FASEB Journal* Accepted March 2010.

In Submission

Psaltis PJ, Carbone A, Leong DP, Lau DH, Nelson AJ, Jantzen T, Manavis J, Williams K, Sanders P, Gronthos S, Zannettino ACW, Worthley SG. Assessment of myocardial fibrosis by endoventricular electromechanical mapping in experimental nonischemic cardiomyopathy. *In submission* March 2010.

Psaltis PJ, Carbone A, Nelson AJ, Lau DH, Manavis J, Williams K, Jansen T, Itescu S, Sanders P, Gronthos S, Zannettino ACW, Worthley SG. Pleiotropic reparative effects of allogeneic mesenchymal precursor cells delivered transendocardially in nonischemic cardiomyopathy. *In submission* March 2010.

Abbreviations

αMEM	Alpha modification of Eagle's medium	DMEM	Dulbecco's modification of Eagle's medium
µg	Microgram	DMSO	Dimethyl sulphoxide
µL	Microlitre	DNA	Deoxyribonucleic acid
µm	Micrometre	EC	Endothelial cells
µM	Micromolar	EDD	End-diastolic dimension
ABP	Arterial blood pressure	EDTA	Ethylenediaminetetraacetic acid
AnnV	AnnexinV-Fluos	EDV	End-diastolic volume
ANOVA	Analysis of Variance	EF	Ejection Fraction
AUC	Area under the curve	ELISA	Enzyme-linked immunosorbent assay
BM	Bone marrow	EPC	Endothelial progenitor cells
BMC	Bone marrow cells	ESC	Embryonic stem cells
BV	Bipolar voltage	ESD	End-systolic dimension
CBFA1/ Runx2	Core factor binding protein	ESV	End-systolic volume
CD	Cluster of differentiation	FACS	Fluorescence-activated cell sorting
cDNA	Complementary deoxyribonucleic acid	FCS	Foetal calf serum
CFSE	Carboxyfluorescein diacetate succinimidyl ester	FITC	Fluorescein isothiocyanate
CFU-F	Colony forming units-fibroblast	FS	Fractional Shortening
CI	Confidence interval	g	Gram
cm^2	Centimetre squared	GFP	Green fluorescent protein
CM	Conditioned medium	Hb	Haemoglobin
CMC	Cardiac muscle cells	HBSS	Hanks' balanced salt solution
CMR	Cardiac magnetic resonance imaging	HGF	Hepatocyte growth factor
CXCL12	Stromal cell-derived factor 1 (SDF-1)	HR	Heart rate

Abbreviations (continued)

HSC	Haematopoietic stem cells	nm	Nanometre
IGF	Insulin-like growth factor	P(n)	Passage (number)
IHD	Ischaemic heart disease	PAP	Pulmonary arterial pressure
IL	Interleukin	PBS	Phosphate buffered saline
iPS	Induced pluripotent stem cells	PCWP	Pulmonary capillary wedge pressure
i.u.	International units	PFA	Paraformaldehyde
kg	Kilogram	PI	Propidium iodide
LLS	Linear local shortening	RAP	Right atrial pressure
LV	Left ventricle (or left ventricular)	RNA	Ribonucleic acid
mAb	Monoclonal antibody	ROC	Receiver operating characteristic
MACS	Magnetic-activated cell sorting	RV	Right ventricle (or right ventricular)
MAP	Mean arterial blood pressure	RVP	Right ventricular pressure
mg	Milligram	SD	Standard deviation
MI	Myocardial infarction	SEM	Standard error of the mean
mL	Millilitre	STRO-1	Stromal precursor antigen-1
MNC	Mononuclear cells	STRO-3	Tissue nonspecific alkaline phosphatase
MPC	Mesenchymal precursor cells	TERT	Human telomerase reverse transcriptase (TNSALP)
mRNA	Messenger ribonucleic acid	TGF	Transforming growth factor
MSC	Mesenchymal stromal/stem cells	TNSALP	Tissue nonspecific alkaline phosphatase (STRO-3)
Msx	Msh homeobox	UV	Unipolar voltage
mV	Millivolt	VEGF	Vascular endothelial growth factor
N	Sample number	v/v	Volume per volume
NICM	Nonischaemic cardiomyopathy	w/v	Weight per volume