CELL THERAPY FOR THE REPAIR OF THE MAMMALIAN HEART

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by

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ABSTRACT

Cell Therapy for the Repair of the Mammalian Heart

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There are immense interests in utilising cell therapy for myocardial repair. Although bone marrow cells are the most extensively studied cell type to-date, evidences of the benefits of using these cells are conflicting. In this thesis, I explore the methodologies used for in-vitro and in-vivo studies of cell therapy, and define the potential of bone marrow cells for myocardial repair. I have identified a potential drawback with the use of green fluorescent protein for the cell therapy studies. The use of cardiac explants was also not suitable for my in-vitro studies of cell therapy. I further demonstrated that there were limitations in the current microscopic techniques used for identifying myocyte nucleus, and this can be a potential source of error in in-vivo studies in which myocyte nuclear events, such as proliferation, and transdifferentiation are evaluated. With this in mind, lineage tracing was used to show that bone marrow cells did not differentiate into myocytes when transplanted immediately after acute myocardial infarction. In addition, no functional improvement was also observed on echocardiography. To compliment my pre-clinical work, two randomised trials were conducted. The first trial showed that intramuscular or intracoronary injections of bone marrow cells into scarred myocardium did not contribute to any meaningful regeneration. The second trial investigated the potential cardioprotective paracrine effects of bone marrow cells, and showed that they did not provide additional cardioprotection when used as an adjunct during cardiopulmonary bypass. While these studies contributed to our understanding and planning of future work in this field, many key questions remain unanswered – whether bone marrow cells can truly benefit the heart, and if so, what are the mechanisms? When and to whom it should be given? Hopefully, my final study on novel biomarkers will eventually aid the identification of patients who will benefit from cell therapy.

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LIST OF ACRONYMS

%FAC	Percent fractional area change
%FS	Percent fractional shortening
%SFT	Percent systolic fractional thickening
ANF	atrial natriuretic factor
Ang-1	angiopoietin-1
ASE	American Society of Echocardiography
AUC	area under the curve
AVR	aortic valve replacement
BMC	bone marrow cell
BNP	B-type natriuretic peptide
BSA	body surface area
CABG	coronary artery bypass grafting
CCS	Canadian Cardiovascular Society angina class
CI	confidence interval
CK	creatinine kinase
CPB	cardiopulmonary bypass
CSF	colony stimulating factor
DSE	dobutamine stress echocardiography
EDC	explant derived cell
EDV	end diastolic volume
EF	ejection fraction
EOA	effective orifice area
EuroSCORE	European System for Cardiac Operative Risk Evaluation
GDF-15	Growth Differentiation Factor 15

GFP	green fluorescent protein
HGF	hepatocyte growth factor
HSC	haematopoietic stem cell
IL	interleukin
IQR	interquartile range
Isl 1	islet 1
LAD	left anterior descending
LV	left ventricle/ventricular
LVIDd	left ventricular internal diameter, in end-diastole
LVM	left ventricular mass
LVMI	left ventricular mass index
LVOT	left ventricular outflow tract
MAPC	multi-potential adult progenitor cell
MCP	moncyte chemoattractant protein
MHC	myosin heavy chain
MHC-nLAC	myosin heavy chain nuclear localised β-GAL
MLC	myosin light chain
MNC	mononuclear cells
MRI	magnetic resonance imaging
MSC	messenchymal stem cell
NTproBNP	N-terminal pro-hormone fragment, BNP
NYHA class	New York Heart Association dyspnea class
PCI	percutaneous coronary intervention
PWTd	posterior wall thickness, in end-diastole
ROC	receiver operator characteristics

Sca	stem cell antigen
SD	standard deviation
SDF	stem cell derived factor
SEM	standard error of the mean
SWTd	inter-ventricular septal wall thickness, in end-diastole
TEM	transmission electron microscopy
TGF	transforming growth factor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor
VTI	velocity time integral
WGA	wheat germ agglutinin
X-GAL	5-bromo-4-chloro-3-indolyl-d-galactoside
β-GAL	β-galactosidase

LIST OF PUBLICATIONS ARISING FROM THIS THESIS

Ang KL, Shenje LT, Srinivasan L, Galinanes M. Repair of the damaged heart by bone marrow cells: from experimental evidence to clinical hope. *Ann Thorac Surg*. 2006;82:1549-58

Shenje LT, Field LJ, Pritchard CA, Guerin CJ, Rubart M, Soonpaa MH, Ang KL, Galinanes M. Lineage tracing of cardiac explant derived cells. *PLoS One*. 2008;3:e1929.

Ang KL, Shenje LT, Reuter S, Soonpaa MH, Rubart M, Field LJ, Galinanes M. Limitations of conventional approaches to identify myocyte nuclei in histologic sections of the heart. *Am J Physiol Cell Physiol*. 2010;298:C1603-9

Ang KL, Chin D, Leyva F, Foley P, Kubal C, Chalil S, Srinivasan L, Bernhardt L, Stevens S, Shenje LT, Galinanes M. Randomized, controlled trial of intramuscular or intracoronary injection of autologous bone marrow cells into scarred myocardium during CABG versus CABG alone. *Nat Clin Pract Cardiovasc Med*. 2008;5:663-70

Lai VK, Ang KL, Rathbone W, Harvey NJ, Galinanes M. Randomized controlled trial on the cardioprotective effect of bone marrow cells in patients undergoing coronary bypass graft surgery. *Eur Heart J*. 2009;30:2354-9

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The leading cause of morbidity and mortality in the western world is heart failure. It affects approximately five million people in the United States[1] and at least 10 million people in Western Europe[2]. More than 50% of the reported cases of heart failure was due to ischaemic heart disease[3].

Once end-staged heart failure set in, the therapeutic options available are limited. While heart transplantation currently offers the best outcome in terminal failure[2], it is not widely available because of inadequate donor organs. Furthermore, due to the need for life-long immunosuppression to prevent organ rejection, and its associated complications, transplantation is not the ideal treatment.

Current therapy for heart failure is based on the traditional belief that the heart is unable to generate new myocytes to replace failing or dying myocytes, but instead adapts to new stresses by myocyte hypertrophy and cardiac remodelling. This conventional view was challenged by recent observations of proliferation markers within the adult cardiac tissue[4]. Furthermore, it was also shown that cardiac proliferation can be induced by manipulating the cardiac cell cycle in transgenic mice[5]. Concurrently, there were also studies that reported on-going cell death in the healthy human heart. In one study, the myocyte loss was reported to occur at a frequency of 1-10 myocyte per 100000 cells at any given instant[6]. Based on these figures, the study also inferred that there must be on-going cellular regeneration to maintain the structure and survival of the heart. All these studies suggest the cellular haemostasis between cell growth and death is a far more dynamic process than previously thought. This concept is further supported by the demonstration of some degree of myocardial regeneration from the native heart

tissue[7] as well as from extra-cardiac sources[8]. This observation has attracted immense interest to explore stem cell therapy as potential treatment for cardiac disorders, since the native regenerative capacity may be inadequate in a pathological heart. So far, several different potential sources of cell therapy ranging from embryonic/fetal tissue, umbilical tissue, skeletal muscles, adipose tissue, blood, bone marrow and even the heart have been investigated. Their advantages and disadvantages are elegantly summarised in Figure 1-1.

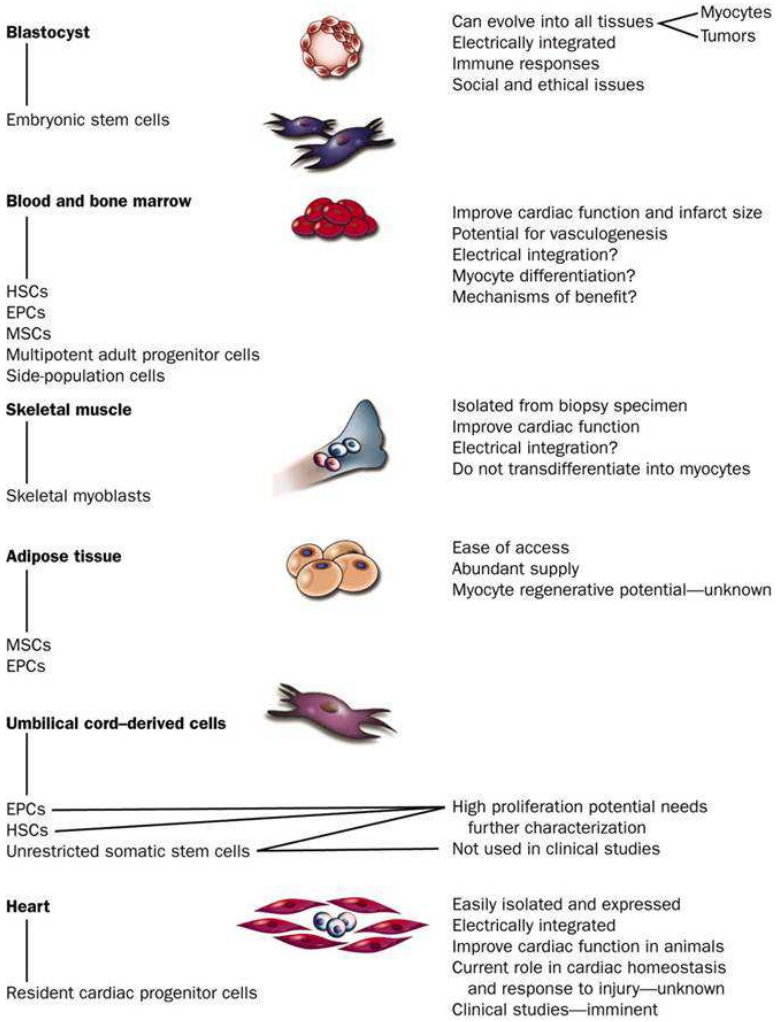


Figure 1-1: Advantages and disadvantages of different tissue sources for cardiac cell therapy. (Gersh *et al*[9])

Autologous bone marrow-derived cells (BMCs), in particular, present an attractive source of stem cells as their multi-lineage differentiation and multi-organ engraftment

potential has been previously demonstrated[10]. They are also easy to harvest and prepare, simple to administer, and do not require additional immunosuppressive treatment. Furthermore, their application does not raise the ethical controversies associated with the use of embryonic stem cells. This chapter presents an overview of the different stem cell populations found in BMCs, the current experimental evidence on how they may contribute to myocardial repair, and their applications into the clinical arena.

1.2 OVERVIEW OF DIFFERENT STEM CELLS WITH THE BONE MARROW

Traditionally, bone marrow was thought to contain two main groups of stem cells: haematopoietic stem cells (HSCs) and their supporting mesenchymal stem cells (MSCs). It is now known that bone marrow also contains endothelial progenitor cells (EPCs)[11, 12], as well as an unique population of cells with the capacity to generate all three germ layers called multi-potent adult progenitor cells (MAPCs)[13, 14]. A summary of their main features is provided in Table 1-1.

As more is learnt about BMCs, their identification and classification become more crucial for understanding their physiologic and therapeutic relevance. However, this is challenging, as different stem cell populations contributing to different cell lineages may be present within an organ like the bone marrow, yet sharing similar markers. The lack of consistent data on stem cell lineage markers[15] further complicates this process. In the context of myocardial repair, it will be important to establish the contribution of different populations of BMCs in improving cardiac function. It is also vital to dissect the mechanisms by which they can improve cardiac function to optimise therapeutic interventions.

Table 1-1: Key features of the different stem cells within the bone marrow (Ang *et al*[16])

Types of stem cells	Key features
Haematopoietic stem cells (HSCs)	• have been used in the treatment of haematological disorders, due to their ability to reconstitute all the blood forming elements in irradiated mammals. • haematological lineage negative (lin^-) HSCs expressing stem cell antigen-1 (Sca-1), c-kit (also known as CD117), CD34 and CD38 are now recognised to have the ability to differentiate into other tissue types[17-19]
Mesenchymal stem cells (MSCs)	• Mainly CD45 negative cells which express a myriad of markers including: $CD34^{low/-}$, Sca-1, stromal cell derived factor 1 (SDF-1), CD29, CD44, CD77, CD90, CD106, CD120, SH2, SH3, and SH4[20-23] • are multipotent[13, 24] and an important source of cytokines (see section on "Other effects of BMCs")
Endothelial progenitor cells (EPCs)	• may originate for a precursor called the primitive haemangioblast • primitive EPCs express CD133 and can indistinguishable from primitive HSCs. • mature EPCs express a variety of markers including CD34, VE-cadherin, CD31, von Willebrand factor and a VEGF receptor called fetal liver kinase[25, 26].
Multi-potent adult progenitor cells (MAPCs)	• One population expresses minimal levels (less than 3%) of CD90, CD105, and CD117, and are capable of differentiating into cells forming all germ layers including those expressing cardiac specific genes[14]; • Another population of MAPCs expresses CD34, CD45, c-kit, Sca-1, Flk-1, Thy-1, CD13 and stage-specific antigen I however did not shown similar range of transdifferentiation[13]

1.3 CAN BONE MARROW CELLS TRANSDIFFERENTIATE INTO MYOCYTES?

One of the primary aims of BMC transplantation is to replenish dying myocytes by transdifferentiating into functional myocytes. For this, they have to acquire a cardiac morphology and express cardiac specific markers and sarcomeric structures. They must demonstrate automaticity and coupling with host myocytes, and more importantly, work in a functional syncytium with the rest of the cardiac muscle. Whether BMCs can differentiate into functional myocytes remains the most controversial and debated topic in this field at present.

1.3.1 In-vitro studies

The earliest in vitro demonstration that BMCs can differentiate into beating cells with cardiac phenotype was by treating immortalised murine MSCs with 5-azacytidine[27]; 30% of the cells formed myotube-like structures expressing myocyte specific genes. They also exhibited spontaneous beating, and measurable action potentials. In addition, when these cells were implanted into myocardial scar tissue, they were associated with improvement in heart function[28]. Subsequently, it has been shown that cardiac specific gene expression can also be induced in a population of human bone marrow-derived MAPCs expressing low levels of CD90, CD105, and CD117, as well as in blood derived EPCs[29], when co-cultured with live myocytes. Despite these favourable studies, reports of successful in vitro transdifferentiation of BMCs into myocytes are scarce, suggesting that this process is complex and not easily reproduced for scalable therapeutic transplantation of myocytes. A closely regulated in vitro environment may be necessary for the occurrence of this transdifferentiation process, in

which direct cell contact and paracrine cross talk with myocytes may also be essential[29, 30]. Even if transdifferentiation does occur in vitro, these findings still need to be substantiated by in vivo studies.

1.3.2 In-vivo studies

Using immunofluoresence and confocal microscopy, it has been reported that lin- c-kit+ BMCs can transdifferentiate into myocyte-like cells when injected into infarcted mice hearts[31], and contribute to the regeneration of up to 65% of the damaged wall. The injected cells also developed endothelial and smooth muscle phenotypes, accompanied by an increase in neovascularisation, and overall cardiac function. However, similar experiments performed using BMCs carrying reporter transgenes that would be switched on upon cardiac transdifferentiation of any cell have failed to reproduce these findings [32, 33]. Reporter gene failure was excluded by the use of several reporters and the successful expression of reporter genes in fetal derived cardiac cells injected into the hearts of adult mice[32]. Instead, the transplanted cells adopted a mature haematopoietic phenotype in the myocardial scar, accompanied by a minor but statistically significant improvement in cardiac function[33].

One possible explanation for these discrepancies could be the difficulties in distinguishing myocyte nuclei from non-myocyte nuclei by conventional confocal microscopy when the cells overlap. Even with the aid of advanced three-dimensional image re-construction, the cytoplasm of adjacent myocytes can still be mistaken as a donor cell structure, and perceived as a transdifferentiation event[34].

Another possible explanation is the phenomenon of cell fusion. Cell fusion occurs when a cell from one lineage fuses and adopts the phenotype of a host cell from a different lineage to form a heterokaryon, which can be mistaken for a transdifferentiation event. BMC fusion with myocytes has been previously observed at a very low frequency following their transplantation in the heart using cre-lox technology [35, 36]. Nevertheless, false positive interpretation is also possible using this technology as a result of metabolic cooperation[37], if the cre recombinase from donor cells leaks into a recipient cell and activates the reporter gene without the occurrence of cell fusion. However, in these studies the heterokaryons were shown to have double the complement of diploid DNA. While the observation of irreversible growth arrest after the fusion of bone marrow and Purkinje cells questions the contribution of this phenomenon in cell regeneration[38], other investigators have showed that fused myocyte were capable of cell cycle reentry[36]. Currently, the physiological importance of fusion remains unclear, but its therapeutic potential appears limited. These conflicting results have stimulated considerable debate as to whether myocyte differentiation occurs, and whether the effects of bone marrow cells on myocardial repair are mediated via other mechanisms.

1.4 BONE MARROW CELLS AND CARDIAC NEOVASCULARISATION

Neovascularisation describes the formation of new blood vessels, either from pre-existing vascular network (angiogenesis) or through a de novo process from the circulating primitive endothelial precursors (vasculogenesis). In the damaged myocardium, enhanced neovascularisation is believed to improve the natural repair and re-modelling process by maintaining the perfusion to the remaining viable tissue in the watershed areas and limiting the extent of myocyte loss. It may also provide the necessary circulatory support to any newly formed myocytes.

The identification and isolation of cells participating in neovascularisation from the bone marrow provides another means by which cellular therapy can enhance cardiac function. EPCs from BMCs have been shown to express endothelial phenotypes in vitro[12, 26, 39]. When a subset of EPCs was administered into animal models of myocardial infarction, it induced significant neovascularisation within the ischaemic regions, accompanied by reduced myocyte apoptosis, and improved overall cardiac function[40]. These cardioprotective effects and the degree of neovascularisation have been found to be dependent on the numbers of cells delivered[41]. On microscopy, some of the administered EPCs were localised predominantly within the vessels of the peri-infarcted area, suggesting their direct participation in the neovascularisation process. It is worth noting that certain populations of BMCs within HSCs[31, 42], MSCs[13] and MAPCs[14, 26] have also demonstrated similar capacity to express endothelial phenotypes in vitro and co-localise amongst capillaries of ischaemic tissue.

However, as with studies investigating the ability of BMCs to transdifferentiate into myocytes, there are concerns whether the above observations obtained using current microscopic techniques are robust enough as evidence for the functional incorporation by BMCs into the vessel structures. These doubts are supported by a study in which no green fluorescent protein (GFP) signals were detected within the growing vessels after GFP-positive BMCs were transplanted into mice hindlimb ischaemic model[43]. Instead GFP positive cells, in the form of fibroblasts, pericytes and leukocytes, were found in the tissues around growing collateral arteries, coinciding with areas of high cytokine concentration. These findings suggest that BMCs may have an important supportive role by interacting or optimising the milieu for host tissue response to ischaemic insults.

A supportive role of BMCs in neovascularisation is further substantiated by reports demonstrating the ability of different sub-populations of BMCs[42, 44-46] to secrete angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and angiopoietin-1 (Ang-1). These factors play a vital role in neovascularisation, and have been directly administered in models of ischaemia with favourable results[47-49], with VEGF showing the greatest effect in the clinical setting[50-52]. Comparable improvement in vascularity has also been shown in animal studies [53, 54], by augmenting the production of these factors through gene therapy. Using this approach, several clinical trials have been undertaken to investigate their effects on neovascularisation in ischaemic heart disease[55, 56] and peripheral vascular diseases[57].

1.5 OTHER POTENTIAL EFFECTS OF BONE MARROW CELLS

As the debate on whether BMCs can transdifferentiate into myocytes or vascular tissue continues, there is increasing evidence suggesting that they have an indirect supportive role in repair. Apart from promoting neovascularisation indirectly through its secretion of angiogenic factors as previously discussed, BMCs also secrete other cytokines and growth factors (see below), that may influence the loss of myocytes in response to injury and the regeneration of cardiac tissue by native heart cells.

1.5.1 Bone marrow as a source of cytokines

In addition to Ang-1, VEGF, HGF, and FGF, several studies [44, 45, 58] have shown that BMCs are capable of secreting an array of cytokines, such as: interleukin(IL)-1, IL-6, tumour necrosis factor α (TNF-α), transforming growth factor-β (TGF-β), monocyte chemoattractant protein (MCP-1), placental growth factor, stem cell derived factor (SDF-1), metalloproteinases, plasminogen activator, granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF. It is recognised that some of the cytokines like IL-1, IL6, TNF-α and TGF-β play a pivotal role in tissue responses to injury and are upregulated in the BMCs during hypoxic injury[45]. Other cytokines like GM-CSF and G-CSF are believed to orchestrate the recruitment, mobilisation and homing of different bone marrow-derived stem cells during tissue repair[59]. This ability of GM-CSF and G-CSF has been used in animal and clinical studies as a mean to increase the number of circulating bone marrow-derived stem cells, although they may influence other tissue regeneration processes[60-62]. As with most cytokines, their effects may depend on the microenvironment of the target tissue, and on the interactions with other cells and cytokines. A detailed discussion of their effects is beyond the scope of this review,

however, it can be inferred from the existing literature that BMCs play a part in modulating tissue response to injury through various mechanisms that may influence the degree of myocyte loss and native cell regeneration in the heart.

1.5.2 Reduction of myocyte loss

The administration of MAPCs and EPCs has been associated with reduced apoptosis following myocardial injury in animal models[14, 40]. The mechanism of this effect is unclear; but it has been shown that improved neovascularisation may provide the critical perfusion to the viable myocytes around the peri-infarct areas, which would have otherwise undergone apoptosis[40, 41]. Our laboratory has recently demonstrated that BMCs are able to directly reduce necrosis and apoptosis of the human myocardium in an in vitro model of simulated ischaemia via the PKC and p38 MAPK pathways[63]. Therefore, it is possible that the BMCs may contribute to a reduction in myocyte loss by direct and indirect mechanisms. Clearly, this is an important area that warrants further investigations.

1.5.3 Stimulate the proliferation of native heart cells?

The potential existence of native cardiac progenitors is one of the most topical issues in cardiovascular science in recent years, as it opens the door for new therapeutic opportunities for heart disease. So far, the identified putative cardiac stem cells are small round cells, with a high nuclear to cytoplasm ratio, and express c-kit, and Sca-1 protein[64]. These cells are clonogenic, self-renewing, pluripotential, and are grouped in niches especially in the atria and the apex of the heart. Although in culture conditions, these cells do not develop full myocyte phenotype, they can develop a

mature cardiac phenotype when transplanted into the border zone of the infarcted heart, and regenerate the entire myocardium[64]. It remains uncertain whether these cells share any similarities to another population of heart derived Sca-1 cells which do not express cardiac, endothelial or haematopoietic markers[65], but exhibit similar capabilities.

Another group of putative cardiac progenitors that express islet 1 (lsl 1), a LIM domain transcription factor found predominantly in embryonic and fetal development[66] has been described. Most of these cells are found in the outflow tract, right heart, and left atrium during early development. However, as cells differentiate, lsl 1 is switched off, and they become virtually undetectable in adulthood, making it difficult to establish their physiological significance in the adult heart.

It is possible that the transplantation of BMCs into the heart can stimulate the development of these putative cardiac progenitors. So far, there is no information on this potential mechanism. However, it can be speculated that the growth factors and cytokines produced by BMCs may play a part in such an effect. Indeed, the elucidation of this potential mechanism also requires more investigations, in order to reap its full potential in clinical practice. In particularly, it is essential to fully characterise the morphological and physicological properties of these putative stem cells.

In the search for cardiac progenitors, our laboratory has hypothesized that if cardiac progenitors exist, they should be able to be obtained from culturing heart tissue explants from biopsy. This will provide a useful in-vitro model to study the cellular dynamics of the heart, as well as its interaction with other cell types, such as the bone marrow cells.

More importantly, it can be exploited as a new therapeutic option to provide autologous cardiac progenitor transplantation. Using heart tissue explant cultures, our laboratory have previously reported the presence of small round cells derived from explant culture in 2001. Similar observations were also reported by Messina *et al*[67] using slightly different methodologies, and they proposed that these small round cells are putative stem cells. Interestingly, studies into the progeny of the small round cells observed in our laboratory revealed potential limitations associated with the use of transgenic technology, which will be addressed in this PhD.

1.6 CLINICAL TRIALS ON BONE MARROW TRANSPLANTATION TO REPAIR THE DAMAGED HEART

Following early encouraging experimental results, there was great enthusiasm to apply cell transplantation to repair the damaged heart in clinical settings, even before the underlying mechanisms were understood. One of the first reports of such application in cardiac disease utilised skeletal myoblast transplantation in patients with poor ventricular function receiving concurrent coronary artery revascularisation[68, 69]. Even though there was improvement in ventricular function in those cell transplant recipients, a few patients experienced severe ventricular arrhythmias requiring the insertion of implantable defibrillators[69]. Subsequently experimental studies observed failure of electromechanical coupling of the skeletal myoblasts with resident myocytes[70] which may account for the fatal ventricular arrhythmias. By contrast, the safety and feasibility of transplantation of autologous BMCs for the treatment of poor cardiac function in myocardial disease was rapidly demonstrated in several phase 1 trials[71-74].

There are a number of key questions that need to be addressed by clinical trials. First and uppermost, can BMCs improve myocardial function in the failing heart? If so, which groups of patients will benefit from it? What is the optimal time for cell transplantation following an acute myocardial infarction? What is the optimal number of cells required to attain maximum benefit? Which is the best route of administration? How many times should the cells be administered? Should cell transplantation be carried out concurrently with coronary revascularisation? At the start of my PhD, most of the published trials have been non-randomised, and therefore the results have to be

interpreted with caution. The clinical trials reported also differed significantly in methodology, making comparisons between trials difficult.

1.6.1 Bone marrow transplantation following acute myocardial infarction (Table 1-2)

The BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial was one of the first randomised control trial to investigate the effects of intra-coronary autologous BMCs delivery within a week after percutaneous coronary intervention (PCI) for acute myocardial infarction[75]. Sixty patients were randomised to become control or to be treat with autologous BMCs via coronary artery delivery within a week of their successful PCI. The primary endpoint was the change in the patient's left ventricular ejection fraction (LVEF) between the baseline and 6 month follow-up cardiac MRI. After this period, a significant increase in the mean global LVEF was observed in the patient receiving bone marrow cells as compared with controls. There was also enhancement of the systolic function in myocardial segments adjacent to the infarcted areas in the bone marrow cells treated group, supporting the findings of most earlier case-control studies [73, 76]. A subsequent randomised study on BMC therapy in patients with acute myocardial infarction has also corroborated these results[77]. Though the infarct size and left ventricular end diastolic volume (LVEDV) remained unchanged in the BOOST trial, some studies have demonstrated reduced infarct size in patients treated with bone marrow cells[73, 77]. Only two studies to date did not observe any significant hemodynamic improvement – a small observational study on five patients[78], and the ASTAMI randomised control trial[79, 80]. In the preliminary results of the ASTAMI study, no significant improvement in LVEF was observed, even though earlier non-randomised control trials suggested that it was possible to have

regional wall motion improvement in the absence of significant change in the global LVEF. These discrepancies in the reported results could be partially due to variations in the trial protocols, such as the methods of assessing myocardial function, study endpoints, patient selection criteria and BMC preparation. Although recent meta-analyses[81-83] favour the use of bone marrow cells in acute myocardial infarction, these findings still need to be interpreted with caution in view of the methodology diversity used in the trials. To establish the true benefits of BMC transplantation in acute myocardial infarction, more randomised studies will be necessary, using a "common" methodology to facilitate better comparative analysis.

1.6.2 Bone marrow transplantation in chronic ischaemic cardiac disease (Table 1-3)

In patients with chronic ischaemic cardiac disease (see Table 1-3), a small randomised control study and several preliminary non-randomised studies, have studied the effects of bone marrow cells administrated to viable ischaemic myocardium and reported various degree of functional improvements[72, 84-87]. For non-viable myocardial areas, one of the earliest phase I studies performed by our group has showed that when autologous BMCs were administered via transepicardial intramuscular injections, significant improvement in regional wall motion was observed only in those injected areas that were simultaneously revascularised[74, 88]. This suggests that in the non-viable myocardium, restoration of normal myocardial perfusion is essential to obtain the beneficial effects of BMCs. This was taken into consideration in the planning of a subsequent randomised trial, in which the efficacy of the intra-myocardial versus intra-coronary administration of bone marrow cells was also investigated[89]. The final results of which will be presented later in this thesis.

1.6.3 Route of administration

In the ideal scenario, the administration of the BMCs should be simple, as minimally invasive as possible, and permit the delivery of the optimal numbers of BMCs needed to obtain maximal clinical benefits. Different routes of administration are possible: transvenous, intra-coronary, or intramuscular (either via transepicardial or transendocardial injections). Although transvenous administration of BMCs is the simplest and least invasive method, a MRI study using tagged BMCs[90] has shown a significantly lower retention of BMCs on the heart by this method than with intra-coronary administration, which is the most common method used in trials of patients with acute myocardial infarction. As mentioned above, whether intra-coronary administration of BMCs is comparable or superior to intramuscular delivery via the trans-epicardial route was studied in a randomised control trial, and will be presented later in this thesis[89]. Theoretically, intramuscular injection of BMCs can accurately delivers a higher local concentration of BMCs to the area requiring treatment under direct vision. However, its main disadvantage is that it is more invasive, and because of this, it has been usually performed in conjunction with open-heart surgery. Recently, the application of electromechanical mapping to guide transendocardial injection[84, 91] has provided a potentially less invasive way to deliver BMCs intramuscularly.

Table 1-2: Summary of clinical trials on bone marrow transplantation following acute myocardial infarction published at the start of this thesis (Ang *et al*[16])

Study	Study Type	Patient number		Time lapse from AMI to cell therapy	Cell types	Delivery route	Follow-up (mths)	Cardiac assessment	Results & comments
		Tx	C						
Strauer *et al* 2002[73]	NR	10	10	5-9 days	MNCs	*ic* + *PTCA*	3	• Angiogram • Ventriculogram • DSE • Scintigraphy	• Improved global function • Improved perfusion
TOPCARE-AMI study 2002[92], 2004[93]	R	59	-	4.9±1.5 days	MNCs or CPCs	*ic*	12	• Ventriculogram • MRI • DSE	• Randomised study comparing MNC (n=30) vs EPC (n=29) – improved global function & reduce infarct size in both groups
Kuethe *et al* 2004[78]	NR	5	-	6-7 days	MNCs	*ic*	12	• Angiogram • Ventriculogram • DSE	• No significant change in global & segmental function
Fernandez-Alives *et al*	NR	20	13	14±6 days	MNCs	*ic*	6-21	• Angiogram • DSE	• Improved global and segmental function

2004[76]								• MRI	
BOOST study 2004[75], 2006[94]	R	30	30	<5 days	MNCs	*ic*	18	• MRI	• Improved global function & some segmental improvement during first 6 mths, no significant further improvement at 18 mths
Chen *et al* 2004[77]	R	34	35	Around 26 days	MNCs	*im*	6	• Echocardiogram • PET • EMM	• Improved global and segment function • Improved perfusion • Improved myocardial mechanical function on EMM
ASTAMI study 2005[79]	R	24	25	5-8 days	MNCs	*ic*	12	• PET • SPECT • MRI • DSE	• 1 patient developed ventricular fibrillation 24 hrs after transplantation • No final published report yet
Bartunek *et al* 2005[95]	NR	19	16	11.6±1.4 days	CD133	*ic*	6	• Angiogram • Ventriculogram • SPECT	• Improved global and segment function • Improved perfusion

		• PET	• ? increase incidence of in-stent restenosis

Abbreviations: Tx – treatment group; C – control group; mths – months; R – Randomised; NR – non-randomised; AMI – acute myocardial infarction; MNCs – mononuclear cells; CPCs – Circulating blood-derived progenitor cells; *im* – intramuscular; *ic* – intracoronary; SPECT- single-photon emission computed tomography; DSE – dobutamine stress echocardiogram; ^{18}F-FDG – 18F-fluro-deoxy-glucose; PET – photon emission tomography, PTCA – percutaneous transluminal coronary angioplasty; EMM – Electromechanical mapping; BOOST – BOne MarrOw transfer to enhance ST-elevation infarct regeneration; ASTAMI – Autologous Stem cell Transplantation in Acute Myocardial Infarction; TOPCARE-AMI – Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction

Table 1-3: Summary of clinical trials on bone marrow transplantation in chronic ischaemic cardiac disease published at the start of this thesis (Ang *et al*[16])

Study	Study Type	Patient number		Viability of target myocardium	Cell types	Delivery route	Follow-up (mths)	Cardiac assessment	Results
		Tx	C						
Hamano *et al* 2001[72]	NR	5	-	Viable	MNCs	*im**	12	• Angiogram, SPECT • Echocardiogram	• Safety confirmed • Improved perfusion in treated myocardium
Fuchs *et al* 2003[85], 2006[96]	NR	12	-	Viable	MNCs	*im*	12	• Echocardiogram • SPECT	• Improved perfusion in treated areas
Tse *et al* 2003[97]	NR	8	-	Viable	MNCs	*im*	3	• SPECT • MRI	• Improved global & regional function • Improved perfusion
Perin *et al* 2003[91]	NR	14	7	Viable	MNCs	*im*	12	• Ventriculogram • Echocardiogram • SPECT • EMM	• Improved global function • Improved mechanical function in treated myocardium
Stamm *et al*	NR	6	-	Viable - infarct	CD133	*im**	3-10	• SPECT	• Improved LVEF

2003[71]				border				Echocardiogram	• Improved perfusion
Silva *et al* 2004[84]	NR	5	-	Viable	MNCs	*im*	6	• SPECT • Echocardiogram • Treadmill	• LVEF – not significant change • Improved myocardial volume oxygen consumption
Galiñanes *et al* 2004[74], 2006[88]	NR	14	-	Non-viable	MNCs	*im**	24	• DSE	• Improved segmental function in injected myocardium that was also revascularised
Blatt *et al* 2005[98]	NR	6	-	Viable	MNCs	*ic*	6	• DSE	• Improved global and segmental function
ICAT study 2005[86]	NR	18	18	Uncertain (single vessel infarction)	MNCs	*ic*	3	• Ventriculogram • SPECT • PET • spiroergometry	• Improved global & segmental function • Reduced infarcted size • Improved ^{18}F-FDG uptake
Patel *et al* 2005[87]	R	10	10	Viable – perinfarct area	MNCs	*im**	6	• Angiogram • Echocardiogram • SPECT	• Improved global function

* during concurrent coronary artery bypass grafting

Abbreviations: Tx – treatment group; C – control group; mths – months; R – Randomised; NR – non-randomised; MNCs – mononuclear cells; *im* – intramuscular; *ic* – intracoronary; SPECT- single-photon emission computed tomography; DSE – dobutamine stress echocardiogram; ^{18}F-FDG – 18F-fluro-deoxy-glucose; PET – photon emission tomography; EMM – electromechanical mapping

In an attempt to limit the invasiveness of cellular therapy, cytokines like G-CSF have been used to mobilise BMCs for myocardial repair after a myocardial infarction[61, 99]. Initial results have shown improvement in cardiac function and perfusion in patients receiving revascularisation and G-CSF therapy early after a myocardial infarction. However, it is uncertain if this was due to the mobilised stem cells or to the direct effect of cytokines on injured myocardium. There were nevertheless concerns about its safety after some patients experienced early re-stenosis of stented coronary vessels following G-CSF administration[61].

1.6.4 The ideal cell type

BMCs are usually harvested from the iliac crest, and the bone marrow mononuclear layer cells are separated using a density gradient for transplantation in most studies. These cells are a composite mixture of different stem cell subtypes including HSCs, EPCs, MSCs and MAPCs, all of which have the potential to improve myocardial function. While some studies had attempted to expand these cells by culturing them for a period prior to their administration[73, 77], others had tried to isolate a subgroup of cells to promote a specific role, like the isolation of CD133+ cells to promote angiogenesis[71]. However, until a more accurate classification and isolation system is devised, it may not be possible to isolate pure population of the various sub-types of stem cells. These technical constraints make the precise evaluation of their individual contribution in myocardial regeneration difficult, and therefore the selection of the optimal cell type for transplantation.

Based on the results of present clinical trials, the use of bone marrow transplantation in both acute myocardial infarction and in chronic ischaemic heart failure appears safe, and beneficial in most of the studies. However, this benefit needs to be confirmed by larger randomised control trials. The best route of administration and the optimal cell-type also need to be determined by more detailed studies, in addition to a better understanding of the underlying mechanism of action.

1.7 HYPOTHESES AND AIMS OF THIS THESIS

Although autologous BMC transplantation represents an attractive and promising treatment for heart failure due to its relatively easy preparation process and established clinical safety profile, conflicting results have been obtained from both pre-clinical and clinical studies regarding its potential benefits. Even if beneficial effects have been reported, the mechanisms of action remain unclear. Therefore, this thesis aims to test the main hypothesis that BMC therapy can contribute to myocardial repair and improve cardiac function.

From this review, it is clear that there are limitations associated with the current methodologies used to study cell transplantation in myocardial repair, and they may contribute the differences in results observed between earlier studies. For these reasons, I will first have to assess fidelity of the current methodologies using both in-vitro and in-vivo studies, before I test the hypothesis BMCs can improve cardiac function in the laboratory and clinical setting. As there is an increasing need to define patients who will benefit from cell therapy, I will conclude by exploring the potential of new investigative tools for such purposes.

Hence, the aims of my studies are:

1. To investigate whether transgenic protein can be retained after cell death and taken up by surrounding cells?
2. To characterise the fidelity of current microscopy techniques used in the study of cell therapy;
3. To investigate whether BMCs can regenerate infarcted myocardium in mice?

4. To investigate whether BMCs can improve cardiac function of chronic scarred myocardium in the clinical setting and whether this depends on the route of administration?
5. To investigate whether BMCs can confer additional cardioprotection in patients undergoing cardiac surgery with cardiopulmonary bypass?
6. To explore the potential of biomarkers for identifying and monitoring patients with cardiac diseases that may benefit from cell therapy.

CHAPTER 2

LIMITATIONS OF TRANSGENIC TECHNOLOGY – EXPERIENCE FROM LINEAGE TRACING OF CARDIAC EXPLANT-DERIVED CELLS AND THEIR POTENTIAL AS AN IN-VITRO MODEL FOR CELL THERAPY STUDIES

2.1 INTRODUCTION

There are studies suggesting that BMCs can exhibit cardiac phenotypes using in-vitro cultures of BMCs either by chemical induction or co-cultures with live myocytes. However, these studies are not easily replicated and reports of successful expression of cardiac phenotypes in-vitro by BMCs are scarce. While variation in culture conditions are often cited as one of the potential reasons for these differences in results, the mis-identification by current microscopic techniques particularly when BMCs were co-cultured with myocytes, is another plausible explanation. This latter explanation was further supported by recent reports of cells derived from cultured cardiac explants have stem cell markers and cardiogenic potential [67, 100].

Our laboratory have previously observed a similar highly refractile population of small round cells (Figure 2-1) budding from mouse cardiac explants after 3 weeks in culture (results of which have since been published in an article I co-authored[101]).

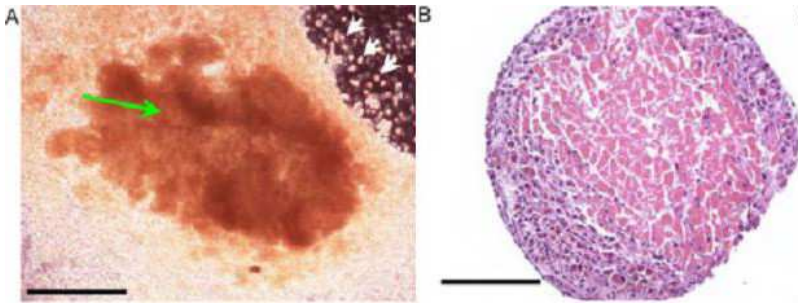


Figure 2-1: Cardiac explants cultured for 3 weeks. (Shenje *et al*[101])

(a) Phase contrast image of numerous highly refractile EDCs (white arrows) from a mouse ventricular explant (green arrows) in culture for 3 weeks (scale bar, 100 μm). (b) Haematoxylin and eosin staining of a mouse ventricular explant (scale bar, 100 μm).

These explanted-derived cells, here termed EDCs, expressed smooth muscle actin, GATA4, vimentin, and α-sacromeric actinin but not stem cell markers stem cell antigen (Sca-1) or c-kit (Figure 2-2).

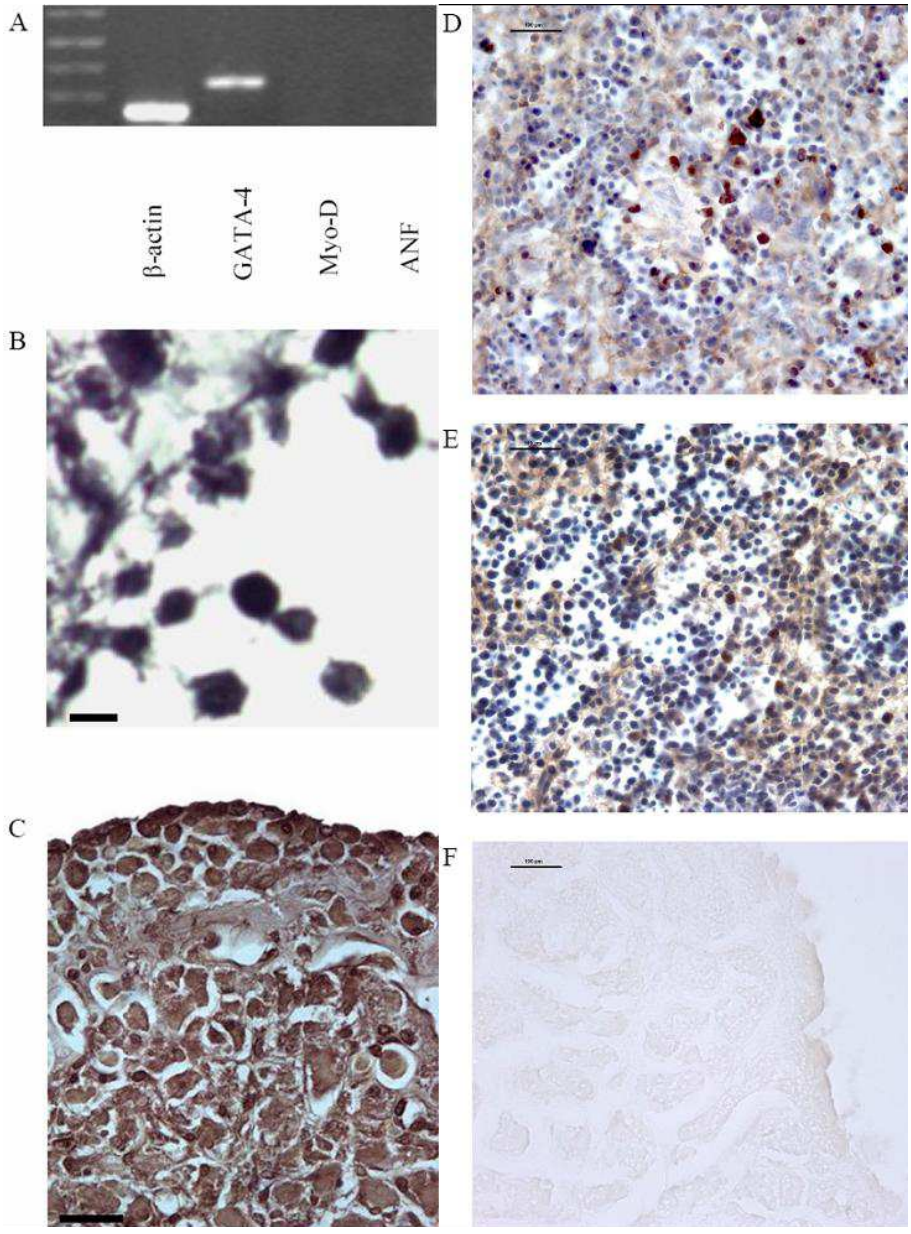


Figure 2-2: Expression profile of EDCs. (Shenje *et al*[101])

(A) RT PCR analysis of EDCs gene expression: Transcripts encoding GATA4 were detected by no MyoD and ANF transcripts. (B) Vimentin (scale bar, 5 μm) and (C) α-sarcomeric actinin (scale bar, 20 μm) immune reactivity, respectively, in cultured EDCs. Positive controls showing c-kit (D) and Sca-1 (E) expressions in mice spleens. Cardiac explant showed no evidence of c-kit (F) immuno activity and Sca-1 expression. [Figure not shown but similar to that in (F); Scale bar, 100μm]

In an effort to perform cardiac specific lineage tracing for these cells, cardiac explants from the double heterozygous MLC2v-Cre/ZEG reporter mouse were used. In this transgenic mouse, the ZEG transgene in non-myocytes expresses β-galactosidase (β-GAL) via a ß-geo insert[102] which is flanked by lox-p sites; whilst the promoter of the ventricular isoform of myosin light chain drives the expression of cre-recombinase[103] resulting in the excision ß-geo and expression of GFP exclusively in ventricular cardiac myocytes. Initial results were promising as GFP was detected on immunohistochemisty in the EDCs cultured from explants of this transgenic mouse (Figure 2-3). However, further PCR analysis surprisingly failed to detect the expected Cre-recombinase recombination products within the EDCs (Figure 2-4).

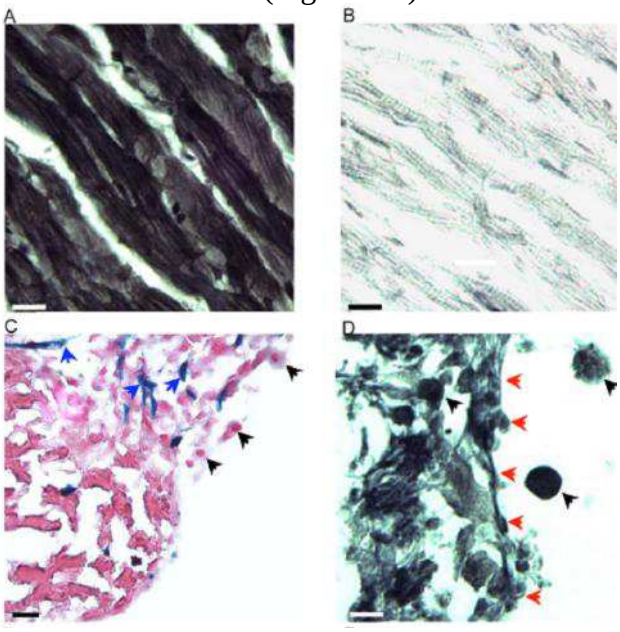


Figure 2-3: Cardiac Explants and cells from MLC2v-Cre/ZEG mice. (Shenje *et al*[101])

(A) GFP immune reactivity by nickel-enhanced DAB-reaction (blue/black) in fresh uncultured MLC2v-Cre/ZEG heart tissue (scale bar, 10 μm). (B) Minus antibody negative control (C) Cultured MLC2v-Cre/ZEG cardiac explants were stained simultaneously with the chromogenic dye X-GAL (generates a blue signal in the presence of β-GAL) and nuclear fast red counterstain. The spindle-shaped fibroblast-like cells were β-GAL positive (blue arrows) whilst the EDCs were β-GAL negative (scale bar, 20 μm) (D) nickel-enhanced DAB GFP immune reactivity in EDCs (black arrows). Red arrows indicate the edge of the explant (scale bar, 5 μm).

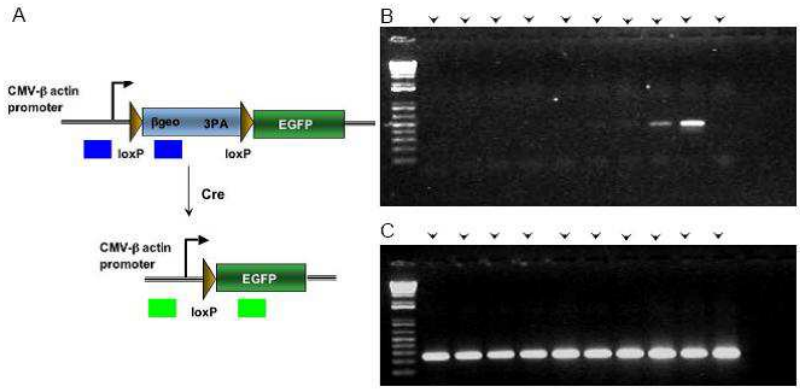


Figure 2-4: Analysis of DNA from MLC2v-Cre/ZEG mice. (Shenje *et al*[101])

(A) Structure of the ZEG reporter transgene before and after Cre recombinase-mediated deletion of the β-GAL encoding sequences. Blue and black arrows indicate the positions of the PCR amplification primers used to distinguish native (450 bp amplification product) and recombined (450 bp amplification product) transgenes. (B) PCR amplification products using oligonucleotide primers to detect the Cre-mediated recombination event. DNA was from EDCs (lanes 1-4) and their corresponding explants (lanes 5-8), a positive control double transgenic mouse heart (lane 9) and a negative control double transgenic mouse kidney (lane 10). (C) PCR amplification products using oligonucleotide primers to detect the native ZEG reporter transgene. DNA samples were as in panel B.

This raised the question as to how it is possible to have GFP immuno-reactivity in the absence of Cre-mediated recombination of the ZEG transgene? It has been observed in our laboratory when cardiac explants were cultured, that the cells within the core underwent progressive cell death over time. Therefore, one possibility was that the GFP observed could come from dead myocytes which was eventually taken up by the EDCs. To test this hypothesis, I designed a series of experiments described in this chapter. First, I investigated whether it is possible for GFP to be retained a few weeks after myocyte death? This was done by analysing the MLC2v-Cre/ZEG reporter mouse heart about three-four weeks after inducing an area of myocardial cell death. In addition, electron microscopic analysis was performed to determine whether the GFP present in EDCs was due to uptake of retained GFP?

2.2 MATERIAL AND METHODS

The investigations described in this thesis conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) as well as the United Kingdom Animals (Scientific Procedures) Act 1986.

2.2.1 Transgenic mice

MLC2v-Cre/ZEG double heterozygous transgenic mice were bred in-house by crossing the MLC2v-Cre mouse (generously provided by Dr C.A. Pritchard) with ZEG reporter mouse (purchased from Jackson laboratories, UK). The presence of double heterozygous transgenes was detected by first testing a small piece of ear snip for β-GAL. This was done by incubating it in X-GAL solution [comprising of 1 mg/ml 5-bromo-4-chloro-3-indolyl--d-galactoside (X-GAL), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride in PBS – all reagents from Sigma, UK], at 37 ºC overnight. Specimens with positive β-GAL signals were further then tested for presence of MLC2v-Cre transgene by PCR analysis.

2.2.2 Myocardial infarction

To investigate whether GFP can be retained after myocardial death, an area of myocardial infarction was induced in the MLC2v-Cre/ZEG mice. The mouse was first anaesthetised using Ketamine/xylazine (2:1) mixture. Once the mouse was anaesthetised, it was intubated and ventilated via a mouse ventilator. The chest was shaved and left thoracotomy was performed. The left anterior descending artery (LAD) was permanently ligated with the aid of operating microscope as previously

described[32] to generate an area of myocardial cell death. After 3 weeks the hearts were excised for immunohistochemical analysis for retained GFP within the infarct area. The hearts were fixed with 10% formalin for 48 hours prior to paraffin embedding. The resulting blocks were then cut at a thickness of 5 μm using standard protocols[104].

2.2.3 Immunohistochemistry and microscopy

Heart sections were deparaffinised in 2 washes of xylene (5 minutes each) and rehydrated serially in reducing percentages of Industrial Methylated Spirit (IMS) solution (100%, 90% and 70%), followed by double distilled water. Forty minutes of microwave antigen retrieval (800W, full power) in 0.1%EDTA solution was performed. The sections were blocked with 1% bovine serum albumin for 20 minutes, and then incubated with the Anti-GFP (Abcam plc, Cambridge, UK; used in 1:200 dilution) primary antibody overnight.

To detect the primary antibody, goat secondary antibody conjugated with Alexa Fluor®488 (Invitrogen, Paisley, UK), was then incubated overnight at a dilution of 1:200. DAPI (Molecular Probes catalogue number D1306) was used to identify nucleus, while wheat germ agglutinin (WGA) Alexa Fluor® 647nm conjugate (Molecular Probes catalogue number W32466) was used to delineate outer cell membrane and fibrosis.

Due to the superior optical sectioning capabilities of confocal microscopy[105], the imaging was performed using a Leica Confocal SP5 system (Leica Microsystems, Wetzlar, Germany) through Apocromat x40 and x63 oil immersion Leica lens Pinhole diameter was set to 1 Airy unit.

2.2.4 Explant cultures

The mouse was first anticoagulated with 200 units of heparin intraperitoneally. Fifteen minutes later the animal was sacrificed by cervical dislocation, and heart was dissected. The Isolated adult mouse heart was diced into small tissue pieces less than 1 mm^3 in size and vigorously triturated with calcium free Hanks buffered saline solution. The explants were then resuspended and cultured in DMEM HAM/F12 supplemented with 10%FCS and LIF (cardiac explant culture medium). The media was changed every 4 days for up to five weeks.

2.2.5 Transmission Electron Microscopy

For Transmission Electron Microscopy (TEM – Simens 102, Simens, UK), EDCs were fixed in 2.5% glutaraldehyde in Dulbecco's PBS and post-fixed in 0.5% osmium tetroxide in PBS. After serial dehydration in graded alcohol specimen were immersed in propylene, followed embedding in Spurs resin. For immunogold staining tissue was fixed in 4% paraformaldehyde instead and embedded in LR White™ embedding resin before standard labelling with anti-GFP primary antibody and gold-conjugated secondary antibody. This work was done together with Dr Shenje in collaboration of Electron Microscopy at the University of Leicester. My initial work described about provided the ground work to proceed with this part of the experiment and I was involved in the tissue preparation before the Electron Microscopy.

2.3 RESULTS

2.3.1 Characteristics of normal MLC2v-Cre/ZEG heart

Figure 2-5 showed the typical immunohistological staining from the MLC2v-Cre/ZEG mouse. GFP was expressed in majority (94±0.5%) of ventricular cardiomyocytes expressed eGFP (n=3) due to the cardiomyocyte-restricted cre-recombinase as evidenced by the presence of anti-GFP immune reactivity.

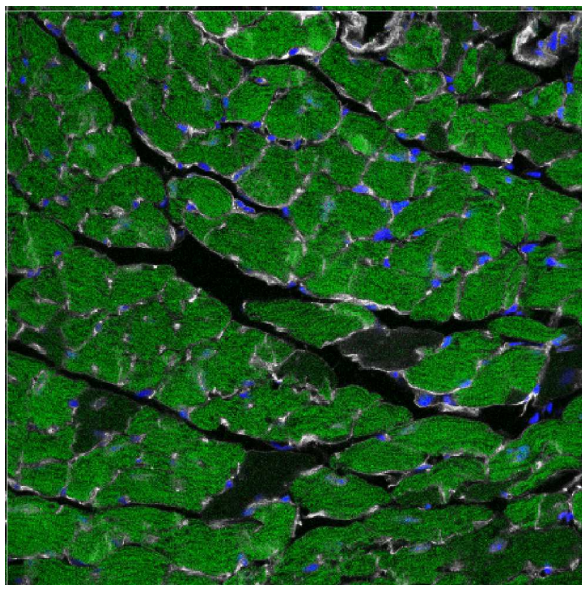


Figure 2-5: Immunohistological characteristics of MLC2v-Cre/ZEG heart.

Pseudo-colouring: Green – GFP, Blue – DAPI, White – wheat germ agglutinin (WGA)

2.3.2 Detection of GFP within infarct scar of MLC2v-Cre/ZEG heart

When the hearts from the MLC2v-Cre/ZEG mice were harvested (n=2) around 3 weeks after the induced injury, there were well-demarcated area of infarct corresponding to area supplied by the ligated LAD territory. On confocal microscopy, the scar and fibrosis were clearly defined by the WGA staining (pseudo-coloured white in Figure 2-6). Within the fibrosis, there were small pockets of GFP immunoreactive that were

not associated with any nucleus or living cytoplasmic structure (yellow arrows). 3-D reconstruction of one these pockets (Figure 2-7) confirmed that the GFP signals was not associated with viable tissue and was completely surrounded by fibrous scar. This was contrary to some nearby GFP signals which still had nucleus and myofilament architecture from surrounding myocytes. These data therefore suggested that it is possible for GFP to be retained for up to 3 weeks after the loss of native cells.

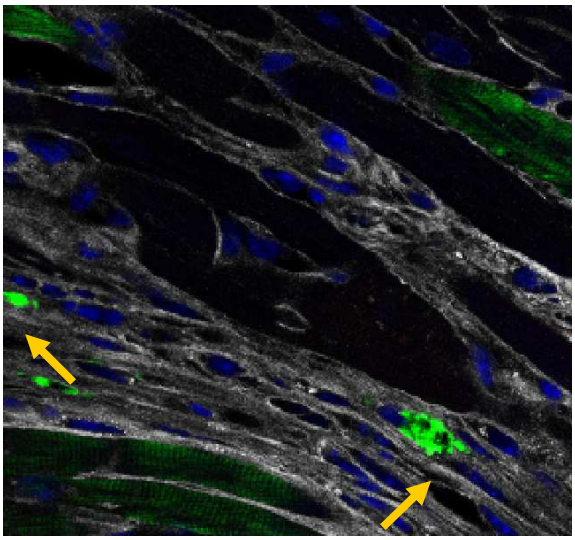


Figure 2-6: Confocal image demonstrating retained GFP (yellow arrows) within infarct scar.

Pseudo-colouring: Green – GFP, Blue – DAPI, White – wheat germ agglutinin (WGA)

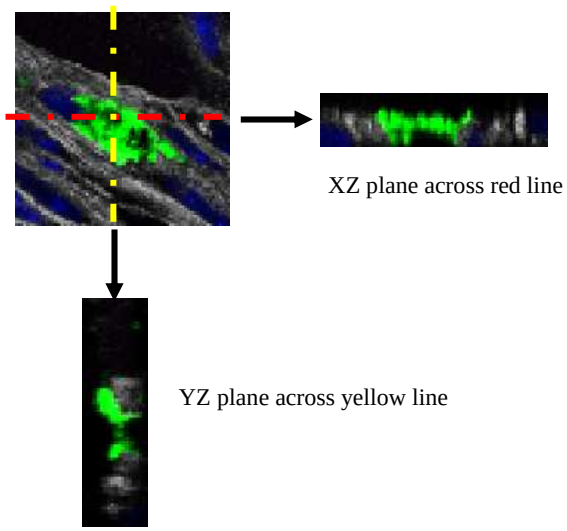


Figure 2-7: 3-D close-up of a retained GFP, with views of XZ & YZ planes

The retained GFP is without nucleus and completely surrounded with fibrosis tissue. Pseudo-colouring: Green – GFP, Blue – DAPI, White – wheat germ agglutinin (WGA)

2.3.3 Electron microscopic examination of EDCs for GFP uptake

Following the previous experiment, electron microscopy was performed to study the ultrastructure of EDCs and evidence of GFP uptake. Figure 2-8A showed the electron microscopic features of a typical EDC. It contained many dense cytoplasmic structures and pseudopodia. Several EDCs could be identified on the surface of the cardiac explants (Figure 2-8B: black arrows). Those detaching away from the explant surface appeared to assume a more circular morphology observed in previous immunohistological studies. It was also interesting to note that there were cells arising within the interstitium of explants that shared similar cytoplasmic content and electron density with the EDCs. Further analysis of these EDC-like cells demonstrated apparent phagocytosis of myocyte mitochondria and sarcomeric structures. Immunogold labelling was also identified within an endocytic compartment of those EDC-like cells (Figure 2-9), suggesting the uptake of GFP by these cells.

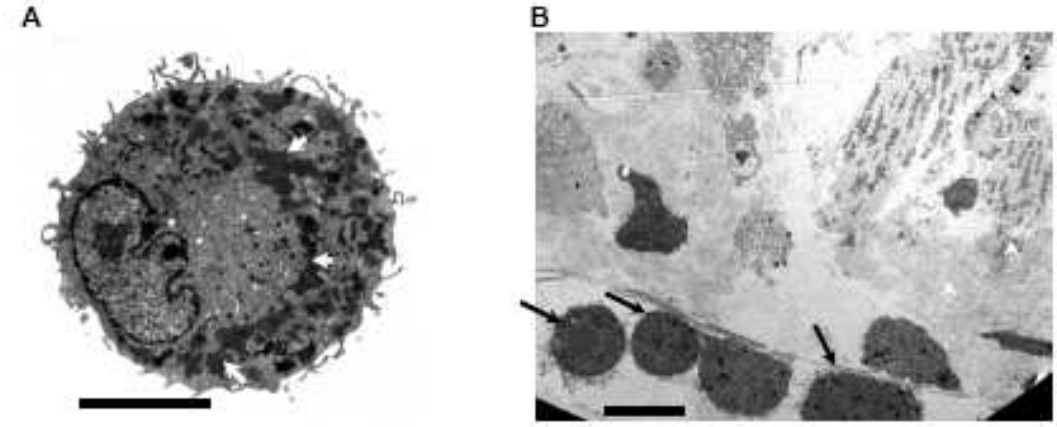


Figure 2-8: Ultrastructural characteristics of EDCs. (Shenje *et al*[101])

(A) Typical EDC – showing electron dense cytoplasmic structures (white arrowheads) and pseudopodia (scale bar, 2 μm). (B) Close-up wiew at the surface of a cardiac explant reveals numerous EDCs (black arrows) on the surface and immediately within the explant (scale bar, 5 μm).

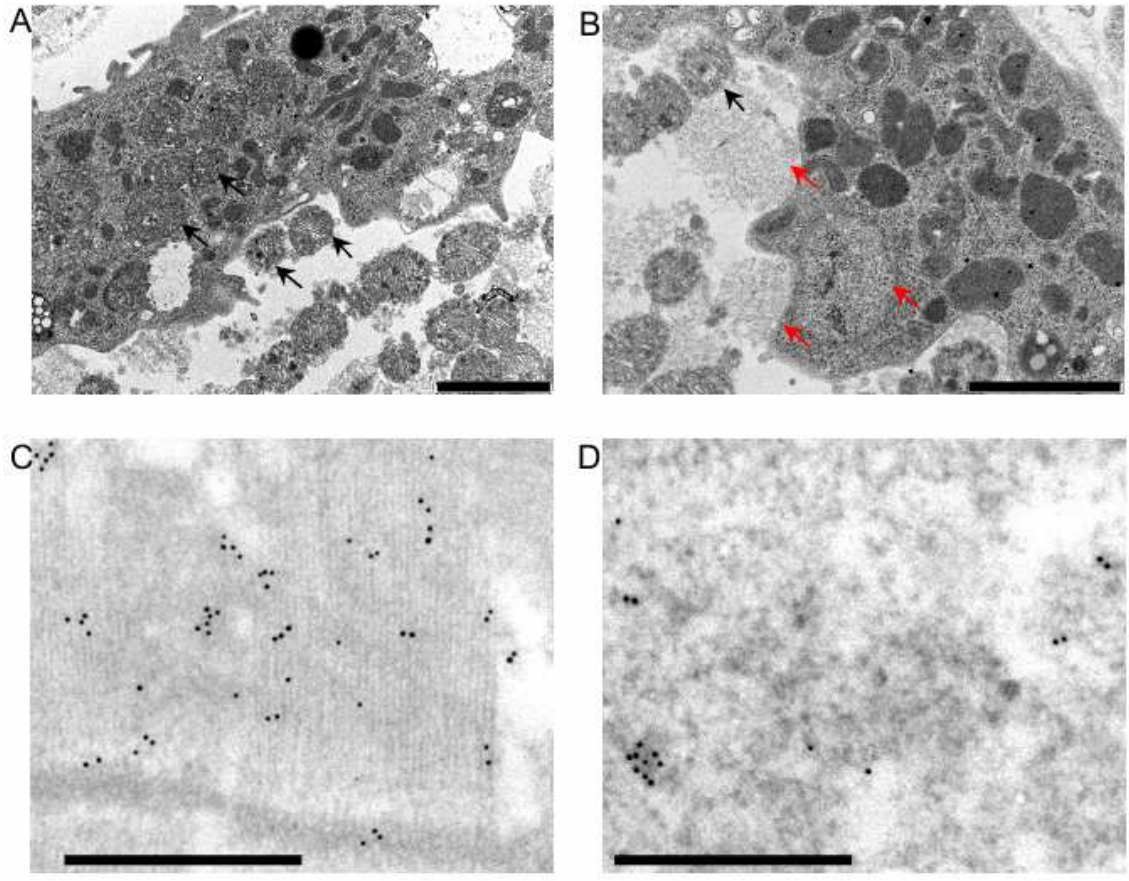


Figure 2-9: Additional ultrastructural and immune cytologic analyses of EDCs. (Shenje *et al*[101])

Apparent phagocytosis of myocyte mitochondria (A: black arrowheads) and sarcomeric structures (B: red arrowheads) by EDC-like cells within the interstitum of a cultured MLC2v-Cre/ZEG cardiac explant (Scale bar, 2 μm). Immunogold labelling of GFP (Scale bars, 0.5 μm) in fresh non-cultured MLC2v-Cre/ZEG cardiac tissue (C), and within an endocytic compartment within an interstitial EDC-like cell (D).

2.4 DISCUSSION

The use of transgenic animals with GFP expression provides a useful means of studying cell therapy. However, the use of GFP is not without its drawbacks. One of which is to distinguish true GFP signal from that of autofluorescence. To overcome this issue, I have used paraffin section and immunohistological staining with anti-GFP primary antibody and a secondary reporter antibody to make sure that the GFP signals were true. Earlier work using anti-GFP primary antibody and DAB staining had also confirmed that the GFP observed in our MLC2v-Cre/ZEG mice (Figure 2-3) was true GFP signal.

This study highlighted another potential problem associated with GFP – that it can be retained within the tissue after the death of the cells. This can lead to potential false positive results, particularly in those reports using transgenic animals ubiquitously expressing GFP to study cell therapy. In experiments relying on specific transgenic activation of GFP for cell lineage tracing, the presence of GFP signal on immunohistochemistry alone will not be sufficient. Additional confirmation such as using DNA analysis is necessary to confirm there is indeed activation of the transgene.

In this study, the GFP detected in EDCs appeared to be related to uptake of retained GFP. Similar apparent phagocytosis of myocardial structures may also explain for the other myocardial proteins observed within the EDCs. These findings, together with the lack of expected recombinant cardiac transgene activation suggests that the EDCs obtained in this culture conditions were unlikely to be cardiac progenitor cells. This raises the other question of what is the nature and progeny of EDCs. To address this, it is necessary to discuss further joint work that was performed in our laboratory.

The view that the EDCs were not cardiac progenitors was also supported by the lack of functional engraftment when they were transplanted into infarct border zone. As phagocytic activities were observed by the EDCs on electron microscopy, additional studies were performed to investigate whether these EDCs have features of neutrophils or macrophages. However, they showed no evidence of CD45 or MAC-1 immunohistochemical staining (Figure 2-10). Interestingly when the hearts were perfused prior to culturing, very few EDCs colonies were observed, suggesting that the origins of these EDCs may be blood-borne. Therefore to establish the true identity of these cells, further separate studies will be needed.

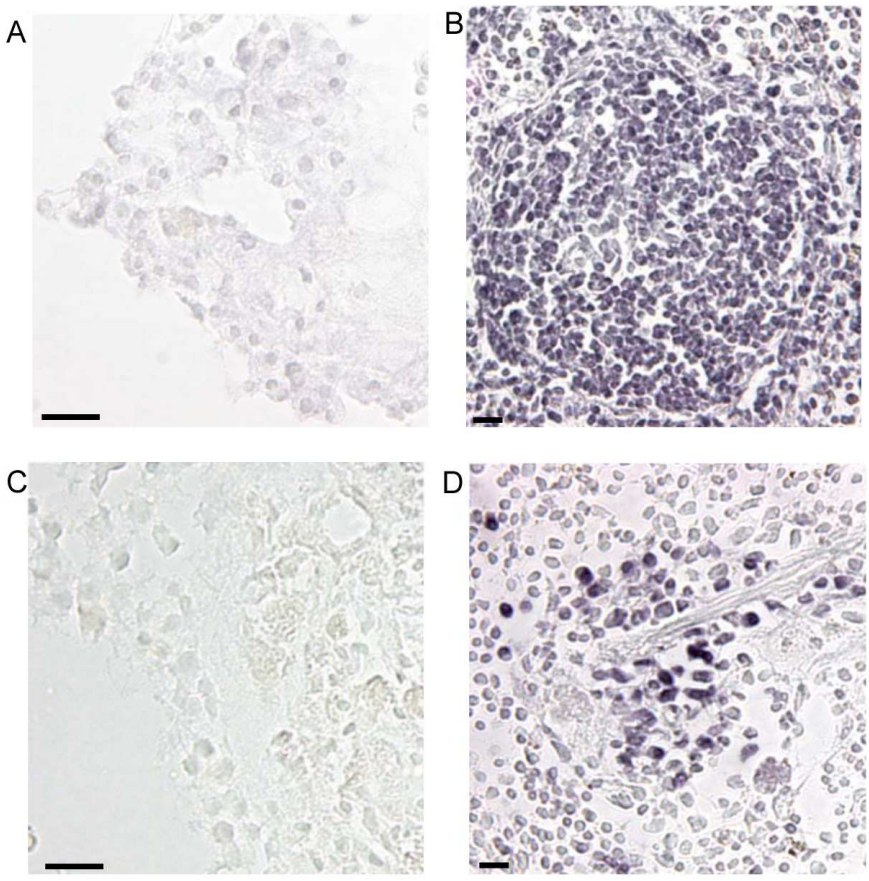


Figure 2-10: Staining of EDCs for neutrophils and macrophage markers. (Shenje *et al*[101])

EDCs lacked anti-CD 45 (A) and MAC-1 staining (B), while positive control mouse spleen germinal centres demonstrated positive signals using a nickel-enhanced DAB reaction for both antibodies (C) & (D) respectively (scale bars, 20 μm) .

In the context of my thesis, the investigations performed in this chapter highlight a potential limitation with transgenic technology that I needed to be aware of in the planning of my future work. It also identified the difficulties of using this in-vitro model for the study of cell therapy. Hence, for my PhD, the pre-clinical study on the use of bone marrow cell therapy for myocardial repair will have to be based on in-vivo model. To do this, I will first need to address one of the main current concerns in the study of cellular therapy and cell dynamic – the accuracy of myocyte nuclei identification by current microscopic techniques. This was achieved using a transgenic mouse in which 99% of myocytes expressed β-GAL nuclear signals in the next chapter.

CHAPTER 3

LIMITATIONS OF CONVENTIONAL APPROACHES TO IDENTIFY MYOCYTE NUCLEI IN HISTOLOGIC SECTIONS OF THE HEART

3.1 INTRODUCTION

Histologic analysis of myocardial regeneration and the pathophysiology of cardiac diseases in many cases relies on scoring events which occur in the myocyte nucleus. Consequently, the accurate identification of myocyte nuclei in histological sections is vital for quantitative analyses. However, distinguishing between a myocyte and a non-myocyte nucleus can be difficult. Even though myocytes occupy 70% of myocardial volume, they only contribute to 20-30% of the total nuclei present in the adult mouse heart[106]. Given the interest in quantitating intrinsic and experimentally induced regenerative growth of the myocardium[34, 107, 108], accurate nuclear identification is crucial to distinguishing the roles of myocytes from those of surrounding cells.

Conventional approaches to identify myocyte nuclei in heart sections typically rely on the use of immune histologic assays with antibodies recognizing myocyte-restricted structural proteins (such as cardiac Myosin Heavy Chain, cardiac actin, or Troponin isoforms) to identify myocyte cytoplasm, and dyes which bind to dsDNA to identify nuclei. Signal can be developed with either chromogenic or fluorescent secondary antibodies followed by light or fluorescence microscopy, respectively. Many studies exploit the optical sectioning capabilities of confocal microscopy[105] in combination with fluorescent read-outs to distinguish myocyte and non-myocyte nuclei in histologic specimens. Although membrane markers such as laminin and wheat germ agglutinin (WGA) have been previously used to aid myocyte demarcation and measurement of myocyte dimensions[109, 110], a systematic comparison testing the impact of such combinatorial approaches has not been performed. Furthermore, the use of nuclear transcriptional factors associated with cardiogenesis such as GATA4 to improve myocyte nuclei identification has also not been quantitatively assessed previously.

The ability to readily manipulate the mouse genome (via traditional transgenesis or gene targeting approaches) has facilitated the generation of numerous lines to monitor specific cell lineages using either constitutive or conditional reporter systems. One model – the MHC-nLAC mice (Figure 3-1), utilized the cardiac myocyte-restricted alpha-cardiac Myosin Heavy Chain promoter to target expression of a nuclear localized β-GAL reporter[111, 112]. Analysis of dispersed cell preparations from adult MHC-nLAC hearts revealed that > 99% of the myocytes exhibited nuclear β-GAL activity, as evidenced by reacting with X-GAL, a chromogenic β-GAL substrate[112].

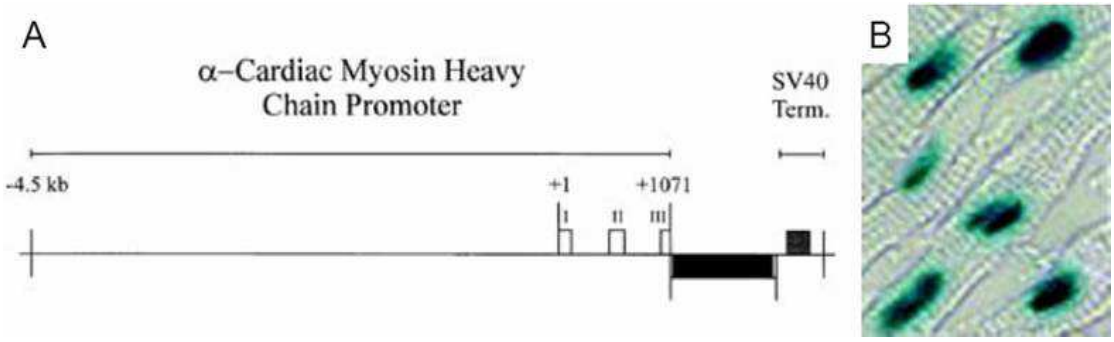


Figure 3-1: The MHC-nLAC Mouse.

The α-cardiac myosin heavy chain promoter drives the expression of β-galactosidase (A). The SV40 Terminus results in nuclear localisation of the β-galactosidase only in cardiac myocytes and the resultant blue nuclei in cardiac myocytes after exposure to x-gal solution as shown in (B). Reproduced courtesy of Professor Loren Field.

In this chapter, I systematically compared the fidelity of current myocyte nuclear identification in MHC-nLAC transgenic heart sections using confocal microscopy in conjunction with anti-Troponin T immune histology in the absence and presence of a

membrane marker (WGA). The values obtained with these assays were then compared with those obtained with anti-β-GAL immune reactivity in the same samples. A similar comparison was also made between anti-GATA4 and β-GAL immune reactivity to assess the accuracy of using nuclear transcriptional factors involved in cardiogenesis, such as GATA4 to identify myocyte nuclei in heart sections.

3.2 METHODS

3.2.1 Tissue Preparation

Generation and characterisation of the MHC-nLAC transgenic mice was described previously[111, 112]. The mice were maintained in an inbred DBA/2J genetic background. Hearts from adult MHC-nLAC mice were harvested and fixed in 10% formalin for at least 48 hours before paraffin embedding. The resulting blocks were then cut at a thickness of 5 μm using standard protocols[104].

3.2.2 Immunohistochemistry

Deparaffinisation, rehydration and microwave antigen retrieval for the heart sections were performed as described in chapter 2. The sections were blocked with 1% bovine serum albumin for 20 minutes, and then incubated with the appropriate primary antibodies overnight.

Anti-β-GAL (Abcam plc, Cambridge, UK: ab616 – 1:200 dilution) and anti-Troponin T (Abcam plc, Cambridge, UK: ab33589 – 1:200 dilution) primary antibodies were used for the study of myocyte identification with and without WGA. Appropriate goat secondary antibodies conjugated with Alexa Fluor®488 and Alexa Fluor®568, respectively (Invitrogen, Paisley, UK), were then incubated overnight at a dilution of 1:200 to detect the primary antibodies. DAPI (Molecular Probes catalogue number D1306) and WGA Alexa Fluor® 647nm conjugate (Molecular Probes catalogue number W32466) were used to identify the nucleus and outer cell membrane, respectively.

For the comparison of GATA4 and β-GAL immune reactivity, the heart tissues were stained using the above techniques except that the primary antibodies used were anti-β-GAL(ab9361 – 1:200 dilution) and anti-GATA4 (Santa Cruz Biotechnology Inc, Santa Cruz, USA: sc-9053).

3.2.3 Confocal laser scanning microscopy and image acquisition

All image volumes were acquired with an Apocromat x63 oil immersion Leica lens (numerical aperture of 1.4) at 1x zoom, using a Leica Confocal SP5 system (Leica Microsystems, Wetzlar, Germany). Pinhole diameter was set to 1 Airy unit. Each image volume was a Z-axis stack comprising 42 steps at intervals of 0.24 μm with an X-Y dimension of 246 x 246 x 10.08 μm (although the blocks were sectioned at 5 μm, thickness increased following hydration).

For the study of the accuracy of myocyte identification with and without WGA, a total of 10 image volumes were acquired, of which 5 contained myocytes aligned predominantly in the transverse orientation relative to the X-Y axis and 5 contained myocytes aligned predominantly in the longitudinal orientation relative to the X-Y axis. For each image volume, 4 channel Z-stack scans were acquired using line sequential laser excitation at 405 nm (to detect DAPI, pseudo-coloured blue), 488 nm (to detect β-GAL immune reactivity, pseudo-coloured green), 561 nm (to detect Troponin T immune or GATA4 reactivity, pseudo-coloured red) and 633 nm (to detect WGA conjugate, pseudo-coloured white).

To test the ability of immune histology to identify myocyte nuclei in the image volumes, the percentage of β-GAL immune reactive nuclei in sections containing myocytes

aligned predominately in the longitudinal orientation was compared to the percentage of nuclei exhibiting X-GAL reactivity in sections with myocytes in a similar alignment. The X-GAL reaction protocol was previously optimised to identify all β-GAL positive nuclei within a section[112]. The values obtained via β-GAL immune reactivity and X-GAL reaction were not significantly different (20.6 + 6% vs. 20.3 + 5%, respectively; mean + SEM, n = 760 cells) indicating that the immune assay was equally efficient at identifying myocyte nuclei in histologic sections. There was no β-GAL immune reactivity detected in wild-type hearts.

3.2.4 Manual Segmentation for Scoring Nuclear Identity with and without WGA

Leica AF software was utilized in post-acquisition mode to generate Z-stacks of the 10 image volumes containing β-GAL, Troponin T, DAPI and WGA. For each image volume, β-GAL myocyte nuclei signals were turned off to generate a 2-channel z-stack (reporting DAPI and Troponin T signals only), as well as a separate 3-channel z-stack (reporting DAPI, Troponin T and WGA signals) in "tiff" format. To avoid bias, each z-stack was then given a coded number and selected in a random order for nuclear identification by different observers. The image sets were imported into Volocity LE (Improvision, Coventry, UK) for the visualisation of different layers of the Z-stacks as well as the generation of orthogonal views. To optimise the identification of myocyte nuclei, all available views were used, namely, all layers of the Z-stack, the maximal projection view and the orthogonal views. A hardcopy of the maximal projection from each 2 channel and 3 channel Z-stack was printed for the observers involved to mark the position of those nuclei that they identified as myocyte nuclei (that is, nuclei residing within Troponin T-positive cytoplasm) without and with the aid of WGA. These results

were then compared to the 4 channel Z-stack images (that is, images in which DAPI, Troponin T, WGA and β-GAL myocyte nuclear signals were activated, see Figure 3-2c and f for representative examples) to establish the identity of the nucleus as defined in Table 3-1. The 4 channel Z-stacks images were only made available at the final analysis.

Table 3-1: Definition of terms used to establish the identity of nuclei scored with and without WGA. (Ang *et al*[113])

	β-GAL Immune Reactivity	
	β-GAL (+)	**β-GAL(-)**
Myocyte	True Positive	False Positive
Non-myocyte	False Negative	True Negative

3.2.5 Nuclear identification with GATA4 immune reactivity

For experiments employing GATA4 immune reactivity, true GATA4 signals were verified by their co-localisation with DAPI signals using all available views described above. Only those GATA4 signals that subsequently co-localized with β-GAL nuclear signals were considered true positive. Those true GATA4 signals without β-GAL nuclear signals were considered non-myocyte nuclei and false positive.

3.2.6 Statistics

Sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy were reported as described below. The level of agreement between different observers was calculated using Fleiss kappa (κ) statistics[114].

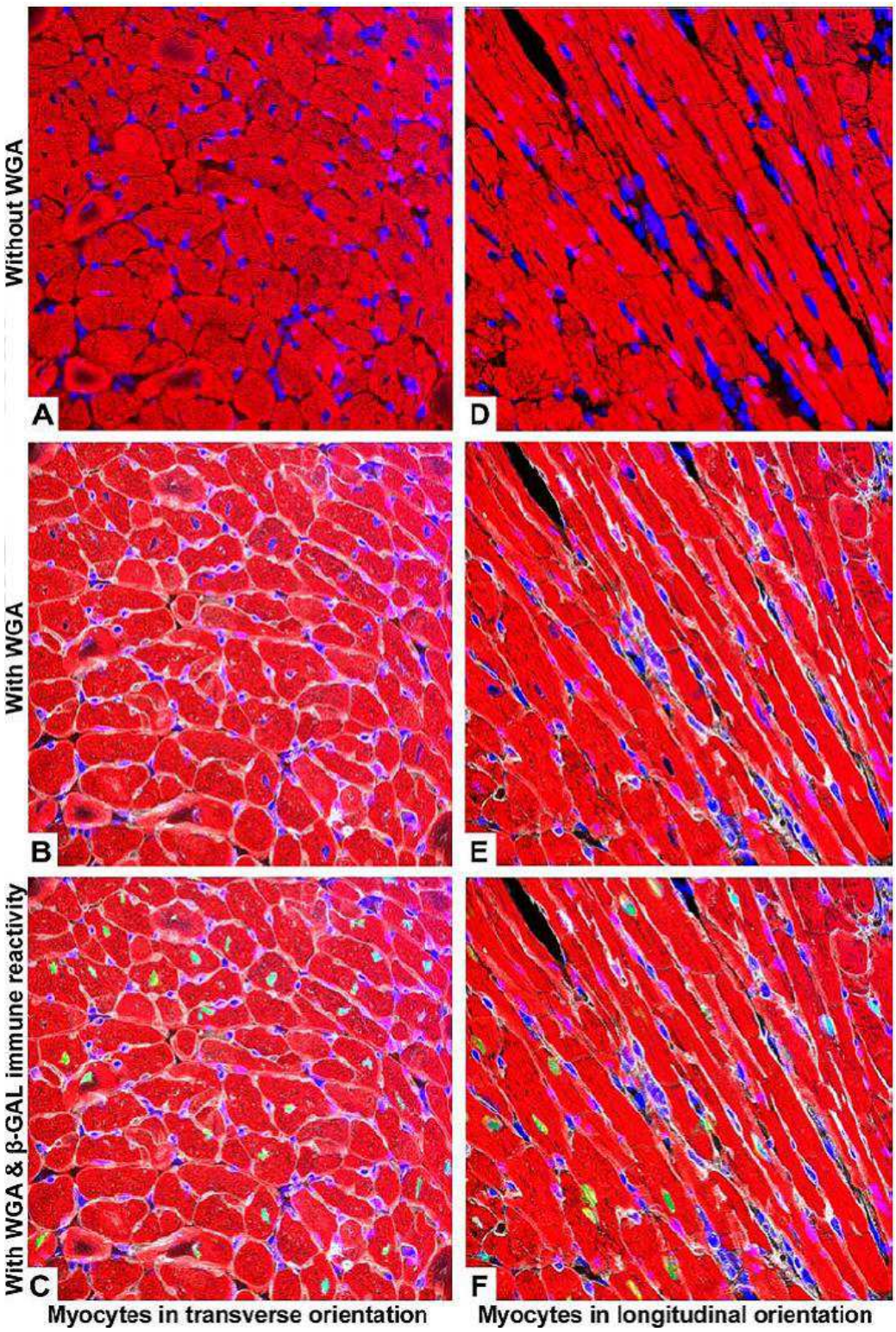


Figure 3-2: Representative maximal projection images used in the study. (Ang *et al*[113])

(A-C): images of myocytes predominantly in the transverse orientation. (D-F): myocytes orientated predominantly in the longitudinal orientation. Pseudo-colouring: Red - Troponin T, Blue - DAPI, White – wheat germ agglutinin (WGA), and Green – β-GAL myocyte nucleus.

3.3 RESULTS

3.3.1 Image volume characteristics

Ten image volumes comprising a total of 6,100,012.8 μm^3 of ventricular myocardium from MHC-nLAC transgenic hearts were acquired. These volumes contained a total of 1503 nuclei, of which 292 nuclei showed β-GAL immune reactivity (19.4%). Figure 3-2 shows the maximal projection for a representative image volume with 2 channels activated (panels a and d; DAPI and Troponin T signals), 3 channels activated (panels b and e; DAPI, Troponin T and WGA signals) and 4 channels activated (panels c and f; DAPI, Troponin T, WGA and β-GAL signals).

3.3.2 Overall sensitivity and specificity with and without WGA

Using Table 3-1, the sensitivity of myocyte nuclear identification is defined mathematically as the number of true positive nuclei / (the number of true positive nuclei plus the number of false negative nuclei). Thus, a method with a higher sensitivity will be better at identifying nuclei of myocyte origin. The specificity of myocyte nuclear identification is defined mathematically as the number of true negative nuclei / (the number of true negative nuclei plus the number of false positive nuclei). Hence, specificity reflects the true negative rate, and how good a method is at correctly excluding non-myocyte nuclei. In the absence of WGA, the overall sensitivity and specificity for all the images ranged from 38 to 48% and 87 to 92%, respectively. With WGA, the sensitivity and specificity improved to 62 to 68% and to approximately 97%, respectively (Table 3-2).

Table 3-2: Sensitivity and specificity of myocyte nuclei identification. (Ang *et al.*[113])

Orientation	Observer	Sensitivity (%) *[CI]*		Specificity (%) *[CI]*	
		without WGA	With WGA	Without WGA	with WGA
Transverse	1	**45.1** *[37.7 - 52.8]*	**64.6** *[57.1 - 71.5]*	**90.1** *[87.6 - 92.1]*	**98.4** *[97.1 - 99.1]*
	2	**48.8** *[41.2 - 56.4]*	**68.9** *[61.5 - 75.5]*	**89.5** *[86.9 - 91.6]*	**99.0** *[97.9 - 99.5]*
	3	**54.3** *[46.6 - 61.7]*	**72.6** *[65.3 - 78.8]*	**94.4** *[92.4 - 95.9]*	**98.4** *[97.1 - 99.1]*
Longitudinal	1	**28.9** *[21.8 - 37.3]*	**59.4** *[50.7 - 67.5]*	**83.4** *[80.0 - 86.3]*	**94.0** *[91.7 - 95.7]*
	2	**34.4** *[26.7 - 43.0]*	**56.3** *[47.6 - 64.5]*	**84.5** *[81.2 - 87.4]*	**95.5** *[93.4 - 97.0]*
	3	**39.8** *[31.8 - 48.5]*	**61.7** *[53.1 - 69.7]*	**88.5** *[85.5 - 90.9]*	**95.3** *[93.2 - 96.8]*
Overall	1	**38.0** *[32.6 - 43.7]*	**62.3** *[56.6 - 67.7]*	**87.1** *[85.1 - 88.9]*	**96.5** *[95.3 - 97.4]*
	2	**42.5** *[36.9 - 48.2]*	**63.4** *[57.7 - 68.7]*	**87.3** *[85.3 - 89.0]*	**97.4** *[96.4 - 98.2]*
	3	**48.0** *[42.3 - 53.7]*	**67.8** *[62.2 - 72.9]*	**91.7** *[90.1 - 93.2]*	**97.0** *[95.9 - 97.8]*

Abbreviations: WGA – wheat germ agglutinin;CI – Confidence interval

Table 3-3: Predictive power of myocyte nuclei identification in the presence and absence of WGA. (Ang *et al.*[113])

Orientation	Observer	Positive Predictive power (%) *[CI]*		Negative Predictive power (%) *[CI]*	
		without WGA	With WGA	without WGA	with WGA
Transverse	1	**52.5** *[44.3 - 60.5]*	**90.6** *[83.9 – 94.7]*	**87.1** *[84.4 - 89.4]*	**92.0** *[89.7 - 93.7]*
	2	**53.0** *[45.0 - 60.8]*	**94.2** *[88.4 – 97.1]*	**87.8** *[85.1 - 90.0]*	**92.9** *[90.8 - 94.6]*
	3	**70.1** *[61.6 - 77.4]*	**91.5** *[85.5 - 95.2]*	**89.5** *[87.0 - 91.5]*	**93.6** *[91.6 - 95.2]*
Longitudinal	1	**29.4** *[22.1 - 37.8]*	**83.1** *[79.7 - 86.0]*	**70.4** *[61.2 - 78.2]*	**90.7** *[88.0 - 92.8]*
	2	**34.7** *[26.9 - 43.3]*	**84.4** *[81.1 - 87.2]*	**75.0** *[65.5 - 82.6]*	**90.2** *[87.4 - 92.3]*
	3	**45.1** *[36.3 - 54.3]*	**86.1** *[82.9 - 88.7]*	**76.0** *[66.9 - 83.2]*	**91.3** *[88.6 - 93.3]*
Overall	1	**41.6** *[35.8 - 47.6]*	**85.4** *[83.3 - 87.2]*	**80.9** *[75.2 - 85.5]*	**91.4** *[89.7 - 92.8]*
	2	**44.6** *[38.9 - 50.5]*	**86.3** *[84.2 - 88.1]*	**85.7** *[80.3 - 89.7]*	**91.7** *[90.1 - 93.1]*
	3	**58.3** *[52.0 - 64.4]*	**88.0** *[86.1 - 89.6]*	**84.6** *[79.4 - 88.7]*	**92.6** *[91.0 - 93.9]*

Abbreviations: WGA – wheat germ agglutinin; CI – Confidence interval

3.3.3 Overall positive and negative predictive power with and without WGA

Positive predictive power is defined mathematically as the number of true positive nuclei / (the number of true positive nuclei plus the number of false positive nuclei). It describes the proportion of myocyte nuclei that are correctly identified, and reflects the probability of a positive myocyte nuclear identification. On the other hand, the negative predictive power is defined mathematically as the number of true negative nuclei / (true negative nuclei plus the number of false negative nuclei). Thus, a high negative predictive power will indicate that the probability of accurately excluding a non-myocyte is higher. The use of WGA improved the overall positive predictive power from a range of 42 to 58% to a range of 85 to 88%. There was also marginal improvement in the overall negative predictive power from 81 to 85% without WGA to 91 to 93% with WGA (Table 3-3).

3.3.4 Overall diagnostic accuracy with and without WGA

Diagnostic accuracy is defined mathematically as (the number of true positive nuclei plus true negative nuclei) / total nuclei. It describes the proportion of myocyte and non-myocyte nuclei that are correctly identified. The use of WGA improved the overall diagnostic accuracy from 78-83% to 90-91% (Table 3-4).

Table 3-4: **Diagnostic accuracy of myocyte nuclei identification in the presence and absence of WGA. (Ang *et al*[113])**

Orientation	Observer	Diagnostic accuracy without WGA (%) *[CI]*	Diagnostic accuracy with WGA (%) *[CI]*
Transverse	1	**81.4** *[78.6 - 83.8]*	**91.8** *[89.7 - 93.4]*
	2	**81.5** *[78.7 – 84.0]*	**93.1** *[91.2 - 94.6]*
	3	**86.5** *[84.0 - 88.7]*	**93.3** *[91.4 - 94.8]*
Longitudinal	1	**72.9** *[69.4 - 76.2]*	**87.4** *[84.6 - 89.7]*
	2	**74.9** *[71.5 – 78.0]*	**88.0** *[85.3 - 90.2]*
	3	**79.1** *[75.8 – 82.0]*	**88.9** *[86.3 - 91.0]*
Overall	1	**77.6** *[75.4 - 79.6]*	**89.8** *[88.2 - 91.2]*
	2	**78.6** *[76.4 - 80.6]*	**90.8** *[89.3 - 92.2]*
	3	**83.2** *[81.3 – 85.0]*	**91.4** *[89.8 - 92.7]*

Abbreviations: WGA – wheat germ agglutinin; CI – Confidence interval

3.3.5 Impact of myocyte orientation on the identification of myocyte nuclei.

The data above indicates that use of WGA improved the sensitivity and specificity of myocyte nuclear identification (Table 3-2). However, sensitivity of nuclear identification with or without WGA in myocytes with transverse orientation (without WGA: 45 to 54%; with WGA: 65 to 73%) were better than that of myocytes with longitudinal orientation (without WGA: 29 to 40%; with WGA: 56 to 62%). In myocytes with transverse orientation, the specificity of myocyte nuclear identification in the presence of WGA was also superior to that of images with myocytes in longitudinal orientation. In terms of predictive power, images with myocytes in the transverse orientation have a higher positive predictive power (53 to 70%) than those in longitudinal orientation (29 to 45%) in the absence of WGA. The use of WGA

improves this value for myocytes in transverse orientation up to 94%. The negative predictive power and diagnostic accuracy of images with predominantly transverse myocytes are slightly better than those orientated longitudinally both in the presence and absence of WGA (Table 3-3 & Table 3-4).

3.3.6 Interobserver variability with and without WGA

The percentage of β-GAL nuclei identified by all 3 observers was higher when WGA was used (Table 3-5). Without WGA, more β-GAL nuclei were missed by all 3 observers. The interobserver agreement was also better with WGA, although Fleiss κ values for both methods (WGA 0.592 vs without WGA 0.466) suggested at best moderate agreement[115] between observers.

Table 3-5: Interobserver agreements in the presence and absence of WGA. (Ang *et al*[113])

	Without WGA		**With WGA**	
	N	**%**	**N**	**%**
Number of β-GAL nuclei that were not identified	108	37.0	67	22.9
Number of β-GAL nuclei that were identified by 1 observer	56	19.2	30	10.3
Number of β-GAL nuclei that were identified by 2 observers	59	20.2	52	17.8
Number of β-GAL nuclei that were identified by 3 observers	69	23.6	143	49.0
Fleiss κ	0.466		0.592	

3.3.7 Diagnostic performance of GATA4 immune reactivity

Although GATA4 signals co-localized with approximately 90% of β-GAL nuclei, up to a third of the total GATA4 signals were β-GAL negative. This gives an overall sensitivity of 91.2% (confidence interval, CI: 89.1-94.3%), specificity of 88.7 (CI: 86.9-90.3%), positive predictive power of 72% (CI: 67.7-75.4%) and negative predictive power of 97.3% (CI: 96.2-98.1%). The diagnostic accuracy for myocyte nuclei (89.5%) was comparable to those using WGA.

3.4 DISCUSSION

This study highlights the limitations of conventional approaches to identify myocyte nuclei in histologic sections of the heart. In the absence of membrane markers, the identification of myocyte nuclei using confocal microscopy in conjunction with myocyte-restricted structural protein immune cytology can be difficult, and the overall sensitivity of identifying myocyte nuclei with confocal microscopy averaged 43% in this study. The use of membrane markers such as WGA improved the sensitivity of myocyte nucleus identification to an average of 65%. The use of WGA improved specificity from an average value of 89% to 97%. Similar trends were observed for the overall diagnostic accuracy and predictive power calculations. Although the ability to identify myocyte nuclei was relatively consistent between different observers, the fact that any discrepancy was observed indicates at least some degree of subjectivity in the process. Importantly, the degree of inter-observer variability was decreased and the overall accuracy was increased with the use of WGA.

Problems typically encountered in nuclear identification in histologic sections are illustrated in Figure 3-3. In the absence of WGA, the nucleus identified by the yellow arrow appears located centrally within a myocyte cell body and was mis-identified by investigators as being of myocyte origin. Although the use of membrane markers can aid myocyte identification (compare panels 2a and 2c), it is still far from ideal. For example, the nucleus identified by the white arrow in Figure 3-3 appeared to lie within the periphery and interstitial space and was mis-identified as being of non-myocyte origin. These examples illustrate the intrinsic difficulty in nuclear identification, despite the use of confocal approaches.

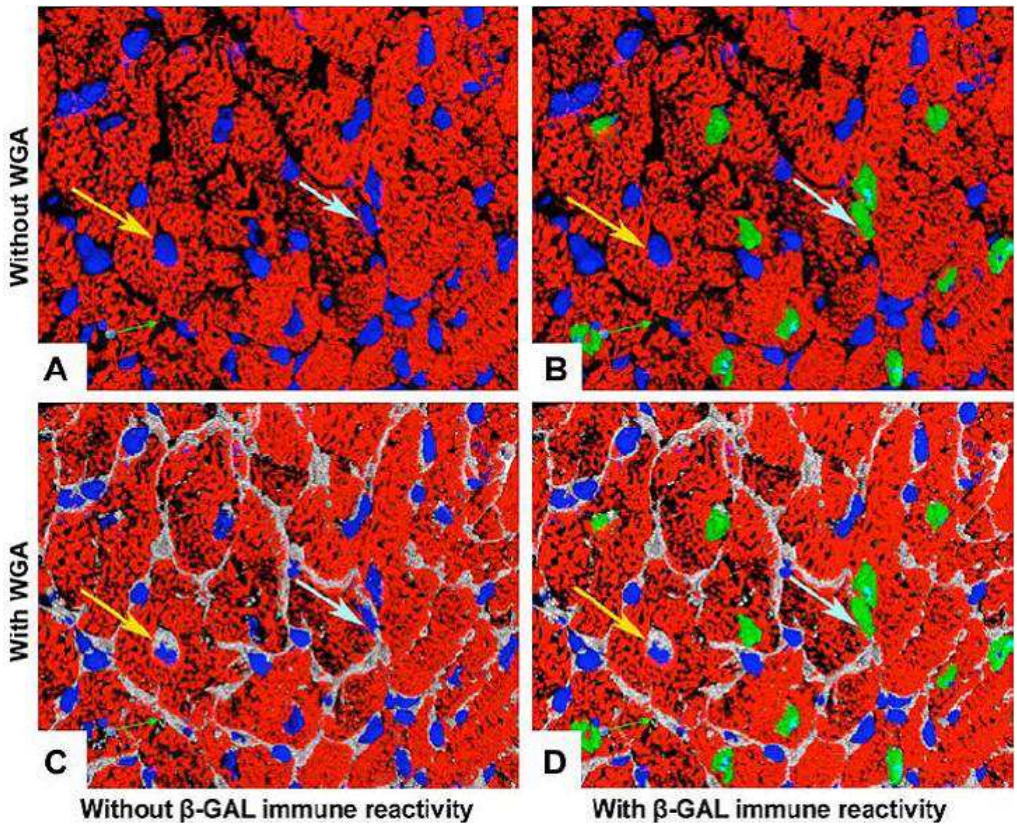


Figure 3-3: Typical problems encountered in nuclear identification. (Ang *et al*.[113])

Yellow arrow illustrates a non-myocyte nucleus surrounded by the myocyte cytoplasm which was thought to be of myocyte origin. White arrow points to a mis-identified myocyte nucleus that was located in the periphery of a myocyte. (See text for detailed explanation of arrows). Pseudo-colouring: White – cell membrane delineated by wheat germ agglutinin (WGA), Red – Troponin T, Blue – DAPI nuclear counter stain and green is β-GAL myocyte nucleus.

Interestingly, this study also demonstrates that the plane of section can influence the accuracy of myocyte nucleus identification. The sensitivity and specificity were higher in myocytes aligned predominantly in the transverse orientation relative to the X-Y axis, with or without the aid of a membrane marker. Given that, in the transverse orientation, the longest dimension of the myocyte is perpendicular to the plane of section, the probability that a non-myocyte will overlie a myocyte is reduced. This likely

contributes to the higher values for sensitivity and specificity as well as diagnostic accuracy for transverse sections.

Perhaps it is not surprising that the use of confocal microscopy failed to correctly identify all myocyte nuclei when used in conjunction with a cytoplasmic myocyte marker. Although the theoretical lateral and axial resolution of confocal microscopy are in the order of ~0.2 and 0.5 μm, respectively, a number of factors conspire to reduce resolution in practice[105]. These include differences in the refractive index of the embedding medium vs. that of the immersion medium, differences in excitation and emission wavelengths, imaging depth, and coverglass thickness. One can calculate the 3D theoretical point spread function for the imaging conditions utilized, and from that determine the lateral and axial resolution (which is set at the full width at half maximum (FWHM) of the point spread function). For this study, at an imaging depth of 0 μm, the theoretical lateral and axial resolution were 0.18 μm and 0.43 μm at an excitation wavelength of 405 nm, and 0.28 μm and 0.51 μm at an excitation wavelength of 633 nm, respectively (see http://www.svi.nl/support/wiki/HuygensCommand_stat). In reality, however, the theoretical and the experimental value for the axial FWHM markedly differ. For example, Centonze and White measured an axial FWHM of 0.85 μm using comparable imaging conditions to the present study, a value approximately double the theoretical value. Given the close proximity of non-myoycte nuclei and myocyte cytoplasm (often less than 0.5 μm)[34], and given the intrinsic and practical physical limitations of confocal microscopy, errors in myocyte nucleus identification can readily be explained.

The use of nuclear transcriptional factors such as GATA4 and Nkx 2.5 for myocyte identification also has its limitations. From the results of this study, there is a 1 in 3 chance a GATA4 signal belongs to a non-myocyte nucleus in the adult heart (Figure 3-4). These results are consistent with previous reports that GATA4 and Nkx 2.5 are not exclusively expressed in myocyte nuclei in the adult heart[116-119].

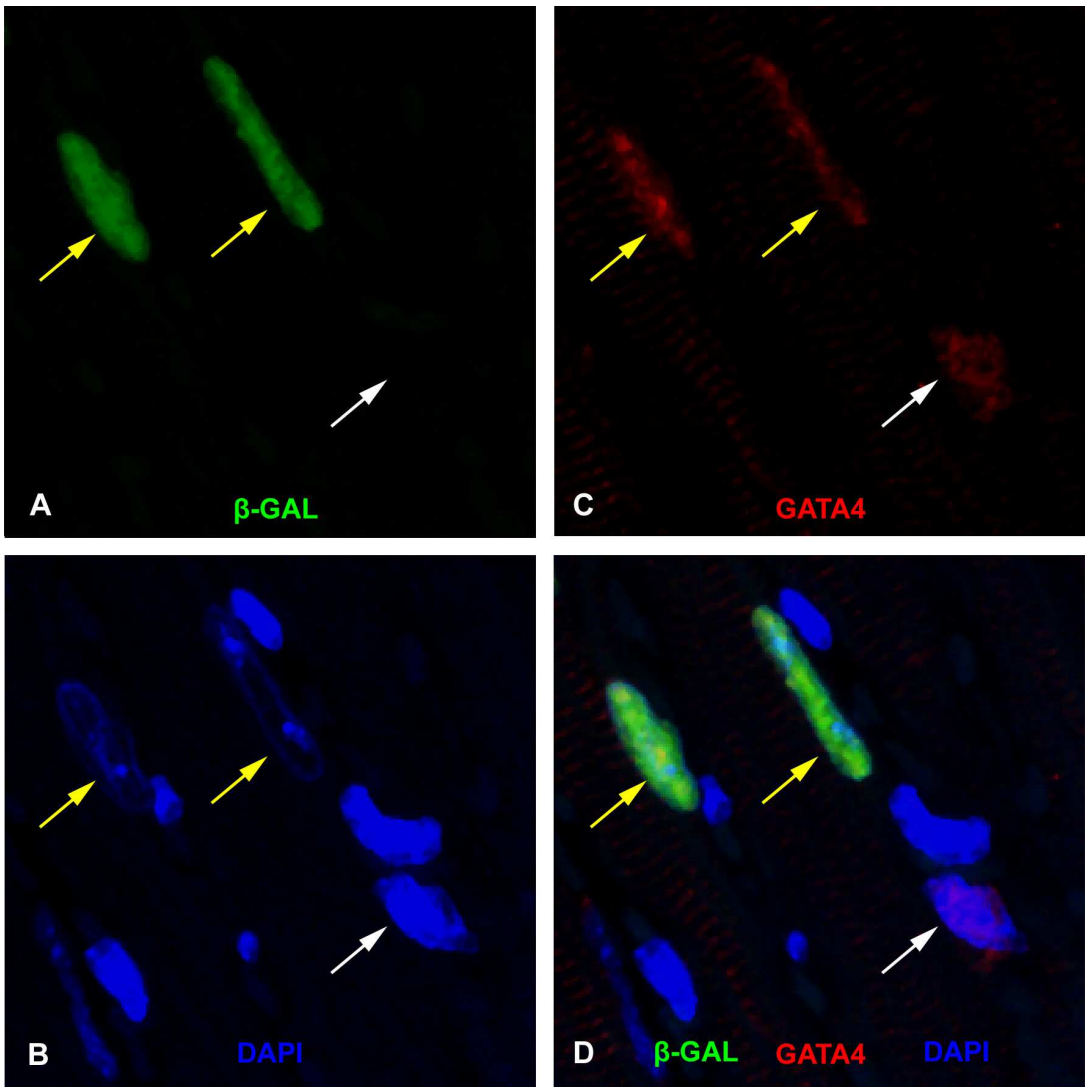


Figure 3-4: Typical problems encountered in nuclear identification. (Ang *et al.*[113])

Yellow arrow illustrates a non-myocyte nucleus surrounded by the myocyte cytoplasm which was thought to be of myocyte origin. White arrow points to a mis-identified myocyte nucleus that was located in the periphery of a myocyte. (See text for detailed explanation of arrows). Pseudo-colouring: White – cell membrane delineated by wheat germ agglutinin (WGA), Red – Troponin T, Blue – DAPI nuclear counter stain and green is β-GAL myocyte nucleus.

Taken into context of these results, if the chance of misindentifying a myocyte nucleus with current methodologies ranges from one in three to one in ten, then accurate quantification of myocyte nuclear events cannot be obtained, particularly when the events are rare (such as myocyte proliferation). These data support the notion that transgenic lineage reporters are useful for monitoring nuclear events in myocytes, especially in these circumstances. Indeed, previous studies utilized the MHC-nLAC reporter system to monitor myocyte DNA synthesis in uninjured adult hearts[111]. These analyses employed a single injection of tritiated thymidine, followed by a 4 hour chase period. Hearts were then harvested and sectioned, and the sections were reacted with X-GAL and subjected to autoradiography. Only 0.0005% of the myocytes were observed to be synthesizing DNA (as evidenced by the presence of silver grains over blue nuclei). If one normalizes this value for daily accumulation, and then for annual accumulation, these data would suggest a myocyte turnover rate of roughly 1.1% per year (i.e., 0.0005% x [24 hours per day / 4 hour chase period] x 365 days / year).

This result for the mouse myocardium was remarkably similar to data recently obtained by Frisen and colleagues for the human myocardium[120]. This latter study used cardiac samples from individuals who were born prior to or after the ban on above ground nuclear testing, and relied on the incorporation of ^{14}C-labeled carbon into the biosphere (and, subsequently, into newly synthesized DNA) to birth date new cells. Cardiac myocyte nuclei were isolated via fluorescence activated cell sorting using an antibody recognizing a myocyte nuclear-restricted motif. A myocyte turnover rate of 1% per year for individuals at 20 years of age was reported. Finally, it is of interest to consider the source of myocyte renewal. The mouse experiment noted above utilized a four hour chase after isotope exposure, and as such only captured DNA synthesis

occurring in pre-existing myocytes. In contrast, the human experiment relied on the cumulative incorporation of isotope, and as such would capture pre-existing myocytes which were synthesizing DNA as well as cycling stem cells which subsequently differentiated into myocytes. Potential species differences notwithstanding, the similar annual turnover rates obtained with these two studies suggest that myocyte proliferation, rather than myogenic neo-differentiation, is the major mechanism giving rise to myocyte renewal in the uninjured adult mammalian heart.

In conclusion, this study illustrates the intrinsic limitations encountered when using conventional approaches to identify myocyte nuclei in histological sections. The sensitivity, specificity and diagnostic accuracy achieved with confocal approaches in combination with image segmentation may not be sufficient for correct identification of rare myocyte nuclear events such as proliferation, apoptosis and trans-differentiation. By accurately determining the predictive values for individual observers (as was done here using the MHC-nLAC model), it should be possible to determine if the nuclear events being studied occur at a sufficiently large frequency such that they can be scored with confidence when using segmentation of confocal images. This has important implications for my pre-clinical study investigating the potential and mechanisms of bone marrow cells for myocardial repair. Given the above diagnostic limitations, accurate assessment of any bone marrow cells transdifferentiation events such as myocyte differentiation for my next study will need a transgenic mice model with a myocyte differentiation reporter.

CHAPTER 4

CAN BONE MARROW CELLS

REGENERATE INFARCTED MYOCARDIUM?

4.1 INTRODUCTION

Whether bone marrow cells (BMCs) can regenerate damaged myocardium remains an on-going controversy in our quest to utilise cell therapy for myocardial repair. Despite some promising in-vitro and in-vivo studies suggesting that BMCs can form myocytes[27, 28, 31, 121, 122], this has been thrown into doubt after several subsequent studies have failed to replicate these results[32, 33, 123]. This has also prompted debates as to whether BMC transplantation can truly benefit the damaged heart, and whether other mechanisms such as neovascularisation, and "paracrine" effects may contribute to any improvements observed[16]. Some have quoted the failure of using the appropriate lineage tracing techniques as a source of different results obtained[32, 33]. As illustrated in chapter 2, the phagocytosis of retained GFP can cause problems with false-positive interpretation. Thus, even with the use of transgenic models, thorough cross-checking of results is required[101]. Another potential source for the discrepancies in observed results is the possibility of mis-identifying transdifferentiation events associated with the use of conventional microscopic techniques for the stem cell therapy, which is well illustrated in chapter 3[113].

Regardless the potential mechanisms in which BMCs may benefit the damaged heart, this benefit must be measurable and clinically relevant. With the increasing use of the mice model for the study of cardiovascular diseases, especially when different relevant transgenic models can be generated with relative ease; there are increasing attempts to utilise current clinical methodologies to assess the cardiac function in mice. Traditionally, cardiac functions in mice were measured by invasive cannulation of its carotids or aorta, with the resultant sacrifice of the animal at the end of the experiment. The development of echocardiographic probes suitable for scanning mouse hearts has

facilitated the application of clinical echocardiography for non-invasive and serial studies in mouse experiments. While several detailed techniques of assessment have been developed for mice[124, 125], they are not widely embraced for mouse research as they require significant echocardiogram expertise. Most of reported echocardiographic evidence in stem cell studies relied mainly on measurement from M-mode, which have their limitations[126, 127]. Clearly, there is a need for a more simplified yet reliable methodology if echocardiography is to become widely used.

With the above consideration in mind, I conducted a study first to investigate whether BMC can form myocytes in acute myocardial infarction, by transplanting BMCs obtained from MLC2v-Cre/ZEG transgenic mouse into wild-type mouse after permanent left anterior descending (LAD) ligation. As described earlier in chapter 2, this model is unique in that only myocytes expressed GFP, while all other cells expressed β-GAL. This will enable the tracking of any BMCs by staining for their β-GAL markers after their transplantation to a wild-type heart; while any myocyte transdifferentiation will be reported by the expression of GFP. I also attempt to quantify any potential functional benefits from BMC transplantation using echocardiography to replicate the clinical setting, after first characterising the echocardiographic features of the mouse permanent LAD ligation model.

4.2 METHODS

4.2.1 Bone marrow cell harvesting

On the day of cell transplantation, donor double transgenic MLC2v-Cre/ZEG mice were anticoagulated with 200 units of heparin intraperitoneally and culled as previously described. Under aseptic technique, both tibiae and femurs were carefully dissected off all remnants of muscle and fat tissues and rinsed in 3 washes of Phosphate Buffered Saline (PBS) to avoid any contamination by skeletal myoblasts and potential adipose stem cells. By cannulating the epiphyseal ends of each shaft, the BMC were flushed out using fresh sterile PBS. They were then resuspended in 10 ml of PBS with 2% foetal calf serum in a 15 ml Falcon tube using a Pasteur pipettes to obtain a single cell suspension, and filtered through a serial of cell strainers of reducing particle sizes 100 μm, 70 μm and 40 μm (BD Biosciences, UK). The strained cells were pelleted at 1200 rpm and transffered in 1 ml of PBS to a 1.5ml eppendorf tube. This was spun at 2000 rpm in a microcentrifuge for 10 minutes. The final cell pellet (approximately 20 $x10^6$ cells), was resuspended in 250 μl of PBS, and kept on ice in preparation of injection into the myocardium.

4.2.2 Myocardial infarction and bone marrow cell injection

Anterior myocardial infarction was induced in wild-type C57 mice by permanent LAD ligation as previously described. The mice were divided into 2 groups: Control group (receiving 20μl of sterile PBS), and BMC group (receiving 20 x 10^6 cells). The injection was performed in the pale infarct area in the anterior wall. To replace incessible fluid as a result of the surgery, 100 μl of normal saline was administered

intraperitoneally. The chest was closed in layers and the mouse extubated. Temgesic was administered by subcutaneous injection for post-operative pain relief and the mice were recovered in a recovery area until the mice started moving their whiskers.

4.2.3 Mouse echocardiography

The Sequoia Acuson 512 with a 14MHz liner probe (Siemens Healthcare, UK) was used for echocardiography studies. For assessment of percent fractional shorterning (%FS), measurement of left ventricular (LV) diastolic diameter and LV systolic diameter were obtained perpendicular to the long axis parasternal view between the tip of the mitral leaflet and the papillary muscle tip. As the tip of the apex and four chamber view were difficult to obtain in mice, one parasternal long axis and 3 short axis echo slices (basal, mid-papillary and apical) were obtained at baseline, 24 hours and then weekly up to 4 weeks to assess post-infarct remodeling (n=6). Regional short axis percent fractional area change (%FAC) during end-diastole and end-systole was obtained offline using OSIRIX™ free software. The mice were sedated for the echocardiography to optimise the image acquisition. The same anaesthetic agents used for performing the above mouse cardiac surgery, and echocardiography, so that the preoperative imaging can be compared with subsequent imaging obtained at week 1, 2, 3 and 4 after surgery.

4.2.4 Histological preparation

For histological analysis, the heart was harvested in diastole after culling the mice at day 3, 7 and 28 after acute myocardial infarction, as previously described. The heart was then fixed in 10% formalin for at least 48 hours before paraffin embedding.

Transverse sections of the heart at a thickness of 5 μm required for staining were obtained by cutting the paraffin block starting from the apex at an interval of 1mm up to the junction between the mid-papillary zone and base of the heart.

4.2.5 Immunohistochemistry and microscopy

Immunohistochemistry and microscopy were performed as described in chapter 2 and 3. Tissue sections also stained for Mason's Trichrome to illustrate post-infarct fibrosis.

4.2.6 Statistics

Normal distributed data were represented as mean ± standard deviation. For comparison between control and bone marrow treated groups, generalised linear model for repeated measures were performed.

4.3 RESULTS

4.3.1 Fate of bone marrow cells after transplantation

Successful BMC transplantations were demonstrated with immunohistochemistry, by the presence of β-GAL cells within the myocardium from histological sections of hearts harvested 3 days after the myocardial infarction and BMC transplantation (Figure 4-1). The number of β-GAL signals declined over time, and was not detectable by the end of the week 4 after the procedure. No GFP signals were detected in any of the sections taken over this 4 weeks period, suggesting a lack of transdifferentiation of the transplanted BMCs.

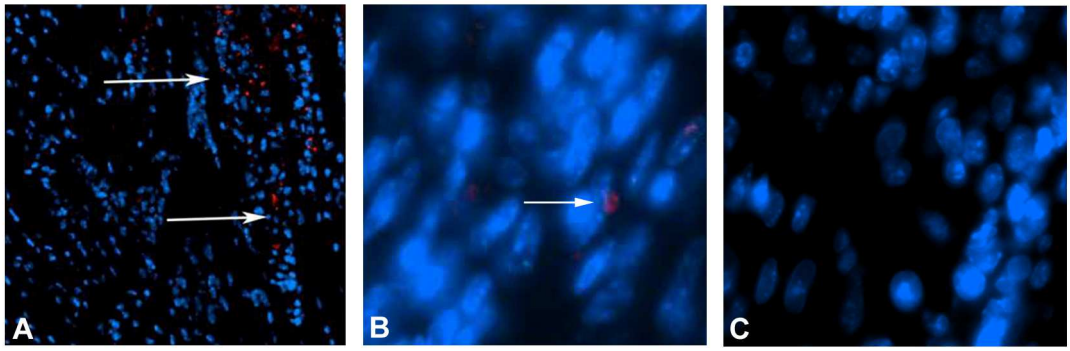


Figure 4-1: Fate of β-GAL BMC (white arrows) at the site of injection after myocardial infarction and BMC transplantation.

(A: 3 days after transplantation, β-GAL signals were easily seen at x40 magnification; B: By the 2nd week, fewer β-GAL signals were present, x60 magnification, C: No β-GAL nor GFP signals were detected at 4 weeks, x60 magnification)

4.3.2 Characterisation of echocardiographic features of mouse myocardial infarction

Following a permanent LAD ligation in mice, histological and echocardiographic examination revealed that the entire apex of the heart was most widely affected by

infarction, with circumferential transmural scarring and dilatation. At the mid-papillary level, transmural scarring tended to affect mainly the anterior and its part of its adjacent left ventricular wall, while the base of the heart was least affected (Figure 4-2).

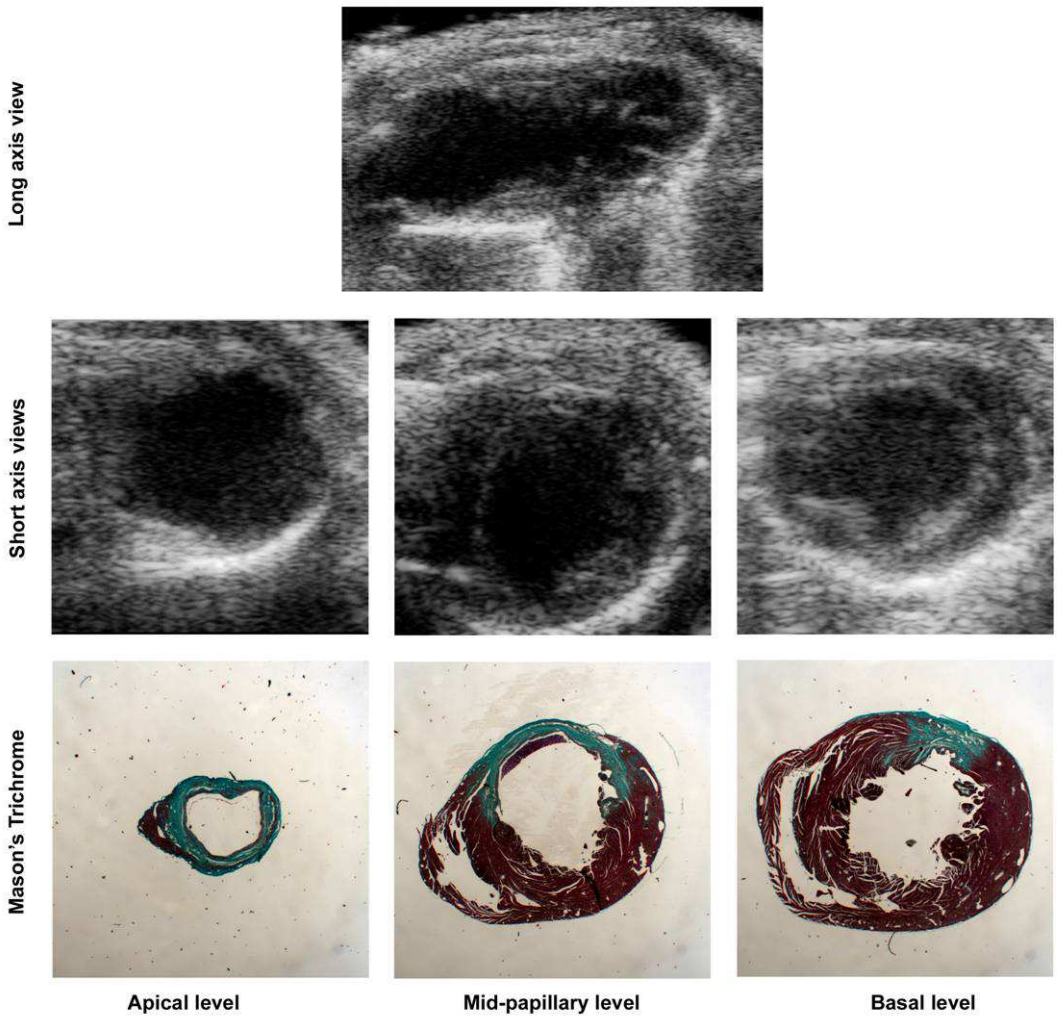


Figure 4-2: Echocardiographic and histological features after permanent LAD ligation.

The fibrotic scar areas were stained green on Mason's Trichrome.

Given these observations, the traditional M-mode measurements used for calculating %FS and ejection fraction (EF) might not reflect changes mid-papillary and apex in the mouse permanent LAD ligation model. To verify this, %FS was compared with %FAC at apex, mid-papillary and base of the heart in sham-control mice measured at baseline, and weekly during the first 4 weeks after permanent LAD ligation. Figure 4-3 shows

that there was a progressive but statisticial non-significant deterioration in %FS 4 weeks after permanent LAD ligation. A similar pattern of decline was also observed by %FAC at the base of the heart. However, there was a decline in the %FAC at the apex and at the mid-papillary region as early as the 2nd day after myocardial infarction, which was not reflected by %FS measurements.

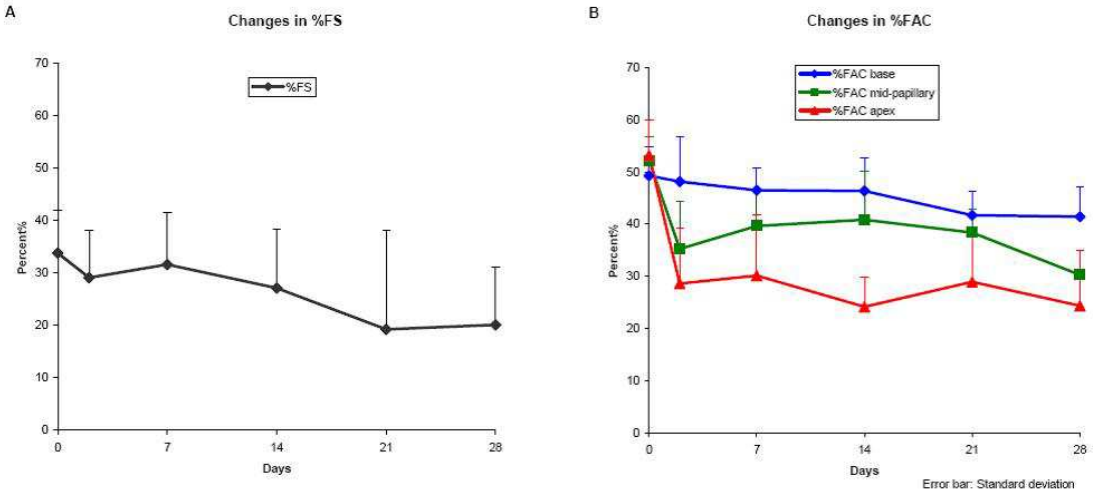


Figure 4-3: Changes in Percentage fractional shortening (%FS, panel A) and fractional area change (%FAC, panel B) during the first 4 weeks after permanent LAD ligation (n=4)

This data suggested confirmed that %FS obtained by M-mode measurements was not sufficient for detected early changes at the apex of the heart in the mouse permanent LAD ligation model. To compare the effects of BMC transplantation in this model, I will need to utilise %FAC at apex, mid-papillary and base of heart to measure of the heart. Wall motion score were also utilised assessed regional function in scar regions according the American Society of Echocardiography (ASE) 16 segment models.[128]

4.3.3 Echocardiographic assessment of the effects of bone marrow cells

There was no statistical significant difference in the %FAC at all three levels (Figure 4-4) in the BMC treated mice compared with sham operated mice. The injection of BMC did not prevent the early deterioration in apical and mid-papillary function (Figure 4-4). There was also no improvement in the wall motion assessment in the infarct segments receiving BMCs, with all the infarct segments remaining in akinesis or dyskinesis.

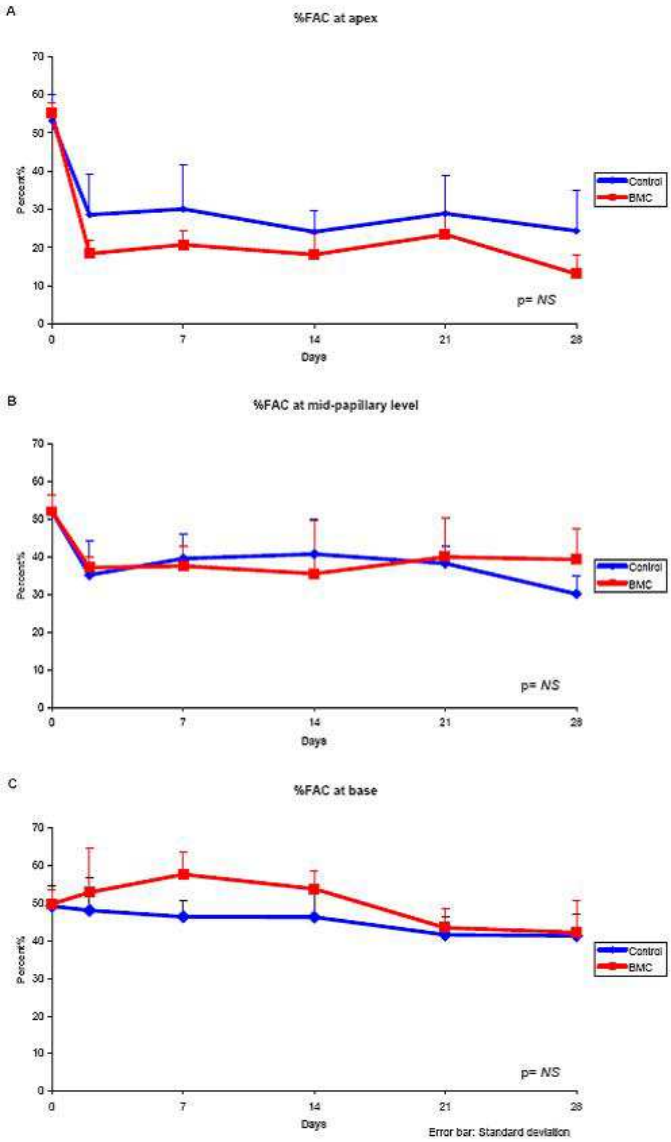


Figure 4-4: %FAC at base (panel A), mid-papillary (panel B) and apex (panel C) in control and BMC-treated mice after permanent LAD ligation (n=4).

4.4 DISCUSSION

This is a unique study as the model permits the dual tracking of transplanted cells and specific lineage tracing for myocyte transdifferentiation. It avoids the potential misinterpretation due to "Cre" leakage or cell fusion associated with the present Cre-lox technology[35-37]. It provides strong evidence that BMC were not cardiogenic, when transplanted into the peri-infarct area in the mouse acute infarct model; and are consistent with previous reports in mouse using specific myocyte lineage tracing tracing techniques.

The successful transplantation of BMCs was confirmed by the presence of β-GAL cells within the first week of transplantation, excluding transplantation failure[31] as a source for the difference in results observed from positive studies. It was interesting to note that the numbers of transplanted BMCs declined to a negligible level by the end of 4 weeks, which was in agreement with several other studies[129, 130]. This was in contrast to earlier studies that suggested BMCs can proliferate and regenerate the myocardium[31].

Given these results and my earlier observations in chapter 3, the mis-identification of a non-myocyte nucleus event as one from myocyte nucleus is a highly plausible explanation for the discrepancies in the results observed so far. While precise assessment of BMC influence on myocyte events such as native myocyte proliferation and death is important, this is not possible unless it is performed in collaboration with a separate transgenic model such as MHC-nLAC mice used in this study. The resources for the latter in terms of time and financial constraints, unfortunately will not be feasible within the remits of this PhD.

In order to assess whether BMC transplantation contributes to any meaningful myocardial repair, irrespective of its potential mechanisms, any functional improvement was measured with echocardiography – a technique widely used in the clinical setting. I have illustrated the limitations of functional assessment from M-mode measurements utilised in certain mouse studies of BMC transplantation. As the early deterioration in circumferential apical function in the mouse acute myocardial infarction model was not detected by the single plane M-mode measurement, a modified multiple plane approach was used to assess the effects of BMC transplantation. With this approach and regional assessment of the infarct area, no improvement was observed with BMC transplantation. However, the echocardiographic assessment of cardiac function used in this study is far from ideal. Due to the asymmetrical remodelling in the mouse heart following myocardial infarction, it was not possible to accurately assess the left ventricular volumes, ejection fractions, and infarct scar volumes, with the parasternal and 3 short-axis views used for this study. These data were also difficult to assess with histological sections, due to processing artefacts in my experience. While %FAC at different levels can provide some measures of regional function, it may not be accurate enough to quantify any improvement in small area of left ventricular wall. Even though, this can be overcome by using wall motion assessment, such assessment is qualitative and can be subjective.

Recent advancements in imaging technology have provided several new methodologies for the assessment of mouse myocardial function for further studies of cell therapy. Notably, the development of mouse cardiac Magnetic Resonance Imaging (MRI) offers the opportunities to accurately measure LV volumes, ejection fraction and even infarct

scar size. As cardiac MRI is already an established gold standard for such measurements in humans[131, 132], it has been proposed as a method of choice for the study of cell therapy in mice[133]. The main restriction of this approach is that it will require special equipment and set-up, which may not be accessible for all laboratories. The use of other imaging modalities used in the clinical settings such as Single-Photon Emission Computed Tomography (SPECT) in small animals[134] can also face similar limitations. On the other hand, echocardiography can be readily set-up in any laboratory. The application of 3D echocardiography in humans can also overcome some of its earlier limitations in global left ventricular assessments, and be eventually extended for mouse and small animal studies[135]. The new software development such as speckle tracking to aid the assessments of regional function[136] can further enhance the future role of echocardiography in our study of cell therapy.

In summary, this experiment suggested that BMCs did not transdifferentiate into myocytes when injected immediately after acute myocardial infarction. However, it does not exclude the possibility that BMC can contribute to myocardial repair by other mechanisms like neovascularisation or paracrine-mediated cardioprotection in the acute setting. These are areas for further studies. In addition, the role of BMCs in chronic myocardial ischaemia also needs further investigations. Potentially, the same animal model here can also be utilised to study whether BMC can form myocytes in chronic ischaemia or scars. However, the need of re-operation makes it difficult to gain ethical approval in the UK for such studies, and indeed identical studies exploring the optimal timing for BMC injections after myocardial infarction. Ironically, due to the fact that BMC transplantation is already an established therapy for other conditions and is relative safe, it is readily tested clinically for a range of heart conditions, following

some favourable earlier pre-clinical results. Therefore, in the next chapter, I will present a clinical trial, conducted to investigate whether BMCs can contribute to myocardial repair when injected into chronic infarct scar, and whether this is dependent on the route of administration.

CHAPTER 5

RANDOMISED CONTROLLED TRIAL OF INTRAMUSCULAR OR INTRACORONARY INJECTION OF AUTOLOGOUS BONE MARROW CELLS INTO SCARRED MYOCARDIUM

5.1 INTRODUCTION

Following encouraging animal studies [28, 31], increasing attention has focused on the possible roles for autologous bone marrow cells (BMCs) in myocardial repair in the clinical setting. This was facilitated by the fact that this type of stem cell is easy to harvest, simple to administer, ethically acceptable and does not require immunosuppression. Studies have shown global and regional functional improvements when BMCs were injected into viable, peri-infarct areas of chronically ischaemic myocardium[87, 137, 138]. The efficacy of BMCs in restoring function of scarred myocardium within an established infarct, however, has not been explored.

This unit has previously demonstrated the safety of injecting BMCs into myocardial scar tissue during surgical revascularisation[74]. In this study, I investigate whether the administration of BMCs, either by intramuscular or intracoronary injection, during coronary artery bypass grafting (CABG) improves contractile function of nonviable scarred myocardium when compared with CABG alone.

5.2 METHODS

This clinical trial is registered on Clinicaltrials.gov (registry number NCT00560742 assigned on 16 November 2007). The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki.

5.2.1 Study population

Patients aged 18–80 years who were candidates for elective CABG were considered for the study. The inclusion criteria were the presence of at least one chronic, irreversible myocardial scar [defined as areas of akinesia or dyskinesia with no contractile reserve on dobutamine stress echocardiography (DSE), identifiable >6 weeks after myocardial infarction, and confirmed at surgery], and the availability of a graftable coronary artery supplying the scarred segments. Exclusion criteria were valvular heart disease requiring concurrent valve surgery, cardiogenic shock (systolic blood pressure <80 mmHg, requiring intravenous inotropes or intra-aortic balloon pump), hepatic or renal failure, evidence of malignancy, pre-existing bone marrow conditions, and contraindications to cardiac Magnetic Resonance Imaging (MRI).

All participants gave written informed consent at enrolment, and received the same standard treatment and rehabilitation regimens available to other patients undergoing CABG.

5.2.2 Study design

All eligible patients underwent DSE for the identification of myocardial scarring[139]. During surgery, performed by a single surgeon, patient suitability was confirmed by the presence of epicardial scarring on visual inspection.

Patients were randomly assigned in equal number to the control group (which received no BMCs or vehicle injection) or to groups administered BMCs via either intramuscular or intracoronary routes. The physicians treating the patients during the postoperative period, as well as the investigators performing the examinations and interpreting the results, were blind to which group patients had been assigned. At 6 months after intervention, all patients underwent clinical follow-up and DSE assessment. Cardiac MRI was available for the last 33 consecutive patients entering the study and was performed (equal sample from each group) before and 6 months following treatment to quantify myocardial scarring[131] as well as global left ventricular volumes and function[132].

The primary end point was improvement in systolic function of scar segments 6 months after treatment. The secondary end points were reductions in infarct size, global end-diastolic volume and end-systolic volume, and improvement in stroke volume and left ventricular ejection fraction. Information was also collected regarding postoperative complications, troponin I levels within 24 hours of surgery and clinical evaluation (assessment of functional status and adverse events) at 6 months.

5.2.3 Bone marrow cell preparation and administration

Before surgery, 50 ml blood was taken from each patient to obtain serum. After anaesthesia but before CABG, 80 ml bone marrow was aspirated from the patient's iliac crest into preservative-free heparin (10 U/ml) and diluted with normal saline. The BMCs were isolated by density centrifugation with Lymphoprep® (AXIS-SHIELD PoC AS, Oslo, Norway), and the separated BMC layer was added to the autologous serum. The viability of BMCs after processing and immediately before administration was more than 95%. To optimise cell contact and retention in patients receiving BMCs 10 ml serum containing diluted BMC solution was injected into each scar segment on completion of coronary artery anastomoses, before the release of the aortic cross-clamp and whilst the heart was still arrested[140]. To cover the whole scar area evenly in the intramuscular group, 20 injections of 500 µl each were administered, approximately 1 cm apart, into the mid-depth of the scar under the guidance of transesophageal echocardiography; in the intracoronary group, the BMCs were delivered via the graft conduit supplying the scar.

5.2.4 Troponin I assessment

Troponin I levels were measured with a Tosoh Bioscience Troponin I assay (Tosoh Bioscience, Belgium) in venous blood samples taken within 24 hours of surgery.

5.2.5 Dobutamine stress echocardiography

All patients underwent DSE before and 6 months following surgery. Calcium antagonists and β-blockers were discontinued 48 h before DSE. Images were acquired with Philips Sonos 5500® and IE33® systems (Philips Medical Systems, Surrey, UK)

before and during infusion of low-dose dobutamine (10–20 μg/kg/min). Cardiac stress was reversed with intravenous atenolol (2.5-5mg). Digitised images were analyzed off-line by an independent assessor who was unaware of the coronary anatomy and intervention performed.

Left ventricular segmental wall motion was qualitatively assessed at rest and during low-dose DSE according to the 16-segment model of the American Society of Echocardiography[128], and assigned to one of four grades (normokinesis, hypokinesis, akinesis, dyskinesis). To increase the specificity for the diagnosis of transmural fibrosis, a scar area without functional reserve was defined as akinetic or dyskinetic if it demonstrated no improvement in wall motion during low-dose DSE. An improvement in wall motion after intervention was defined as a change in segmental function from either akinesis or dyskinesis to either hypokinesis or normokinesis. A change in wall motion between akinesis and dyskinesis was not deemed functionally relevant.

In order to address the recognised concerns about the reproducibility of DSE[141, 142], percent systolic fractional thickening (%SFT) was calculated for all scar segments, both at rest and during low-dose DSE, before and after intervention. The midsegmental diastolic and systolic wall thicknesses were measured at each stage; the respective %SFT was calculated with the formula: %SFT = [(systolic thickness – diastolic thickness)/diastolic thickness] x 100%.

5.2.6 Cardiac MRI

Images were acquired on a 1.5 Tesla Signa® scanner (General Electric, Slough, UK) using a phased array cardiac coil during repeated 8 s breath-holds. A short-axis stack of

left ventricular images was acquired using a steady state in free precession sequence (repetition time 3.0–3.8 ms; excitation time 1.0 ms; image matrix 224 x 224; field of view 36–42 cm; flip angle 45°) in sequential 8 mm slices (2 mm interslice gap) from the atrioventricular ring to apex. Left ventricular volumes, ejection fraction and mass (myocardial density = 1.05 g/cm^3) were quantified by planimetry of all short-axis steady state in free precession sequence cine images with MASS analysis software (Medis, Leiden, Netherlands). The observers were blinded to all other clinical details of the patients, including the outcome measures.

Quantification of myocardial scarring was undertaken in all cardiac MRI. To this end, 0.1 mmol/kg gadolinium-diethylenetriamine penta-acetic acid was administered intravenously and images were acquired after 10 min with a segmented inversion-recovery technique in identical short-axis slices. Inversion times were adjusted to null normal myocardium (260–400 ms). Quantification of myocardial scarring was carried out by planimetry of hyperenhanced tissue on short-axis images. Infarct volume was calculated in cubic centimeters by multiplying the planimetered area in each segment by the slice thickness. The percent volume of myocardial scar was then derived by expressing infarct volume as a percentage of left ventricular myocardial volume in the diastolic phase.

Scar transmurality was assessed on contrast-enhanced images and matched to echocardiogram segments. Scar transmurality was a measure of the transmural extent of the scar as a percentage of left ventricular wall thickness. A scar was considered to be transmural if its scar transmurality exceeds 51%[131].

5.2.7 Power calculation and statistical analysis

As this was a novel study, we did not know the variability associated with the primary outcome or its minimal clinically relevant difference at the start of the study. Therefore, to determine the sample size, we specified that we wished to detect whether improvement was as much as 1 standard deviation (SD) better in a treated group than in the control group[143]. Assuming 80% power and that $\alpha = 0.05$, 17 scar segments were required per group to show this difference. Allowing for 20% withdrawals and assuming each patient has at least 1 scar segment, we calculated that 63 patients had to be enrolled in total.

Continuous variables that were normally distributed are presented as mean (± SD). For baseline characteristics, ANOVA was used to compare the means between the treatment groups. The effects of different interventions were analyzed with a generalized linear model that compared the mean differences between groups before and 6 months after treatment, adjusting for baseline values. Variables that were not normally distributed are expressed as medians (interquartile range) and were compared by the appropriate nonparametric tests, such as the Kruskal–Wallis test. Categorical variables were analyzed with the χ^2 or Fisher's exact test as appropriate.

For segmental data on %SFT and scar transmurality, the mean values were calculated for each patient. Summary measures were analyzed by the Generalised Linear Model to enable several observations in the same patient (clustering). Scar segments within the BMC treatment groups that were revascularised but could not be treated by BMCs were not included in the analysis such as those septal segments that were inaccessible to BMC injection. The χ^2 test was used to compare the proportion of patients with at least

one scar segment that showed contractile improvement based on segmental wall motion assessment between treatment groups.

All tests were two-sided and $P<0.05$ was deemed significant. Analyses were performed with SPSS software version 14 (SPSS Inc, USA) or SAS software version 9.1 (SAS Institute Inc, USA).

5.3 RESULTS

5.3.1 Baseline characteristics and operative data

A total of 63 patients were included in the study. One patient in the control group withdrew from the study before the surgery. Another patient in the control group who underwent cardiac resynchronisation therapy was deemed unsuitable for further follow-up. The preoperative and operative characteristics were similar in the three study groups (Table 5-1). From this cohort of patients, 154 scar segments were suitable for analysis (45 in the intramuscular BMC group, 59 in the intracoronary BMC group and 50 in the control group).

5.3.2 Clinical outcomes

A mean of 84 ± 56 x 10^6 and 115 ± 73 x 10^6 BMCs (P = 0.184); and 142 ± 166 x 10^3 and 245 ± 254 x 10^3 CD34+/CD117+ cells (P = 0.239) were injected in the intramuscular and intracoronary groups, respectively. The indices for classification of physical activity for cardiac patients, given by the NYHA and the Canadian Cardiovascular Society, improved in all three groups following treatment (Table 5-2). No adverse events associated with BMC injections were observed throughout the study (Table 5-2).

Table 5-1: Patient's characteristics (Ang *et al*[144])

Characteristics	Control (n=20)	IM (n=21)	IC (n=21)	P value
Demographics:				
Mean (SD) Age (years)	61.3 ± 8.3	64.7 ± 8.7	62.1 ± 8.7	0.415
Male sex – no. (%)	18 (90.0)	15 (71.4)	19 (90.5)	0.188
Mean (SD) Body Mass Index (kg/m2)	29.2 ± 4.4	27.2 ± 4.8	26.8 ± 3.1	0.140
Risk Factors:				
Hypertension – no. (%)	12 (60.0)	9 (42.9)	10 (47.6)	0.528
Diabetes – no. (%)	6 (30.0)	4 (19.0)	5 (23.8)	0.708
Hyperlipidemia – no. (%)	16 (80.0)	14 (66.7)	15 (71.4)	0.680
Smoking history – no. (%)	15 (75.0)	12 (57.1)	12 (57.1)	0.409
Pre-op medication:				
Aspirin – no. (%)	17 (85.0)	13 (61.9)	17 (81.0)	0.227
Clopidogrel – no. (%)	10 (50.0)	10 (47.6)	11 (52.4)	0.953
Beta-blockers – no. (%)	13 (65.0)	15 (71.4)	18 (85.7)	0.298
ACE inhibitors – no. (%)	13 (65.0)	13 (61.9)	16 (76.2)	0.582
Statins – no. (%)	17 (85.0)	16 (76.2)	18 (85.7)	0.768
Diuretics – no. (%)	9 (45.0)	9 (42.9)	7 (33.3)	0.717
Operative details:				
Mean (SD) Bypass time (minutes)	97.6 ± 35.5	101.5 ± 21.0	110.5 ± 36.1	0.409
Mean (SD) Cross-clamp time (minutes)	50.9 ± 20.7	55.0 ± 12.0	65.7 ± 23.4	0.051
Median [IQR] No. of grafts	3[2]	3[3]	3[3]	0.778

Abbreviations: IM – intramuscular; IC – intracoronary; SD – standard deviation; IQR – interquartile range; ACE – angiotensin converting enzyme

Table 5-2: Clinical outcomes (Ang *et al*[144])

Clinical Parameters	Control (n=20)	IM (n=21)	IC (n=21)
CCS class:			
% of patients with CCS >2 Preop	35.0	33.3	14.3
% of patients with CCS >2 Postop	0.0	0.0	0.0
NYHA class:			
% of patients with NYHA III-IV Preop	15.0	19.0	14.3
% of patients with NYHA III-IV Postop	0.0	0.0	0.0
Mean (SD) Troponin I, µg/L	1.4 ± 1.3	2.8 ± 2.6	1.7 ± 2.0
Complications (numbers of patients):			
New Q wave myocardial infarction	0	0	0
Ventricular arrhythmia	0	0	0
Intra-aortic balloon pump support	3	0	0
Renal failure	1	1	0
Stroke	1	0	0
Death within 30 days of treatment	1	0	1

Abbreviations: IM – intramuscular; IC – intracoronary; NYHA class – New York Heart Association dyspnea class; CCS class – Canadian Cardiovascular Society angina class.

5.3.3 Effect on myocardial scars

The %SFT measured before and after surgery were negative for all segments and did not show systolic thickening at rest or during low-dose DSE, confirming absent contractile reserve and nonviability (Table 5-3). Moreover, there was no difference between the proportions of patients with contractile improvement in at least one scar

segment after intervention on the basis of segmental wall motion assessment at rest (control 47.1%, intramuscular 12.5% and intracoronary 29.4%; P = 0.092) and during low-dose DSE (control 47.1%, intramuscular 31.3% and intracoronary 41.2%; P = 0.717).

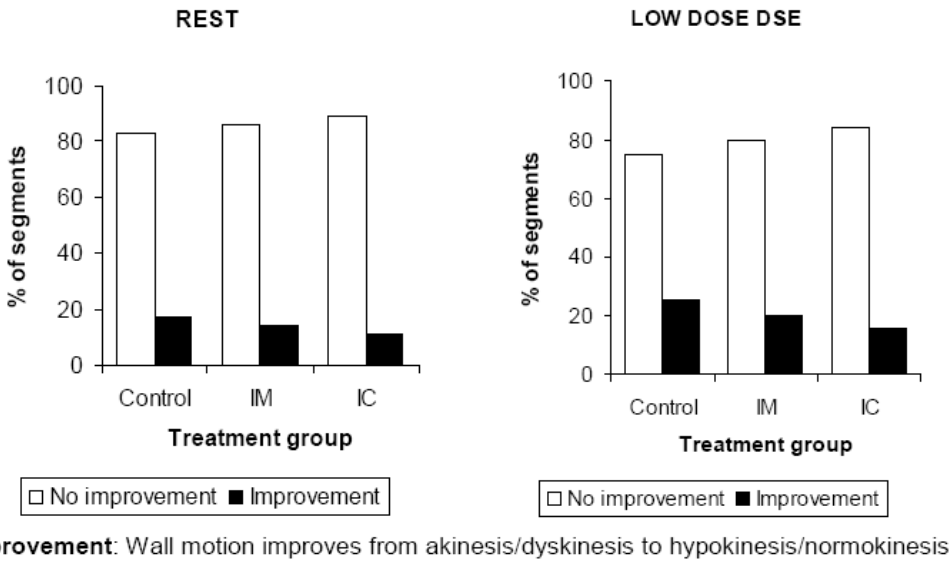


Figure 5-1: Percentage of scar segments with improved wall motion at rest & low-dose DSE

Abbreviations: IM – intramuscular; IC – intracoronary

Scar transmurality was measured in 73 segments in the cohort of patients who underwent cardiac MRI. The mean transmurality before surgery in all groups was greater than 60%; values were not affected by BMC treatment (Table 5-3).

5.3.4 Effect on global left ventricular function

Of the 33 patients who underwent cardiac MRI, the images for four, taken at one or more of the time points, were not suitable for accurate analysis. Comparison of percent volume of the myocardial scar could be accurately measured in only 22 patients. There were no differences between groups in the baseline functions and administration of

BMCs did not affect the percent volume of the myocardial scar, end systolic volume, end diastolic volume, stroke volume or ejection fraction (Table 5-4).

Table 5-3: Effects of BMC administration on scarred myocardium. (Ang *et al*[144])

Summary measures	Number of scar segments assessed	Number of patients	Parameter at baseline and follow-up			P value
			Baseline	6 months after treatment	Mean difference, adjusted for baseline (95% CI)	
Percent systolic fractional thickening at rest:						
Control	42	15	–6.0 ± 4.1	–0.7 ± 7.2	4.3 (0.9 to 7.6)	0.256
IM	45	17	–3.5 ± 5.0	–2.9 ± 6.2	2.0 (–1.3 to 5.2)	
IC	52	17	–5.5 ± 5.3	–4.5 ± 5.8	0.4 (–2.7 to 3.6)	
Percent systolic fractional thickening at low-dose DSE:						
Control	42	15	–4.5 ± 5.2	–1.0 ± 10.7	3.8 (–0.8 to 8.5)	0.920
IM	45	17	–4.7 ± 5.0	–2.3 ± 6.9	2.6 (–1.8 to 7.0)	
IC	48	16	–5.8 ± 5.4	–2.7 ± 9.8	2.7 (–1.8 to 7.3)	
Scar transmurality:						
Control	26	6	68.9 ± 11.3	75.2 ± 10.3	6.4 (–6.5 to 19.4)	0.235
IM	16	6	60.7 ± 20.6	67.1 ± 25.2	5.9 (–7.3 to 19.0)	
IC	31	8	71.0 ± 22.8	56.8 ± 27.6	–14.0 (–25.3 to –2.6)	

Abbreviations: DSE – dobutamine stress echocardiography; IC – intracoronary; IM – intramuscular

Table 5-4: Effect of BMCs assessed by cardiac MRI. (Ang *et al*[144])

Parameter	Number of patients suitable for analysis	Parameter at baseline and follow-up			P value
		Baseline	6 months after treatment	Mean difference, adjusted for baseline (95% CI)	
Percent volume of myocardial scar:					
Control	6	39.0 ± 15.8	41.0 ± 25.9	2.4 (–9.9 to 14.7)	0.713
Intramuscular	8	31.5 ± 18.1	28.2 ± 14.0	–3.5 (–14.0 to 7.1)	
Intracoronary	8	32.3 ± 18.1	33.5 ± 23.4	1.1 (–9.4 to 11.6)	
End systolic volume:					
Control	7	198.6 ± 44.6	216.1 ± 54.7	17.9 (–10.2 to 46.1)	0.095
Intramuscular	10	178.5 ± 46.0	159.5 ± 50.5	–19.3 (–42.6 to 4.1)	
Intracoronary	8	181.6 ± 40.9	191.6 ± 59.9	9.9 (–16.1 to 35.9)	
End diastolic volume:					
Control	7	249.0 ± 41.1	275.4 ± 56.2	27.0 (-6.8 to 60.8)	0.146
Intramuscular	10	237.3 ± 51.5	222.6 ± 54.2	-16.1 (-44.6 to 12.3)	
Intracoronary	8	252.7 ± 47.6	259.7 ± 62.4	8.3 (-23.4 to 40.0)	
Stroke volume:					
Control	7	50.3 ± 18.2	59.3 ± 11.0	3.3 (–6.9 to 13.5)	0.990
Intramuscular	10	58.7 ± 16.8	63.5 ± 15.0	3.9 (–4.3 to 12.1)	
Intracoronary	8	71.1 ± 15.4	68.2 ± 15.3	3.1 (–6.6 to 12.8)	
Ejection fraction:					
Control	7	20.9 ± 8.9	22.3 ± 5.8	0.7 (–3.2 to 4.5)	0.094
Intramuscular	10	25.4 ± 8.1	29.7 ± 9.1	4.3 (1.2 to 7.4)	
Intracoronary	8	28.5 ± 6.5	27.3 ± 7.7	–0.5 (–4.1 to 3.0)	

5.4 DISCUSSION

This randomised, controlled study measured the effects of injecting BMCs into scarred myocardium on the regional contractility of these nonviable areas. In order to avoid the confounding effects of functional improvement in viable myocardium following revascularisation, BMCs were injected directly into myocardial scars and the functional assessments were focused on these scar areas. Because all scars were revascularised, any observed changes in contractility could not be attributed to differences in perfusion.

The results show that BMCs, whether delivered by intramuscular or intracoronary routes, did not improve contractile function in chronically scarred myocardium when compared with revascularisation alone. Moreover, there was no reduction in scar transmurality or volume, or any significant improvement in global left ventricular function.

This study differs from previously published randomised trials performed in patients with myocardial scar (such as established myocardial infarction) where the BMCs were injected into the peri-infarct viable myocardium, but not into nonviable scars. The two randomised studies that injected BMCs enriched for CD34+ or CD133+ cells into the peri-infarct zone concentrated on its impact on global left ventricular function[87, 138]; only that by Hendrikx *et al*[137] looked at the effects of treating these areas with unselected BMCs on regional as well as global function. In that study a modest improvement in regional systolic thickening was observed following BMC treatment. Their cell isolation method was similar in that unselected BMCs were used; however, those investigators cultivated the BMCs overnight before administration. The BMCs were harvested and administered during surgery to minimise the patient's discomfort

caused by aspiration and to avoid the theoretical risk of bacterial contamination from additional manipulation. Although the overnight cultivation step might be a factor for the difference in results, another report[145] suggests that additional processing has little effect on the number, viability and functional capacity of BMCs when compared with freshly harvested BMCs. The regional improvements observed by Hendrikx *et al* might be attributable to recovery of contractile function in the peri-infarct zone rather than the scar itself.

Although the main focus of this study was on the regional function of the scarred myocardium, we did not observe any substantial changes in global left ventricular parameters following BMC treatment. This finding is in agreement with that of Hendrikx *et al*[137] but in contrast to those of other studies of BMCs enriched for CD34+ or CD133+, which reported improved left ventricular function[87, 138]. CD34+ and CD133+ cells are capable of assuming endothelial phenotypes in vitro as well as contributing to neovascularisation and improvement in cardiac function in vivo[12, 39, 146]. This feature might explain the improved global left ventricular function in patients who received BMCs enriched with these cells in the peri-infarct zone[87, 138]. Whether the use of CD34+ or CD133+ enriched cells is beneficial when injected directly into the scar tissue in the clinical setting remains, however, to be seen. Such comparisons across trials should be interpreted with caution, as trial methods differ substantially, namely in the site of injection, the cell types injected and the primary end points of the study.

Whether BMCs can restore myocardial function and, if so, what the mechanisms of action involved are remain subjects of intense debate. This study was not specifically

designed to investigate the mechanism of action of BMCs, but the lack of functional improvement in injected scar segments and no substantial reduction of infarct size indicate an absence of meaningful myocardial regeneration by BMCs. Orlic *et al*[31] reported that BMCs can regenerate up to 60% of the infarcted myocardium in mice; subsequent studies have not, however, replicated this finding[32, 33]. Similarly, there are conflicting data regarding the role of BMCs in neovascularisation[12, 39-41, 43, 146]. Clearly, further studies are required to clarify the role of BMCs in the context of myocardial repair, both in the acute and chronic phase.

Another possibility for the lack of improvement in scar function could be failure of BMCs to engraft in the chronic ischaemic myocardium. Although investigation of the engraftment potential would be difficult in the clinical settings, in a rat model of chronic myocardial ischaemia BMCs grafted well following intramuscular or intracoronary administration[129]. Despite engraftment, however, there was no clear evidence of differentiation. Skeletal myoblasts have also been shown to engraft in the myocardium after transplantation, but the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial showed no functional improvement following administration of these cells into the myocardial scar[147].

This data confirm those from previous studies[72-74, 91, 92, 138] showing the safety of both routes of BMC administration. Neither intramuscular nor intracoronary BMC injections led to myocardial damage, as shown by the lack of substantial elevation in postoperative troponin I levels and the absence of new Q-wave infarction. Furthermore, neither route of administration triggered severe ventricular arrhythmia, which had been a concern of clinical trials of myoblast transplantation in the heart[69].

In conclusion, I have shown that BMCs, administered via intramuscular or intracoronary routes, even though safe, do not restore contractile function in chronically scarred myocardium. This study only targeted chronically scarred non-viable myocardium and therefore, therefore, these findings are not transferable to other clinical settings such as acute myocardial infarction or chronically ischaemic but viable myocardium. Further clinical trials are needed to specifically address the role of BMCs and its mechanisms of action in these settings. Given that this laboratory had previously demonstrated that a potent cardioprotective effect by BMCs in an in-vitro model of human ischaemia[148], the aim of my next study was to investigate whether this beneficial effect can be translated into clinical practice.

CHAPTER 6

RANDOMISED CONTROLLED TRIAL ON THE CARDIOPROTECTIVE EFFECT OF BONE MARROW CELLS IN PATIENTS UNDERGOING CORONARY BYPASS GRAFT SURGERY

6.1 INTRODUCTION

Given that there was little evidence of meaningful myocardial regeneration by BMCs in my previous animal and clinical studies, I proceeded to explore the other potential mechanisms through which BMCs may participate in myocardial repair.

In recent years, there has been increasing evidence that BMCs may have cardioprotective properties. This laboratory had previously demonstrated that BMCs have a potent effect against ischaemic injury of the human myocardium in an in-vitro model of acute ischaemia, by reducing creatinine kinase (CK) release, apoptosis and necrosis[148]. This was in agreement with other studies in animal models whereby BMCs have been shown to decrease the expression of pro-apoptotic proteins and reduce apoptosis[149-151].

As myocardial ischaemic injury during cardiac surgery remains a major cause of post-operative complications and morbidity [152], this presents an area in which the potential cardioprotective effects of BMCs can be harnessed in the clinical setting. Therefore, a randomised controlled trial was conducted to investigate whether the administration of autologous BMCs as an additive to cardioplegia solution during cardiac surgery can provide additional cardioprotection.

6.2 METHODS

6.2.1 Study population

Patients undergoing first-time elective coronary artery bypass grafting (CABG) by a single surgeon, were considered for the study. For inclusion in the study, the patients must have 3 graftable diseased coronary vessels and a left ventricular ejection fraction greater than 40%. The exclusion criteria were: unstable angina, cardiogenic shock (systolic blood pressure<80mmHg, requiring intravenous inotropes or an intra-aortic balloon pump), percutaneous coronary intervention during the preceding 3 months, pre-existing bone marrow conditions, bleeding disorders, hepatic or renal failure, diabetics, chronic inflammatory disease, previous neoplasm, chronic treatment with oral antibiotic agents or the K_{ATP} channel opener, nicorandil.

The study was approved by the local ethics committee and was conducted in accordance with Medicine for Human Use (Clinical Trials) Regulations 2004 and EU Clinical Trials Directive in the UK. The clinical trial is also registered on the International Standard Randomised Control Number Register (ISRCTN 22639386).

6.2.2 Study design

Using computer-generated block randomisation, patients were randomised either to receive BMCs during each dose of cardioplegia (BMCs group) or to be control. Physicians treating the patients as well as the investigators analyzing the results were blinded to the randomisation.

The primary end-point was a reduction in myocardial injury during the first 48 hours after the release of aortic cross-clamp. The secondary end-point was an improvement in cardiac function during the first 24 hours after aortic cross-clamp release. Information was also collected regarding any postoperative complications prior to the patient's discharge.

6.2.3 Operative procedure

All patients underwent CABG under standard anaesthetic protocol, operative techniques and post-operative care. Briefly, patients were given Temazepam 20 mg and ranitidine 150 mg as premedication 2 hours before their scheduled operation. In the induction room, intravenous and intra-arterial accesses were established and the patients were monitored with ECG, pulse oximetry and arterial line pressure tracing. After a period of pre-oxygenation, anaesthesia was then induced with fentanyl 5-10 µg/kg, midazolam 0.05-0.1 mg/kg and rocuronium 1 mg/kg, and maintained with O_2/air mixture and isoflurane to achieve a Bispectral Index System reading of less than 50. Patients were then intubated. A central venous catheter as well as Swan Ganz pulmonary artery flow catheter were also inserted. All operations were performed through a median sternotomy using standard techniques with cardiopulmonary bypass (CPB) under full heparinisation (3-4 mg/kg intravenously), and regular doses of cold blood cardioplegia, constituted using 1 part of blood with 4 part of St. Thomas' cardioplegic solution No.1. A litre of cardioplegia was given during the first dose, followed by a further 500 ml at 15-20 minutes interval, coinciding with the completion of each bypass graft. Bone marrow was aspirated from all patients under general anaesthesia before the start of CABG. In patients assigned to the BMCs group, BMCs were given together with the cardioplegia at the

end of each dose of cardioplegia (see below). After their operation, patients were transferred to the Cardiac Intensive Care Unit for further care, keeping their cardiac filling pressures (central venous pressure between 8 and 12 mmHg and pulmonary capillary wedge pressure between 12 and 16 mmHg with appropriate transfusion), heart rate (between 70 and 90 beats/minute with atrial pacing if required) and systemic vascular resistance index (between 1200 and 1800 units using vasodilators such as GTN and vasoconstrictors as norepinephrine and vasopressin if required) within the physiological range. Patients were extubated and cared for postoperatively as per unit standard routine procedures until discharge.

6.2.4 Bone marrow cell preparation and administration

After anaesthesia but before CABG, 120 ml of bone marrow were aspirated from the patient's iliac crest into preservative-free heparin (10 U/ml) and diluted with normal saline. The BMCs were separated by density centrifugation with Lymphoprep® (AXIS-SHIELD PoC AS, Oslo, Norway), and diluted in 31 ml of cardioplegia solution (30 ml for administration and 1 ml for cell characterisation described below). The viability of BMCs (assessed by trypan blue exclusion) was more than 94%. 10 ml of diluted BMCs was delivered at the end of each cardioplegia infusion antegradely into the coronary circulation via a side-arm of the administration set close to the aortic root.

6.2.5 Bone marrow cell characterisation by flow cytometry

The BMC suspension (1 ml) was further diluted with PBS and re-distributed equally so that each flow cytometry analysis tube contained 2ml of resconstituted BMCs. The

BMCs were pelleted by centrifuge at 1500rpm for 10 minutes and resuspended in 200µl of PBS. For bone marrow cell characterisation, they were then incubated with the following antibodies in a dark room for 30 minutes according to manufacturer's instructions: CD34 FITC (BD Biosciences IgG1 #345801), CD117 PE (BD Biosciences IgG1 #332785), and CD133 PE (Miltenyi IgG1 #130090801). After two washes with 2 ml of PBS, the cells were fixed with 4% paraformaldehyde in preparation for flow cytometry analysis. Flow cytometry was performed using FACScan flow cytometer (BD Biosciences, UK) and analysed offline using freeware WinMDI 2.8 (J Trotter 1993-1998, Purdue University Cytometry Laboratories).

6.2.6 Assessment of myocardial injury

Blood samples were taken before surgery and at 4, 12, 24 and 48 hours after the cross-clamp release for the determination of plasma levels of Troponin I and CK-MB. Troponin I and CK-MB were measured using ADVIA Centaur CP system (Siemens, Deerfield, USA) based on direct chemiluminescence technology. The minimal detection limits of the assay were 0.04 ng/ml and 3.03 ng/ml for Troponin I and CK-MB, respectively.

6.2.7 Assessments of cardiac function

Cardiac index was measured by thermodilution using a Swan-Ganz pulmonary artery flow catheter at different time points: before surgery and 1, 2, 4, 8, 12 and 18-24 hours after aortic cross-clamp release.

6.2.8 Statistics and expression of results

As this was a novel study, we did not know the variability associated with the primary end-point at the start of the study. Therefore, to determine the sample size, we specified that we wish to be able to detect whether the treated group were as much as one standard deviation better off than the control group[143]. Assuming 80% power, an alpha=0.05 and an estimated 20% drop-out rate, 22 subjects per group were required. Continuous variables that were normally distributed were presented as mean ± standard deviation (SD), and differences between 2 groups were compared using independent t-tests. For non-parametric data, the χ^2 test or the approximate non-parametric Mann-Whitney test was used. For the analysis of myocardial injury and cardiac function between groups, area under the curve (AUC) was calculated for Troponin I, CK-MB, and cardiac index over their respectively time points, and compared using the appropriate tests. Analyses were performed using SPSS software version 14 (SPSS Inc, USA).

6.3 RESULTS

6.3.1 Baseline characteristics and operative and postoperative data

A total of 44 patients were recruited for the study. Two patients (one from each group) withdrew from the study before the surgery. A mean of 155 ± 78 x 10^6 BMCs were administered in the treated group, comprising of a mean of 24.9 ± 30.0 x 10^3 CD34/117+ cells, 6.8 ± 9.4 x 10^6 CD45+ cells, 78.4 ± 130.8 x 10^3 CD34+ cells and 20.3 ± 22.3 x 10^3 CD133+ cells. The baseline characteristics and operative date were similar in both study groups (Table 6-1). There was no identifiable complication directly associated with the BMCs administration. One patient in the control group developed post-operative atrial fibrillation requiring medical treatment. In the BMCs group, a patient had low systemic resistance requiring noradrenaline support for 48 hours. Another patient was re-intubated on the first postoperative day and mechanically ventilated for a further 24 hours due to poor gas exchange after initial successful extubation.

Table 6-1: Patients' characteristics and operative data (as published with Lai *et al*[153])

Characteristics	Control	BMCs	P value
Demographics:			
Age (years)	62.2 ± 11.2	64.4 ± 8.8	0.49
Male sex – (%)	76.2	76.2	1.00
Risk Factors:			
Hypertension – (%)	71.4	61.9	0.51
Hyperlipidemia – (%)	61.9	57.1	0.75
Smoking history – (%)	61.9	57.1	0.75
Family history of CAD– (%)	57.1	66.7	0.53
Pre-op medication:			
Aspirin – (%)	85.7	95.2	0.61
Clopidogrel – (%)	33.3	9.5	0.13
Beta-blockers – (%)	57.1	76.2	0.19
ACE inhibitors – (%)	52.4	23.8	0.11
Statins – (%)	81.0	90.5	0.66
Diuretics – (%)	19.0	19.0	1.00
Operative details:			
Bypass time (minutes)	108.5 ± 19.3	108.6 ± 14.5	0.99
Cross-clamp time (minutes)	62.3 ± 12.0	64.3 ± 11.6	0.57

Abbreviations: BMCs – bone marrow cells; CAD- coronary artery disease

6.3.2 Cardiac enzymes

As shown in Figure 6-1A-B, the plasma Troponin I and CK-MB level were equally raised after surgery and followed a similar profile during the first postoperative 48 hours in the BMCs treated and control groups. Analysis of the AUC for the first 48 hours after surgery confirmed that the plasma levels for Troponin I and CK-MB in the BMCs treated group were not significantly different from those seen in the control group (Troponin I: 165.6 ± 153.2 vs 188.2 ± 213.8 µg/L in control and CK-MB 639.6 ± 714.9 vs 567.1 ± 454.2 µg/L in control; p=NS in both instances).

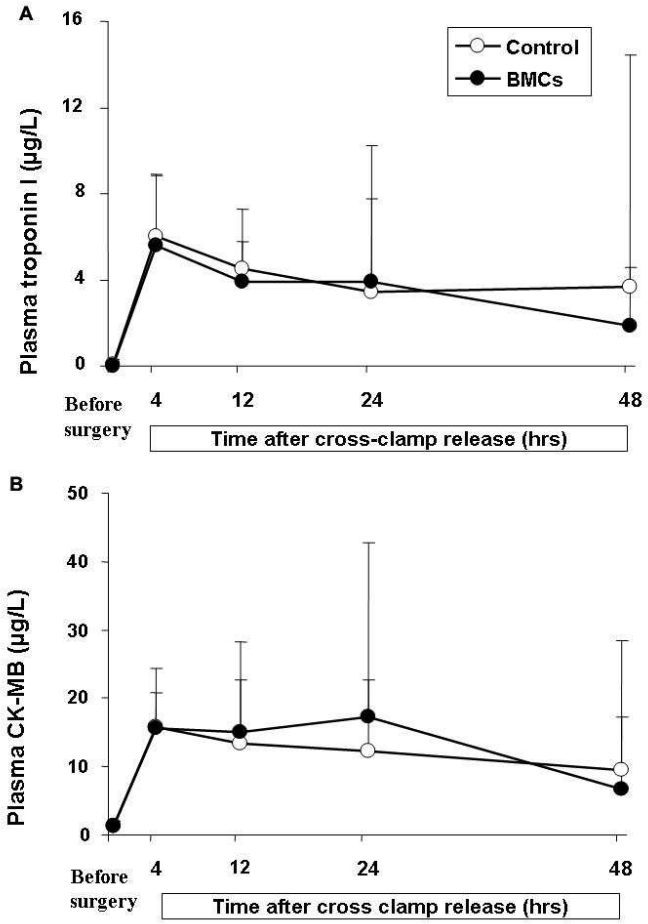


Figure 6-1: Plasma Troponin I (A) and CK-MB (B) in control and BMCs-treated patients before and during the first 48 hours following aortic cross-clamp release (as published with Lai *et al*[153])

6.3.3 Cardiac function

As seen in Table 6-2, the mean cardiac index in the BMCs treated group was similar to those in the control group before surgery and values were almost identical in both study groups in the first 24 hours after surgery.

Table 6-2: Cardiac index (±SD) before and after coronary bypass grafting (as published with Lai *et al*[153])

Groups	Time after cross clamp release (hrs)							P Value
	Baseline	1	2	4	8	12	24	
Control	2.51 ± 0.59	3.63 ± 0.54	3.54 ± 0.68	2.86 ± 0.74	3.26 ± 0.81	3.42 ± 0.73	3.08 ± 0.62	0.47
BMCs	2.31 ± 0.60	3.29 ± 0.86	3.24 ± 0.79	2.94 ± 0.87	3.26 ± 0.63	3.31 ± 0.56	3.11 ± 0.39	

6.4 DISCUSSION

There are still controversies as to whether BMCs contribute to myocardial repair and if so whether is by myocyte regeneration, neovascularisation and other paracrine effects. Having previously demonstrated the cardioprotective effect of BMCs against ischaemic injury in an in-vitro model in this laboratory[148], this is the first randomised study to explore this potential beneficial effects in limiting myocardial ischaemic injury during cardiac surgery. This clinical setup was chosen because the initiation of myocardial ischaemia and the timing of reperfusion are controlled. Furthermore, to reduce study variability, patients with good LV function and those requiring only 3 bypass grafting were selected. Hence, all patients received the same dose of cardioplegia. The bypass and cross-clamp time were also similar in all study patients.

The results have shown that the use of BMCs as an adjunct to the cardioplegic solution does not afford additional cardioprotection, as assessed by the plasma cardiac enzymes and overall cardiac function. This was in contrary to our earlier promising studies using human atrial model of stimulated ischaemia. The explanation for the lack of benefit in this study could be found in the fact that the BMCs were given after CPB was initiated. When atrial tissues taken after CPB were subjected to in-vitro model with stimulated ischaemia, it was subsequently shown that the CK release and cell death by the tissue were significantly lower compared with those from muscles taken before CPB[153]. This suggested that the initiation of CPB itself can induce cardioprotection. These results therefore clearly demonstrated that BMCs were not able to provide additional myocardial protection once cardioprotection is activated by CPB.

It is worth noting that the study was performed under clinical conditions where double blinded randomisation could not be achieved. Furthermore, as the inter-subject variability in cardiac enzymes was large, the power of the study was limited for moderate or smaller treatment effects. In addition, the assessment of end-points only up to 48 hours, and the absence of additional evaluations of left ventricular and regional function may represent limitations of the study.

In conclusion, the use of BMCs as an additive to cardioplegia during CPB did not confer additional cardioprotection above and beyond that of cardioplegia. However, the in-vitro study has demonstrated that BMCs can induce cardioprotection in the absence of stress conditions such as CPB that per se, may have already protected the heart. These results are of clinical relevance and need to be taken into consideration for the planning of future clinical trials, particularly in relation to the timing of BMC administration, to fully benefit from BMCs. It is equally important for the future to identify those patients who will potentially benefit from cell therapy in future trial, especially those at risk of adverse events. Therefore, in the next chapter, I sought to explore the role of novel biomarkers a monitoring tool as well as an investigative test to identify patients at risk of adverse outcome, and in whom cell-based therapies could be potentially beneficial.

CHAPTER 7

NOVEL BIOMARKERS

FOR MONITORING TREATMENT

AND PREDICTING ADVERSE OUTCOME

7.1 INTRODUCTION

One of the challenges facing our clinical studies in cell therapy is selecting the appropriate patients who will benefit most from cell therapy and are at risk of adverse events. In this connection, biochemical markers have emerged in recent years as valuable diagnostic and prognostic tools for heart failure and ischaemic heart disease. Such biomarkers often outperform or complement existing clinical scoring methods. Not surprisingly, some clinical studies on cell therapy had since incorporated biomarkers as part of their study end-points[154-156]. However, before biomarkers can used as routinely as a tool for patient selection or monitoring of patient's disease progression and response to treatment, we need to have a better understanding of its role in different cardiac diseases. An area in which its role is still ill-defined is aortic valve diseases.

Most of the current studies on aortic valve diseases have focused on B-type natriuretic peptide (BNP) or its surrogate marker - the more stable pro-hormone N-terminal fragment, NTproBNP. BNP is secreted predominantly from the left ventricle (LV) in response to increased LV tension and stress and is now an established diagnostic and prognostic marker for LV dysfunction and ischaemic heart disease[157-161]. In aortic stenosis, studies have shown that BNP and NTproBNP levels correlate with the severity and progression of disease[162-165], and potentially can be used to monitor and guide the treatment in asymptomatic aortic valve diseases. Its application as a prognostic marker for mortality after aortic valve replacement (AVR) has also been reported[166]. Recently, several new markers of heart failure and ischaemic heart disease such as ST2 and Growth Differentiation Factor 15 (GDF-15) have emerged. To date, there are no studies on their potential roles in aortic valve disease.

ST2 is a member of interleukin (IL)-1 receptor family that is markedly upregulated during mechanical strain of cardiac myocytes[167]. The ST2 gene encodes 2 isoforms, namely the receptor which together with its natural ligand interleukin-33 (IL-33) reduces cardiac hypertrophic and fibrotic responses to mechanical stress; and also a truncated soluble version (henceforth referred to as ST2) detectable in the serum which conversely may act as a decoy for IL-33, thereby having a detrimental role[167]. Elevated soluble ST2 has been associated with an adverse short term prognosis in ST-elevation myocardial infarction and heart failure[168-173]. GDP-15 is a member of the transforming growth factor-β (TGF-β) cytokine superfamily[174, 175], thought to be involved in the cell survival, proliferation, differentiation, and protection from ischaemic/reperfusion injury[176]. Although their mechanisms and roles are yet to be fully elucidated, both GDF-15 and ST2 have been implicated in coronary artery disease and heart failure and are now recognised as independent predictors of mortality in these groups of patients[168, 170-173, 177-179].

As aortic valve diseases impose strain and stress on the myocardium resulting in heart failure, these changes may be reflected by both markers. For both markers to be used universally for identifying patients at risk for all cardiovascular diseases, their role in this respect needs to be defined in aortic valve conditions to compliment their recognised association as mortality predictors in ischaemic heart diseases and heart failure. In this study, I explored the relationship between the pre-operative clinical features and the plasma levels of both biomarkers before surgery. I also investigated whether the pre-operative plasma levels of ST2 and GDF-15 were associated with

mortality after elective aortic valve replacement, and compared them with NT-proBNP and Logistic EuroSCORE, an established operative risk stratification tool[180-182].

7.2 METHODS

7.2.1 Study population

Between June 2001 and September 2004, patients referred for AVR with or without concurrent coronary artery bypass grafting (CABG) were recruited. Exclusion criteria were previous cardiac surgery, requirement for other procedures other than CABG and history of malignancies at the time of recruitment. The study was approved by the local ethics committee and was conducted in accordance with Declaration of Helsinki. All patients participating in the study gave written informed consent.

7.2.2 Surgical Procedures

The AVRs were performed using standard cardiopulmonary bypass and operative techniques, with warm blood cardioplegia. The aortic valves used included: Carbomedics, Pericarbon, Freedom (from Sorin Group, Italy), Aspire and Elan (from Vascutek, UK). Valve sizing and replacements were performed using the guidelines from the manufacturer. CABG was performed concurrently if the patient had significant coronary artery disease. All patients received standard post-operative care as per our unit protocol. Patients receiving mechanical valves were anti-coagulated. Those receiving tissue valves were given anti-platelet therapy (aspirin 75mg od) after surgery and were only anti-coagulated if they developed prolonged atrial fibrillation (AF).

7.2.3 Pre-operative echocardiogram parameters

Transthoracic echocardiograms were performed prior to surgery. Left ventricular (LV) ejection fraction was calculated by the modified Simpson's biplane method of discs. LV mass (LVM) was calculated by American Society of Echocardiography (ASE) criteria using the equation: $LVM = 0.8x\{1.04[(LVIDd+PWTd+SWTd)^3-(LVIDd)^3]\} + 0.6g$, where LVIDd was the left ventricular internal diameter, PWTd the posterior wall thickness, and SWTd the inter-ventricular septal wall thickness, all measured in end diastole. The aortic valve pathology was qualitatively graded according to ASE guidelines. Aortic valve effective orifice area (EOA) was calculated using the continuity equation: $EOA = [3.14x(LVOTD/2)^2 \times LVOT\ VTI]/(AV\ VTI)$, where LVOTD was the LV outflow tract diameter in mid-systole, LVOT VTI was the LVOT velocity time integral measured with pulsed wave Doppler at the LVOT, and AV VTI was the aortic valve velocity time integral measured with continuous wave Doppler aligned to systolic colour flow across the aortic valve. Body surface area (BSA) was estimated using the formula of Mosteller: $BSA\ (m^2) = [(weight_{kg} \times height_{cm})/3600]^{½}$. The left ventricular mass index (LVMI), was derived by LVMI = LVM/BSA. EOA was similarly indexed to the BSA to give EOA index.

7.2.4 Clinical Data

The occurrence of death was obtained by reviewing the data from the Office of National Statistics Registry and contacting each patient or their general practitioners. Preoperative clinical, echocardiographic and biochemical data as well as their follow-up information were also collected from participating patients.

7.2.5 Plasma samples

Blood samples were taken during patient's pre-operative work-up, and plasma was extracted and stored at -80°C until assayed in a single batch for determination of plasma GDF-15, ST2 and NTproBNP as described below, in a blinded fashion.

7.2.6 Biochemical assays

Commercially available antibodies (R and D systems, Abingdon, Oxfordshire, UK) were used for determination of GDF-15 and ST2 as detailed below, and NT-proBNP was assayed using in-house antibodies. All assays were based on a two-site non-competitive assay format[183].

The assay for NTproBNP has been previously reported[183]. Briefly, sheep antibodies were raised to the N-terminal of human NTproBNP, and monoclonal mouse antibodies were raised to the C-terminal. Samples or NTproBNP standards were incubated in C-terminal IgG-coated wells with the biotinylated N-terminal antibody for 24 hours at 4°C. This was detected with methyl-acridinium ester–labelled streptavidin (MAE-streptavidin) using an MLX plate luminometer (Dynex Technologies Ltd, Worthing, UK)[183].

For the GDF-15[178] and ST2 assays, specific mouse monoclonal antibodies for these peptides were coated onto ELISA plates (200 ng/100 µL). After incubation for 24 hours, all plates were washed and blocked using 10% FCS. Plasma samples were pipetted into the wells (10 and 20 µL per well for the GDF-15 and ST2 respectively), together with appropriate standards, and allowed to incubate for another 24 hours. At the end of this,

plates were washed and biotinylated goat antibodies pipetted into the wells (5 or 10 ng/100 µL for GDF-15 and ST2 respectively). After an additional 2 hours of incubation, plates were washed and developed with MAE-streptavidin as above.

7.2.7 Statistical Analysis

Statistical analyses were performed on SPSS Version 16 (SPSS Inc, USA). Normality of continuous variables was assessed by the Kolmogorov–Smirnov test. Comparisons of normally distributed continuous variables were made using t-tests, while non-normally distributed or ordinal variables were made using the Mann–Whitney U test. Proportions were compared by using the χ^2 test. Spearman's correlations were performed. Multivariate stepwise linear regression was used to identify pre-operative factors that may influence pre-operative biomarker levels. Using death from all causes as an end-point, the predictive value of the pre-operative levels of biomarkers was assessed by Cox proportional hazards univariate and multivariate analysis after log transformation of the biomarker levels. Hazards ratio and 95% confidence intervals (CI) for risk factors and significance level (likelihood ratio test) are given. Receiver Operator Characteristics (ROC) curves were used to determine the optimal discriminatory biomarkers levels for overall mortality. Kaplan–Meier cumulative survival curves were also constructed and compared by the log-rank test for trend. A two-tailed P-value less than 0.05 was deemed to be statistically significant.

7.3 RESULTS

7.3.1 Patient's characteristics

Two hundred and thirty-five patients participated in the study and Table 7-1 summarises their pre-operative and post-operative characteristics. Seventy nine percent of patients suffered primarily from aortic stenosis. 9% had aortic regurgitation and remainder had mixed diseases. The median length of follow-up was 2556 days (IQR 718 days, range 9 – 3207 days). There was a total of 53 deaths, giving an overall mortality during this period of 22.6%. The 30 day and 1 year mortality were 1.7% and 5.1% respectively.

7.3.2 Biomarkers and pre-operative clinical features

NTproBNP was positively correlated with age (r=0.303, p<0.001), history of AF (r=0.176, p=0.007) and LVMI (r=0.383 p<0.001); and negatively correlated with estimated glomerular filtration rate (eGFR, r=-0.309, p<0.001), ejection fraction (r=-0.384, p<0.001) and valve EOA (r=-0.159, p=0.025). Unlike NTproBNP, GDF-15 only had significant correlation with age (r=0.296, p<0.001) and eGFR (r=-0.236, p<0.001); while ST2 is only weakly correlated to eGFR (r=-0.167, p= 0.01). In addition, GDF-15 also demonstrated weak correlation with NTproBNP (r=0.251, p<0.001) and ST2 (r=0.201, p=0.002). On stepwise linear regression, pre-operative NTproBNP was dependent on age, eGFR, pre-operative ejection fraction, LVMI and aortic maximal gradient; while GDF-15 was dependent on and ST2 levels. However, pre-operative levels of both GDF-15 and ST2 were not dependent on any echocardiographic parameters in this group of patients. There were also no significant differences in pre-operative biomarker levels amongst patients with different valve pathology.

Table 7-1: Patient's pre-operative and operative characteristics

Variable	Value
Nos of patients	235
Age, years old, median[IQR]	70 [13]
Male,%	62.55
NHYA III-IV, %	39.15
Pre-op AF, %	15.32
Diabetic, %	10.53
Logistic EuroSCORE, median[IQR]	4.29 [3.81]
Hemoglobulin, g/dL, median[IQR]	14.20 [2.13]
eGFR, mean±SD	67.32±16.26
Ejection fraction, %, median[IQR]	60 [14]
Left ventricular mass index, g/m^2, mean±SD	152.58±50.72
Effective orifice area, cm^2, median[IQR]	0.78 [0.37]
Aortic peak gradient, mmHg, mean±SD	73.98±29.63
Aortic mean gradient, mmHg, mean±SD	43.99±19.07
NTproBNP, pmol/L, median[IQR]	550.70 [1001.41]
GDF-15, µg/L, median[IQR]	1277.57 [1126.85]
ST2, ng/L, median[IQR]	509.58 [291.21]
Mechanical AVR, %	23.40
Concomitant CABG, %	28.09
CPB, minutes, median[IQR]	104.00 [36.00
AXC, minutes, median[IQR]	75.50 [34.00]
Post-op Hospital stay, days, median[IQR]	9 [6]

Abbreviations: IQR – interquartile range; SD – standard deviation; Pre-op – Pre-operative; Postop – Post-operative; CCS – Canadian Cardiac Society; NYHA – New York Heart Association; eGFR – glomerular filtration rate; CABG – coronary artery bypass grafting; CPB – cardiopulmonary bypass; AXC – aortic cross clamp; GDF-15 – Growth differentiation factor 15

7.3.3 GDF-15, ST2 and NTproBNP as predictors of overall mortality

The pre-operative NTproBNP was significantly raised in non-survivors compared with those who were still alive. Similarly, ST2 and GDF-15 were elevated in those non-survivors (Table 7-2).

Table 7-2: Pre-operative biomarker levels in non-survivors vs survivors

Biomarkers	Non-survivor		Survivor		P value
NTproBNP, pmol/L, median[IQR]	925.32	[1703.25]	493.88	[828.82]	<0.001
GDF-15, µg/L, median[IQR]	1703.69	[2377.58]	1213.80	[990.88]	0.001
ST2, ng/L, median[IQR]	592.57	[294.52]	497.16	[283.58]	0.001

Abbreviations: IQR – interquartile range; GDF-15 – Growth differentiation factor 15

On Cox univariate analysis, NTproBNP, GDF-15 and ST2, were significant predictors of death (Table 7-3) along with age, New York Heart Association (NYHA) class, history of AF, eGFR and Logistic EuroSCORE. However, some of the recognised predictors of mortality reported in the literature such as pre-operative aortic gradient, effective valve orifice area, left ventricular ejection fraction and mass index were not significant predictors in this group of patients and neither was concomitant CABG, type of valve used, post-operative patient prosthetic mismatch (defined by EOA index of less than 0.85), operative cardiopulmonary bypass and aortic cross clamp duration. Even after correcting for age, NYHA class, previous history of AF, and eGFR by Cox multivariate model, all three biomarkers remained significant independent predictors of overall mortality (Table 7-4). When their relative predictive values were compared with Logistic EuroSCORE in a separate Cox multivariate regression model, all 3 biomarkers were once again significant independent predictors of mortality after standardisation for their IQR[184]. Interestingly, Logistic EuroSCORE was not a significant predictor of mortality in this model (Table 7-4).

Table 7-3: Cox univariate analysis of mortality predictors

Predictors	Hazard Ratio (HR)	95.0% CI for HR		p value
		Lower	Upper	
Pre-op CCS class	1.18	0.85	1.64	0.319
Pre-op NYHA class 3 or 4	1.72	1.00	2.95	0.048
Pre-op atrial fibrillation	1.92	1.03	3.60	0.041
Gender	0.75	0.44	1.30	0.310
Age	1.07	1.03	1.11	0.000
Endocarditis	0.05	0.00	8708.67	0.625
Logistic EuroSCORE	1.08	1.03	1.14	0.002
Underlying valve pathology	089	0.59	1.37	0.617
Pre-op eGFR	0.97	0.95	0.99	0.001
Pre-op ejection fraction	0.99	0.97	1.02	0.581
Pre-op left ventricular mass index	1.00	0.99	1.00	0.619
Pre-op aortic peak gradient	1.00	0.99	1.01	0.797
Pre-op aortic mean gradient	1.00	0.98	1.01	0.870
Pre-op effective orifice area	0.81	0.47	1.41	0.460
concomitant CABG	1.50	0.85	2.63	0.159
Types of valve	1.08	0.90	1.30	0.423
CPB time	1.00	0.99	1.01	0.653
AXC time	1.00	0.98	1.01	0.642
Patient prosthetic mismatch	1.48	0.70	3.13	0.299
Pre-op Log NTproBNP	2.55	1.50	4.32	0.001
Pre-op Log GDF-15	2.26	1.37	3.72	0.001
Pre-op Log ST2	9.77	2.33	40.93	0.002

Abbreviations: Pre-op – Pre-operative; CCS – Canadian Cardiac Society; NYHA – New York Heart Association; eGFR – glomerular filtration rate; CABG – coronary artery bypass grafting; CPB – cardiopulmonary bypass; AXC – aortic cross clamp; GDF-15 – Growth differentiation factor 15cardiopulmonary bypass; AXC – aortic cross clamp; GDF-15 – Growth differentiation factor 15

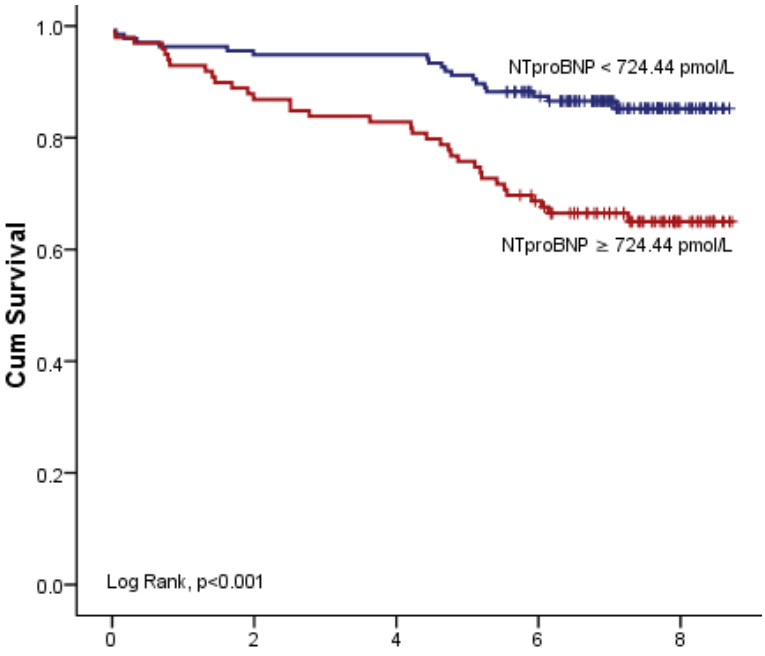
Table 7-4: Cox multivariate analysis of mortality

Predictors	Hazard Ratio (HR)	95.0% CI for HR		p value
		Lower	Upper	
Model - exploring the predictive values of each biomarker, adjusted for significant univariate predictors*				
Log NTproBNP*	1.91	1.08	3.38	0.025
Log GDF-15*	1.86	1.03	3.35	0.040
Log ST2*	8.15	1.82	36.57	0.006
Model – exploring the predictive values between all biomarkers with Logistic EuroSCORE				
Standardised Log NTproBNP†	1.62	1.09	2.42	0.018
Standardised Log GDF-15†	1.31	1.06	1.64	0.014
Standardised Log ST2†	1.54	1.08	2.20	0.017
Logistic EuroSCORE	1.05	0.99	1.11	0.142

* after adjusting for age, New York Heart Association class, history of pre-operative AF, and estimated Glomerular Filtration Rate

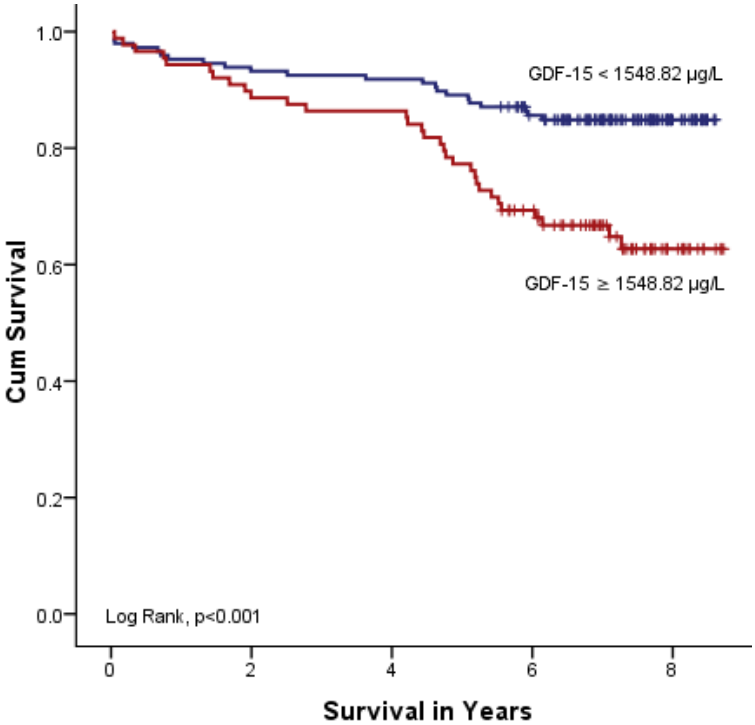
† Standardised by dividing the units of measurements by its interquartile range[184]

Using ROC curves, the optimal cut-off values for NTproBNP, GDF-15 and ST2 to discriminate overall mortality were 724.44 pmol/L, 1548.82 μg/L and 426.58 ng/L respectively. Kaplan-Meier survival analysis revealed a significantly better outcome in patients with NTproBNP, GDF-15 & ST2 below these cut-off values (Figure 7-1, Figure 7-2 & Figure 7-3) and supported the findings obtained in Cox proportional hazard model. Patients with all 3 biomarkers above the cut-off values also had a higher mortality (Hazard Ratio of 7.18, 95% CI of 2.48 – 20.79, $p<0.001$) compared with those with all biomarker levels below the cut-off values (Figure 7-4).



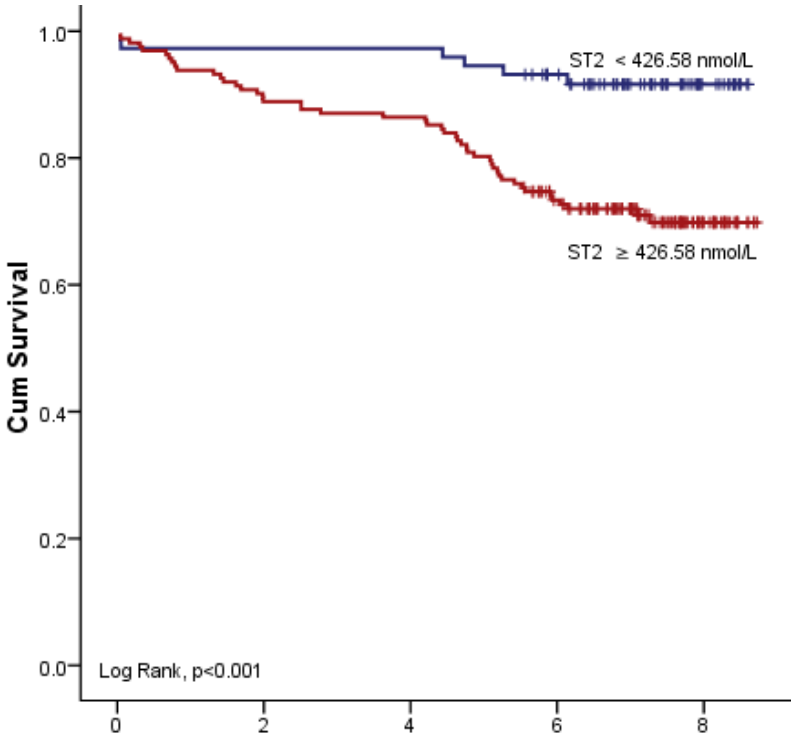
Nos at risk:					
NTproBNP<724.44pmol/L	136	129	129	106	22
NTproBNP≥724.44pmol/L	99	86	82	65	19

Figure 7-1: Kaplan-Meier Survival Curve for NTproBNP



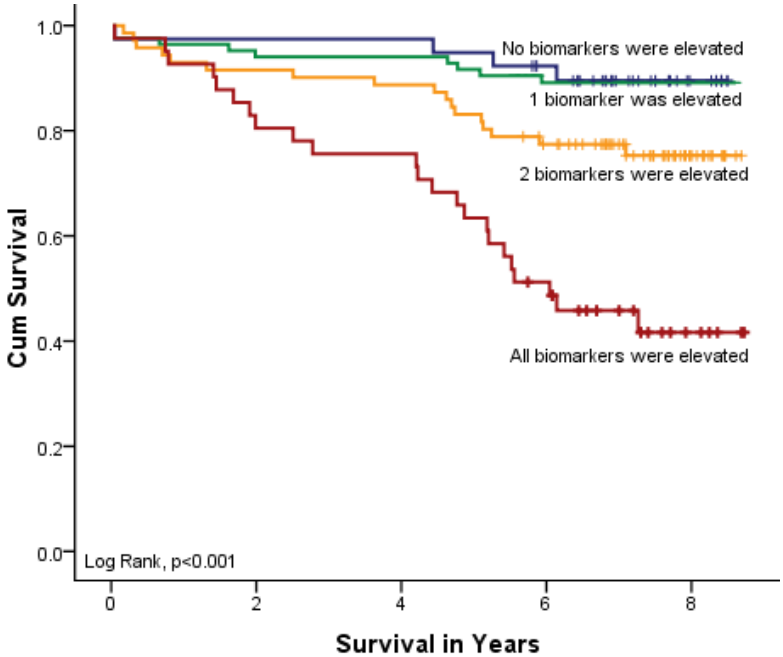
Nos at risk:					
GDF-15<1548.82µg/L	147	137	135	115	28
GDF-15>1548.82µg/L	88	78	76	56	13

Figure 7-2: Kaplan-Meier Survival Curve for GDF-15



Nos at risk:					
ST2<426.58ng/L	73	71	71	62	15
ST2≥426.58ng/L	162	144	140	109	26

Figure 7-3: Kaplan-Meier Survival Curve for ST2



Nos at risk:					
No biomarkers elevated	39	38	38	33	8
1 biomarker elevated	84	79	79	66	13
2 biomarkers elevated	71	65	63	52	15
All biomarkers elevated	41	33	31	20	5

Figure 7-4: Kaplan Meier Curves comparing different combination of biomarkers

7.4 DISCUSSION

This is the first study to investigate the role of GDF-15 and ST2 in a group of elective patients undergoing AVR with or without coronary artery bypass grafting. I have shown that both the pre-operative GDF-15 and ST2 levels, like NTproBNP, were significant independent predictors of overall mortality in these patients in univariate and multivariate analyses. It is worth noting that apart from age, NYHA class, history of AF, and eGFR, the other previously reported predictors of mortality[185] were not significant predictors in our study. Notably, when compared with a widely validated risk stratification tool – the Logistic EuroSCORE[180-182], all three biomarkers were found to be significant independent predictors of overall mortality in the multivariate Cox regression model. However, Logistic EuroSCORE was not a significant mortality predictor in this model. Given that EuroSCORE was initially set up for operative risk evaluation[181] and that it is now recognised that the performance of the EuroSCORE system varies across different surgical subgroups[186-188], this was not a surprising finding. With the growing interest in pre-operative risk stratification in cardiac patients and recognition of the limitations of the current models, there are increasing demands for a separate risk stratification modelling for different surgical procedures[189-192]. To this end, this study supports earlier work on BNP in patients undergoing surgery for aortic stenosis by Pedrazzini et al[166], that there is a role for the use of biomarkers, such as NTproBNP, GDF-15 and ST2 as part of the risk stratification strategy that is complementary to pre-operative clinical parameters.

In line with published work[162-165], the pre-operative NTproBNP levels correlated with patients' age, eGFR, preoperative symptom status, and relevant cardiac parameters such as LV function, LV mass and aortic valve gradients. NTproBNP may therefore be

used as a potential marker for monitoring patient's disease progression in addition to the prognostic information provided.

Although the preoperative GDF-15 levels in our patients shared weak correlations with age and eGFR, GDF-15 was not correlated with preoperative clinical and echocardiographic parameters, in contrast to such correlations that had been demonstrated in patients with heart failure and acute myocardial infarction[178, 179]. A potential explanation for this could lie in the difference in patient's population. There is clearly a need for further studies to define the role and release GDF-15 in aortic valve diseases to explain these discrepancies, and explore their potential role in the monitoring of aortic valve diseases.

Interestingly, ST2 was not related to any cardiac parameter or age. Similar observations in ST2 levels were found in patients from the Clopidogrel as Adjunctive ReperfusIon TherapY–Thrombolysis in Myocardial Infarction 28 (CLARITY-TIMI 28) trial[168], whereby ST2 levels were independent of recognised cardiac clinical factors and NTproBNP levels. This suggests that the pathway for ST2 secretion might differ from that of NTproBNP and other biomarkers. Given that ST2 was a strong independent predictor in our study as well as in the CLARITY-TIMI 28 study despite a lack of association with cardiac clinical factors, it may potentially be useful for identifying patients who are at risk of poor outcomes. It can also be exploited to complement other biomarkers to refine our risk stratification accuracy. While NTproBNP can provide information about the patient's clinical status and progression of aortic valve disease, its application in combination with ST2 and GDF-15, can facilitate the future identification of patients at high clinical risk who may not benefit from aortic valve surgery,

particularly those with all biomarkers above cut-off values. This could a potential group of patients that will benefit from novel therapeutic approaches such as cell therapy in the future to reverse the poor prognosis, and is an area for further research.

This was a single-centre study limited to patients undergoing elective aortic valve procedures, its findings relate only to this group of patients. However, the study is one of the largest series, with the longest follow-up, exploring the role of pre-operative biomarker levels after elective aortic valve procedures reported to date. Although there is no significant difference or association between the underlying valve pathology in this cohort of patients, to fully appreciate the role of these markers in the context of aortic valve diseases, the results need to be replicated in a larger patient population – including those undergoing emergency or redo procedures, as well as those with asymptomatic aortic valve diseases under clinical surveillance based on current clinical recommendations. As the primary aim of the study was to investigate the role of pre-operative biomarker levels in patients undergoing elective aortic valve procedures, we did not investigate the change in biomarker levels after surgery. Further work in this area would be beneficial to fully understand the role of these markers in myocardial remodelling after relief of aortic valve dysfunction. Their applications for risk stratification in aortic valve diseases also need to be validated larger population to ascertain their accuracy and discriminatory abilities over any existing risk model.

In conclusion, pre-operative GDF-15, ST2 and NTproBNP levels are strong independent predictors of long-term mortality after elective aortic valve procedures in this preliminary study. They can be potentially be used in future alone or in combination for the identification of patients at risk of adverse events in aortic valvular

diseases. As there is now a growing interest to use biomarkers in cell therapy studies [154-156], it is hope that this pilot work will provide the foundation for further studies so that their application can eventually be extended for identifying patients who may benefit from cell therapy as well as monitoring their responses to therapy.

CHAPTER 8

CONCLUSIONS

8.1 CONCLUSIONS

Since the first clinical study using cell therapy for myocardial repair was reported in 2001[72], there were great hopes and expectations for its application as a therapeutic option for treating heart diseases. However a decade later, its potential and expectation as a treatment for myocardial repair remains to be fully fulfilled. Skeletal and bone marrow cells were almost the earliest and most widely studied cell types for myocardial cell transplantation. While there were concerns about the increase risk of fatal dysrhythmia and lack of functional integration associated with myoblast transplantation[147], the safety profile of BMC therapy for cardiac diseases is now well established. Evidence for their benefits were not conclusive, with conflicting findings from both pre-clinical and clinical studies. Even if beneficial effects were observed, controversies surround the mechanisms involved (See chapter 1). Methodological differences were thought to be the main cause of most of the discrepancies observed in laboratory and clinical studies. The thesis attempts to address some of these discrepancies.

In chapter 2, I have demonstrated that potential errors can still occur even with the use of transgenic models, and highlight the need of thorough cross-checking when we are investigating the potential and mechanisms involved in cell therapy. Reliance of immunohistochemistry with fluorescent readouts and PCR findings (which were commonly used in other cell therapy studies) was not adequate to establish the true identity of our EDCs. Additional cross-checking with functional studies as well as ultrastructural analyses with electron microscopy was vital in to avoid any mis-interpretations.

While it is important to confirm the presence of GFP with non-fluorescent-based immunohistochemical read-out and exclude the possibility of autofluorescence, the demonstration of GFP within the phagocytic compartment of EDCs in the absence of transgenic activation has another important implication in transgenic model relying on GFP. It highlights that the possibility of GFP uptake in neighbouring phagocytic cells when GFP tagged cells are transplanted. Theoretically, this can happen with any transgenic-derived protein tagging system, and can be a source of potential mis-interpretation and mis-identification which should be borne in mind.

A further source of potential mis-interpretation and mis-identification was also identified in the conventional immunohistochemical techniques frequently used in cell therapy studies for myocardial repair. Myocyte nuclei events related to cell therapy were frequently identified using immunohistochemical labelling of the myocyte cytoplasm or nuclear markers associated with cardiogenesis such as GATA4, to distinguish them from non-myocyte nuclei with the aid of microscopy. This methodology formed one of the scientific basis of many positive studies on the ability of different cell types to regenerate myocardium[31, 121, 122, 193, 194], and has been questioned as a source of mis-identification. In my thesis, I have shown that even with confocal microscopy and further refinement of current techniques using a cell membrane marker or GATA4, its diagnostic accuracy is not adequate for precise myocyte nuclear identification. There is a chance as high as 1 in 3 of labelling a non-myocyte nucleus as one from myocyte origin. This can contribute to significant error when the myocyte nuclei events occurred at a very low rate compared with non-myocyte, such as proliferation. This supports previous observations that the presence of a proliferating supporting cell or a tagged cell can easily be misidentified as a

proliferating myocyte or a transdifferentiation event[34]. While there may be situations whereby transgenic models with myocyte nuclear tagging is not possible, this work provides a reference for determining whether the myocyte nuclear event of interest occurs at a sufficient frequency for it to be identified confidently.

Although there are limitations with transgenic model, it can be effectively utilised as a model for investigating cell therapy upon careful consideration of their strengths and limitations against the objectives of the study as demonstrated in chapter 3 and 4. However, as illustrated in chapter 2, thorough cross-checking is necessary to avoid mis-interpretation of results due to any intrinsic limitations associated with the transgenic model used. For the study of cell differentiation, the reporter gene for the transgenic animal used should ideally be exclusively-expressed only as a result of the transdifferentiation event. In addition, this should also be confirmed with functional assessment. While I have tried to utilise echocardiography in this thesis to assess murine cardiac function after acute myocardial infarction, and cell therapy, it is hoped that several recent developments in the assessment of myocardial function in small animals[133, 134] will help to further refine our current methodologies in the near future.

It is interesting to note similar conflicting results were also obtained in the clinical studies of bone marrow cells therapy (see chapter 1). Differences in trial methodologies were widely quoted as a source for the differences in results, and making cross trials comparison difficult. The other issue was a lot of the earlier trials were initiated before the mechanisms of action of bone marrow cells are fully dissected. Given this scenario, carefully designed negative studies are as valuable as positive studies in our

understanding of the potential mechanisms and role of bone marrow cells or other cell types for myocardial repair. This holds true for pre-clinical studies as well.

In my thesis, I did not find any evidence that BMC contributed to myocyte formation in mouse acute myocardial infarction, or improved myocardial function when injected into myocardial scars in a randomised trial. This suggests that myocyte transdifferentiation is unlikely to contribute to any meaningful regeneration, at least under those circumstances tested. It is still possible that BMC can repair the heart in other situations and through other mechanisms. There are growing evidences suggesting that the BMC can exert beneficial paracrine effects to the heart and is an area which warrants future studies. In this thesis, the cardioprotective effects of BMC was tested in another randomised trial, and showed that it can be influenced by cardiopulmonary bypass. These results further our understanding of BMC, and are useful for the planning and refinements of future laboratory and clinical trials to avoid unnecessary wastage of resources and time in "re-inventing the wheel". The methodological limitations observed during my thesis are also important considerations for all future cell therapy studies for cardiac repair, particularly when there are still many unknowns – such as whether and how cell therapies can truly benefit cardiac function? If so, when it should be given? These are vital questions for further studies, some of which can be addressed using the models from this thesis.

Finally, of importance as well, is who will benefit most? Towards the end of my thesis, I explore the use of novel biomarkers for cardiovascular disease in hope it will pave the way for its further research and application as a tool for identifying patients who are going to benefit from cell therapy as well as for monitoring of treatment.

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APPENDICES

Publications arising from this thesis:

Ang KL, Shenje LT, Srinivasan L, Galinanes M. Repair of the damaged heart by bone marrow cells: from experimental evidence to clinical hope. *Ann Thorac Surg*. 2006;82:1549-58

Shenje LT, Field LJ, Pritchard CA, Guerin CJ, Rubart M, Soonpaa MH, Ang KL, Galinanes M. Lineage tracing of cardiac explant derived cells. *PLoS One*. 2008;3:e1929.

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