BONE MARROW STROMAL CELLS IN THE TISSUE REPAIR PROCESS FOLLOWING MYOCARDIAL INFARCTION

A Thesis Submitted to the Faculty of Graduate Studies

and Research in Partial Fulfillment of the Requirements of the

Degree of Master of Science in Surgical Research

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PREFACE:

The experiments leading to this thesis were all performed in the University Surgical Clinic of the Montreal General Hospital and funded by a grant from Bioheart, Inc. of Weston, Florida.

When the author started her year of academic enrichment in the laboratory, she followed in the footsteps of numerous surgical residents who had preceded her. In previous years, this same laboratory headed by Dr. Ray C-J Chiu had successfully pioneered the current clinical applications of skeletal dynamic cardiomyoplasty, TMR (trans-myocardial revascularization) and more recently, skeletal myoblast transplantation.

Previously, the laboratory had concentrated mainly on cell therapy with skeletal myoblast transplantation, but always looking for new endeavours, switched gears to the new and exciting field of bone marrow stem cell (MSC) transplantation. As is outlined in this thesis, a series of experiments using marrow stromal cells were conducted, and as nature performed its magic, we were able to observe and realize the tremendous physiologic roles of the MSC and its potential clinical applications.

The results from these experiments were presented at the McGill Annual Biomedical Graduate Conference in February 2001. In addition, these results were granted the second prize for best presentation at the McGill Fraser Gurd Research day in May 2001 as well as the Dr. Wilfred Bigelow prize in basic science research at the 2001 Terrence Donnelly Research Day at the University of Toronto.

These results were also presented at the 2001 Scientific Sessions of the AHA held in Anaheim, California in November 2001 and for consideration in the Vivien Thomas Young Investigator Award competition. A full manuscript was submitted for publication in the peer-reviewed journal *Circulation.*

ACKNOWLEDGEMENTS:

I am indebted to the gratitude of my supervisors Dr. Dominique Shum-Tim and especially Dr. Ray C-J Chiu. Their constant supervision and advice both about experimental details and life's lessons was always appreciated.

I have to thank Ms. Salome Piquer for helping me initiate the pilot project last year and for much technical assistance at that time. Dr. Jin-Qiang Kuang assisted in the surgeries and taught me to apply my operative skills to rats with much patience and perseverance. My colleage Abdulaziz Al-Khaldi helped with the Lac Z labelling and to perfect the staining technique. Mrs. Minh Duong diligently helped with the cell culturing and her expertise in countless days of staining is greatly appreciated. A special thanks also goes to Mrs. Irene Sidorenko for making all of this possible.

This has been a wonderful year and an experience second to none. To be able to work in the warm atmosphere of C9 and especially with Dr. Chiu, is an opportunity which I am fortunate to have experienced.

ABSTRACT:

Marrow stromal cells (MSCs) are pluripotent stem cells which may participate in the repair of damaged organs. We tested the hypothesis that MSCs can be recruited to the heart upon myocardial infarction, and play pathophysiological roles in the subsequent healing and adaptation process. An animal model was created with labeled MSCs in its bone marrow and then a myocardial infarction produced. Donor MSCs from isogenic Lewis rats were harvested, multiplied in culture and labelled. Labelled cells were intravenously injected into the recipient rats and one week later upon the engraftment of these labelled MSCs within the bone marrow, rats underwent a coronary artery ligation or sham operation. The hearts were removed at various time points and the presence of labelled cells in the heart was confirmed and their phenotypes identified. We confirmed the presence of labelled cells in the rat bone marrows and the presence of labelled cells in the infarcted myocardium at all time points studied, but not in the normal hearts. There was evidence for myogenic differentiation with some labelled cells expressing smooth muscle/myofibroblast phenotypes and appearing to participate in vasculogenesis. Our evidence is consistent with the hypothesis that myocardial infarction may send a signal to recruit MSC's to the injured heart, where they undergo milieu-dependent differentiation. The ability of these cells to express various phenotypes may allow them to participate in the pathophysiology of post-infarct remodeling and angiogenesis. Therapeutic implantation of MSCs thus may further enhance such effects.

RESUMÉ

Les cellules médullaires mésenchymateuses (CMM) sont des cellules souches pluripotentes qui semblent participer à la réparation d'organes endommagés. Nous avons testé l'hypothèse selon laquelle les CMM peuvent être recrutées par le coeur suite à un infarctus du myocarde et par la suite jouer un rôle physiopatholgique dans les processus de guérison et d'adaptation. Un modèle animal a été conçu, ses CMM situées à la moëlle épinière ont été marquées, puis un infarctus du myocarde a été induit à l'animal. Les CMM données, provenant de rats Lewis isogènes, ont été cultivées, multipliées in vitro puis marquées. Ces cellules ont été injectées de façon intraveineuse dans les rats receveurs. Une semaine après la prise de greffe des CMM dans la moëlle osseuse, les rats ont subi une ligature de l'artère coronaire ou bien une opération fictive. Les coeurs ont été enlevés à différents moments et la présence des CMM marquées a été confirmée et leur phénotypes identifiés. Nous confirmons la présence de CMM marquées au niveau do la moëlle osseuse et également au niveau du myocarde infarci à chaque moment étudié, et leur absence dans le cas des coeurs normaux. Il a indication de différenciation myogène pour certaines cellules marquées, ces dernières exprimant les phénotypes de muscles lisses/myofibroblastes et apparaissant participer au développement du système vasculaire. Notre preuve este consistante avec l'hypothèse que l'infarctus du myocarde envoie possiblement un signal pour recruter des CMM au coeur atteint, où ces cellules subissent une différentiation dépendemment du milieu. L'habileté don't elles font preuve concernant l'expression de différents phénotypes, peut possiblement leur permettre de participer au niveau de la physiopathologie du remodelage post-infarctus ainsi qu'au

niveau de l'angiogénèse. L'implantation thérapeutique des cellules médullaires mesenchymateuses peut possiblement stimuler des effets semblables.

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

Congestive Heart Failure:

Myocardial injury caused by ischemia leads to cardiomyocyte death and is a leading cause of morbidity and mortality.[34] Congestive heart failure (CHF) is a progressive disorder which is often a complication which follows a myocardial infarction as there is a loss of functioning cardiomyocytes and the myocardium is unable to generate force and contract normally. Despite current therapeutic options, CHF remains a major medical problem as the management issues for these patients are complex.[15] While pharmacological therapy of CHF with digitalis, ACE inhibitors, diuretics, beta blockers and inotropic agents has significantly improved outcome and slowed disease progression,[2] end-stage disease still occurs.

There are four main surgical therapies available depending on the cause and the severity of heart failure. These include: conventional corrective surgery (coronary artery bypass surgery), cardiac transplantation, ventricular assist devices or total artificial heart transplantation and dynamic cardiomyoplasty. While surgical revascularization remains the treatment of choice for ischemic myocardium, those with diffuse ischemic disease or end-stage heart failure may not be appropriate candidates for this procedure. Conventional surgery also provides symptomatic relief and longevity to patients, but does not prevent the progression of heart failure or the need for future palliative reoperations.

Allograft transplantation requires lifelong immunosuppression and the only benefits so far are short term.[2] Long term results have been limited by the development of rejection episodes, the sequelae of immunosuppression and transplant

coronary arteriopathy. Xenotransplantation of hearts, which is another option in the chronic organ shortage that exists is still being developed and clinical experience is still anecdotal at present.

Mechanical assist devices and mechanical hearts, while important bridges to transplantation, must overcome ongoing problems with coagulopathy, cost and infection before they can be more widely implemented.[33] Dynamic cardiomyoplasty is suited for those not eligible for heart transplantation and for those who do not respond to alternate therapies, however, the survival advantage has not been shown.[15]

So the available medical therapy has limited efficacy, and surgical therapy is still being developed, so continued investigation is needed for alternative treatment strategies. The ideal therapy should restore normal cardiac function, be free from immunosuppression, infection and bleeding complications, and be inexpensive enough to be used for the large numbers of patients who could benefit. It has only been recently that cellular-based therapeutic options have shown the potential in fulfilling these needs.

Cellular Therapies to Augment Cell Numbers in the Heart:

Cellular therapy attempts to restore myocardial function through the transplantation of healthy, competent cells and thereby increase the biological mass available for systolic contraction.[49, 68] Unlike adult skeletal muscle, which contains a subpopulation of satellite cells for repair, the heart is devoid of such precursor cells.[74] Therefore cardiomyocyte loss which results after injury such as a myocardial infarction is irreversible.[53] Cellular cardiomyoplasty, as it has also been termed, has

been used to transplant immature cells into injured myocardium in the hopes of restoring new functional tissue. [50, 83, 84] Several cell types including embryonic stem cells, satellite cells and marrow stromal stem cells have been shown to generate viable tissue in a variety of chronic injury models.[5, 6, 18, 54, 70, 71, 72] However, the optimal source of these "cells" for such valuable therapies remains to be determined. (Figure 1)

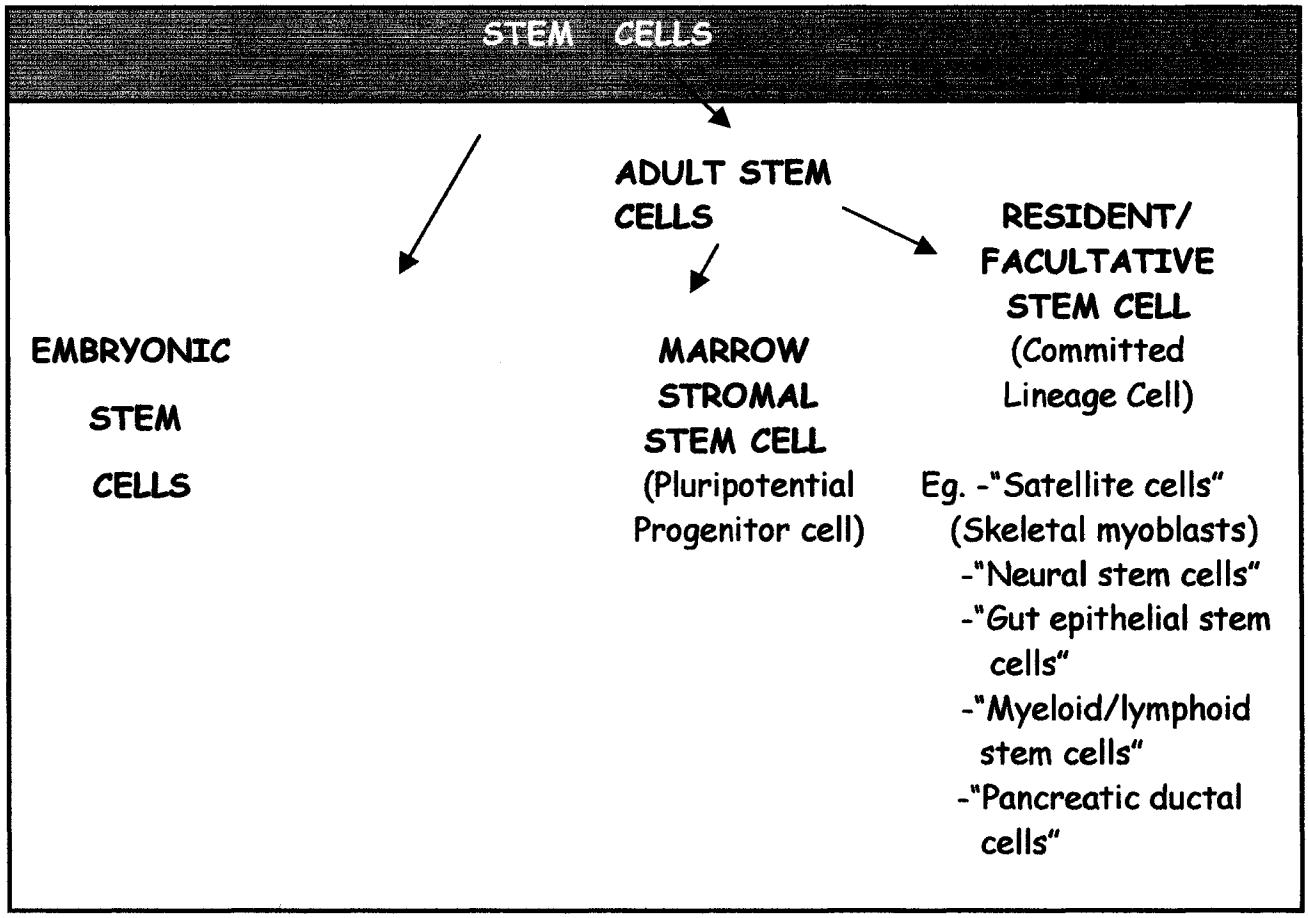

STEM CELLS
ADULT STEM CELLS
RESIDENT/ FACULTATIVE STEM CELL (Committed Lineage Cell)
EMBRYONIC STEM CELLS
MARROW STROMAL STEM CELL (Pluripotential Progenitor cell)
Eg. -"Satellite cells" (Skeletal myoblasts)
-"Neural stem cells"
-"Gut epithelial stem cells"
-"Myeloid/lymphoid stem cells"
-"Pancreatic ductal cells"

Figure 1

A) Embryonal Stem Cells:

Embryonic stem cells are nearly totipotent, reserving the privilege to choose among most, if not all of the differentiation pathways that specify the animal. The successful culturing and propagation of human embryonic stem cells and primordial germ cells was first reported in 1998 when Shamblott and co-workers [76] isolated and cultured the gonadal ridges of 5- to 9- week old aborted human fetuses. However, from the moment these reports appeared, the use of these human stem cells has come under serious ethical scrutiny.

Since then, initial studies in the heart have shown that it is feasible to transplant fetal cardiomyocytes into the myocardial scar.[53] Field and associates [40, 78] reported that fetal cardiomyocytes can be engrafted and integrated within the normal myocardium of mice. A subsequent study by this group reported the formation of stable fetal cardiomyocyte grafts as long as 10 weeks after engraftment in the myocardium of dystrophic mice and dogs.[38] However, they were not able to prove that the implanted embryonic cardiomyocytes proliferated and differentiated, and myocardial function after transplantation of the tissue was not assessed.

Allogenic fetal cardiomyocytes [46, 75] as well as allogeneic cardiomyocytes from embryonic stem cells have been transplanted into myocardial scar. It was shown that normal fetal cardiomyocytes transplanted into the subcutaneous fibrous tissue of adult rat legs formed cardiac tissue, which contracted regularly and spontaneously for as long as three months. The contractile tissue was organized in sarcomeres and linked by junctions [51] and had initiated angiogenesis as seen by capillary ingrowth from the surrounding skeletal muscle into the contractile tissue.

Although this data demonstrated that transplanted fetal cardiomyocytes can form functional cardiac tissue within fibrous tissue, significant hurdles to clinical application remain. It has been shown that despite daily immunosuppression, these fetal cells are still subject to rejection and the potential for infections and neoplastic complications still remains.[46] Fetal cardiomyocytes, however, seem to be highly sensitive to ischemic injury, and may require cardioprotective genes or drugs before they are considered successful therapies in the future.[70] The ethical/moral dilemma of using fetal cardiomyocytes for research will also continue to be an issue and recent research has therefore focused on more autologous sources of cell transplantation.

B) Satellite Cell Transplantation:

Each mature skeletal myofibril contains a few myogenic cells known as satellite cells, which remain in an undifferentiated state near the basal lamina.[37] These cells are activated following skeletal injury, enter the mitotic cycle and later fuse with injured myofibers, thus restoring continuity and function of skeletal muscle.[84] These cells have been isolated from skeletal muscle and transplanted into the myocardial scar without the need for immunosuppression. In addition, compared to cardiac myocytes, skeletal muscle cells as a source of cell therapy are more resistant to ischemia,[45] and when transplanted into hibernating myocardium post-injury, may be more resistant to adverse environmental influences than other cells types. Since the successful transplantation of exogenous cells into the canine heart was reported by Marelli et al[50] in 1992, this area has been one of active

investigation. Canine satellite cells have been successfully autografted in a canine cryoinjury model [18] as well as autologous myogenic cells in rodents.[40, 71]

Whether skeletal muscle cells could appropriately communicate with the underlying myocardium and express cardiac contractile proteins however, remains another controversy. Due to the histological differences between skeletal and cardiac myocytes, the inability to electromechanically couple and generate a cardiac syncitium necessary for contraction might limit the functional use of the satellite cell. Another limitation is obtaining the adequate number of cells required for transplantation. Because the percentage of mammalian skeletal muscle cells decreases with age,[94] a large quantity of muscle is required in order to obtain sufficient numbers of cultured skeletal myoblasts to improve heart function. Satellite cells are also sensitive to tissue culturing techniques, and since they are not transformed cells, their lifespan is limited. After the cell harvesting process, some cells may become damaged and unable to be mitotically active, often being rapidly overgrown in culture by fibroblasts.[73]

So despite the presence of certain characteristics of cardiomyocytes which satellite cells display after implantation into the myocardium, they still remain largely skeletal muscle. Further studies are also needed in order to characterize the in vivo proliferation of satellite cells, as very few investigators have been able to show that myogenic cells affect ventricular compliance following myocardial infarction.[6]

C) Bone Marrow Stromal Cells:

The bone marrow contains an autologous source of cells which have the properties of stem cells, and which are referred to as marrow stromal cells (MSC's). MSC's were previously referred to as mesenchymal stem cells and were known as early as 1968 through the work of Friedenstein and his coworkers.[26, 27, 28] These MSC's derived from the marrow of postnatal animals have been shown to be multipotential, [64, 65, 66, 67, 85, 86] and under specific culture conditions to differentiate into osteocytes [28], chondrocytes [36], adipocytes [26], early astrocyte cells [92] and even myocytes[23]. The differentiation of MSC's into multiple lineages, not just mesenchymal in origin, has been demonstrated in vivo both by implantation directly into tissues and indirectly by systemic infusion. [20] (Figure 2)

As a result, this developmental plasticity demonstrates a previously unsuspected role of MSC's in the development and repair of adult mammalian tissue. Because these cells are relatively easy to aspirate from the marrow of patients and can be multiplied in large numbers in vitro and reinjected without the need for immunosuppression, they offer an attractive option for cell-based therapies. They are also relatively easy to genetically engineer, and offer another vehicle for gene therapy.[32]

Over the past few years, several studies have focused on the plasticity of MSC's and have challenged their ability to select atypical lineages when placed in non-hematopoietic environments. When implanted into the brain of rats, human MSC's did not differentiate into neural cells, but they did use some of their stromal cell characteristics and migrated along neuronal pathways into successive layer of the

brain.[7] Additionally, MSC's from male mice could repopulate bone, cartilage and the lung of lethally irradiated female recipient mice, [59,60] and in similar assays, bone-marrow derived myogenic progenitors populated the regenerating muscle of a wounded recipient animal [23] and the muscle of dystrophin defective mice. [29] Although work with the myocardium has been limited, both Wang [88,89] and Tomita [87] have shown the potential of using MSC's in treating the chronically injured heart. Recent studies [56] have shown the therapeutic advantages of utilizing MSC's immediately after myocardial infarction, thereby potentially preventing or slowing the cascade of events leading to heart failure.

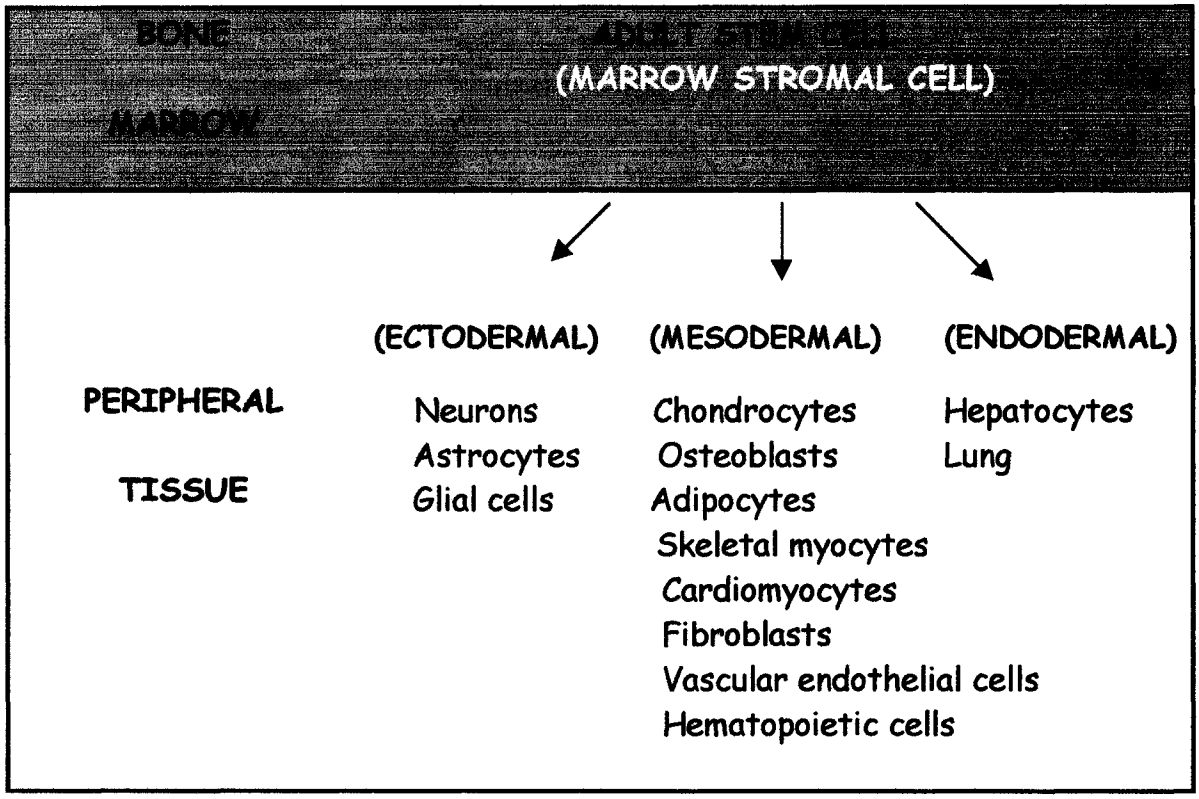

BONE MARROW
(MARROW STROMAL CELL)
(ECTODERMAL)
(MESODERMAL)
(ENDODERMAL)
PERIPHERAL TISSUE
Neurons
Astrocytes
Glial cells
Chondrocytes
Osteoblasts
Adipocytes
Skeletal myocytes
Cardiomyocytes
Fibroblasts
Vascular endothelial cells
Hematopoietic cells
Hepatocytes
Lung

Figure 2

Pathophysiology of Myocardial Infarction (MI)

Early Myocardial Response to Injury:

Cardiac muscle requires approximately 1.3 ml of oxygen/100 grams of muscle tissue/minute in order to remain alive. Normally, 8 ml of oxygen/100 grams of muscle are delivered to the normal resting left ventricle every minute.[30] If even as much as 15-30% of normal resting coronary blood flow is provided, the muscle will not die. [30] Immediately after an acute coronary occlusion, blood flow ceases in the coronary vessels beyond the occlusion, and is supplemented only by small amounts of collateral flow from surrounding vessels. The occlusion results in ischemia throughout the anatomic regions supplied by that artery, most pronounced in the subendocardium.

Acutely ischemic myocardium undergoes progressive biochemical, functional and morphologic changes- the outcome of which largely depends on the severity and duration of flow deprivation. The principal biochemical consequence of myocardial ischemia is the onset of anaerobic glycolysis within seconds, leading to inadequate production of high-energy phosphates. [79] Soon after the onset of infarction, small amounts of collateral blood permeates into the infarcted area, and this with progressive dilatation of local blood vessels causes the area to become filled with stagnant blood. The muscle fibers use the last vestiges of the oxygen in the blood, causing the hemoglobin to become reduced and dark blue in colour. The infarcted area takes on a dark brownish-blue hue and the blood vessels of the area appear to be engorged despite the lack of blood flow.

In the later stages, ultrastructural damage develops with the vessels becoming highly permeable and leaking fluid. The tissue becomes edematous and the cardiac muscle cells and mitochondria begin to swell because of diminished cellular metabolism and glycogen depletion. Within a few hours of almost no blood supply, the cells die. Function becomes abnormal within one minute after the onset of ischemia and myocardial coagulation necrosis occurs after 20-40 minutes of severe ischemia.[30] Multiple, dynamic, structural changes occur after infarction to maintain cardiac output. These abnormalities of ventricular function that follow an MI are not homogeneous and have been characterized by numerous investigators as stunning, hibernation, infarct size, expansion and remodeling and will be outlined in the following.[48]

Remodelling in the Subacute and Chronic Phases Post-infarction.

A) Gross structural changes associated with ventricular remodelling:

Infarct expansion continues as a regional hemodynamic event in these inflammatory and post-inflammatory phases. The overall shape of the ventricle becomes increasingly spherical or globular.[63] Soon after an MI, there is elongation and thinning of the infarcted region over days to weeks. Over time, ventricular hypertrophy develops with further dilatation and thinning and the whole heart becomes more spherically shaped.

Abnormal contours of the left ventricle are an important determinant of patient outcome.[62] In patients who have suffered a myocardial infarction, left ventricular remodelling is associated with higher morbidity and mortality rates.[91]

Lamas et al.[43] also found that after an MI, patients with a spherical-shaped ventricle had lower ejection fractions, decreased exercise tolerance and an increased incidence of heart failure. This data supports the idea that left ventricular remodeling contributes to the development of heart failure.

Remodeling also involves large increases in end-diastolic volume and end-systolic volumes, both having been associated with increased mortality rates.[31] Initially, the wall stress is maintained within normal limits because the hypertrophy and increased ventricular wall thickness serve to offset the increased ventricular dilatation according to the law of LaPlace. However, over time, the increased intraventricular dimension becomes disproportionate to the increase in wall thickness, and wall stress is markedly increased.

B) Cellular changes associated with remodelling:

During the early stages of healing, the infarcted heart is a cell rich tissue, containing a variety of inflammatory cells, fibroblasts and endothelial cells.[79] Although the chemotactic attraction of circulating blood cells, or precursors is crucial to the formation of granulation tissue and subsequent repair, proliferation of resident myocardial cells is also important. The debate as to whether the cardiomyocyte is capable of division after reaching maturity and whether the heart is infact a post-mitotic organ has resurfaced after recent evidence from Beltrami et al. [8] They were able to show that after an MI, proliferating cells were derived from adult cardiomyocytes present within the infarcted region itself. But the origin of the

myocyte as resident cardiomyocytes or from circulating stem cells still remains unanswered.

Recently, focus has been on the non-myocyte cells, which play an equally important role in the pathophysiology and progression of left ventricular remodelling. These cells include the alpha smooth muscle actin myofibroblasts, endothelial cells, smooth muscle cells and immune cells. These cells may act alone or in concert with one another in order to alter the biology of the heart. They are also important sources of cytokines and growth factors necessary to support fibroblast proliferation and neovessel formation.

The majority of proliferating cells in the healing heart were found to be myofibroblasts in a canine myocardial injury model. [24] These nonvascular, spindle-shaped cells are present in human myocardial scars 4-6 days after an infarction [25] and are the predominant source of collagen mRNA in healing myocardial infarcts. Myofibroblasts appear transiently during granulation tissue formation and become apoptotic when the scar matures.

In addition to these well known cellular responses, the previously unsuspected role of MSC's in the development and repair of adult mammalian tissue has enabled the MSC to be considered a new player in the pathophysiology of myocardial infarction. The development of the MSC both into cardiomyocytes [89] and endothelial progenitor cells [3, 4] have important implications in the remodelling and reparative processes following an MI. The following discussions will further define the contributions of this new cellular player.

CHAPTER TWO

METHODS

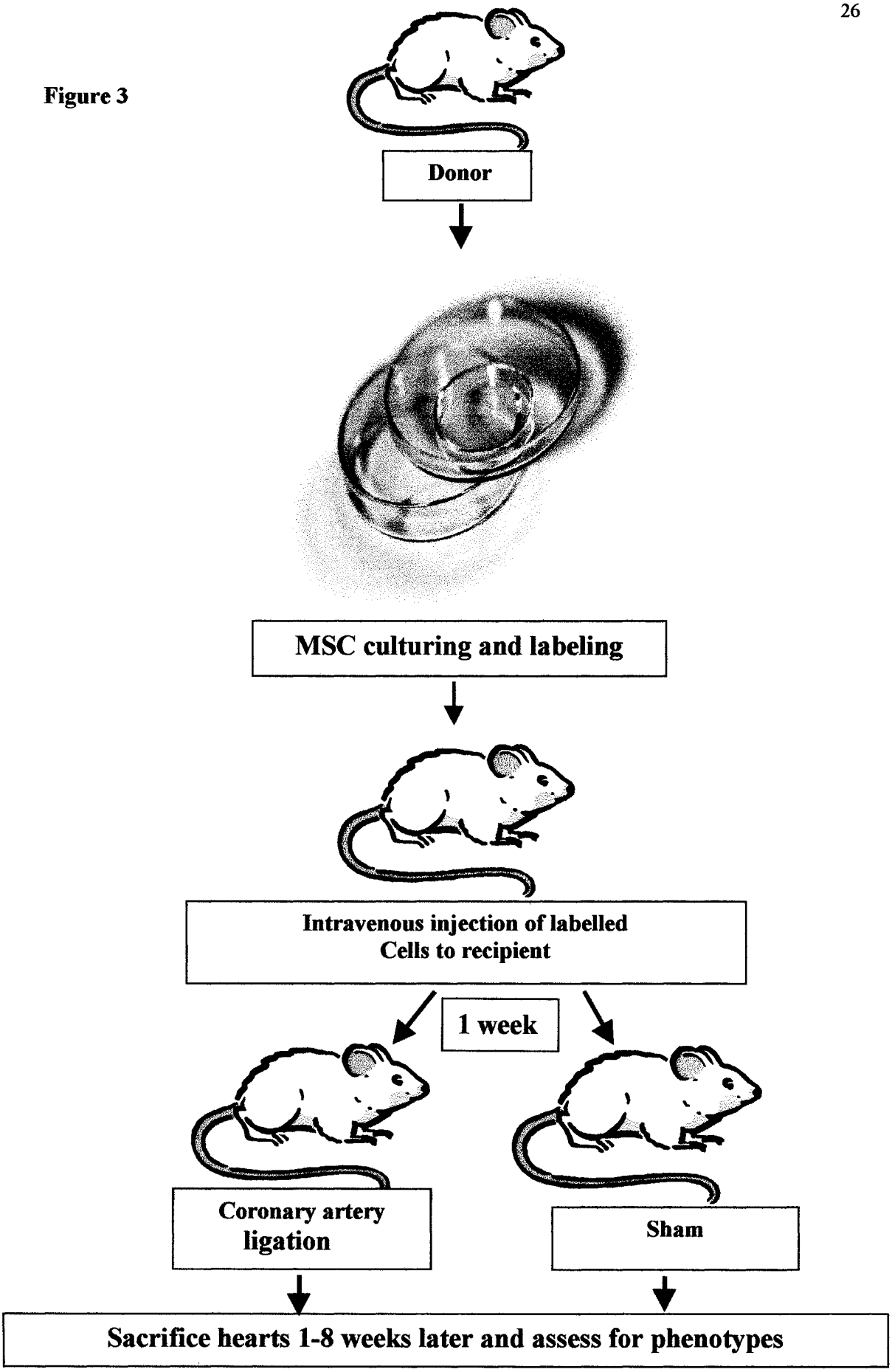
METHODS

Study Rationale and Experimental Design:

Previous studies within our laboratory have shown that MSC's could effectively traffick into the coronary circulation of rat hearts and remain viable after six weeks. [88] The rationale for this study was to confirm through an animal model that MSC's could be recruited from the bone marrow by an ischemic stimulus sent from the injured myocardium. Once present within the heart, they could be influenced by the cardiac microenvironment to develop into phenotypes such as cardiomyocytes, endothelial progenitor cells, smooth muscle cells or myofibroblasts, in order to aid in ventricular remodelling and repair. (Figure 3)

First, in order to confirm that the labelled cells from the bone marrow migrated to the ischemic myocardium, an animal model was created with labelled MSC's within their bone marrows. These animals were then subjected to either coronary artery ligations or sham operations. The presence of the labelled cells was then reconfirmed within the circulation, within the bone marrow and within the hearts at various time points after injection. Once the presence of MSC's was confirmed, their phenotypes were identified with immunohistochemical markers.

Figure 3


Donor
MSC culturing and labeling
Intravenous injection of labelled
Cells to recipient
1 week
Coronary artery
ligation
Sham
Sacrifice hearts 1-8 weeks later and assess for phenotypes

Animals:

A) Pilot Study. In order to confirm the feasibility of MSC injection, 20 male isogenic Lewis rats from Charles River laboratories were initially used as recipients of labelled MSC's. Their bone marrow was harvested at various time points in order to confirm the presence of viable, labelled cells. (Table 1).

B) Coronary Artery Ligation study. 22 male isogenic Lewis rats were used for coronary artery ligation and 8 rats were used for the sham operation. Bone marrow and hearts were harvested at various time points to verify the presence of labelled cells. (Table 2).

Table 1. Individual Rats Used for Confirmation of Labelled CellsTrafficking to Bone Marrow

RAT NUMBER	NUMBER OF CELLS INJECTED ON DAY 1	NUMBER OF CELLS INJECTED ON DAY 2	AMOUNT OF TIME AFTER INJECTIONS WHEN RAT WAS SACRIFICED	PRESENCE OF LABELLED CELLS IN BONE MARROW
1	6×10^6 (3)	6×10^6 (3)	1 week	None. Cells died in incubator due to contamination
2	6×10^6 (3)	6×10^6 (3)	2 hours	Rat died 2 hours after second injection
3	5×10^6 (3)	0	1 week	None. Cells died in incubator due to lack of CO_2 infusion
4	5×10^6 (3)	5×10^6 (3)	1 week	+ (6/6 plates)
5	5×10^6 (3)	5×10^6 (3)	24 hours	+ (6/6 plates)
6	5×10^6 (3)	5×10^6 (3)	1 week	+ (6/6 plates)
7	5×10^6 (3)	5×10^6 (3)	1 week	+ (6/6 plates)
8	5×10^6 (3)	5×10^6 (3)	5 days	+ (6/6 plates)
9	5×10^6 (3)	5×10^6 (3)	3 days	+ (6/6 plates)
10	5×10^6 (3)	5×10^6 (3)	24 hours	+ (6/6 plates)
11	5×10^6 (3)	5×10^6 (3)	3 days	+ (6/6 plates)
12	5×10^6 (3)	5×10^6 (3)	5 days	+ (6/6 plates)
13	5×10^6 (3)	5×10^6 (3)	1 week	+ (6/6 plates)

14	5×10^6 (3)	5×10^6 (3)	2 weeks	+ (6/6 plates)
15	5×10^6 (3)	5×10^6 (3)	2 weeks	+ (6/6 plates)
16	5×10^6 (3)	5×10^6 (3)	3 weeks	+ (6/6 plates)
17	5×10^6 (3)	5×10^6 (3)	3 weeks	+ (6/6 plates)
18	5×10^6 (3)	5×10^6 (3)	4 weeks	+ (6/6 plates)
19	5×10^6 (3)	5×10^6 (3)	6 weeks	+ (6/6 plates)
20	5×10^6 (3)	5×10^6 (3)	6 weeks	+ (6/6 plates)

Legend:

( )- Numbers in parentheses indicate the rating which was given for the quality of penile vein intravenous injections:

3- 100% intraluminal injection of medium.

2- 50% of medium infiltrated, then needle repositioned and remainder of medium injected intraluminally.

1- Complete infiltration of medium initially, and even after repositioning. Likely not intravenous delivery of cells.

Table 2. Individual Rats Used for Coronary Artery Ligation Study

RAT NUMBER	NUMBER OF CELLS INJECTED ON DAY 1	NUMBER OF CELLS INJECTED ON DAY 2	OR	TIME SACRIFICED AFTER OPERATION	PRESENCE OF LABELLED CELLS IN BONE MARROW	PRESENCE OF LABELLED CELLS IN HEART
A1	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+
A2	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+
A3	5×10^6 (3)	5×10^6 (3)	Coronary ligation	3 weeks	+ (6/6 plates)	+
A4	5×10^6 (2)	5×10^6 (2)	Coronary ligation	3 weeks	+ (6/6 plates)	+
A5	5×10^6 (2)	5×10^6 (3)	Coronary ligation	2 weeks	+ (6/6 plates)	+
A6	5×10^6 (3)	5×10^6 (3)	Coronary ligation	1 week	+ (6/6 plates)	+
A7	5×10^6 (3)	5×10^6 (3)	Coronary ligation	1 week	+ (6/6 plates)	+
A8	5×10^6 (3)	5×10^6 (2)	Coronary ligation	4 weeks	+ (6/6 plates)	+
A9	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+(DAPI)
A10	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+(DAPI)
A11	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+
A12	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+
A13	5×10^6 (3)	5×10^6 (3)	Coronary ligation	4 weeks	+ (6/6 plates)	+
A14	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+(DAPI)
A15	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+(DAPI)
A16	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+(DAPI)
A17	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+
A18	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+

A19	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+
A20	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+(GFP)
A21	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+(GFP)
A22	5×10^6 (3)	5×10^6 (3)	Coronary ligation	6 weeks	+ (6/6 plates)	+(GFP)
B1	5×10^6 (3)	5×10^6 (3)	Sham	1 week	+ (6/6 plates)	-
B2	5×10^6 (3)	5×10^6 (3)	Sham	2 weeks	+ (6/6 plates)	-
B3	5×10^6 (3)	5×10^6 (3)	Sham	3 weeks	+ (6/6 plates)	-
B4	5×10^6 (3)	5×10^6 (3)	Sham	4 weeks	+ (6/6 plates)	-
B5	5×10^6 (3)	5×10^6 (3)	Sham	4 weeks	+ (6/6 plates)	-
B6	5×10^6 (3)	5×10^6 (3)	Sham	6 weeks	+ (6/6 plates)	-
B7	5×10^6 (3)	5×10^6 (3)	Sham	6 weeks	+ (6/6 plates)	-
B8	5×10^6 (3)	5×10^6 (3)	Sham	4 weeks	+ (6/6 plates)	-(DAPI)

Legend:

( )- Numbers in parentheses indicate the rating which was given for the quality of penile vein intravenous injections:

3- 100% intraluminal injection of medium.

2- 50% of medium infiltrated, then needle repositioned and remainder of medium injected intraluminally.

1- Complete infiltration of medium initially, and even after repositioning. Likely no intravenous delivery of cells.

Isolation and Culture of Marrow-Derived Stromal Cells:

Isolation and primary culture of MSC's from the donor rats was performed according to Caplan's method.[16] After an overdose of pentobarbital (100 mg/kg) given intraperitoneally, the femoral and tibial bones were collected and both ends of the long bones were cut away from the diaphyses. The bone marrow plugs were hydrostatically expelled from the bones using complete medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing selected lots of 10% calf serum and antibiotics (100U/ml penicillin G, 100ug/mg streptomycin, 0.25 ug amphotericin B, all obtained from Gibco laboratories) in a humidified atmosphere of 5 % CO_2. The marrow plugs were disaggregated and the dispersed cells were centrifuged and resuspended twice in complete medium. These cells in 10 ml of complete medium were then introduced into tissue culture dishes. Medium was replaced every 3 days and the non-adherent cells discarded. Each primary culture was passaged twice to 3 new plates and the cell density of the colonies grown to approximately 90% confluence. After these twice passaged cells were nearly confluent, they were labelled in the following three methods: Lac Z, DAPI and Green Fluorescent Protein (GFP).

MSC Labelling

A) Lac Z:

In order to determine the presence of MSC's within the rat's circulation and to identify any phenotypical changes they underwent, three labelling techniques were used. They included Lac Z, DAPI and GFP (green fluorescent protein). Lac Z GP+AM12 amphotropic retrovirus producer cells were obtained from Dr. Jacques Galipeau's Laboratory (McGill University, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada). These cells produce a replication defective retrovirus containing the reporter (Lac Z) gene that encodes for the bacterial ß-galactosidase enzyme. These cells were cultured in DMEM with 10% Fetal Bovine Serum (FBS) and antibiotics (50 U/ml Penicillin G and 50ug/ml Streptomycin from Wisent Inc.) The cells were allowed to proliferate until at least a 70% confluence was achieved before using them for transduction in order to achieve a high retrovirus titer. Twenty-four hours before transduction, the MSC's were trypsinized with 0.05 % Trypsin + 0.53 mM EDTA (Gibco Laboratories) and replated. The following day, these cells were transduced with LacZ retroviral particles twice per day for three consecutive days with lipofectamine (3uL of lipofectamine 2 mg/ml solution for each 1 ml of virus medium). At each transduction, the MSC medium was replaced with the supernatant from the Lac Z GP+AM12 cells , after being filtered through a 0.45 um filter. Seventy-two hours after the last transduction, MSC's were trypsinized and part of the cells were plated in a 35 mm dish for histochemical staining for ß-galactosidase activity, in order to determine the percentage of cells expressing ß-galactosidase. The medium was aspirated from the plates and the cells rinsed with phosphate buffered

saline (PBS). The cells were fixed at 4° C in fix solution (2% formaldehyde and 0.2 % glutaraldehyde in PBS) for 15 minutes and re-rinsed with PBS. Staining for ß-gal activity was performed with a solution containing 1mg/ml 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-gal), 2 % dimethylsulfoxide, 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6.3H_2O$ and 2 mM Magnesium chloride. This solution and all other solutions containing X gal were titrated to a pH above 8.5 prior to staining. The cells were then incubated at 37 °C and protected from light for 16 hours. The presence of blue-labelled cells was then confirmed under phase microscopy.

B) ***MSC Labelling with DAPI:***

The medium was removed from the plates containing the cultured MSC's and each plate was rinsed with Hank's Basic Salt Solution (HBSS). The HBSS was removed and DAPI (4' 6-diamidino-2-phenylindole) solution (50ug/ml in DMEM) was added to each plate and incubated for one hour in a 37^0 C incubator humidified with 5% CO_2. The DAPI solution was then removed and the plate rinsed several times with HBSS. The attached cells were trypsinized with 0.25% trypsin for 3-4 minutes in a 37^0C incubator. 2 ml of 10% DMEM was added to stop the reaction and the cells collected in a 50 ml sterile tube. The cells were centrifuged at 2700 rpm for 5 minutes and the supernatant removed. The pellet of cells was then resuspended in 1 ml DMEM and the cells counted and prepared for injection. Following the DAPI reaction and prior to trypsinization, a subset of plates were also rinsed with HBSS and viewed under a fluorescent microscope in order to confirm the labelling efficiency. The labelling efficiency for DAPI was nearly 100% using this method.

A) MSC Labelling with Green Fluorescent Protein (GFP):

MSC's were grown to 90% confluence and plates selected for labelling. The cells were counted in each plate in order to determine the labelling efficiency. AdenoGFP was purchased from Quantum Biotechnologies (Montreal, Quebec, Canada) for labelling. The concentration of stock solution was 1 x 10^9 plaque-forming units (PFU)/ml. 10 ul of stock solution was added to each plate for 24 hours in a 37^0C incubator with 5% humidified CO_2. The following day, the supernatant was removed and the plates viewed under a fluorescent microscope in order to determine the labelling efficiency. AdenoGFP achieved outstanding nuclear labelling efficiency with almost 100% of cultured cells positively labelled with this technique.

Preparation of Cells for Injection:

Cells which were labelled with Lac Z, DAPI or GFP were cultured in tissue dishes in preparation for injection. The medium was aspirated and the cells in each dish were washed with 6 ml of HBSS. The HBSS was aspirated and 2 ml of trypsin-EDTA was added to detach the cells from the bottom of the dish. The detached cell suspension was then placed in a flask with 2 ml of complete medium and placed in a hemocytometer for counting. A volume consisting of 5 x 10^6 cells was then collected and centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 0.5 cc of complete medium. The cell suspension was then preserved on ice in preparation for injection.

Creation of an Animal Model with Labelled MSC's in the Bone Marrow:

Recipient rats underwent intravenous injection of labeled MSC's. The recipient Lewis rat was placed in a glass canister and 3 % isoflurane at 1.5 L/min was introduced until anesthesia was achieved. The rat was then removed and placed in a restraining device and intubated with a 16-gauge intravenous catheter and ventilated at a tidal volume of 2.5 ml and a respiratory rate of 85 breaths/min. The skin covering the penis was retracted until the dorsal penile vein was visualized. 5 million labelled cells suspended in 0.5 cc of DMEM were injected into the penile vein with a 28.5 gauge needle. Once hemostasis was achieved, the skin was replaced over the penis and the animal extubated when spontaneous respirations resumed. 5 million labelled cells were re-injected in the same manner 24 hours later. Successful engraftment of these labelled MSC's within the recipient bone marrow was confirmed in 10 rats by sacrificing them at various time points ranging from 2 hours to 6 weeks after the procedures. Their bone marrows were harvested, plated and cultured as described earlier. When the cells were approximately 50-60% confluent, they were stained with X-gal for B-galactosidase activity, or the plates viewed under a fluorescent microscope in order to confirm the presence of DAPI or GFP.

Upon establishment of this animal model, a series of 30 rats were again implanted with labelled MSC's, as described above (21 with Lac Z labelled MSC's, 3 with GFP labelled MSC's, and 6 with DAPI labelled MSC's). One week later, they were divided into the coronary artery ligation group (myocardial infarction group) and the sham operated group (control group).

Operative Procedures:

30 recipient rats underwent either a coronary artery ligation (22/30) or a sham (8/30) operation. The rats were anesthetized with isoflurane (MTP Pharmaceuticals). Rats were intubated and ventilated at 85 breaths/min with a Harvard Rodent Ventilator. In the coronary artery ligation group, the heart was exposed via a left thoracotomy incision and the left coronary artery was identified and ligated proximally using a 7-0 polypropylene suture. Regional myocardial ischemia was confirmed by the occurrence of blanching of the ventricular myocardium due to tissue ischemia and by akinesia in the area supplied by the left coronary artery. The wound was then closed with 3-0 vicryl sutures for both the muscle and skin and the animal extubated. The remaining rats in the sham operated group were anesthetized and ventilated in a similar manner, and underwent a left thoracotomy with no myocardial damage. The muscle and skin layers were then closed with 3-0 vicryl sutures and the animal extubated.

Histology and Immunohistochemical Staining:

A) Beta-galactosidase Activity:

The rats in both experimental and control groups were sacrificed at various time points ranging from one to six weeks after either operation. The animals were overdosed with 2 ml Somnotol (120mg/kg) and both the hearts and bone marrow were harvested. The bone marrow was harvested and stained for ß-galactosidase activity as previously described, and the hearts were harvested and perfused with PBS to remove any blood clots. They were then perfusion fixed in 2% paraformaldehyde in PBS and the whole mount hearts stained for ß-galactosidase activity in the following manner:

The hearts were added to a solution containing 1mg/ml 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-gal), 2% dimethylsulfoxide, 20 mM $K_3FE(CN)_6$, 20 mM $K_4Fe(CN)_6.3H_2O$ and 2 mM magnesium chloride, 0.02 % Nonidet P40 and 0.01% deoxycholate. The hearts were also perfused with this solution several times prior to incubation. The specimens were incubated in a 37^0 C incubator with a humidified atmosphere of 5% CO_2 for 6 hours. The hearts were then fixed in 2% paraformaldehyde overnight. After fixation, the lateral wall of the left ventricle containing the scarred myocardium was separated from the surrounding normal myocardium and both segments embedded in paraffin. Ribbon sections of tissue were cut on microtomes, and the sections then placed in a 20% ethyl alcohol bath and transferred to a 45^0 C water bath. Sections were separated and placed on gelatin-coated slides, and slides were then placed on a warming plate and dried. The coronal sections were then used for various different stains.

B) Hematoxylin and Eosin:

A series of sections from each heart specimen were stained with hematoxylin and eosin in order to show nuclei, cytoplasm and connective tissue. The slides were dewaxed and then stained in the following method:

Hematoxylin and Eosin Staining Procedure:

1.	Xylene	2 minutes
2.	Xylene	2 minutes
3.	100% alcohol	2 minutes
4.	100% alcohol	2 minutes
5.	95 % alcohol	2 minutes
6.	80% alcohol	2 minutes
7.	70% alcohol	2 minutes
8.	Tap water wash	2 minutes
9.	Distilled water	6 dips
10.	Harris' Hematoxylin	Up to 5 minutes if necessary*
11.	Tap water	2 minutes or until clear
12.	Acid alcohol (70% alcohol 1000cc + hydrochloric acid, concentrated 10 cc)	3 dips
13.	Tap water	2 minutes
14.	Ammonia water (2 ml NH_4OH + 1 L of distilled water)	7 dips
15.	Tap water	2 minutes

16. Eosin 1 dip
17. 100% alcohol 2 dips
18. 100% alcohol 2 dips
19. 95% alcohol 2 dips
20. Xylene 2 minutes
21. Xylene 2 minutes
22. Slides were then dried and coverslips applied with Permount mounting media. (Fischer Scientific)

* Slides may be dipped in Hematoxylin for 1-2 minutes at a time, then rinsed under tap water and viewed under a light microscope for adequate staining.

C) Picrosirius Red:

Another series of sections were stained with Picrosirius Red to show the presence of connective and fibrous tissue in the following manner:

Picrosirius Red Stain:

Saturated aqueous picric acid was made by heating 2-3 heaping teaspoons picric acid in 500 ml of distilled water, then cooled. A magnetic stirrer/hot plate was used. If yellow needle-like crystals were not present when cool, then the temperature was not high enough, and more acid was added. 0.1 gram of Sirius red F3BA was added per 100ml, the solution was allowed to stand for 24 hours and 250 ml was used for proper staining procedure.

Staining Technique:

Paraffin embedded, 5 or 10 um thick sections were stained as follows:

1. Xylene	3 minutes
2. Xylene	3 minutes
3. Xylene	3 minutes
4. 100% Ethanol	3 minutes
5. 95% Ethanol	3 minutes
6. 70% Ethanol	3 minutes
7. Tap water rinse	10 minutes
8. Picrosirius stain	90 minutes
9. 70% Ethanol	3 minutes
10. 95 % Ethanol	3 minutes
11. 100% Ethanol	3 minutes
12. 100% Ethanol	3 minutes
13. Xylene	3 minutes
14. Xylene	3 minutes
15. Xylene	3 minutes

16. Slides were then dried and coverslips mounted using Permount mounting medium.

D) Immunostaining:

Immunostaining for gap junctions with anti-connexin 43 , sarcomeric myosin heavy chain molecules with MF20, troponin I-c and anti-alpha smooth muscle actin was performed in the following manner:

I. Dewaxing Paraffin Slides:

1. Incubate slides in Xylene	5 minutes
2. Incubate slides in Xylene	5 minutes
3. 100 % ethanol twice	10 minutes each
4. 95% ethanol twice	10 minutes each
5. Wash in deionized water	5 minutes

II. Heat-induced Epitope Retrieval (for Connexin 43 only):

1. After slides were deparaffinized as described above, they were washed with distilled water 3 times for 2 minutes each time.

2. Slides were put in a slide rack and placed in a 1 L glass beaker containing 500 ml of 0.01 M citrate buffer.

3. The beaker was placed on a hot plate and the solution heated until it boiled for 10 minutes.

4. After heating, the beaker was removed from the hot plate and allowed to cool down for at least 10-20 minutes at room temperature.

5. Slides were rinsed with PBS and the immunostaining protocol started.

III. Immunoperoxidase Staining Procedure:

All steps were carried out at room temperature in a humidified chamber.

A.Blocking Endogenous Peroxidase Activity:

1. Slides were incubated for 10 minutes in 0.3 % hydrogen peroxide diluted in methanol at room temperature to quench endogenous peroxidase activity. (Methanol/Peroxide: 1.0 ml 30% H_2O_2 with 99 ml methanol).
2. Slides were washed in PBS twice for 5 minutes each time.
3. A tissue adhesion pen was used to encircle all specimens on the slide.

B. Blocking Non-Specific Antigenicity:

Sections were incubated for one hour with 1.5% blocking serum in PBS. Excess blocking serum was blotted from all slides.

C. Primary Antibody Application:

Sections were incubated with primary antibody overnight in a humidified chamber at 4^0c. Optimal antibody concentrations were determined by titration, diluted in 1.5% blocking serum in PBS. Optimal antibody concentrations for all antibodies used were 1 ug/ml. Antibodies used included MF20 (Developmental Studies Hybridoma Bank) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences); Connexin 43 (Zymed laboratories Inc, San Francisco, California); troponin I-c (Santa Cruz Biotechnology Inc.); and anti-alpha smooth muscle actin (Sigma laboratories).

D. Secondary Antibody Application:

1. Slides were washed with PBS 3 times, for 5 minutes each time after the application of primary antibody. Sections were incubated for one hour with 10 ul of biotinylated secondary antibody at a concentration of 1 ug/ml. (Secondary antibody-biotinylated alpha-rabbit IgG made in goat by Vector laboratories).

Note: When DAPI or GFP was used as the labelling technique, fluorescent conjugated secondary antibodies conjugated to fluorescein for anti-connexin 43 and conjugated to Texas Red for MF20 were used. All previous steps were carried out in a dark room and 5 ug/ml of AlexaFluor (568) goat anti-Rabbit IgG (H+L) conjugate for C43 or 5ug/ml AlexaFluor (488) goat anti-Rabbit IgG (H+L) conjugate for MF20 was added. The sections were incubated for one hour and rinsed with 3 changes of PBS for 5 minutes each. Sections were then viewed under a fluorescence microscope with the appropriate filters, or combined DAPI and Texas Red, GFP and Texas Red or DAPI and fluorescein images were made using simultaneous excitation filters under the reflected light fluorescence microscope.

2. Sections were washed with 3 changes of PBS for 5 minutes each time.

E. ABC Horseradish Peroxidase Application (Peroxidase Vectastain Kit, Vector):

1. ABC solution was mixed and allowed to stand for 15 minutes prior to use. ABC solution consisted of 1 ul avidin (solution A) in 998 ul of 10% lamb serum/PBS with 1 ul botinylated horseradish peroxidase (solution B).
2. Slides were washed 3 times with PBS.
3. Sections were incubated with 100 ul of (1:1000) ABC solution at room temperature for 60 minutes.
4. Slides were washed with 3 changes of PBS for 5 minutes each time.

F. Chromagen Visualization:

Diaminobenzidine tetrahydrochloride (DAB) solution.

1. DAB solution consisted of 100 ml 0.1 M Tris, pH 7.6; 11.1 mM D-glucose (BDH Inc.); 7.5 mM NH_4Cl (Caledon); 60 units glucose oxidase (added just before using); and 1.16 mM DAB (Sigma).
2. Solution was filtered prior to using.

Slides were incubated in solution at room temperature for 5-10 minutes.

AEC Substrate Kit for Peroxidase

Immediately before use, the substrate solution was prepared as follows:

1. To 5.0 ml of distilled water, 2 drops of Buffer Stock solution was added and mixed well.
2. 3 drops of AEC stock solution were added and mixed well.

3. 2 drops of hydrogen peroxide solution were added and mixed well.
4. Tissue sections were incubated with the substrate at room temperature until suitable staining developed. Development times varied based on the tissue thickness and were determined by incubating sections 2 minutes at a time, stopping the reaction in distilled water and then viewing the intensity of the colored reaction product under light microscopy.

G. Counterstaining after Immunostaining:

Counterstaining after immunostaining was performed with Hematoxylin alone in order to show the morphology of the labelled cells in relation to surrounding myocardium. The procedure is as follows:

1. Distilled water rinse	6 dips
2. Harris' hematoxylin	Up to 5 minutes
3. Tap water	2 minutes or until clear
4. Acid alcohol	Dips
5. Tap water	2 minutes
6. Ammonia water	7 dips
7. Tap water	2 minutes

8. Slides were dried and coverslipped with Permount mounting medium.

Cells derived from X-gal labelled MSC's were identified by their blue nuclei with an Olympus light microscope (BX-FLA, Olympus). Similarly, the green nuclei and blue nuclei of GFP labelled DAPI labelled MSC's respectively were viewed with the appropriate fluorescent filters on the fluorescent microscope (BX-FLA, Olympus).

Digital images were transferred to a computer equipped with Image Pro software (Media Cybernetics, MA) and subsequently printed.

CHAPTER THREE

RESULTS

RESULTS

Cell Culturing and Passaging:

The MSC's in culture were assessed under phase contrast microscopy for proliferation and morphological change at each culture medium change. (Figure 4) The hematopoietic cells were not adherent and mostly removed by the second change of medium. Approximately 3-4 passages after first culturing the cells, the cells were expanded to over 25 million cells from the initial 250-50 which adhered to each culture dish.

Primary cultures reached confluence in about 5 days and plates were split prior to reaching full confluence in order to preserve their proliferative ability. Plates were also grown to full confluence and myotube formation was confirmed after 2-3 weeks. On replating, MSC's slowed their proliferation rate considerably and after the second passaging, stabilized their growth rate and required replating every 6-7 days. Concurrent with this slowing of the growth rate, the MSC's changed their appearance from an initial spindle shape to more rounded cells. (Figures 5, 6) By the end of the first passage, the 'round' cells were reduced in frequency (1-2% of total cells) and were not found after the first passaging.

The uniform attachment and spindle-shaped morphology of MSC's was observed at every passage until the hallmarks of cellular senescence appeared. These usually included cessation of mitotic activity, accumulation of cellular debris and a broad flattened morphology. New cells were harvested for a fresh culture at this time in order to guarantee the successful proliferation of cells. The retention of the MSC

phenotype following serial passaging has been confirmed previously [14] and was not studied here.

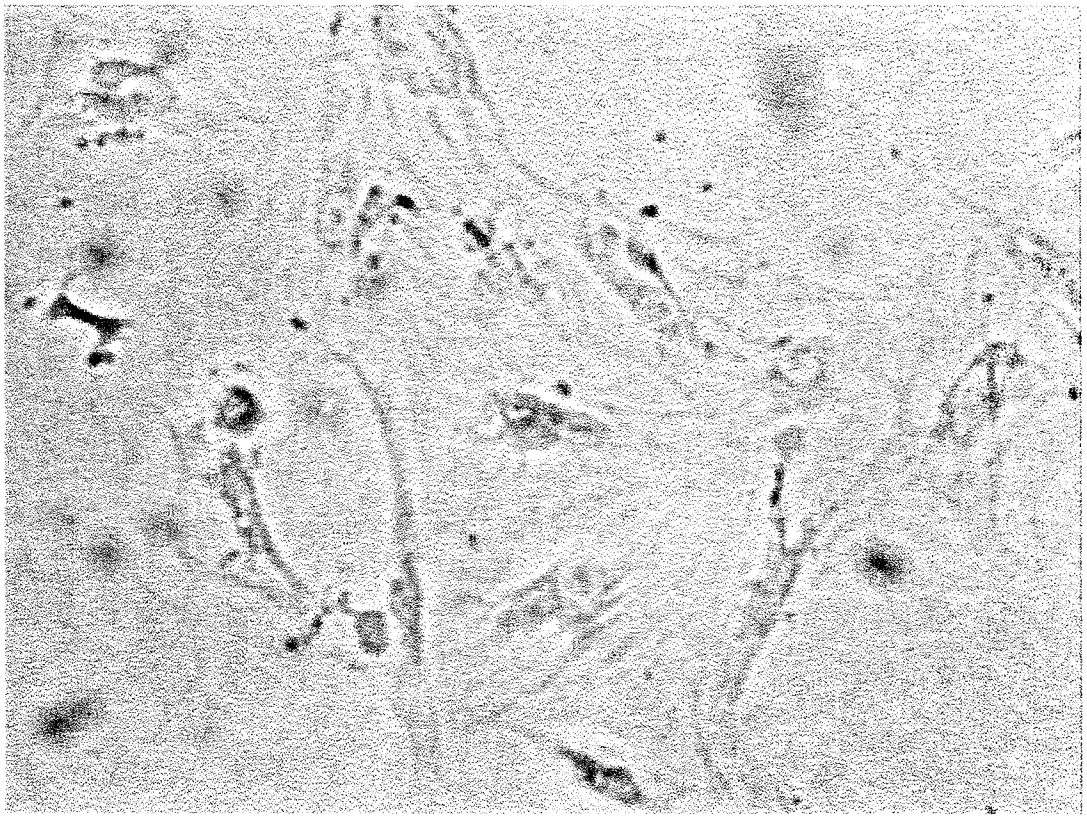


Figure 4. Marrow stromal cells in culture after first passaging on day 6. Note spindle shaped morphology of cells. (Mag. 400X)

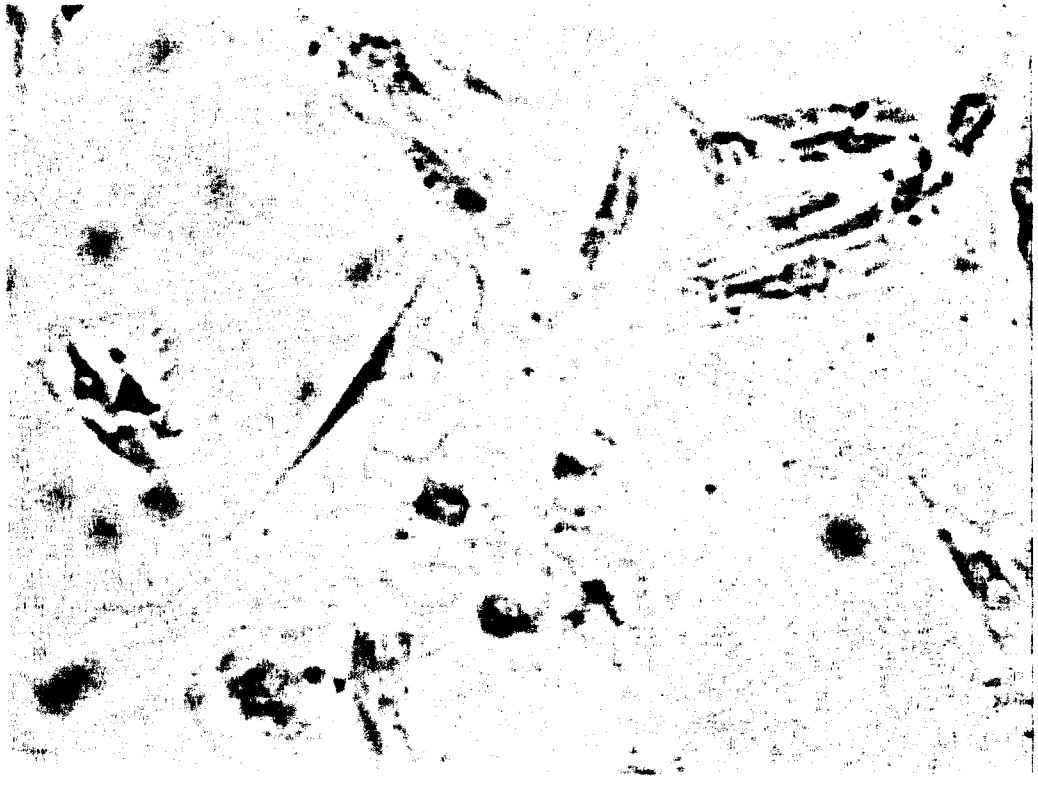


Figure 5. MSC's in culture at Day 8. Note rounded spindle-shaped morphology of cells . (Mag 400X)

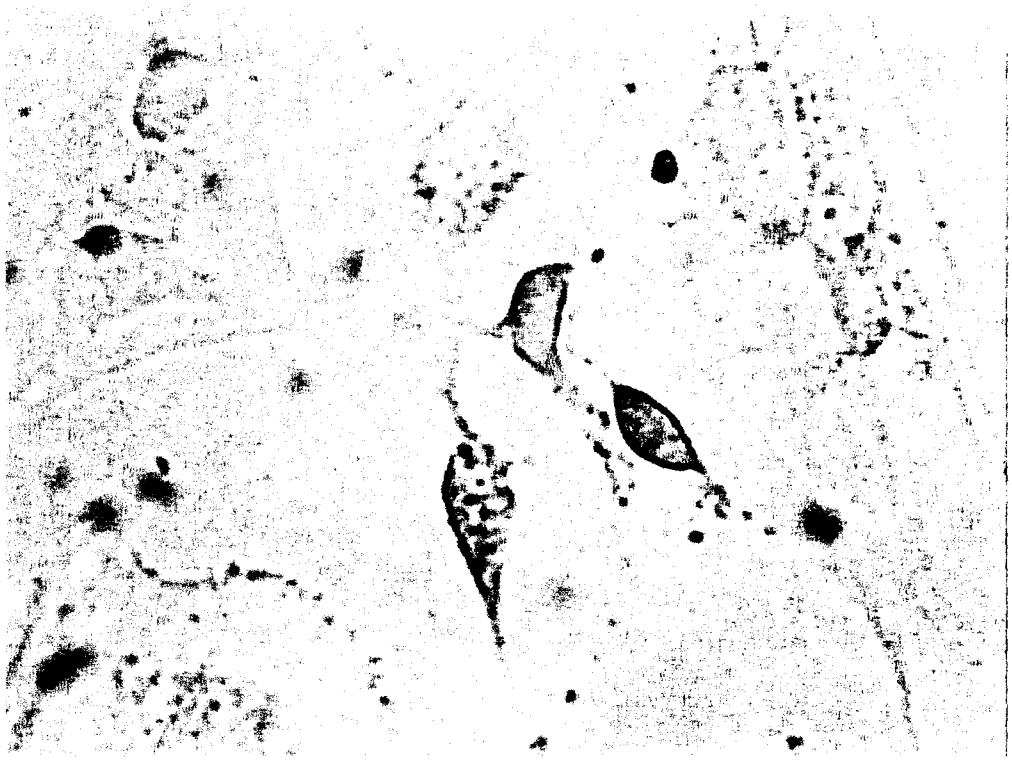


Figure 6. MSC's in culture at Day 14. Note rounded morphology of cells.

(Mag 400X)

Efficiency of Labelling:

The labelling efficiency for all 3 labelling techniques approached 100%. MSC's transfected with Lac Z were counted within the petri dish in order to obtain a percentage of cells which were labelled. The results were 99.8 % for these cells. Both DAPI and GFP also achieved outstanding labelling efficiency of 100%. (Figures 7A, 7B, 7C)

Although the use of the bacterial reporter gene Lac Z has been described as a cell labelling technique, [95] the conditions for its use must be optimized before the results can be interpreted. Reaction conditions including temperature, duration of X-gal exposure, pH of the reaction solution, and different methods of preparing and fixing tissue prior to X-gal exposure all can alter the interpretation of staining results.[90] In addition to testing the appropriate controls to ensure that the X-gal reaction was not due to false positivity, cell labelling was also reconfirmed with both DAPI and GFP.

In order to confirm the true positivity of the X-gal staining both within the heart and for the transfected, harvested MSC's grown within the culture dish, the X-gal solution was tested at varying pH's from < 4 to >10. Since a false positive X gal reaction can be a pitfall in this cell transplantation study, experiments were rigorously performed, maintaining optimal, reproducible conditions. Optimal staining whereby transfected bone marrow was seen with a true positive blue/indigo colour from the nonsoluble, nondiffusible crystalline precipitate characteristic of ß-gal was seen at a pH > 7.5 and <8.5 in the culture dish. (Figure 8A) pH's lower than this created both cytoplasmic as well as nuclear staining, altering cell morphology. (Figure 8B) pH's

> 8.5 caused a dull reaction to appear within the dish with faint coloration of cell nuclei. (Figure 8C) Surprisingly, even normal, non-transfected MSC's were found to stain positive in X-gal solution under a pH of 8.0.

In order to confirm that there was no endogenous Beta-galactosidase activity within the heart, serial cross-sections of normal myocardium were stained with B-gal solution at pH's 4.5, 5.0, 5.5.,6.0, 6.5, 7.0, 7.5, 8.5, 9.0 and 9.5. The precise values of the pH's were titrated using 1.0 N HCl and 1.0 N NaOH. Similar results were seen within the heart as well, where only at a pH > 8.5, the transfected MSC's stained positive. When heart specimens were stained at random from normal hearts that had not been subjected to labelled MSC's, there was still some positive staining evident at pH's < 8.0. This indicated that the heart had some endogenous ß-galactosidase activity which needed to be controlled for.

Therefore, all staining with X-gal was used at a controlled pH > 8.5, to ensure the true positivity of all of our results, and was further verified with results from both DAPI and GFP.

After the expected dates of sacrifice, the bone marrow as well as the hearts were sacrificed from each rat in order to identify the presence of labelled cells. A subset of 9 rats, which had been injected with GFP or DAPI were also injected with 10 million cells over 2 days and random blood samples drawn at 2 hours, 6 hours, 12 hours, 24 hours, 2 days, 5 days and 7 days. The blood was plated and viewed under a fluorescent microscope with the appropriate filter in order to see evidence of labelled cells. There was no evidence of any cells labelled cells seen in the circulation at any of the time points. At all time points, except for 2 hours after injection of cells,

however, there was evidence of the labelled cells within the bone marrow when it was reharvested at various time points. (See Table 1) (Figures 9A, 9B, 9C)

In all rats, the reharvested bone marrow contained an estimated 1-10% of engrafted, labelled cells within each plate. There was no relationship seen between the dose of MSC's injected or the length of time after injection and the percentage of engraftment of these cells.

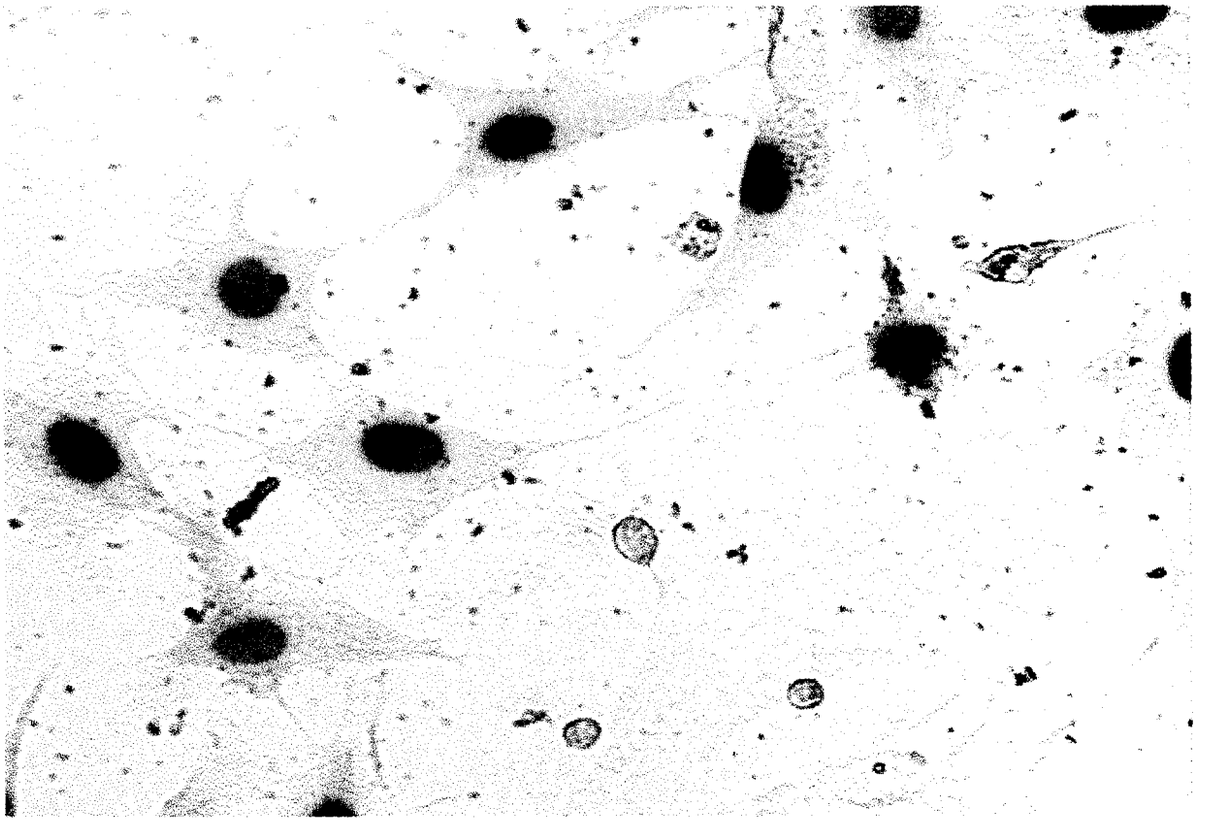


Figure 7A. B-gal labelled MSC's within culture dish. Stained with X-gal solution. Note presence of indigo blue colour in nucleus. (Mag 100X)

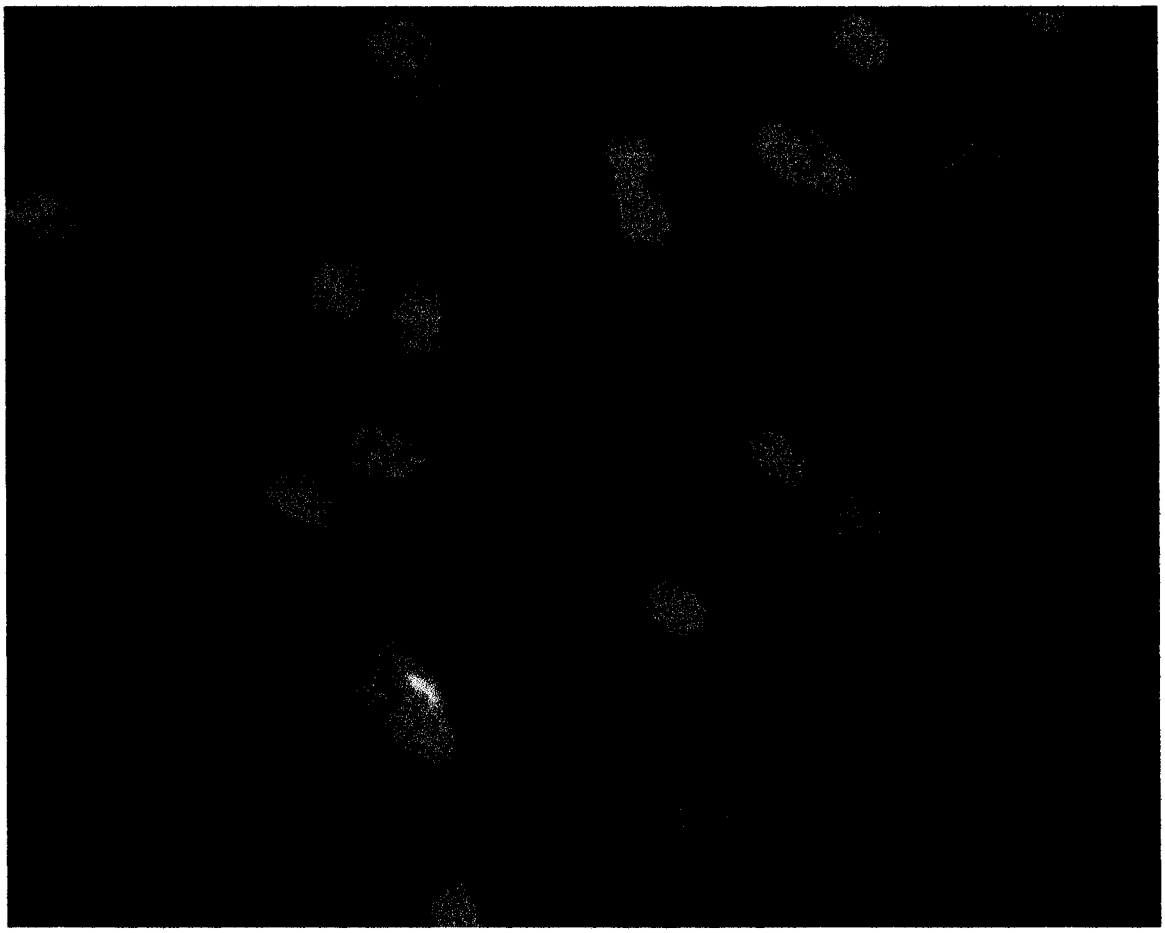


Figure 7B. DAPI labelled MSC's within culture dish as seen under fluorescent light microscope. (Mag 200X)

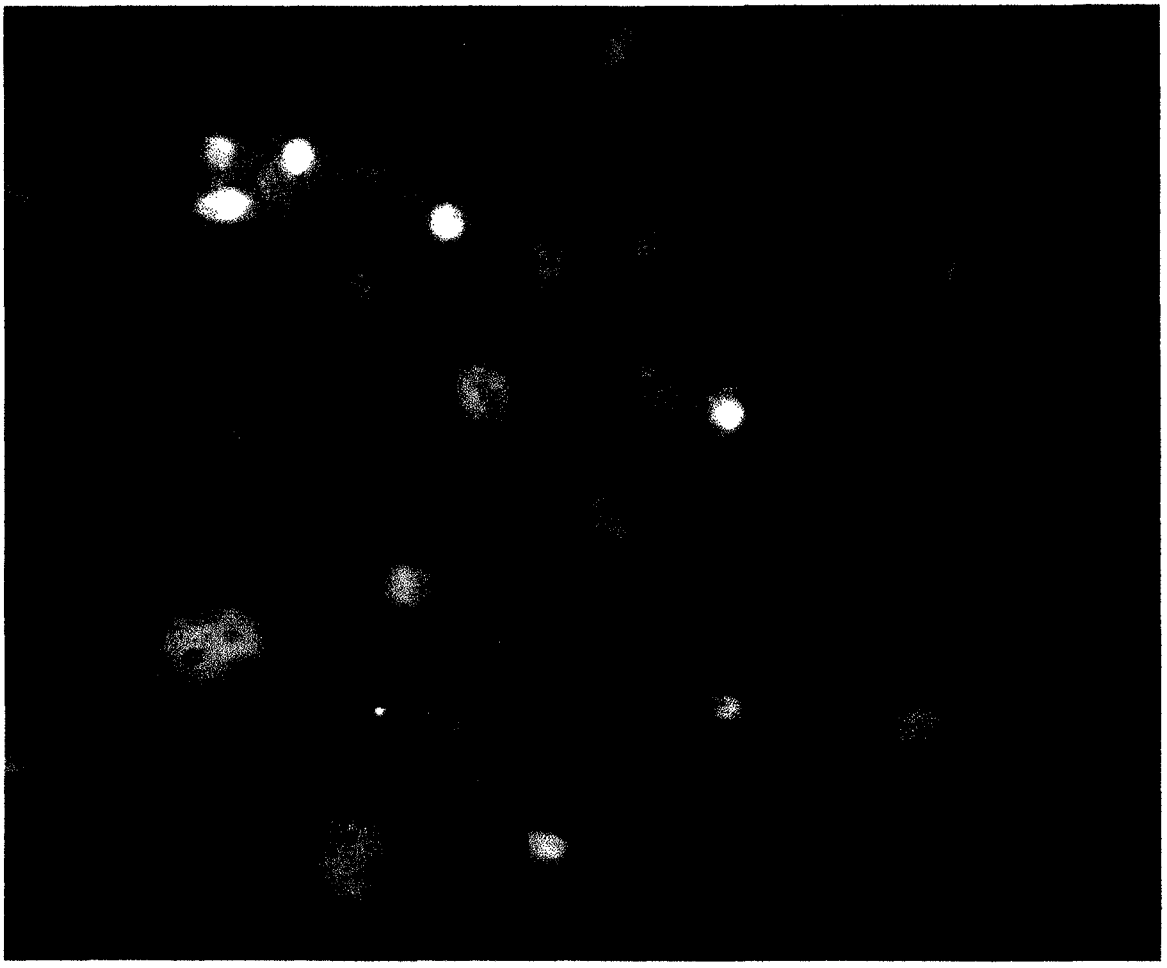


Figure 7C. Green Fluorescent Protein-labelled MSC's within culture dish as seen under fluorescent light microscope. (Mag 200X)

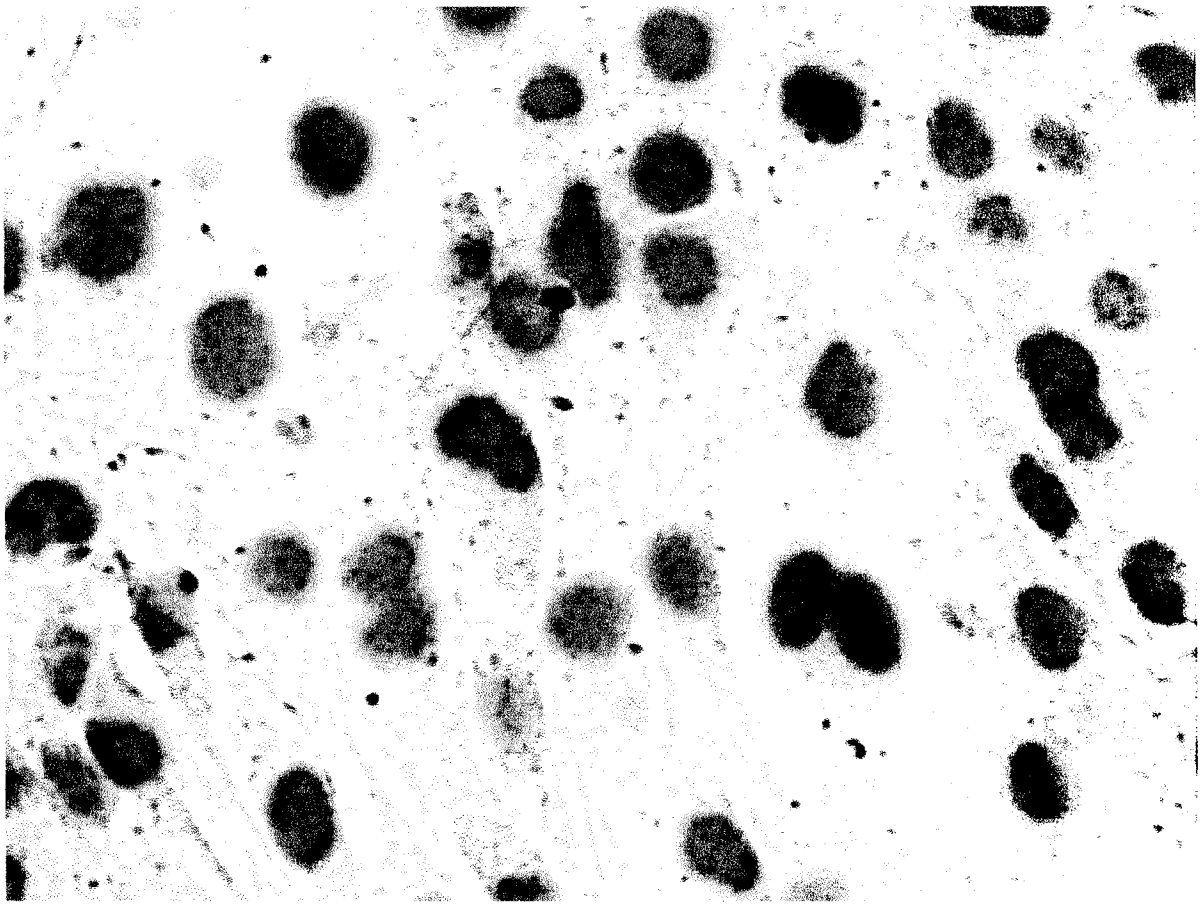


Figure 8A. ß-gal labelled MSC's in culture stained with X-gal solution at pH between 7.5-8.5 (pH=8.03). Note bright indigo-blue colour of stain, which is seen within cell nuclei. (Mag 100X)

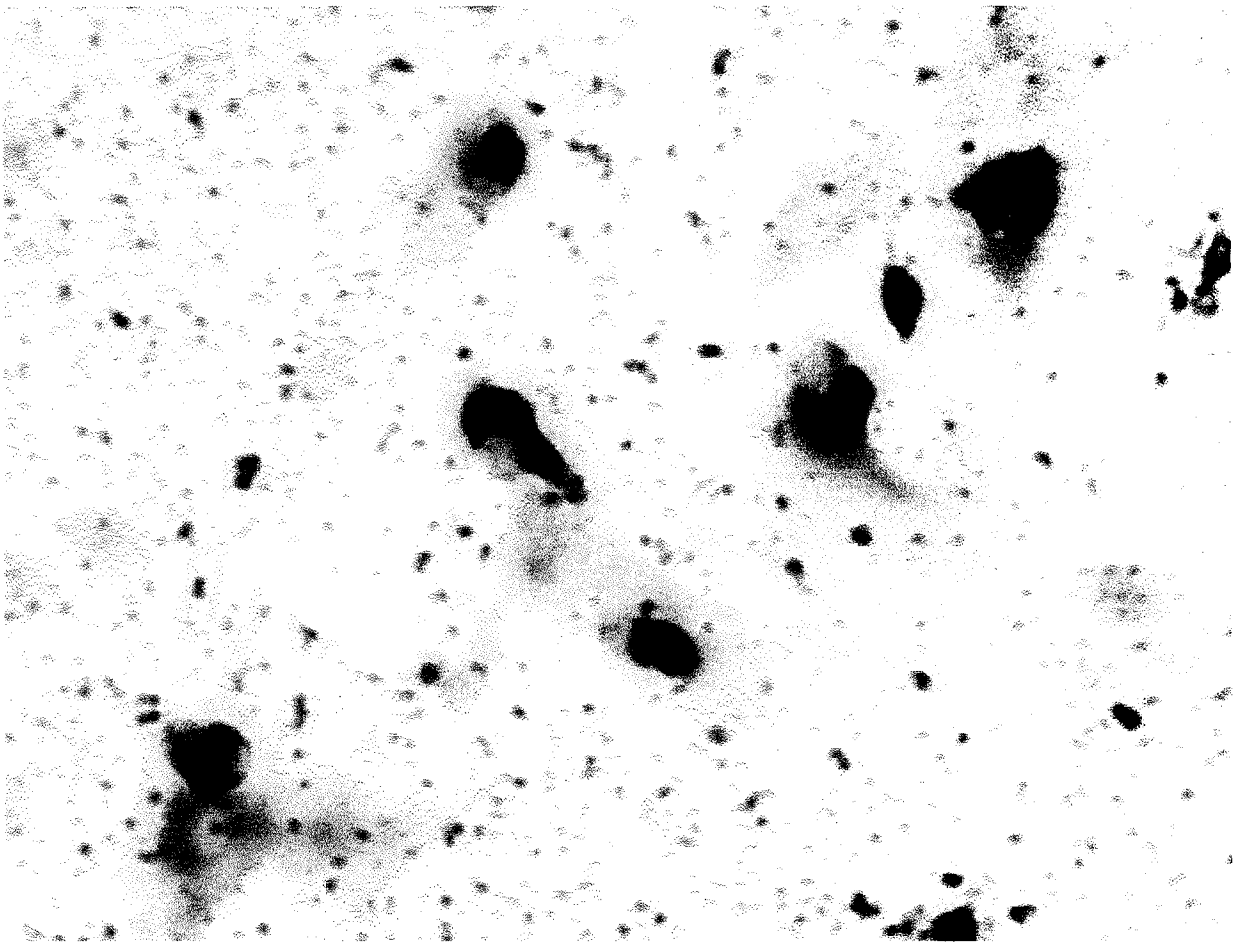


Figure 8B. ß-gal labelled MSC's in culture stained with X-gal solution at pH less than 7.5 (pH = 5.40). Note colour present in both nuclei as well as cell cytoplasm with irregular staining pattern. (Mag 100X)

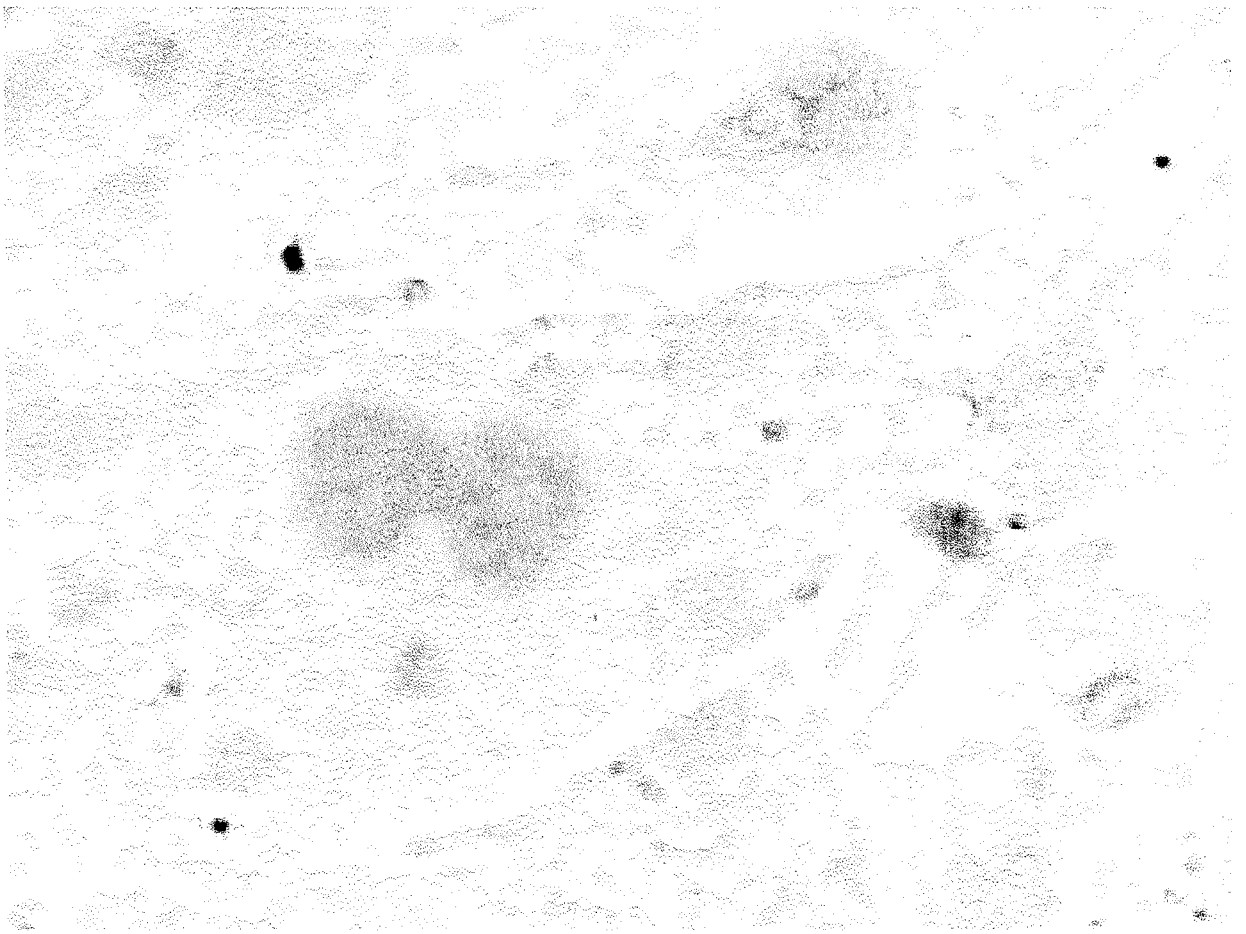


Figure 8C. ß-gal labelled MSC's in culture stained with X-gal solution at pH greater than 8.5. (pH= 8.68). Note dull colour of nuclei. (Mag 200X)

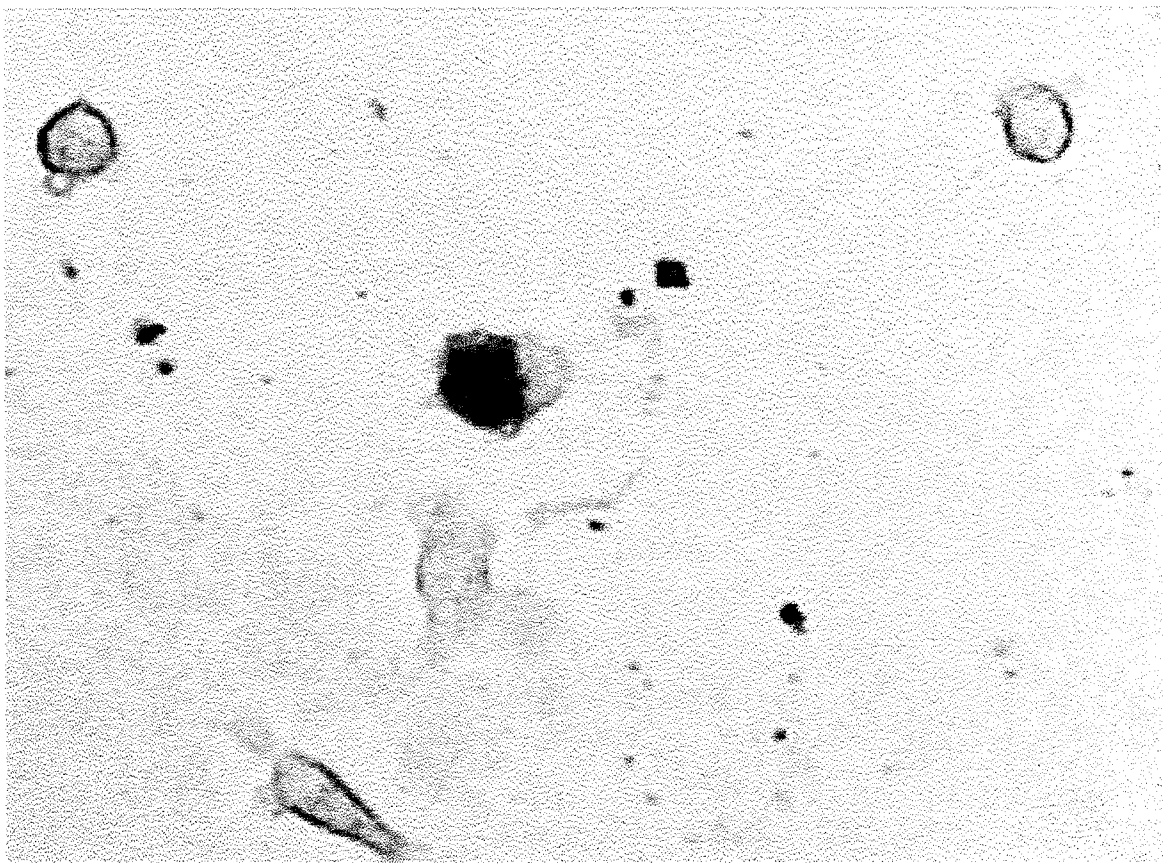


Figure 9A. MSC's harvested from rat which had previously been injected with labelled MSC's. Cells were grown in culture for one week and culture dish stained with X-gal solution. Note unlabelled native MSC's of rat surrounding labelled cell. (Mag 200X)

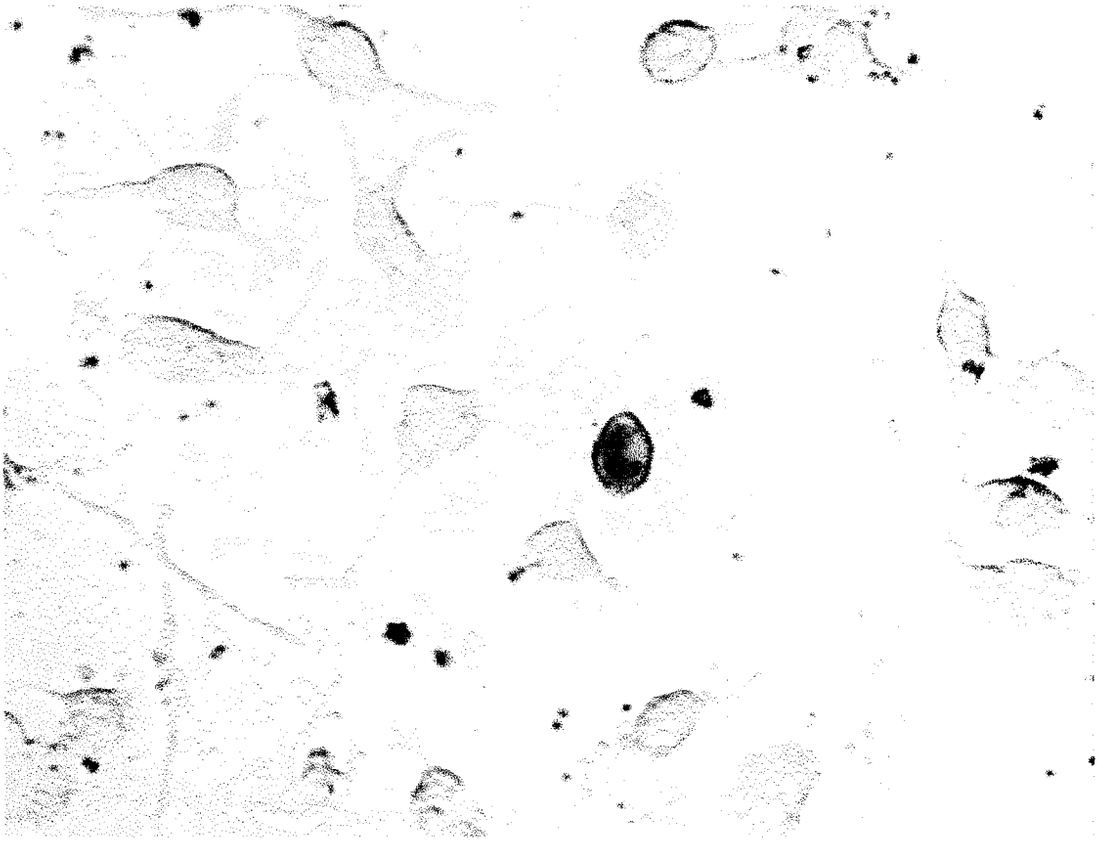


Figure 9B. MSC's harvested from rat which had previously been injected with labelled MSC's. Cells were grown in culture for four weeks and culture dish was stained with X-gal solution. (Mag 200X)

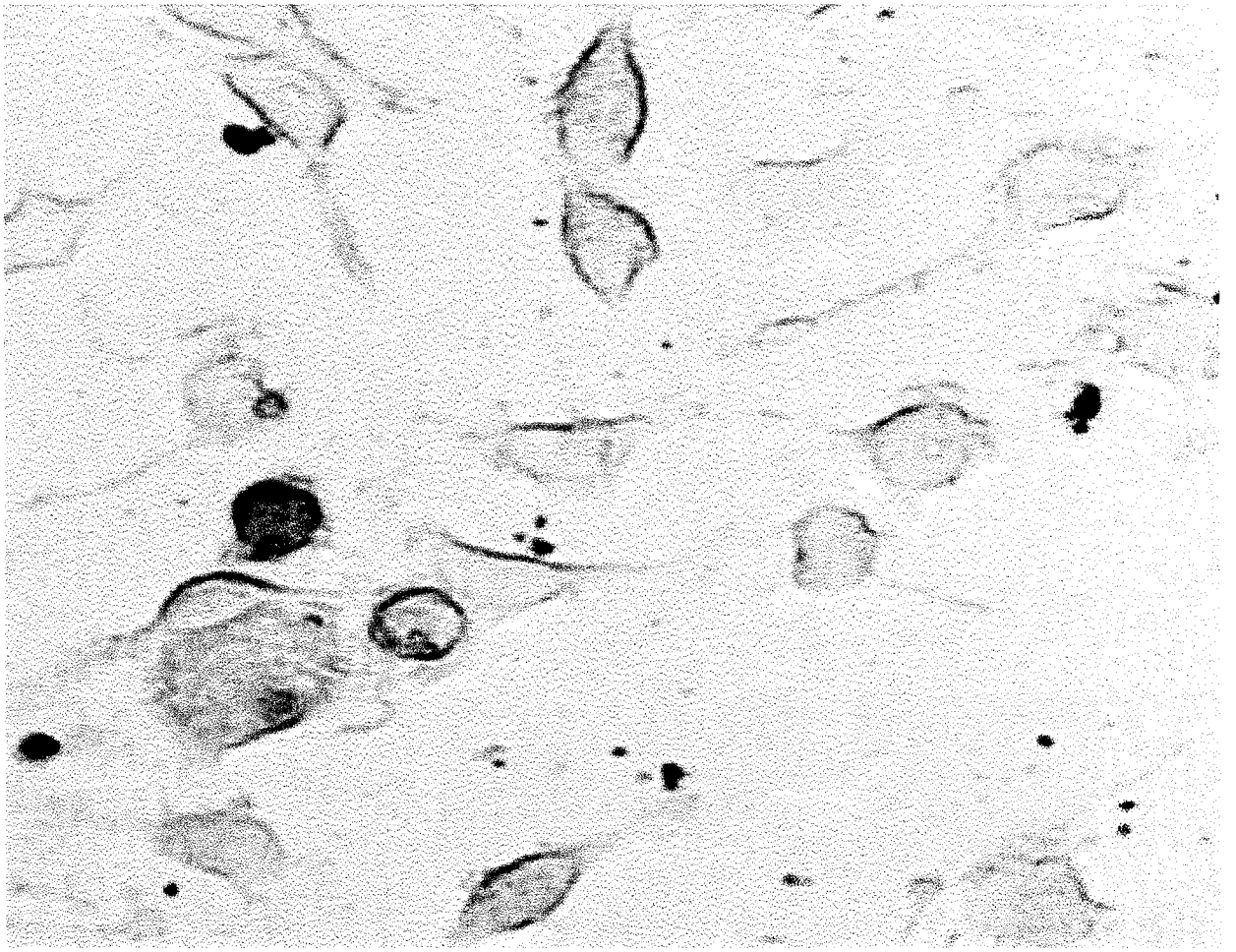


Figure 9C. MSC's harvested from rat which had previously been injected with labelled MSC's. Cells were grown in culture for six weeks and culture dish was stained with X-gal solution. (Mag 200X).

Note no relationship between length of time cells were harvested after injection and the number of cells present within the culture dish.

Operative Results:

The implantation of 5 million cells/day through the dorsal penile vein on 2 consecutive days yielded no procedural mortality and the rats tolerated the procedure well, without any requirement for pain medication. Initially, when the dosing schedule for the number of cells needed to be implanted was being finalized, 3 rats died. These rats received cell dosages as follows:

Rat 1- 10 million cells on day 1

Rat 2- 5 million cells on day 1 and 2 million cells on day 2

Rat 3- 7 million cells on day 1

Both rats 1 and 3 may have experienced an embolic phenomenon when a large bolus of "clumped" cells were introduced into the venous circulation, causing pulmonary emboli. Rat 2 died on the second day due to respiratory depression from an unknown cause, although embolism may have also been possible. All rats survived the coronary artery ligations and sham operations until the expected dates of sacrifice.

Gross Specimens:

The gross examination of the coronary ligated hearts revealed a fibrotic scar in the distribution of the left anterior descending coronary artery in all hearts and a clear delineation between the area of scar and the adjacent normal myocardium. (Figure 10) The gross hearts were stained with X-gal solution and gross examination of all coronary artery ligated hearts revealed preferential blue colour of the X-gal dye in the area of the scarred myocardium. (Figures 11A ,11B) The borderzone between scar and normal myocardium had less blue staining in comparison, with normal

myocardial areas far from the infarcted region with no stain at all. The extent of the engraftment, as seen by the intensity of the blue colour, seemed to correlate with the extent of the myocardial injury and the subsequent scar formation. Hearts with a very small area of scar appeared to have relatively fewer MSC's recruited to the area of injury and appeared less blue in colour. In contrast, all sham operated hearts with an infarction appeared to have no blue discoloration after similar X-gal staining, under the same experimental conditions.

Recent reports have commented and cautioned on the interpretation of results using X-gal as a reporter gene due to the increase in ß-galactosidase activity specifically seen in areas of myocardial damage.[93] It was shown that microinfarction and not the presence of the gene itself had caused a false positive result. Since false positive measurements of ß-galactosidase enzyme activity can be seen in the presence of hemoglobin and blood [57] , 5 control, gross rat hearts were tested for false positivity. These hearts were explanted 4 weeks after coronary artery ligation and were stained with X-gal solution under the same experimental conditions. None of the hearts stained with any blue colour and confirmed our previous histological findings where the only hearts which stained blue, were those where the rats had been previously injected with labelled MSC's. (Figure 12)

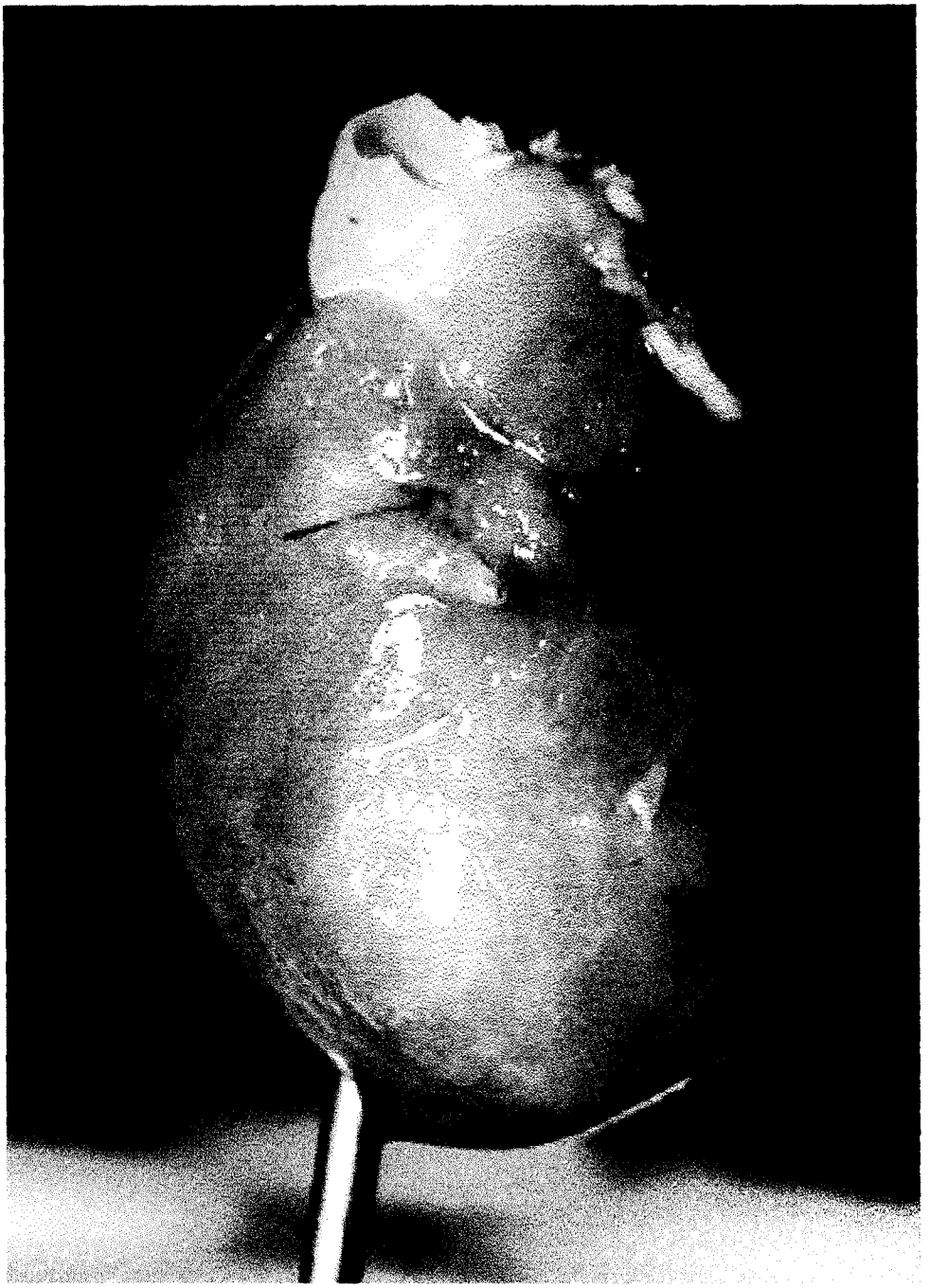


Figure 10. Gross specimen of coronary ligated heart sacrificed after 4 weeks, showing delineation between scarred myocardium and normal myocardium. Note blanching of scarred myocardium below blue prolene suture ligature.

Figure 11A. Frontal view of coronary ligated heart explanted 4 weeks after ligation. Stained with X-gal solution. Note delineation between scar and normal myocardium, which does not appear to stained blue.

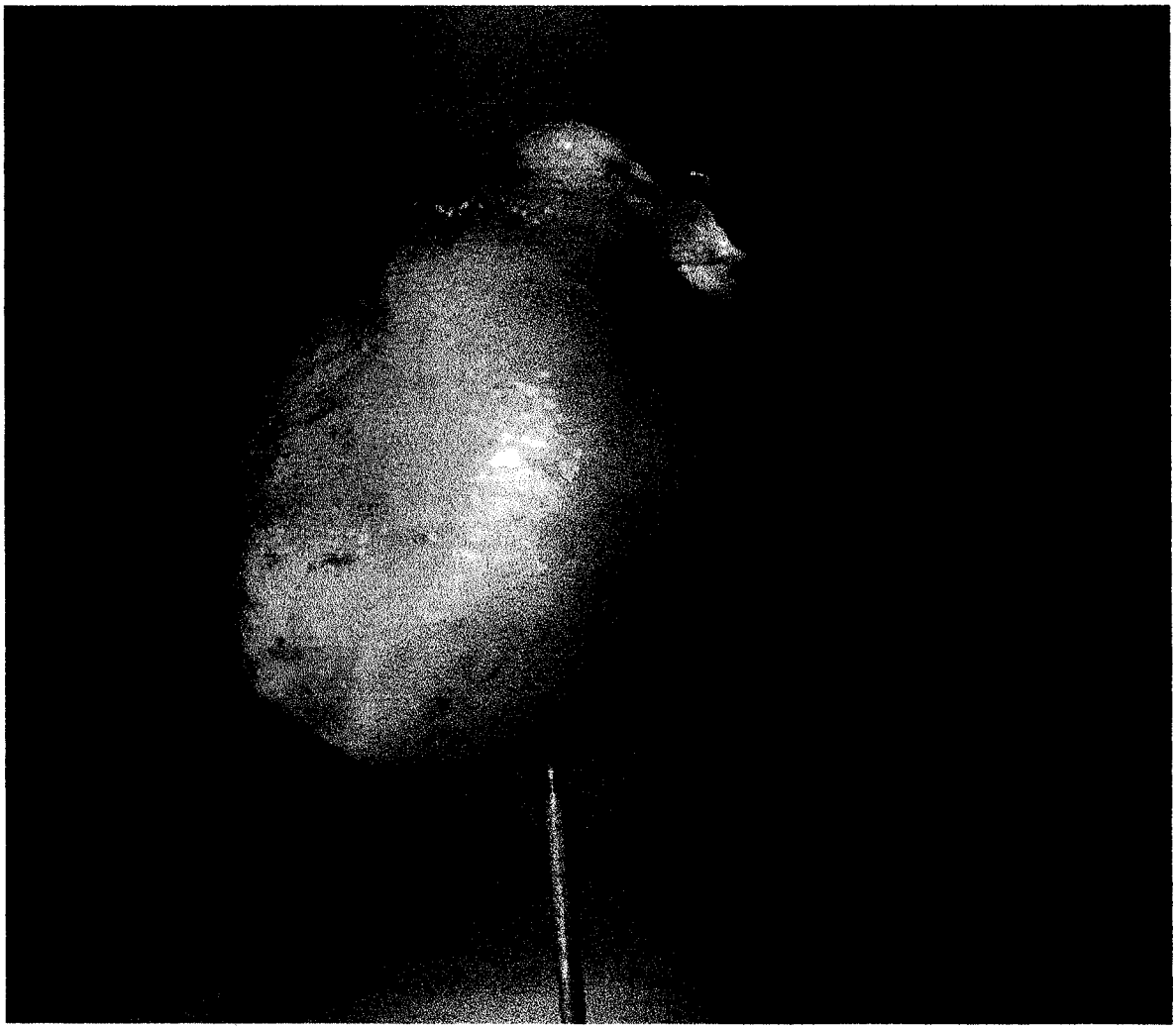


Figure 11B. Oblique view of heart in Figure 11A. Note borderzone between infarcted myocardium, which appears blue and normal heart, which appears unstained.

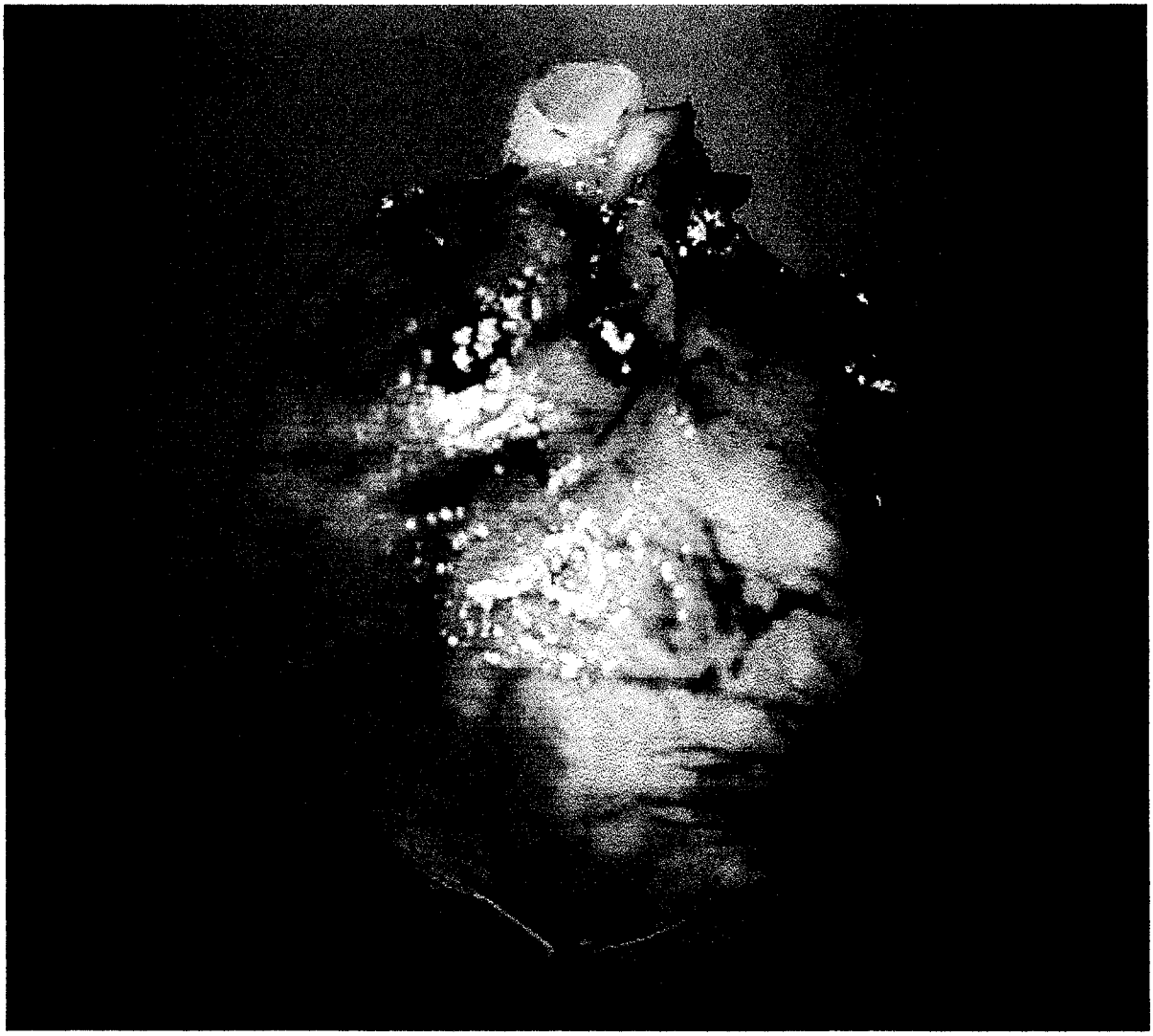


Figure 12. Gross specimen of coronary ligated heart explanted 4 weeks after ligation, with no previous injection of labelled MSC's. Heart was stained with X-gal solution revealing no presence of blue colour both on macroscopic and microscopic examination.

Histologic Results:

A) Hematoxylin and Eosin (H&E) and Picrosirius Red Staining:

When serial cross-sections of the hearts were evaluated, labelled MSC's could be identified within all of the ligated hearts in the area of the scar, but no labelled cells were found in any of the sections observed from the sham operated hearts. The ligated hearts served as their own controls as well, as there was no evidence of any labelled cells in the sections of the normal myocardium bordering the infarction zones.

H& E staining showed labelled cells incorporated within the scar area surrounded by connective tissue/fibrous scar. Cells were varied in morphology and showed more elongation in structure and alignment with one another at later time points. (Figure 13A, 13B) Sections were also stained with Picrosirius Red to show the presence of labelled cells consistently only within the scarred regions. Normal myocardium, seen yellow or white in colour, did not contain any labelled cells. (Figures 14A, 14B)

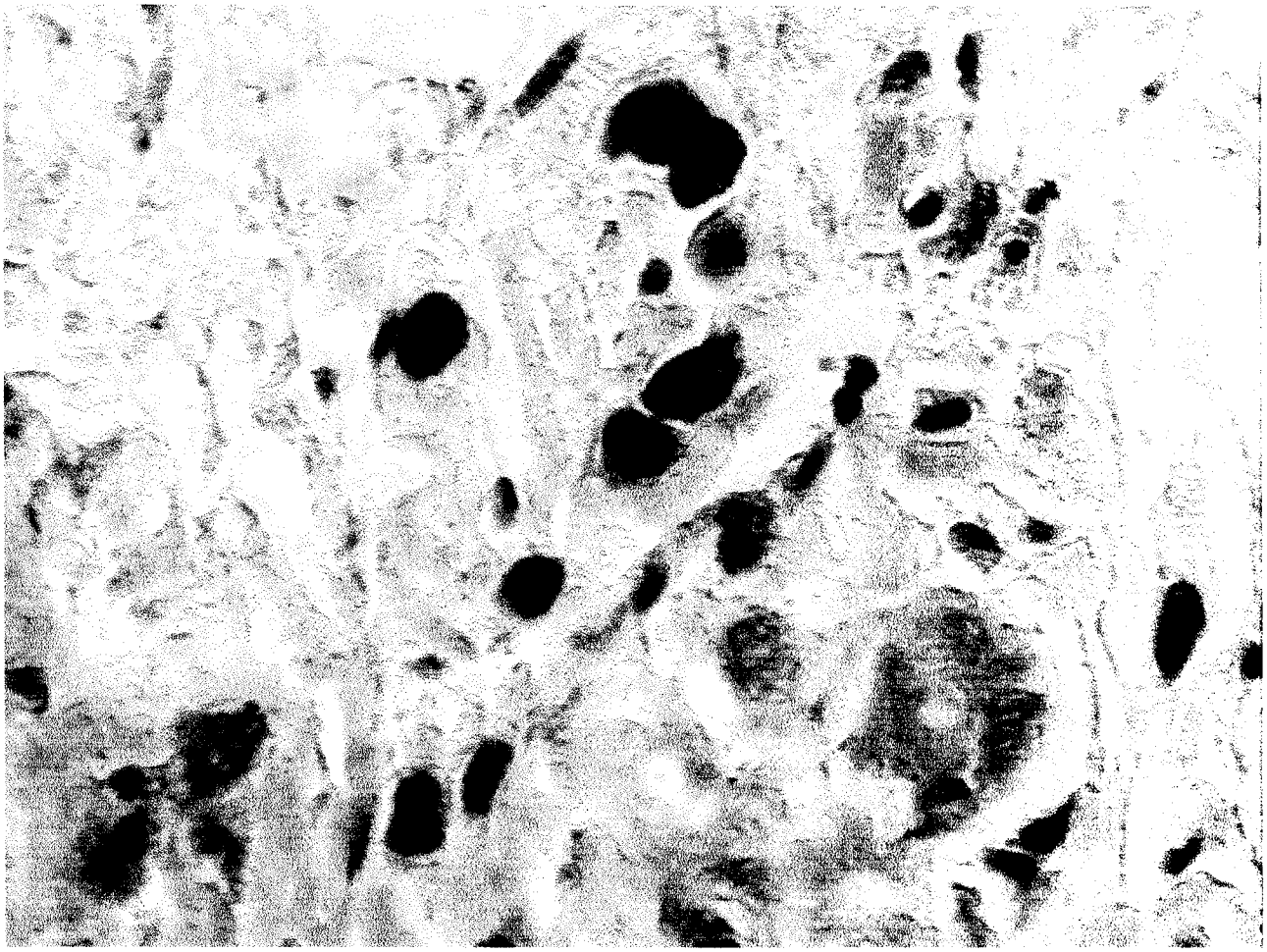


Figure 13A. Cross-section through scar area 4 weeks after coronary artery ligation, containing labelled MSC's. Slide stained with X-gal and H& E. Note cells are round and undifferentiated. (Mag 400X)

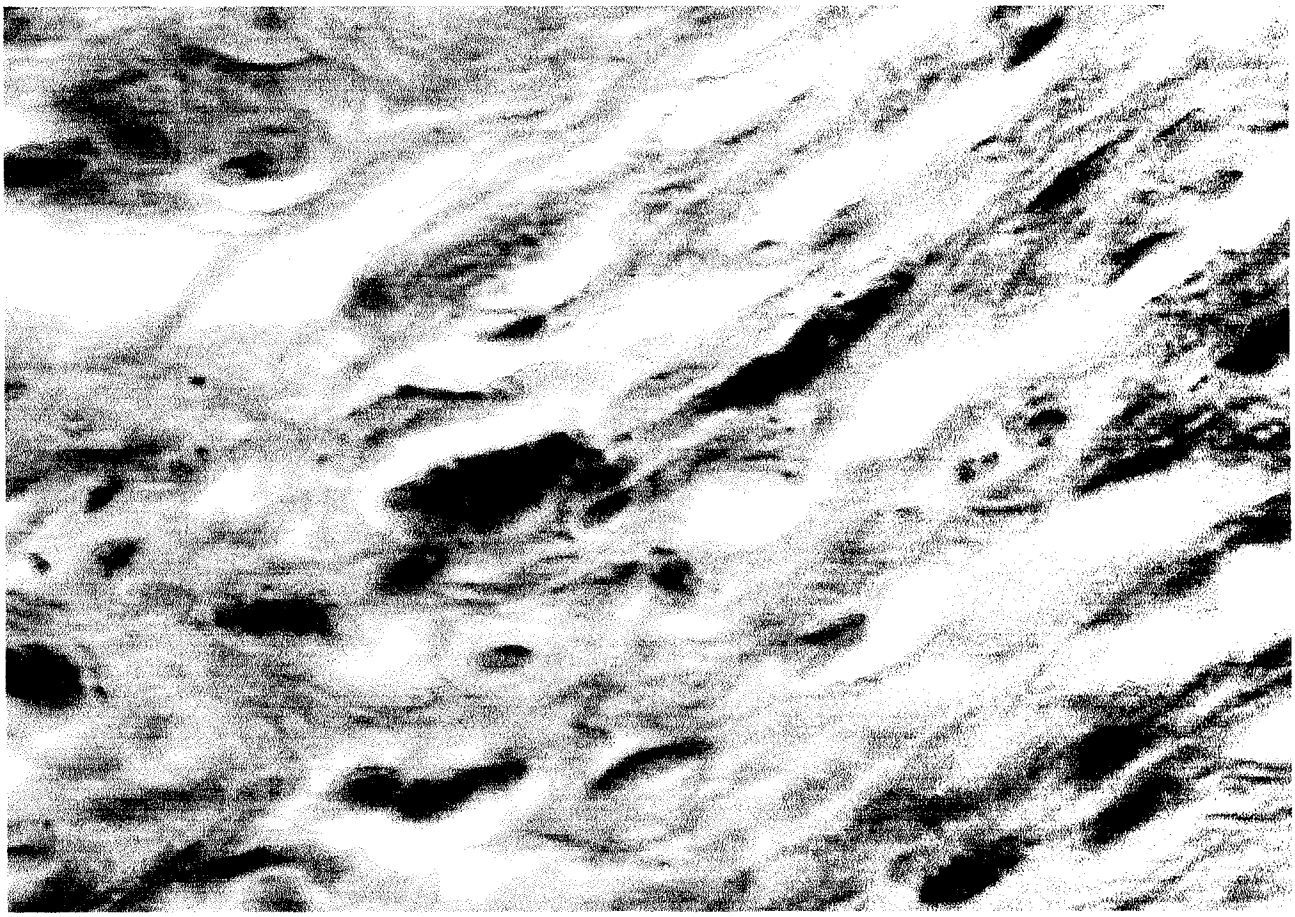


Figure 13B. Cross-section through scar area 6 weeks after coronary artery ligation, containing labelled MSC's. Slide stained with X-gal and H&E. Note cells are relatively more elongated and lining up with one another. (Mag 400X)

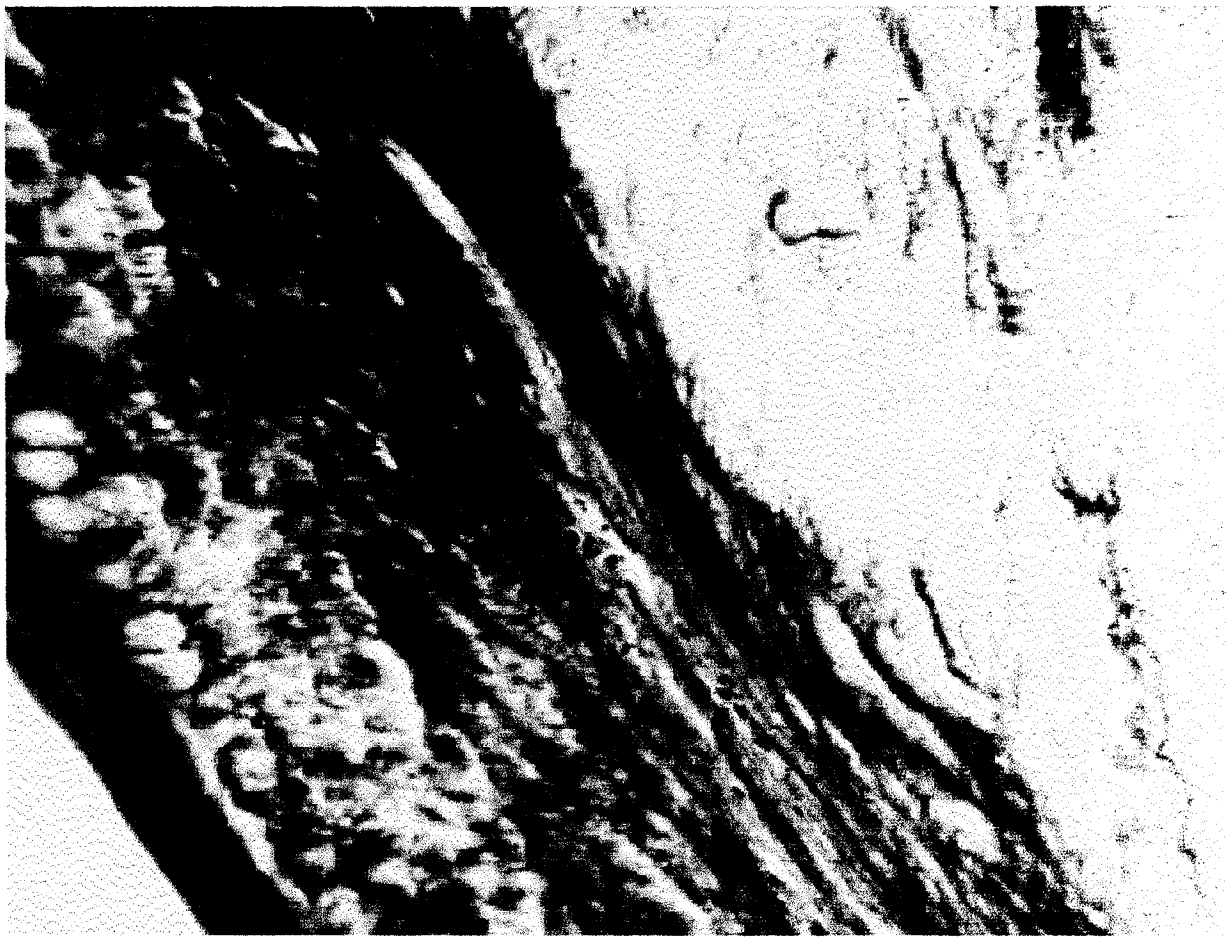


Figure 14A. Slide through cross-section of scar stained with Picrosirius Red Stain. Note labelled MSC's seen within scar area, which appears red. Normal myocardium appears white or yellow. (Mag 200X)

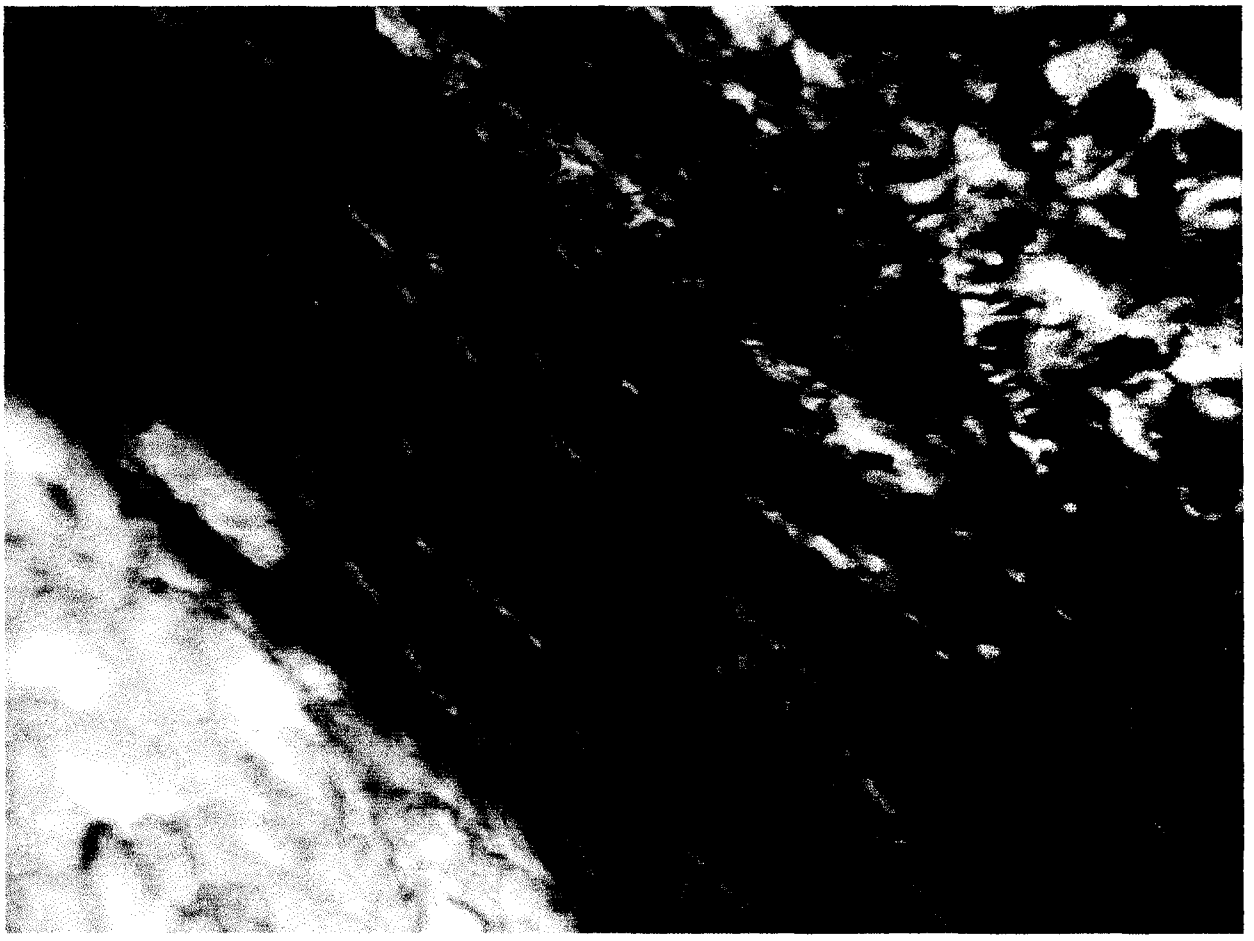


Figure 14B. Slides through cross-section of scar area 6 weeks after coronary artery ligation. Picrosirius red stain shows red colour in area of scar, in the presence of labelled MSC's. Normal myocardium appears white. (Mag 400X)

Immunostaining:

Immunostaining showed the presence of cardiomyogenic differentiation with the presence of troponin I-c, a cardiomyocyte specific protein within the cell's cytoplasm. [1] The presence of troponin I-c was seen as early as 4 weeks, but was more pronounced at 6 weeks. There was also a clear morphological change seen within these cells at the later time points. At 2 and 4 weeks, the cells appeared to be undifferentiated and circular in size, with a large nucleus to cytoplasm ratio. By 6 weeks, the cells had elongated and lined up in relation with one another, in the appearance of myotubes. (Figures 15A, 15B)

Connexin 43, the main constituent protein in the intercalated disks [9] was not seen to be produced at any of the time points. Figure 16 shows the presence of connexin 43 production within the normal myocardium adjacent to the scar, but not in the injured scar area itself surrounding the labelled MSC's. Results with DAPI labelling and production of connexin 43 as seen with fluorescent labelling showed inconclusive results. Some areas of myocardium in the area adjacent to the labelled MSC's showed early gap junction formation. (Figure 17)

In addition to evidence of cardiomyogenic differentiation, other phenotypes were looked for, including smooth muscle and fibrous phenotypes. Figure 18 shows slides stained with anti-alpha smooth muscle actin antibody, and the production of alpha smooth muscle actin protein within the cytoplasm of the labelled MSC's, indicating the development into a smooth muscle or myofibroblast phenotype. Incorporation of labelled cells within small arterioles was also seen as early as 4 weeks, indicating a possible role of these MSC's in angiogenesis. (Figure 19)

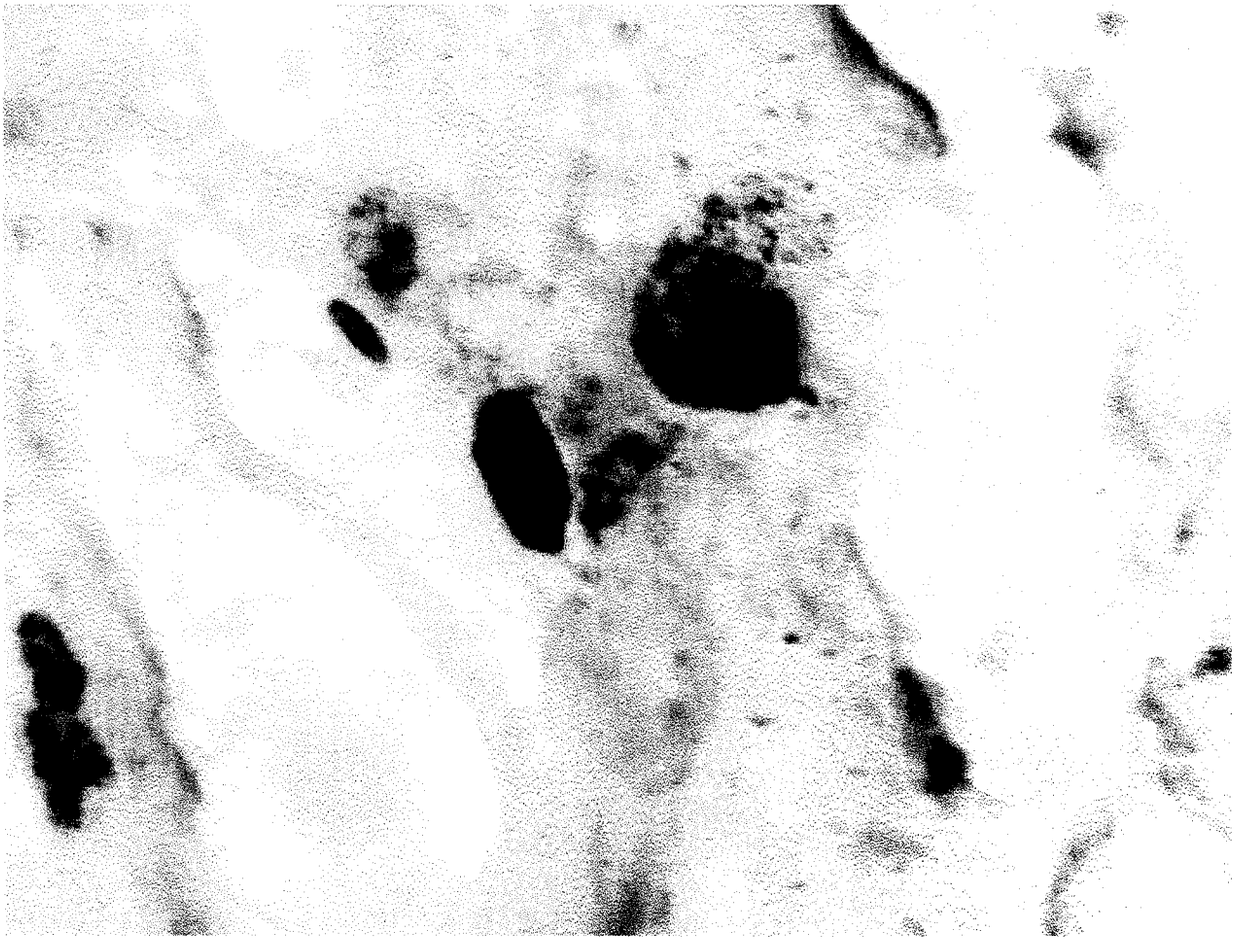


Figure 15A. Troponin I-c staining 4 weeks after coronary artery ligation. Note labelled MSC's appear undifferentiated with large nucleus: cytoplasm ratio and relatively little troponin production seen in cytoplasm, denoted by brown colour. (Mag 400X)

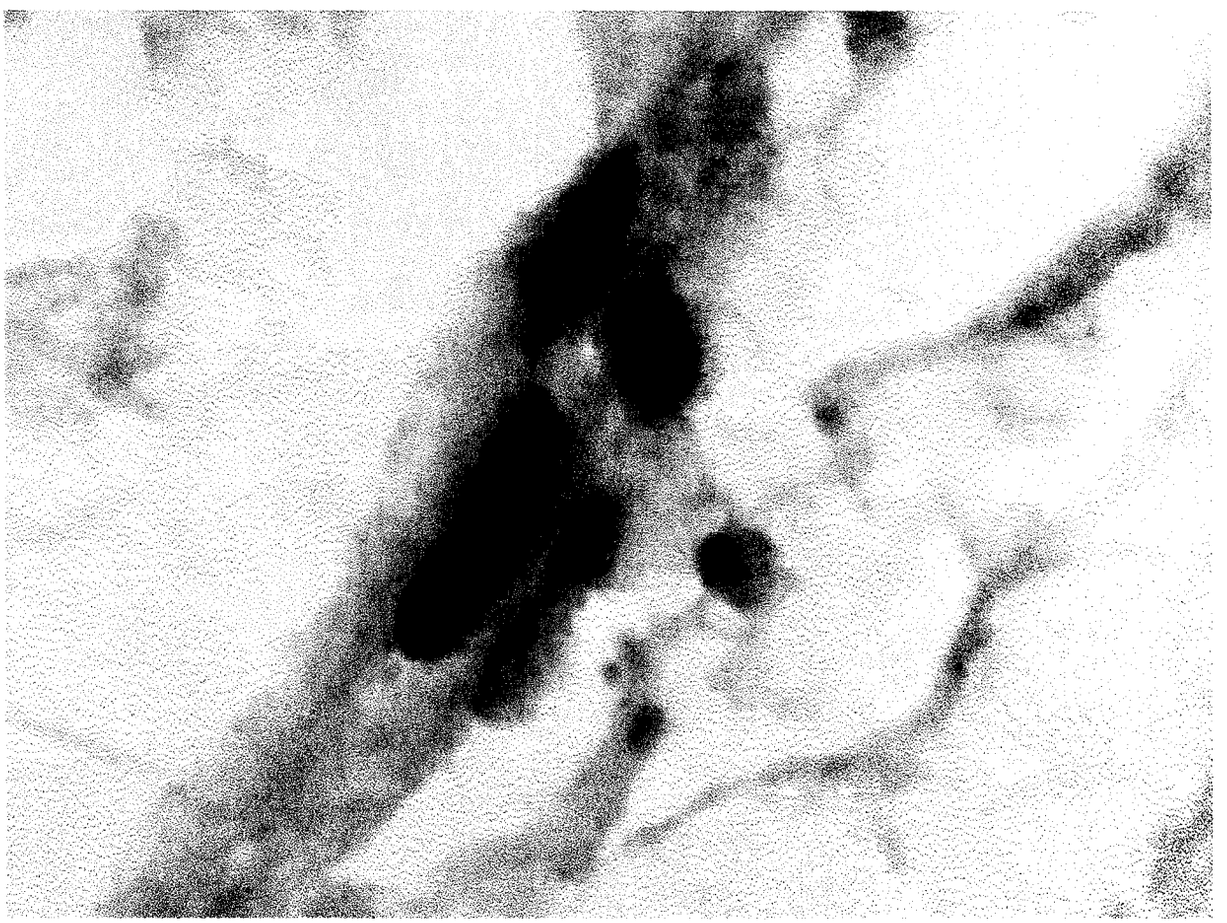


Figure 15B. Troponin I-c staining 6 weeks after coronary artery ligation. Note labelled MSC's appear to be more differentiated with elongated morphology; and more brown pigment in cytoplasm of cells, indicating more troponin production. (Mag 400X)

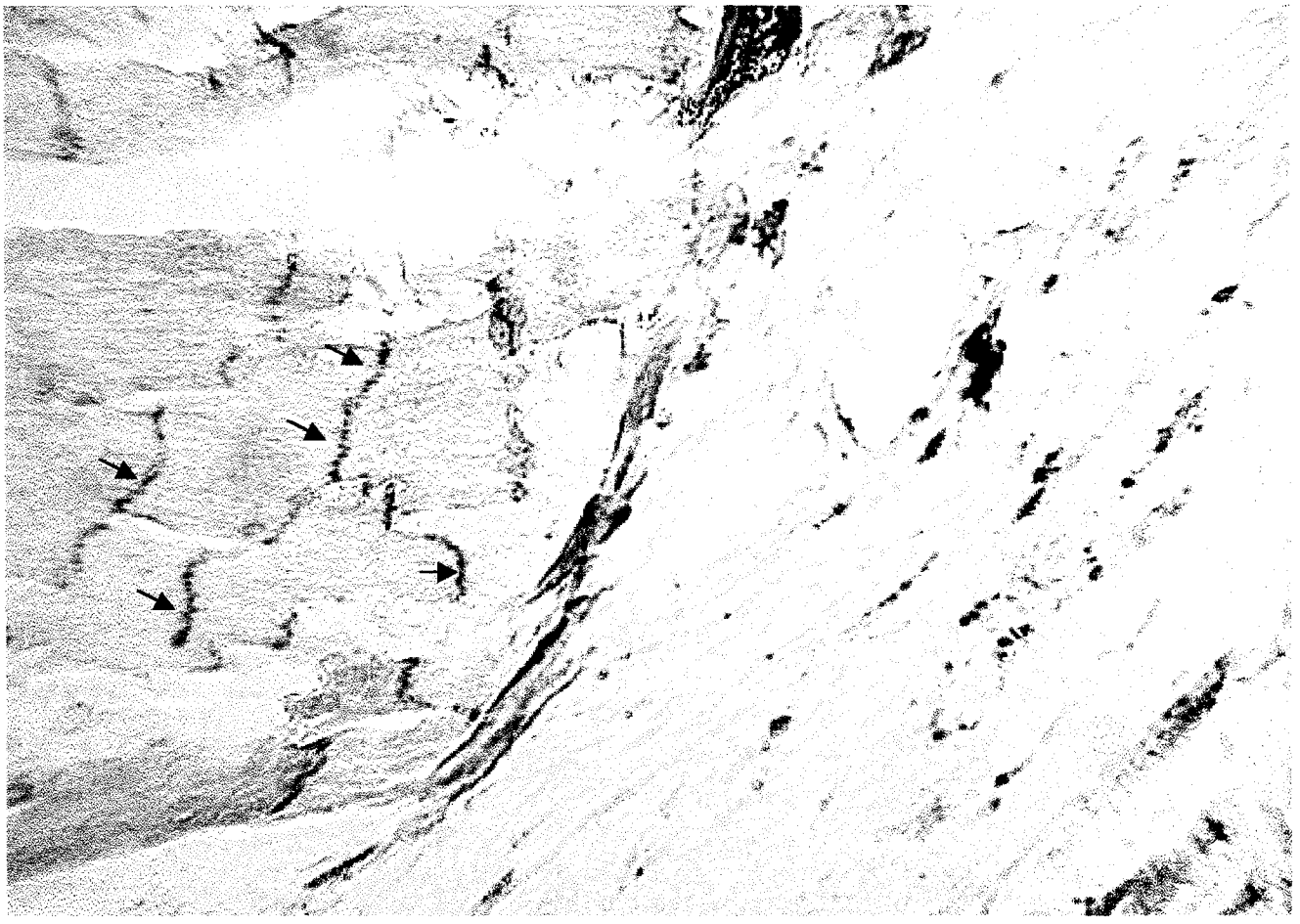


Figure 16. Cross-section through scar and normal myocardium at the border zone at six weeks. Slide stained with X-gal and connexin 43 for evidence of gap junction formation. Note no presence of gap junctions in area surrounding labelled MSC's seen in blue. Arrows denote presence of gap junctions in normal myocardium. (Mag 400X)

Figure 17. Slide of cross-section through scarred myocardium 4 weeks after coronary artery ligation stained with C43 antibody conjugated with Fluorescein. Note DAPI labelled MSC in the centre with evidence of gap junction formation, seen as fluorescent green dots surrounding cell. (Mag 400X)

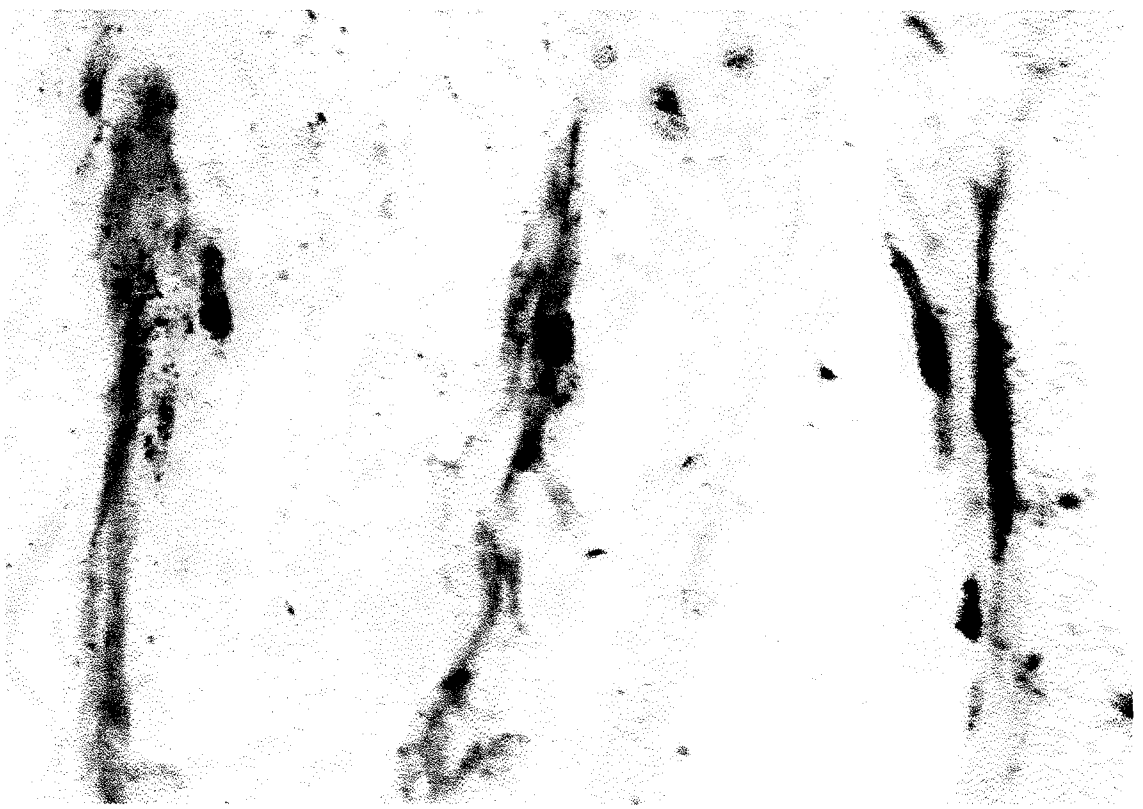


Figure 18. Labelled MSC's seen at 6 weeks. Slide stained with anti-alpha smooth muscle actin . Note presence of brown colour within MSC cytoplasm indicating presence of actin protein and development into a myofibroblast phenotype. (Mag 200X)

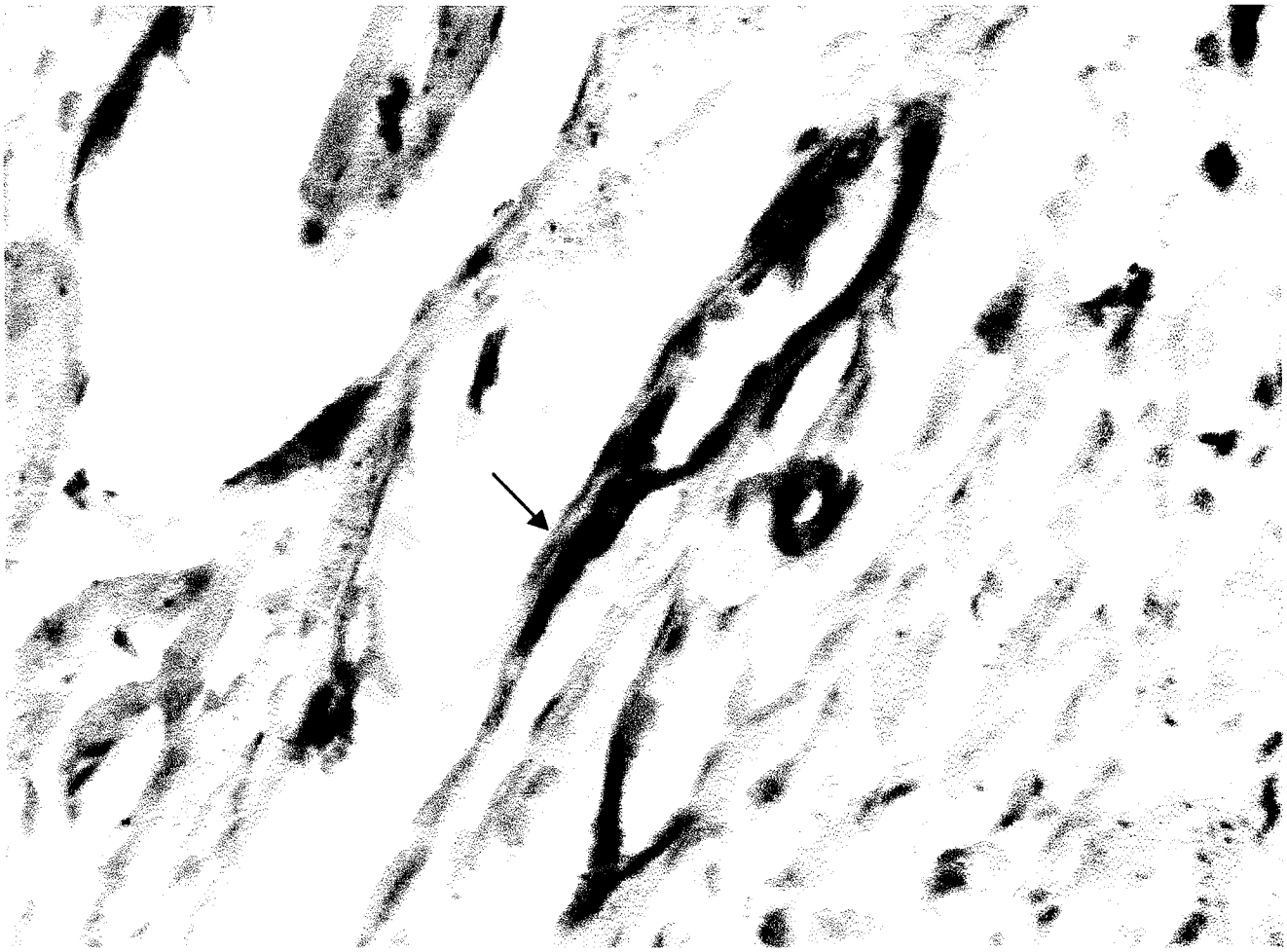


Figure 19. Arrow denotes labelled MSC incorporated into small arteriole after 6 weeks. Slide has been counterstained with anti-alpha smooth muscle actin. Presence of brown colour indicates smooth muscle present in arteriole. (Mag 400X)

CHAPTER FOUR

DISCUSSION

AND

CONCLUDING REMARKS

DISCUSSION:

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Following an MI, a reparative process is quickly initiated in order to counter the loss of necrotic cardiac myocytes, rebuild the infarcted myocardium and maintain ventricular structural integrity.[80] The necrosis eventually causes an increase in load, and a cascade of biochemical intracellular signalling processes is triggered to modulate reparative changes. [79]

The infarction results in a series of cellular responses with the attraction of inflammatory cells, consisting of macrophages, monocytes, neutrophils and mast cells into the infarction zone. These cells infiltrate the healing tissue and provide a source of cytokines and growth factors. As regulatory peptides are released, new blood vessels are formed and fibroblast-like cells appear for ongoing remodelling. The activated macrophages which appear at the site of MI on the first and second days elaborate TGF-B1 and appear to recruit myofibroblasts thereafter, although the signals that determine the appearance of myofibroblasts are not fully understood.[80] These fibroblasts produce the extracellular matrix constituents needed to supply all ingrowth and newly formed blood vessels to carry oxygen and nutrients needed for cell metabolism. [48]

The remodelling process post MI involves all cell types present in the myocardium: the myocyte, the interstitial cells, the vascular endothelium, the immune cells and in particular, the alpha-smooth muscle actin positive myofibrobasts.[25] Although the key roles are played by the cardiac myocyte and fibroblast, this study

strongly indicates that the MSC is also a less well known participant in this response. These MSC's are recruited from the bone marrow in response to myocardial ischemia, migrate to the myocardial lesion and develop into various phenotypes, which subsequently complement the pathophysiological changes which occur following an MI.

There has been increasing evidence over the past decade supporting the pluripotential nature of the MSC. Both intravenous infusion of MSC's [44, 47] as well as direct injection into tissues such as the brain [7, 11, 12, 41] and the heart [87] have shown the ability of these cells to undergo site-specific differentiation into various tissue types. While the fate of MSC's after systemic administration has been studied [69] of more interest has been the response of MSC's following organ injury. Ferrari et al.[23] showed that transplantation of genetically marked bone marrow into immunodeficient mice revealed that marrow-derived cells migrated into areas of muscle degeneration. These cells underwent myogenic differentiation and participated in the regeneration of damaged fibers. Similarly, the recruitment of bone-marrow derived cells by skeletal and cardiac muscle has been shown by Bittner [10] in adult dystrophic mdx mice. The presence of a cell associated with the bone marrow, which may not act as a progenitor to several types of liver cells was also seen after the induction of chemically induced hepatocellular carcinoma.[61]

While the contribution of intravenously administered MSC's to the repair and regeneration of various organs has been studied, especially in the areas of Parkinson's disease and cerebral ischemia [17] their role in cardiac muscle repair has not previously been fully elucidated. Current findings in this study and in those of others [10, 56] have

shown that MSC's may participate in the normal biological repair process which occurs following a myocardial infarction, and may serve as a continuing source of progenitor cells. A subset of these cells may develop into cardiomyocytes with gap junction formation between adjacent cells, while others which have developed into smooth muscle phenotypes may have functions which are likely twofold. One to strengthen the scarred myocardium preventing subsequent scar expansion and rupture; and two, by incorporating into vascular walls, contributing to angiogenesis. These observations support the findings that bone marrow derived endothelial progenitor cells do participate in angiogenesis.[4, 39, 77, 89]

In this study, a rat model of myocardial ischemia was used where 10 million labelled MSC's were systemically introduced, followed by coronary artery ligation one week later. In all of the damaged hearts, there were labelled MSC's present with no labelled cells found in any of the sections of normal (control) hearts observed. In addition, there were no labelled cells found in the sections observed of the normal myocardial areas bordering the scarred regions. However, this does not indicate that labelled cells are not able to migrate to the normal myocardium. Since the heart has to be analyzed microscopically in its entirety in order to locate all of the migrated MSC's, it is possible that even if very small numbers of MSC's were present in the normal myocardium, that they could have been overlooked.

When the MSC's were reassessed at varying time points, by as early as 4 weeks in this study, they had shown evidence for differentiation into cardiomyocytes and smooth muscle cells or myofibroblasts. These phenotypes were also observed at

the later timepoints with ongoing evidence of maturation, structural rearrangement and realignment with neighbouring cells.

Troponin I-c production, as seen in the cytoplasm of these MSC's was our earliest evidence for cardiomyogenic differentiation.[35] As late as 6 weeks, these cells showed no convincing evidence for connexin 43 gap junction formation or the production of sarcomeric myosin heavy chain. These results are not in keeping with the recently reported results by Orlic et al,[56] where connexin 43 was apparent in the cell cytoplasm at the surfaces of closely aligned differentiating cells as early as 9 days. However, there are fundamental differences between their study and the present one. First, they used lineage-negative (Lin-) bone marrow cells from transgenic mice sorted on the basis of C-kit expression [22, 55]; 3 million cells were used for injection and not 10 million; and the cells were injected in the contracting wall bordering the infarct 3-5 hours after infarction, rather than intravenously injecting the cells.

By preselecting for a cell lineage which has proven pluripotential ability as seen with its expression of the C-kit receptor, [55] they have purified their heterogeneous MSC population prior to injection. The injection of cells in the peri-infarcted tissue might have also provided the nutrients and growth factors available in this viable myocardium, which were not present in the bordering scarred myocardium. The presence of this nutrient rich environment might have therefore enabled these cells to differentiate and proliferate, which was not possible in the scarred environment into which the injected MSC's in this study were attracted to.

Connexin 43 expression has been shown to be downregulated after myocardial infarction [52], and factors present in the acute phase of MI, such as lysophosphatidycholine (LPI) [19], high cytosolic calcium, intracellular acidosis, and ATP depletion are all known inhibitors of gap junctional communication.[81] The failure to show the presence of both connexin 43 and sarcomeric myosin heavy chain in our samples may be due to a number of reasons. The labelled MSC's which migrated to the myocardium may not have been programmed to follow a cardiomyogenic differentiation pathway, and therefore were of another lineage type altogether (endothelial precursor, etc). In this instance, they would have never developed into a cardiac myocyte, and other phenotypes might have been positive.

Expression of connexin 43 and myosin heavy chain proteins might also have developed at later time points than were studied and troponin may just have been the earliest one expressed. Although many factors are known to affect the production of these proteins after myocardial damage, there is no real evidence showing the temporal sequence of their production. As well, the scarred myocardium might not have contained the appropriate mixture of trophic factors needed for this expression of phenotypic differentiation to take place.

A number of specimens which were stained for the myosin heavy chain antibody showed the brown pigment indicating the presence of this protein within the cell's cytoplasm. However, the characteristic striations of the myosin heavy chain itself were not identified. This might indicate that these cells are in a transition phase, where they are still developing into cardiomyocytes, and therefore, unable to express the mature phenotype at the timepoints studied. Therefore, it is premature to

conclude that these cells might not be cardiomyocytes altogether, as one must be open to the idea that these premature cells are still developing and unable to express the adult phenotype we are looking for.

In this study, there were positively labelled cells seen within the bone marrows of all rats, but never any seen within the blood samples taken from the systemic circulation of the rats. It is possible that random sampling of small aliquots of blood taken from the rat circulation would have missed the cells which might have been present anywhere within the rat's entire blood volume.

But what are the signalling mechanisms involved which have recruited these MSC's largely to the injured myocardium? The presence of labelled MSC's only seen within the injured hearts but not in the normal hearts, and not even within the normal myocardial segments of the injured hearts indicates the presence of signalling messengers produced or released by the injured myocardium. It appears that in response to myocardial infarction, MSC's are recruited as early as one week in this study, and migrate preferentially to the infarct site. Some may migrate to the normal myocardium itself, or to the border zone, but were not observed in this study. Once in the cardiac microenvironment, they then develop into various cellular phenotypes. Much work needs to be done to determine the surface markers of these multipotential cells and to elucidate the exact chemoattractant to the myocardial injury stimulus.

Krause et al. [42] have shown that the homing of intravenously injected MSC's into the bone marrow increased the expression of CD34 and SCA-1 uniquely on these cells. These same cells demonstrated tremendous differentiative capacity and differentiated into epithelial cells of the liver, lung, skin and gastrointestinal tract.

Whether these injected cells have a pluripotential ability which is only expressed after in-vivo modulation somehow within the marrow stroma is still unknown.

Takahashi [82] was able to show direct evidence of bone marrow derived endothelial progenitor cells mobilized into corneas in response to limb ischemia. Their findings indicated that circulating progenitor cells could be mobilized endogenously both in response to tissue ischemia or exogenously by cytokine therapy to augment neovascularization of ischemic tissues.

By labelling MSC's and by following their migration and phenotypical changes in response to myocardial ischemia, we were able to observe a new dimension of the pathophysiology in myocardial infarction which has never been realized before. Without labelling, this entity is not able to be realized, as these cells are difficult to identify from adjacent native cardiomyocytes or from the inflammatory cell. The pathophysiology following an MI consists of reparative and adaptive changes which include: 1) formation of a fibrous scar; 2) angiogenesis and 3) ventricular remodelling. The contribution of these labelled MSC's has been seen in all three of these processes. Cardiomyogenic differentiation of MSC's contributes new cells to aid in left ventricular remodelling; the formation of smooth muscle-like and myofibroblast phenotypes enables the strengthening of the scar to prevent expansion and rupture, as well as aiding in neovascularization.

One can therefore take advantage of the MSC's pluripotential ability and utilize these cells as a therapeutic modality in order to augment and enhance the natural reparative processes which occur post myocardial infarction. Before clinical trials are in place, further work needs to be done with dose-function relationships in

order to determine the quantity of cells, as well as the optimal time of cell infusion post-infarct, which will optimally enhance cardiac functional recovery. Perhaps pre-selecting for MSC's destined towards a cardiomyogenic or other desirable phenotype and then proliferating these cells in culture with the addition of drugs such as 5-azacytidine [87], or even genetically engineering these cells [21] with angiogenic factors prior to intravenous infusion may prove a more advantageous option.

In this study, we have seen that the intravenous administration of MSC's, subjected to the host's humoral factors prior to arrival at the myocardial site of injury has enabled their conversion into a number of advantageous phenotypes required for cell repair. Previously, in compliance with a milieu-dependent differentiation, when MSC's were directly injected into scarred myocardium, they developed into more scar tissue. [88,89] Interestingly, Orlic et al.[56] showed that MSC's injected directly into the peri-infarct area and allowed to migrate to the scarred myocardium, also developed into cardiomyocytes. Therefore, the site of implantation of these MSC's seems crucial to their ultimate phenotype.

Those MSC's introduced into the circulation or within normal myocardium are influenced by chemokines and other unknown migratory stiumul which enable them to be attracted to an area where they participate in repair. Those directly injected into scarred myocardium will likely not receive any or a relatively smaller gradient of stimulatory factors, thereby making their conversion to cardiomyocytes difficult. These MSC's subsequently respond to the immediate environmental influences surrounding them, and become scar tissue. So if direct injection of MSC's into scar is to remain a viable therapeutic modality, perhaps pre-

programming these cells in vitro with drugs such as 5-azacytidine prior to implantation, may aid their conversion into more therapeutically desirable phenotypes.

These are all important variants which may enhance myocardial function and repair, and need to be explored in future experimental and clinical studies.

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