**TRANSMYOCARDIAL REVASCULARIZATION ENHANCES
MESENCHYMAL STEM CELL ENGRAFTMENT IN INFARCTED HEARTS THROUGH
SCF—C-KIT AND SDF-1—CXCR4 SIGNALING AXES**

by

Uswa Shahzad

**A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of the Institute of Medical Science,
University of Toronto**

© Copyright by Uswa Shahzad (2014)

ABSTRACT

TRANSMYOCARDIAL REVASCULARIZATION ENHANCES MESENCHYMAL STEM CELL ENGRAFTMENT IN INFARCTED HEARTS THROUGH SCF—C-KIT AND SDF-1—CXCR4 SIGNALING AXES

Uswa Shahzad
Master of Science
Graduate Department of the Institute of Medical Science
University of Toronto
2014

Objective: We investigated the roles of stem cell factor (SCF)—c-kit and stromal derived factor-1 (SDF-1)—CXCR4 signaling in transmyocardial revascularization (TMR)-enhanced engraftment of transplanted mesenchymal stem cells (MSCs) in infarcted hearts.

Methods: 3 weeks after LAD ligation, female Lewis rats underwent 10-channel needle TMR, followed by daily IV injections of 1-million male donor MSCs for 5 days, either wild type (WT) or with knockdown of c-kit or CXCR4. Three days and 1 week after transplantation, we evaluated MSC engraftment, SCF—c-kit and SDF-1—CXCR4 expression, and left ventricular (LV) function.

Results: MSC engraftment was affected by both TMR and donor cell type at 3 days and 1 week. LV ejection fraction improved when WT MSCs were infused, but knockdown of either receptor abrogated TMR-mediated augmentation of MSC reparative effect.

Conclusions: Downregulation of either c-kit or CXCR4 on MSC decreased engraftment of circulating MSC and inhibited reparative effects of TMR.

DEDICATION

I would like to dedicate my thesis in loving memory to my father, Shahzad Ahmad. His constant support and sacrifices over the years have been the reason for my motivation. I will always remember his help, guidance, and the love he gave me throughout life.

ACKNOWLEDGEMENTS

I would like to acknowledge the following people who were instrumental in helping me complete my thesis.

First, I would like to acknowledge my supervisor, teacher, mentor, and coach, Dr. Terrence Yau, for all his guidance and support. Without your dedication and your constant encouragement, I would not have been able to complete this thesis. Your faith in my abilities inspired me to overcome the challenges and obstacles I faced during my graduate studies. Thank you for believing in me and reminding me of my strengths. I will forever be grateful to you for providing me with the amazing opportunities to enrich my educational experience.

I would also like to acknowledge the members of my advisory committee, Dr. Ren-Ke Li and Dr. Vivek Rao, for their invaluable input and advice. Thank you for challenging me to do my very best and for offering me continued support.

I am grateful to Dr. Guangming Li for teaching me the skills and techniques needed in the laboratory. I would like to acknowledge his help with RT-PCR and the knockdown construct preparation. I am thankful to Dr. Yaoguang Zhang for his help in performing the animal surgeries and echocardiography. I would like to express my sincere gratitude to Dr. Yasuo Miyagi for training me in echocardiography and Mr. Kota Hatta for teaching me immunohistochemistry.

I greatly appreciate the administrative support and care by Susan Tarnawski, Sara Novak, Katherine Tsang, and Susannah Moore.

I would also like to thank my loving mother, Shamila Shahzad, my sister, Ramsha, and my brother, Moez, for their prayers and numerous sacrifices. Your love and constant encouragement of my interests have made it possible for me to strive for my goals. I will forever be grateful for your love and support.

I am also thankful to Mr. Nasir Haq and Mrs. Tehmina Nasir for their support and prayers, especially during the time I was writing my thesis.

I greatly appreciate the love of my wonderful husband, Moazzam. You have been my source of comfort and guidance all this time. It is with your encouragement that I dare to have big dreams.

Lastly, I would like to thank the following funding sources for providing me with research financial support: Canadian Institutes of Health Research, Cardiovascular Sciences Collaborative Program, School of Graduate Studies, and Institute of Medical Science.

TABLE OF CONTENTS

SECTION	PAGE NUMBER
Title Page	...i
Abstract	...ii
Dedication	...iii
Acknowledgements	...iv
Table of Contents	...vi
List of Tables	...xii
List of Figures	...xiii
List of Abbreviations	...xviii
List of Appendices	...xxv
Chapter One: The Ischemic Heart	...1
1.1 Statistics	...2
1.2 Myocardial Infarction and Heart Failure	...2
1.3 Pathophysiology	...4
1.3.1 Atherosclerotic Plaque Formation	...4
1.3.2 Plaque Disruption	...5
1.4 Consequences of Acute Myocardial Infarction Due to Coronary	...6
Artery Occlusion	
1.4.1 Impairment of Ventricular Function	...6
1.4.2 Metabolic Abnormalities	...6
1.4.3 Electrophysiological Changes	...7

1.4.4 Mechanical and Structural Abnormalities	...7
1.4.5 Cell Death	...8
1.5 Left Ventricular Post-Infarction Remodeling	...9
1.5.1 Collagen Degradation	...9
1.5.2 Neurohormonal Activation	...10
1.5.3 Myocardial Fibrosis	...11
1.6 Treatments	...11
1.6.1 ST Segment Elevated MI	...12
1.6.2 Non-ST Segment Elevated MI	...12
1.6.3 Summary	...13
Chapter Two: Cardiac Regeneration	...14
2.1 Evidence of Cardiac Regeneration	...15
2.1.1 Evidence of Heart Regeneration in Zebrafish and Rodent Hearts	...15
2.1.2 Evidence of Human Heart Regeneration	...16
2.2 Cardiac Regenerative Therapies	...17
2.2.1 Stem Cells and Cell Therapy	...18
2.2.2 Reprogramming Fibroblasts to Cardiomyocytes	...22
2.3 Mesenchymal Stem Cell Transplantation for Cardiac Regeneration	...23
2.3.1 Biology of Mesenchymal Stem Cells	...23
2.3.2 <i>In Vitro</i> Studies	...25
2.3.3 <i>In Vivo</i> Studies	...27

2.3.4 Clinical Trials	...28
Chapter Three: Mesenchymal Stem Cell Trafficking and Homing	...35
3.1 Mesenchymal Stem Cell Trafficking	...36
3.2 Biology of Stem Cell Factor and C-kit	...37
3.2.1 Expression of SCF and C-kit	...37
3.2.2 Isoforms of C-kit	...37
3.2.3 Isoforms of SCF	...38
3.2.4 C-kit Binding to SCF	...39
3.2.5 Functions of SCF and C-kit	...40
3.3 Biology of Stromal-Derived Factor-1 and Chemokine Receptor	...41
Type 4	
3.3.1 Expression of SDF-1 and CXCR4	...41
3.3.2 Isoforms of CXCR4	...42
3.3.3 Isoforms of SDF-1	...42
3.3.4 CXCR4 Binding to SDF-1	...43
3.3.5 Functions of SDF-1 and CXCR4	...44
Chapter Four: Transmyocardial Revascularization	...45
4.1 Procedure and Mechanism of Action	...46
4.2 Patency of the TMR Channels	...47
4.3 Cell Therapy and TMR	...48
Chapter Five: Transmyocardial Revascularization and Mesenchymal Stem Cell Engraftment	...50
5.1 Study Rationale	...51

5.2 Objectives	...52
5.3 Hypothesis	...52
5.4 Experimental Protocol	...52
Chapter Six: Materials and Methods	...55
6.1 Animal Characteristics	...56
6.2 Left Anterior Descending (LAD) Coronary Artery Ligations	...56
6.3 Transmyocardial Revascularization (TMR)	...57
6.4 Mesenchymal Stem Cell (MSC) Isolation	...57
6.5 Cell Transplantation	...58
6.6 Procuring the Hearts	...58
6.7 CXCR4 Receptor Knockdown	...59
6.8 C-kit Receptor Knockdown	...65
6.9 DNA Isolation	...67
6.10 Quantification of the Y Chromosomal DNA by Real-Time	...67
PCR	
6.11 RNA Isolation	...68
6.12 Quantification of C-kit, SCF, CXCR4, and SDF-1 by Real-time	...69
PCR	
6.13 Flow Cytometry	...70
6.14 Immunohistochemistry	...70
6.15 Vascular Densities	...72
6.16 Left Ventricular Function	...72
6.17 Statistical Analysis	...74

Chapter Seven: Results	...75
7.1 Efficiency of the Knockdown Construct	...76
7.1.1 <i>In Vitro</i> mRNA Expression of C-kit or CXCR4	...76
7.1.2 <i>In Vitro</i> Cell Surface Expression of C-kit or CXCR4	...79
7.2 <i>In Vivo</i> Donor MSC Engraftment After Delivery	...84
7.2.1 Quantification of Donor MSC Engraftment via mRNA Expression of sry2	...84
7.2.2 Qualitative Analysis of Donor MSC Engraftment via Immunohistochemistry	...88
7.3 <i>In Vivo</i> mRNA Expression of Stem Cell Signaling Axes After MSC Delivery	...91
7.3.1 <i>In Vivo</i> mRNA Expression of Stem Cell Factor (SCF)	...91
7.3.2 <i>In Vivo</i> mRNA Expression of C-kit	...93
7.3.3 <i>In Vivo</i> mRNA Expression of Stromal-Derived Factor-1 (SDF-1)	...95
7.3.4 <i>In Vivo</i> mRNA Expression of Chemokine Receptor Type 4 (CXCR4)	...97
7.4 Vascular Densities After MSC Delivery	...99
7.4.1 Capillary Density (Factor VIII)	...99
7.4.2 Arteriolar Density (α -SMA)	...99
7.5 Left Ventricular Function	...102
Chapter Eight: Discussion	...104
8.1 Summary	...105

8.2 Knockdown Construct Reduces <i>In Vitro</i> mRNA and Cell Surface Expression of C-kit and CXCR4	...106
8.3 TMR Increases Donor MSC Engraftment	...108
8.4 TMR Increases Expression of Signaling Cytokines	...111
8.5 Effect of TMR on Vascular Densities	...114
8.6 TMR Improves Left Ventricular Function Only When SCF—C-kit and SDF-1—CXCR4 Axes are Intact	...115
8.7 Conclusions and Clinical Implications	...117
8.8 Study Limitations	...118
8.9 Future Directions	...119
Chapter Nine: References	...120
9.1 References	...121
Appendix 1	...135
Transmyocardial Revascularization Induces Mesenchymal Stem Cell Engraftment in Infarcted Hearts	...136

LIST OF TABLES

Table 1: Summary of selected stem cell clinical trial results.

...Page 34

Table 2: *In vitro* mRNA expression of c-kit, CXCR4, and 18s rRNA (housekeeping) genes one and two weeks after knockdown.

...Page 78

LIST OF FIGURES

- Figure 1:** C-kit or CXCR4 knockdown mesenchymal stem cell transplantation with or without transmyocardial revascularization experimental study outline.
...Page 54
- Figure 2:** Summary of the CXCR4 receptor knockdown process.
...Page 64
- Figure 3:** Summary of the c-kit receptor knockdown process.
...Page 66
- Figure 4:** The above flow cytometry dot plots show a representative distribution of GFP+ MSCs and c-kit+ MSCs in only one sample per group. On average, the percentage of both GFP+ and c-kit+ MSCs was more than eight fold lower in the c-kit shRNA+plasmid+lentivirus group, as compared to the empty cassette and untreated MSCs at both 1 and 2 weeks time points (N=3/group).
...Page 80
- Figure 5:** The c-kit shRNA+plasmid+lentivirus construct was significantly able to knockdown the average cell surface expression of c-kit compared to untreated MSCs and empty cassette group at both 1-week and 2-week time points ($P<0.01$) (N=3/group).
...Page 81
- Figure 6:** These flow cytometry dot plots show a representative distribution of GFP+ MSCs and CXCR4+ MSCs in only one sample per group. On average, the percentage of both GFP+ and CXCR4+ MSCs was approximately 10-fold lower in the CXCR4

shRNA+plasmid+lentivirus group, as compared to the empty cassette and untreated MSCs at both 1 and 2 weeks time points (N=3/group).

...Page 82

Figure 7: The CXCR4 shRNA+plasmid+lentivirus construct was significantly able to knockdown the average cell surface expression of CXCR4 compared to untreated MSCs and empty cassette group at both 1-week and 2-week time points ($P<0.01$) (N=3/group).

...Page 83

Figure 8: Three days after the last MSC injection (N=6/group), the number of MSCs that had homed into the infarct was affected by both TMR and donor cell type (TMR, cell type and interaction, all $P<0.01$) Male donor MSC engraftment was greater in groups that underwent TMR. However, the knockdown of c-kit or CXCR4 receptor resulted in decreased MSC engraftment despite TMR.

...Page 86

Figure 9: One week after the last MSC injection was performed (N=6/group), the number of MSCs that had homed into the infarct was affected by both TMR and cell type (TMR, cell type, both $P<0.05$; interaction, $P=0.37$). Male donor MSC engraftment was still greater in groups that underwent TMR. However, the knockdown of c-kit or CXCR4 receptor resulted in decreased MSC engraftment despite TMR.

...Page 87

Figure 10: Pictomicrographs (200X magnification) of scar tissue from 3-day recipient hearts (N=6/group), immunostained to visualize engraftment of male donor MSCs, using antibodies directed against GFP (green), and DAPI (blue). The highest engraftment occurred

in the no TMR—WT and TMR—WT groups (A, B). The loss of c-kit (C, D) or CXCR4 (E, F) receptors resulted in decreased MSC engraftment, with or without TMR.

...Page 89

Figure 11: Pictomicrographs (200X magnification) of scar tissue from 1-week recipient hearts (N=6/group), immunostained to visualize engraftment of male donor MSCs, using antibodies directed against GFP (green), and DAPI (blue). The highest engraftment occurred in the no TMR—WT and TMR—WT groups (A, B). The loss of c-kit (C, D) or CXCR4 (E, F) receptors resulted in decreased MSC engraftment, with or without TMR.

...Page 90

Figure 12: At three days, the mRNA expression of SCF (N=6/group per time point) was significantly upregulated due to TMR and MSC cell type (TMR and cell type $P<0.01$; interaction $P=0.41$). At 1 week, the expression of SCF dropped across all groups, but was upregulated in groups that received TMR (TMR $P=0.02$; cell type $P=0.47$; interaction $P=0.78$).

...Page 92

Figure 13: The mRNA expression of c-kit (N=6/group per time point) was significantly upregulated at three days in the groups that underwent TMR (TMR $P<0.01$; cell type $P=0.26$; interaction $P=0.84$). At 1 week, the expression of c-kit decreased across all groups, and was affected only by the type of cells that were transplanted (TMR $P=0.79$; cell type $P=0.02$; interaction $P=0.58$).

...Page 94

Figure 14: Three days after the last MSC injection, the expression of SDF-1 (N=6/group per time point) was upregulated due to both TMR and cell type (TMR $P<0.01$; cell type

P=0.04; interaction P=0.43). At 1 week, the expression of SDF-1 was upregulated in the groups that received TMR (TMR P<0.01; cell type P=0.64; interaction P=0.69).

...Page 96

Figure 15: The mRNA expression of CXCR4 (N=6/group per time point) was significantly upregulated at 3 days due to TMR and cell type (TMR P<0.01; cell type P<0.03; interaction P=0.44). At 1 week, all of the groups experienced a dramatic drop in the expression of CXCR4, but was still upregulated in the groups that received TMR (TMR P<0.01; cell type P=0.02; interaction P=0.37).

...Page 98

Figure 16: No significant differences were detected in capillary density (factor VIII) amongst groups at both 3 days (TMR P=0.52; cell type P=0.54; interaction P=0.97) and at 1 week (TMR P=0.89; cell type P=0.41; interaction P=0.73) (N=5x5/group).

...Page 100

Figure 17: No significant differences were detected in α -smooth muscle actin (α -SMA) amongst groups at both 3 days (TMR P=0.71; cell type P=0.64; interaction P=0.92) and at 1 week (TMR P=0.48; cell type P=0.44; interaction P=0.96) (N=5x5/group).

...Page 101

Figure 18: In the 3-day rats (N=6/group), before the LAD ligation, LVEF was different due to cell type (TMR P=0.84; cell type P=0.02; interaction P=0.99). Three weeks after the LAD ligation was performed, all the groups experienced a similar decline in LVEF (TMR P=0.58; cell type P=0.20; interaction P=0.67). Three days after the last MSC injection, no significant differences were detected in LVEF (TMR P=0.70; cell type P=0.31; interaction P=0.94). In the 1-week rats (N=6/group), before the LAD ligation was performed, LVEF was different

due to cell type (TMR $P=0.75$; cell type $P=0.03$; interaction $P=0.08$). All of the groups experienced a similar decline in LVEF (TMR $P=0.48$; cell type $P=0.91$; interaction $P=0.46$) one week after ligation. However, one week after the last MSC injection, LVEF was improved due to TMR and cell type (TMR $P=0.01$; cell type $P<0.01$; interaction $P=0.54$).

...Page 103

LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ϵ	Epsilon
ζ	Zeta
κ	Kappa
μg	Microgram
μL	Microliter
μM	Micrometer
kg	Kilogram
mg	Microgram
mL	Milliliter
ρM	Picomoles per liter
ABC	ATP-binding cassette
ACE	Angiotensin-converting enzyme
AMI	Acute MI
Ang II	Angiotensin II

ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ARB	Angiotensin II receptor blocker
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
bFGF	Basic fibroblast growth factor
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
BMC	Bone marrow cell
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
C	Carbon
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CD	Cluster of differentiation
CDC	Cardiosphere-derived cell
cDNA	Complementary DNA
CO ₂	Carbon dioxide
CSC	Cardiac stem cell
CSP	Cardiac side population cell
CVD	Cardiovascular disease
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T

Ctrl	Control
CXCL12	Chemokine ligand type 12
CXCR4	Chemokine receptor type 4
CXCR7	Chemokine receptor type 7
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	Distilled deionized water
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EC	Endothelial cell
ECG	Electrocardiography
ECM	Extracellular matrix
EDV	End-diastolic volume
EGF	Epidermal growth factor
eGFP	Enhanced GFP
EF	Ejection fraction
EPC	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinase
ESV	End-systolic volume
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FAS	Fractional area shortening
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
FS	Fractional shortening
G-CSF	Granulocyte colony-stimulating factor
GATA4	GATA binding protein 4
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
HEK	Human embryonic kidney
HF	Heart failure
HGF	Hepatocyte growth factor
HIF-1 α	Hypoxia-inducible factor-1 α
HLA	Human leukocyte antigen
HMGB-1	High mobility group box-1
Ho:YAG	Holmium:yttrium-aluminum-garnet
HSC	Hematopoietic stem cell
HUVEC	Human umbilical vascular endothelial cell
IdU	Iododeoxyuridine
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMDM	Iscoe's Modified Dulbecco's Medium
INF	Interferon
iPSC	Induced pluripotent stem cell
IV	Intravenous

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
kDa	Kilo Dalton
KLF4	Kruppel-like factor 4
LAD	Left anterior descending coronary artery
LB	Lysogeny broth
LV	Left ventricle
LVEF	Left ventricular ejection fraction
LVEDA	Left ventricular end-diastolic area
LVEDD	Left ventricular end-diastolic diameter
LVEDV	Left ventricular end-diastolic volume
LVESA	Left ventricular end-diastolic area
LVESD	Left ventricular end-diastolic diameter
LVESV	Left ventricular end-systolic volume
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEF2C	Myocyte enhancer factor 2C
MHC	Myosin heavy chain
MI	Myocardial infarction
MMLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase

mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NCBI	National Center for Biotechnology Information
NE	Norepinephrine
NF- κ B	Nuclear factor- κ B
NSTEMI	Non-ST elevation MI
Oct4	Octamer-binding transcription factor 4 (or POU5F1)
PBS	Phosphate-buffered saline
PC	Progenitor cell
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFU	Plaque formation unit
PI3K	Phosphoinositide 3-kinase
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RV	Right ventricle
RyR2	Ryanodine receptor 2
SCF	Stem cell factor
SDF-1	Stromal derived factor-1
SERCA2	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SFK	Src family of kinases
shRNA	Short hairpin RNA

SMC	Smooth muscle cell
SOX2	Sex-determining region Y-box 2
Sry	Sex-determining region on the Y chromosome
STAT	Signal transducer and activator of transcription
STEMI	ST elevation MI
TBS	Tris-buffered saline
Tbx5	T-box transcription factor 5
TGF- β 1	Transforming growth factor- β 1
TIMP	Tissue inhibitors of metalloproteinase
TMR	Transmyocardial revascularization
TNF- α	Tumor necrosis factor- α
VBI	VEGF, bFGF, and IGF-1
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
WT	Wild type

LIST OF APPENDICES

Appendix 1: Transmyocardial revascularization induces mesenchymal stem cell engraftment in infarcted hearts

...Page 135

CHAPTER ONE

THE ISCHEMIC HEART

1.1 STATISTICS

Ischemic heart disease (IHD) is one of the leading causes of death in Canada. In 2008, 15% of all deaths in Canada were attributed to IHD.¹ Heart failure was responsible for 2% of all deaths.¹ Furthermore, 54% of all of cardiovascular deaths were due to chronic ischemic heart disease, whereas another 23% were due to acute myocardial infarction.¹ In 2005, there were 160,323 hospitalizations due to IHD, which was the most common reason for hospitalizations in Canada, whereas 60,996 hospitalizations were due to myocardial infarction.² On the other hand, 54,333 hospitalizations were due to congestive heart failure in 2005.² It is estimated that \$22.2 billion were spent on health care costs related to all types of cardiovascular disease (including stroke).² Therefore, ischemic heart disease causes a significant economic burden and as such, constitutes a major public health concern.

1.2 MYOCARDIAL INFARCTION AND HEART FAILURE

Coronary artery disease (CAD), or ischemic heart disease, is a chronic condition that results in a perfusion imbalance between supply and demand of the heart due to a coronary occlusion. A myocardial infarction (MI) is a manifestation of unstable CAD, and occurs when there is immediate loss of function and death of cardiomyocytes due to obstruction of blood flow. While most of the myocardial infarctions occur in the left ventricle (LV), right ventricular (RV) MI can also occur, but are less common. There are two classifications of MI based on the pathology. The NSTEMI, or non-ST elevation MI, usually involves a small area of the subendocardial wall of the left ventricle, intraventricular septum, or the papillary

muscles, and is marked by ST segment depression on the electrocardiogram (ECG). The STEMI, or ST-elevation MI, usually involves a major coronary artery and results in a transmural infarct, extending through the entire thickness of the ventricular muscle. Furthermore, transmural infarcts are characterized on the ECG by abnormal Q waves, hyperacute T waves, an elevation of the ST segment, and a depression of the TP segment.³

Recently, a consensus report published by the joint task force of the European Society of Cardiology, the American College of Cardiology, the American Heart Association, and the World Heart Federation further classified myocardial infarction based on clinical manifestations. Type 1 MI is a spontaneous infarct that results from a primary coronary event, such as plaque rupture, whereas type 2 MI is secondary to ischemia that occurs due to imbalance in oxygen demand and supply. On the other hand, type 3 MI is an infarct that results in sudden cardiac death, including cardiac arrest, that occurs even before cardiac biomarkers can be detected in blood samples. Type 4a is a MI that is associated with percutaneous coronary intervention (PCI), while type 4b occurs due to stent thrombosis. Lastly, type 5 MI is associated with coronary artery bypass graft (CABG). At times, patients may experience more than one type of MI either simultaneously or sequentially.⁴ The clinical manifestation of MI largely determines the course of treatment and management of the infarct.

On the other hand, heart failure (HF) is a potentially fatal syndrome that causes progressive dysfunction of the pump action of the heart. It is characterized by a reduction in cardiac output, an increase in venous pressure, and impaired systolic or diastolic function of the myocardium. Most patients who present clinically with heart failure experience impairment of left ventricular function as a result of dilated cardiomyopathies secondary to

ischemia or hypertensive heart disease. Right heart failure, in comparison, is much less common, and may be secondary due to left-sided heart disease, cor pulmonale, or primary pulmonary hypertension.³ The progressive deterioration of the ventricular myocardium after a MI may lead to heart failure over time.

1.3 PATHOPHYSIOLOGY

1.3.1 Atherosclerotic Plaque Formation

A myocardial infarction occurs due to a coronary occlusion, usually triggered by the rupture of an atherosclerotic plaque. The formation of an atherosclerotic plaque is a slow and gradual process, and is initiated by dysfunction of the arterial endothelium. Chronic injury to the arterial endothelium may occur due to disturbances in blood flow patterns, hypercholesterolemia, dyslipidemia, hyperglycemia, chemical irritants in tobacco smoke, or the presence of other proinflammatory cytokines.^{5,6} The arterial wall injury causes the endothelium to become permeable, allowing leukocyte migration, while augmenting expression of chemoattractants that promote leukocyte adhesion to the arterial wall. Furthermore, smooth muscle cell (SMC) migration from the tunica media to the tunica intima results in secretion of various matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs). The MMPs and TIMPs contribute to plaque formation by modulating geometric remodeling and further destruction of extracellular matrix of the arterial walls.⁷

In a parallel fashion, monocytes infiltrate the tunica intima of the arterial wall, while proliferating and differentiating into macrophages. The macrophages then absorb the low-density lipoprotein molecules from the bloodstream and become “foam cells.” Due to over accumulation of lipids, foam cells undergo apoptosis and form a necrotic core.⁷ Moreover, release of cytokines by the necrotic core induces SMCs to form a fibrous cap over the lesion to prevent it from rupturing, creating a fibrointimal lesion.⁵⁻⁷

1.3.2 Plaque Disruption

It is the disruption of an atherosclerotic plaque with resultant thrombosis that may lead to an acute MI (AMI). Some foam cells may secrete proteases that disrupt the fibrous cap, releasing the contents of the plaque into the bloodstream. As a result of the plaque rupture, thrombogenic elements such as thromboplastin are released into the bloodstream, causing aggregation of platelets at the sites of damaged smooth muscle layer and subsequent formation of a thrombus, resulting in a partial or total coronary occlusion.⁵⁻⁷ Interestingly, some atherosclerotic lesions, mostly stable plaques, repeatedly develop fissures and internalize thrombi, without complete rupture of the fibrous cap. The factors that may completely disrupt an atherosclerotic plaque include: increase in shear forces in the area, sudden changes in intraluminal pressure, or bending/twisting of coronary artery during myocardial contractions. Furthermore, stress from within the plaque combined with stress produced by the disrupted blood flow in the area of stenosis may contribute to plaque rupture.^{5,6}

1.4 CONSEQUENCES OF ACUTE MYOCARDIAL INFARCTION DUE TO CORONARY ARTERY OCCLUSION

1.4.1 Impairment of Ventricular Function

After an acute myocardial infarction, patients generally experience severe angina, followed by the loss of viable cardiomyocytes and impaired pump function of the heart, and may also display signs of arrhythmias. When a coronary occlusion occurs, the disruption in blood flow causes a decrease in contractility, resulting in an imbalance in intraventricular pressure between the normally perfused myocardium and the ischemic region. Therefore, a part of that ischemic region is forced to bulge outward during diastole; consequentially, systolic function of the heart and ejection are significantly impaired.^{3,8}

1.4.2 Metabolic Abnormalities

As the myocardial tissue becomes ischemic after an AMI, it also becomes increasingly hypoxic, and experiences metabolic changes such as cessation of oxidative phosphorylation and a rapid decrease in ATP content. Hence, anaerobic energy production is increased in the ischemic heart, and results in an accumulation of lactate. In addition, byproducts of ATP hydrolysis (protons and inorganic phosphates) begin to build up within the heart, as they cannot be removed due to decreased coronary blood flow, leading to a rapid fall in the pH and subsequent acidification of the heart. The phosphate and lactate molecules are released from ischemic cardiomyocytes and into the extracellular space, followed by an

efflux of potassium cations. The result is a failure of the sodium-potassium channel as well as calcium channel inactivation, leading to abnormal membrane depolarization and possibly arrhythmias.³

1.4.3 Electrophysiological Changes

In addition to arrhythmias, ischemia causes electrophysiological abnormalities that may become evident on the electrocardiogram. In a transmural MI, the ischemic myocardium is depolarized, resulting in an elevated ST segment during diastole. This also causes a depression of the TP segment and abnormal Q waves during ECG recording.³ On the other hand, in a subendocardial MI, the diastolic injury current occurs in the endocardium that is separated from the epicardial surface by a layer of normally perfused endocardium. Therefore, during ECG recording, the partially depolarized ischemic endocardium produces a ST segment depression and TP segment elevation.^{3,8} The electrocardiographic characterization of an AMI may help localize the infarct, which influences the course of treatment.

1.4.4 Mechanical and Structural Abnormalities

An acute myocardial infarction may also cause structural and mechanical abnormalities of the heart. Asynchrony may occur due to the failure of the infarcted region to generate contraction in a timely sequence, whereas akinesis refers to the inability of the segment to participate in ejection. The affected segment may demonstrate hypokinesis, with

reduced inward movement, or dyskinesis, where the ischemic region bulges outward during systole.³ The decrease in blood supply after an AMI may cause the area of the infarct to enlarge, causing infarct extension. In comparison, infarct expansion occurs when the left ventricular wall is dilated because of myofilament slippage. In some cases, high blood pressure may cause the layers of left ventricular wall to separate and cause cardiac rupture. In addition, due to ischemia induced pH changes and calcium overload there is a decrease in contractile cross bridge formation, which may lead to contractile dysfunction of the myocardium.³

1.4.5 Cell Death

The hallmark of a myocardial infarction is cell death due to necrosis, followed by apoptosis. Necrosis is characterized by the failure to maintain cell membrane integrity due to lack of energy supply, accompanied by the inability to maintain oncotic gradient, hence resulting in cell swelling and death. Apoptosis, on the other hand, is triggered by either intrinsic or extrinsic death signaling pathways, activating a series of caspases downstream to execute orderly sequence of cell death and DNA fragmentation.³

At five minutes post-infarction, the spaces between myofibrils start to increase. Fifteen minutes after the myocardial blood supply has been cut off, evidence of cell separation begins to emerge. Between four and five hours post-infarct, myofibrils start to tear and sarcolemma ruptures, leaking intracellular proteins such as transaminases, creatine phosphokinase, and troponin components into the bloodstream. About six hours after the infarct, only a few cells remain viable in center of a transmural infarct region.^{3,9} Besides

necrosis, programmed cell death by activation of pro-apoptotic pathways also contributes to loss of cardiomyocytes after prolonged ischemia. Certain stimuli, such as release of cytochrome C by damaged mitochondria, may activate pro-apoptotic pathways and further contribute to myocardial cell death.³

1.5 LEFT VENTRICULAR POST-INFARCTION REMODELING

1.5.1 Collagen Degradation

The left ventricular remodeling starts almost immediately after an infarct. Loss of viable cardiomyocytes due to necrosis triggers a series of intracellular biochemical signaling pathways that aim to repair the ischemic tissue. Within hours of myocyte injury, neutrophils infiltrate the injured tissue and release MMPs, along with zinc-dependent collagenases and serine proteases. Collagen breakdown begins within three hours of MI and may continue as long as 14 days, leading to slippage of necrotic myofilaments, and subsequently, infarct expansion and ventricular dilatation. The activity of MMPs has been detected as early as 2 days post-infarct, and peaks at 7 days. On the other hand, TIMPs, which inhibit MMPs and restrict collagen lysis, have been shown to peak at day 2 and return to normal at 14 days post-infarct.^{10,11}

1.5.2 Neurohormonal Activation

After an infarct, overall cardiac output is decreased. In order to compensate, the adrenal medullae release norepinephrine (NE) to activate the sympathetic drive and maintain cardiac output. However, this results in increased loading in the left ventricle, further aggravating infarct expansion. Increased serum NE levels contribute directly to LV hypertrophy by stimulating $\alpha 1$ adrenoreceptors and activating G α q-dependent signaling pathway. Norepinephrine may also stimulate $\beta 1$ adrenoreceptors in the juxtaglomerular apparatus to release renin and activate the renin-angiotensin-aldosterone system (RAAS). The RAAS may also be activated directly in the myocardium, and directly affect hypertrophy and fibrosis.¹¹

On the other hand, elevated levels of renin increase production of angiotensin II (Ang II), which promotes secretion of endothelin-1 (ET-1).¹¹ ET-1, combined with other growth-promoting peptides released by ischemic tissue, such as insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), or cardiotrophin I activate cascades of intracellular proliferative and anti-apoptotic signaling pathways like phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathway.^{10,11}

In addition to promoting LV hypertrophy, ET-1 augments production of natriuretic peptides. The release of both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) transiently improves LV function by improving fluid balance. Interestingly, both ANP and BNP have been shown to inhibit LV hypertrophy in the long term.¹⁰

1.5.3 Myocardial Fibrosis

Myocardial fibrosis is primarily driven by cytokines that are released during early remodeling process. The extracellular matrix forms a collagen scar to stabilize distension and prevent deformation of the LV. After MI, transforming growth factor- β 1 (TGF- β 1) levels increase, promoting fibroblast migration and proliferation to the site of the infarct. In addition, formation of a fibrin-fibronectin matrix allows fibroblasts to adhere to it. Almost simultaneously, γ -interferon (γ -INF) activates the macrophage population to produce nitric oxide. As a result, vascular permeability is increased and the inflammatory response is contained within the infarct zone.^{10,11}

Furthermore, co-expression of aldosterone, ANP, ET-1, and Ang II in the presence of nitric oxide induces transcription of collagen type I and type III mRNA. At 7 days post-infarction, type III and type I collagen start to deposit and form scar tissue, and by day 28, almost all of the necrotic myocytes have been replaced by fibrous tissue. The scar formation continues until the infarct is stabilized, and then collagen formation is downregulated and myofibroblasts are signaled to undergo apoptosis.¹¹

1.6 TREATMENTS

The treatment of MI is dependent on the clinical profile and manifestation of the symptoms. The goal of initial treatment is to relieve pain and manage hemodynamic abnormalities. If the patient has a ST-segment elevation MI, s/he may be a candidate for reperfusion therapy.

1.6.1 ST Segment Elevated MI

The diagnosis of ST segment elevated MI indicates a transmural infarct. Therefore, it requires immediate reperfusion, either through intravenous thrombolytic therapy or via percutaneous coronary intervention (PCI). The American College of Cardiology and American Heart Association recommend the use of intravenous fibrinolytic therapy within 30 minutes of when a patient arrives at the emergency department. On the other hand, PCI can be performed within 90 minutes of patient's arrival to the hospital, and can improve reperfusion rate by approximately 90%. In some cases, patients may need immediate CABG surgery.¹²⁻¹⁴

Along with reperfusion therapy, nitroglycerin and morphine are used to relieve chest pain. The patient may be started on an anti-platelet therapy regimen consisting of: aspirin, thienopyridine (platelet ADP receptor blockers), or glycoprotein IIb/IIIa antagonists (block IIb/IIIa receptors that activate fibrinogen). In addition, patients may be given anticoagulants, such as unfractionated heparin or low molecular weight heparin, to prevent reocclusion and reinfarction. Furthermore, to reduce LV remodeling and prevent heart failure, patients may be administered a combination of: β -adrenergic blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), statins, or nitrates.¹²⁻¹⁴

1.6.2 Non-ST Segment Elevated MI

A non-ST segment elevated MI indicates a subendocardial infarct; hence, it must be treated differently than a STEMI. The initial therapy focuses on pain management by morphine, stabilization of hemodynamics, and maintenance of oxygen saturation above 90%.

The patients are also given dual antiplatelet therapy to prevent clot propagation. Moreover, nitrates and β -adrenergic blockers are administered to manage ischemia. Lastly, the patients undergo coronary angiography to determine the extent of stenosis, and are then risk stratified based on the persistence of symptoms and elevated cardiac markers. Patients who have less than 50% of coronary stenoses and small infarcts may be treated with only medical therapy. However, patients who are classified as high-risk with greater degrees of coronary stenoses and significant areas of myocardium at risk may be candidates for revascularization via either PCI or CABG.¹²⁻¹⁴

1.6.3 Summary

Recent improvements in therapeutic options have significantly reduced mortality and morbidity associated with acute coronary syndromes. However, even multimodal therapy consisting of invasive procedures and pharmacotherapies has been unable to replace necrotic cardiomyocytes in ischemic tissue, and restrict further deterioration of LV function and its progression to HF. There is a need for further therapies that seek to replace lost cardiomyocytes and restore LV function so HF can be prevented.

CHAPTER TWO

CARDIAC REGENERATION

2.1 EVIDENCE OF CARDIAC REGENERATION

The heart is one of the least regenerative organs in the body. In fact, it was initially thought that cardiomyocytes were terminally differentiated and that the heart was incapable of regeneration. However, recently, some evidence has emerged which suggests otherwise.

2.1.1 *Evidence of Heart Regeneration in Zebrafish and Rodent Hearts*

Zebrafish are capable of regenerating limbs, appendages, and even internal organs after injury. After injury, they can regenerate *de novo* heart tissue. Jopling and colleagues studied cardiac regeneration in a zebrafish model by surgically amputating the apex of zebrafish hearts.¹⁵ After amputation, the injured area was initially sealed by a fibrin clot. However, 30 days after amputation, the area was replaced by *de novo* regenerated heart tissue. Furthermore, new myocardium in the apex was generated from expansion of pre-existing cardiomyocytes, rather than from progenitor cells.¹⁵

Using a genetic fate-mapping model, Hsieh and colleagues were able to demonstrate cardiac regeneration in rodent hearts.¹⁶ In this model, Cre-loxP system was able to fluorescently label cardiomyocytes with green fluorescent protein (GFP), after a tamoxifen pulse was introduced. Hsieh et al. found that after tamoxifen treatment, approximately 80% of the cardiomyocytes remained GFP+, while other 20% were GFP-. Even after 1 year of normal aging, the ratio of GFP+ cardiomyocytes remained consistent, despite tamoxifen pulse, indicating that progenitor cells did not play a significant role in replenishing the cardiomyocyte pool in normal cells. However, after a myocardial infarction, the number of

GFP+ cardiomyocytes was 65% in the border zone of the infarct, while other 35% of cardiomyocytes were GFP-, which suggested that during injury, progenitor and stem cells play an important role in replenishing cardiomyocytes.¹⁶

2.1.2 Evidence of Human Heart Regeneration

The human heart has limited regenerative capacity. After birth, cardiomyocytes proliferate for at least two months, until the normal human adult number of approximately 2 billion cardiomyocytes is reached. This cardiomyocyte nuclear number remains steady in normal hearts, increasing only in hypertrophied hearts. In comparison, the number of non-cardiomyocyte cells in the heart, such as fibroblasts and SMC, increases linearly with heart weight throughout life. Cardiomyocyte renewal, on the other hand, usually occurs during pathological hypertrophy.¹⁷

Some recent mathematical models have suggested that cardiomyocyte renewal may actually occur throughout an individual's life span. To determine the rate of cardiomyocyte renewal, Bergmann et al. studied the hearts of individuals who were exposed to ¹⁴C during the Cold War.¹⁸ The ¹⁴C had become incorporated into the DNA of these individuals, and therefore, provided a way for the authors to date the cells and determine their origin. As expected, Bergmann et al. found that compared to cardiomyocytes, non-cardiomyocytes were much younger than the patients. Mathematical modeling suggested that in normal human hearts, the non-cardiomyocytes had a ~18% turnover rate per year. In comparison, cardiomyocyte renewal was found to be age dependent, with only an ~1% turnover rate per year in individuals under 20, and by age 75, only 0.4% of cardiomyocytes could be expected

to renew per year. Bergmann et al. proposed that during a normal human lifespan, ~55% of cardiomyocytes could be expected to have survived since birth, whereas another ~45% would be the ones renewed over time.¹⁸

In another study conducted by Kajstura et al., the authors examined post-mortem hearts from individuals who had died from cancer.¹⁹ These patients had been treated with iododeoxyuridine (IdU), which is a thymidine analogue and gets incorporated into nascent DNA. Kajstura et al. were able to detect the IdU signal and identify cardiomyocytes and determine their turnover rate. Based on mathematical modeling, the authors suggested that the cardiomyocytes were being renewed at a rate of 22% per year, whereas fibroblasts were renewed at a rate of 20% per year, and endothelial cells had a turnover of 13% per year.¹⁹ While the data from these two studies conflicts regarding the magnitude of cardiomyocyte renewal in adults, they provide evidence for possible cardiomyocyte proliferation in what were originally thought to be terminally differentiated cells.

2.2 CARDIAC REGENERATIVE THERAPIES

The progressive loss of cardiomyocytes after a myocardial infarction and adverse left ventricular remodeling may cause further deterioration of the left ventricular function and predispose the heart to chronic HF. The ventricular remodeling events may cause infarct expansion, myocardial hypertrophy and fibrosis, cell apoptosis, which may lead to thinning of the left ventricular wall and LV dilatation. The increased demands on the surviving myocardium further develop wall tension and result in an increase in left ventricular end-systolic volume and a decrease in end-diastolic volume. Therefore, the left ventricular

ejection fraction, which represents the volumetric fraction of blood that is pumped out of the left ventricle during each cardiac cycle, decreases.^{10,11}

The current clinical therapies, including reperfusion therapy and myocardial protective agents, have come a long way in preventing against progressive loss of cardiomyocytes. Over time, however, due to the release of proteolytic enzymes by neutrophils, activation of matrix metalloproteinases, and neurohormonal activation, cardiac function continues to deteriorate.^{10,11}

Several therapies are currently being investigated with the aim to replenish cardiomyocytes and regenerate the myocardium. The rationale behind this hypothesis is that by regenerating the damaged cardiac tissue through cell transplantation, the LV wall thinning and infarct size can be decreased, thus improving cardiac function.

2.2.1 Stem Cells and Cell Therapy

Cell transplantation has been investigated extensively as a potential novel therapy for ischemic heart disease. Many different cell types, including pluripotent and adult stem cells, have demonstrated therapeutic value for the repair of infarcted tissue.

The pluripotent stem cells, mainly embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs), possess the capacity to differentiate into almost any cell type, including cardiomyocytes. Cardiomyogenic differentiation from ESCs closely resembles the cardiac development in the embryo. In fact, cardiomyocytes derived from human ESCs have been shown to express early cardiac-specific transcription factors, such as Nkx2.4.

Furthermore, they also express sarcomeric proteins, connexins, calcium channels, and show functional properties similar to those of developing cardiomyocytes.^{17,20}

Similarly, iPSCs have also been demonstrated to differentiate into myogenic cells. The iPSCs have been generated by reprogramming adult somatic cells through the delivery of transcription factors via viral vectors. Although, both iPSCs and ESCs are capable of differentiating into cardiomyogenic cells, there are also challenges associated with using cells with unrestricted pluripotent potential. Both iPSCs and ESCs can undergo uncontrolled proliferation, are tumorigenic, can form teratomas, and may undergo chromatin mutations and epigenetic modifications with later passages. Furthermore, since human ESCs are transplanted allogeneically, they also require immunosuppression. On the other hand, even though iPSCs may be isolated through autologous source, their reprogramming may cause them to become immunogenic.^{17,20} Hence, the adverse events associated with ESC and iPSC transplantation in animal models have raised safety and ethical concerns about the use of these cell sources clinically in regenerative medicine, which are yet to be conclusively settled.

In comparison, adult stem cell transplantation has shown promising results both in animal models and in clinical trials. Endothelial progenitor cells (EPC) have been extensively studied for therapy in ischemic heart disease due to their potential to stimulate angiogenesis and improve vascular repair. Based on the length of culture and the protocols used to obtain these cells, EPCs have been divided into “early EPCs,” and “late EPCs.”²¹ The “early EPCs” have been shown to contribute to vasculogenesis through release of proangiogenic growth factors, whereas the “late EPCs” have the capacity to differentiate into endothelial cells and thus participate directly in angiogenesis.²²⁻²⁴ Because of the variability

in biomarker expression of EPCs, as well as the gene expression overlap between endothelial and hematopoietic progenitor cell phenotypes, it has been challenging to distinguish and define a true EPC population. In addition, the endothelial differentiation potential of EPCs is largely influenced by the surrounding milieu and varies greatly according to local environment. Therefore, the therapeutic applications of EPCs are limited by the current knowledge and functional characterization of these cells.²¹

Bone marrow-derived cells, specifically mesenchymal stem cells (discussed below), have shown beneficial effects in improvement of cardiac function. Another source of multipotent adult stem cells, i.e., c-kit⁺ cardiac stem cells (CSC) have also demonstrated cardiogenic potential. Several distinct classes of CSCs have been identified, including ISL1⁺ progenitor cells, epicardial progenitors, cardiosphere-derived cells (CDCs), Sca1⁺ progenitors, and cardiac side population cells (CSP).²⁵ The CSC population of cells is cardiac-resident and is stored in niches where the stem cells can remain undifferentiated and receive growth signals from supporting cells. It is upon activation that these lineage negative cells proliferate and differentiate into myocytes, vascular SMCs, and endothelial cells (ECs). CSCs have been shown to regenerate cardiomyocytes that possess normal functional and mechanical properties. Furthermore, CSCs are able to repair coronary vessels, restore structural integrity of the infarcted tissue, and improve LV function.²⁵

While the CSC population of cells is capable of cardiac repair after an infarct, due to its limited numbers, it is unable to prevent deterioration of LV function and progression of HF.^{20,25} However, in the SCIPIO randomized open-label trial, intracoronary delivery of a c-kit⁺ CSC population resulted in marked improvement in LV function in patients with heart failure. The CSCs were isolated from patients who were undergoing CABG, expanded *ex*

vivo, and then percutaneously infused into the scar tissue four months later. Four and 12 months after cell infusion, the patients treated with CSCs showed a significant improvement in left ventricular ejection fraction (LVEF), a decrease in infarct size, and an increase in LV viable mass.²⁶ The ability of CSCs to regenerate cardiac tissue and improve LV function makes them an attractive option for cell transplantation.

Another population of adult stem cells, the cardiosphere-derived cells, is also widely studied and has shown great promise in cardiac regeneration. CDCs are derivatives of cardiospheres, which consist of a core of c-kit⁺ cells, several layers of differentiating progenitor cells, cardiac myocyte-like cells, and vascular cells, and an outer sheet of mesenchymal stromal cells.^{25,27} The c-kit⁺ and Sca1⁺ subset of cells from these cardiospheres have demonstrated cardiomyogenic properties. The safety of CDC transplantation has been demonstrated in a randomized, phase 1 trial. In the CADUCEUS trial, CDCs were obtained through endomyocardial biopsy and were infused 3 months after MI into the infarct-related artery through an angioplasty catheter. Six months after coronary infusion, patients that received CDCs showed significant reductions in myocardial scar and increases in viable heart mass, contractility, and systolic wall thickening. However, no significant improvements due to CDC infusion were detected in end-diastolic volume (EDV), end-systolic volume (ESV), and LVEF.²⁸

Aside from CDC, another population of cells that may hold promise in cardiac regeneration is the CSP. These cells are characterized by their ability to actively exclude Hoechst 33342 DNA binding dye through an ATP-binding cassette (ABC) transporter, thereby appearing at the “side” on fluorescence-activated cell sorting (FACS) analysis.^{27,29-30} In both *in vitro* and *in vivo* animal models, CSPs expressed cardiac-specific markers and also

showed spontaneous beating. When delivered intravenously, CSPs migrated in significantly greater numbers in injured hearts in comparison to normal hearts. Moreover, CSPs have been found to differentiate into cardiomyocytes, endothelial cells, or smooth muscle cells.³⁰⁻³¹ Specifically, the Sca1+CD31- CSP population has the ability to differentiate into mature cardiomyocytes, express cardiomyocyte-specific transcription factors, and stimulate contractions by coupling with adult cardiomyocytes.³¹ It is evident that CSP cells hold great promise for therapeutic cardiac regeneration due to their ability to differentiate into mature cardiomyocytes; however, further investigations may help establish their efficacy and safety in preclinical and clinical disease models.

2.2.2 Reprogramming Fibroblasts to Cardiomyocytes

Another approach to inducing cardiac regeneration is by reprogramming fibroblasts and forcing them to transdifferentiate into cardiomyocytes. Fibroblasts can be reprogrammed to become myogenic using combinations of transcription factors that activate transcriptional patterns of cardiomyocytes. Srivastava and colleagues have recently reported that fibroblasts can be reprogrammed using a cocktail of three developmental transcription factors, which included myocyte enhancer factor 2C (MEF2C), GATA binding protein 4 (GATA4), and T-box transcription factor 5 (Tbx5).³²⁻³³ The authors were able to demonstrate contractility and electrophysiological maturation in these cardiomyogenic cells *in vitro*.³² Additionally, *in vivo*, a decrease in infarct size and improvement in cardiac function was also noted.³³

In comparison, Efe et al. reprogrammed mouse embryonic fibroblasts into cardiomyocytes using a cocktail consisting of octamer-binding transcription factor 4 (Oct4 or

POU5F1), sex-determining region Y-box 2 (SOX2), kruppel-like factor 4 (KLF4), and c-Myc, called the “Yamanaka factors.”³⁴ The reprogramming led to generation of spontaneous contracting patches of differentiated cardiomyocytes, without involvement of a pluripotent intermediate.³⁴ While the results from these studies are promising, and reprogramming of fibroblasts seems to be a very attractive option for cardiac regeneration, the retroviral delivery of transcription factors makes this a challenging therapeutic option. Hence, the reprogramming process needs to be investigated further, to improve its efficiency can be improved and to render the process more streamlined.

2.3 MESENCHYMAL STEM CELL TRANSPLANTATION FOR CARDIAC REGENERATION

2.3.1 Biology of Mesenchymal Stem Cells

The most widely studied option for regenerating infarcted myocardium is the use of bone marrow-derived mesenchymal stem cells (MSCs) for transplantation. MSCs have been isolated from a variety of sources, including: bone marrow (BM), adipose tissue, synovial tissue, lung tissue, umbilical cord blood, and peripheral blood. While MSCs are not found in peripheral blood in large numbers, a large population of MSCs can be isolated from the bone marrow. Through a combination of centrifugation, MSCs can be separated from non-nucleated cells of the bone marrow. They are then plated onto plastic culture dishes; the non-adherent monocytes are removed through serial washes, while plastic-adherent MSCs remain plated.³⁵

There are no unique cell surface markers that distinguish MSCs from other hematopoietic stem cells. Therefore, the International Society for Cell Therapy has set criteria for distinguishing a MSC population. A cell population is considered to be of MSC if 1) it is able to adhere to plastic in standard culture conditions, 2) capable of differentiating into osteoblasts, adipocytes, and chondroblasts *in vitro*, and 3) has the following expression profile of cell surface molecules: CD73+, CD90+, CD105+, CD34-, CD45-, HLA-DR-, CD14-, CD11b-, CD79a-, and CD19- of cell surface molecules.³⁵ Two different subtypes of MSCs have been identified based on additional cell surface marker expression. One population of MSCs, that also expresses a neurotrophic growth factor (CD271) marker, has a similar differentiation capacity as regular MSCs, but possesses greater immunosuppressive properties and secretes higher levels of cytokines. MSCs that express Stro-3 constitute another subpopulation that serve as precursors to other MSCs and are capable of extensive proliferation and differentiation.³⁵

What truly makes MSCs such great candidates for cell therapy, in comparison to other cell types, is their immunotolerance. MSCs express human leukocyte antigen (HLA) major histocompatibility complex type I only, and lack the expression of major histocompatibility complex type II and co-stimulatory B7 molecules and the CD40 ligand. The immunologic profile of MSCs allows them to be allogeneically transplanted with reduced concerns about immunogenicity and the need for immunosuppressive therapy. This remarkable characteristic of MSCs allows them to suppress the activity of other immune cells, such as neutrophils, dendritic cells, natural killer cells, eosinophils, mast cells, and macrophages, via cell-to-cell contact. MSCs also inhibit production of proinflammatory

cytokines like tumor necrosis factor- α (TNF- α) and interleukin 2 (IL-2).³⁵ However, MSCs may lose their immunoprivilege properties once they differentiate.³⁶

The differentiation of MSCs is regulated by a variety of growth factors, such as TGF- β , epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) that activate several intracellular signaling cascades like the MAPK/extracellular signal-regulated kinase (ERK) pathway. MSCs themselves are able to secrete many cytokines and growth factors. In fact, paracrine signaling is thought to be the principal mechanism of action of MSCs. They are able to induce angiogenesis by secreting TGF- β , vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1), among others, and promote remodeling of extracellular matrix (ECM) by activating MMP-1, MMP-2, MMP-9, and TNF- α . Lastly, MSCs can recruit and stimulate proliferation of other stem cells through granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and IGF-1.^{35,37} It is evident that MSCs are a versatile population of cells and are capable of inducing regeneration in the infarcted tissue. The results from both preclinical and clinical studies have further validated their regenerative potential.

2.3.2 In Vitro Studies

Bone marrow-derived MSCs have shown cardiomyogenic phenotypes in cultures. In a series of experiments by Xu et al., human MSCs were treated with 5-azacytidine in culture and their differentiation potential into cardiomyocytes was investigated.³⁸⁻³⁹ Two weeks after 5-azacytidine treatment, the myogenic cells that had differentiated from MSCs highly

expressed β -myosin heavy chain (β -MHC), desmin, cardiac troponin T, and α -actin, suggesting a cardiomyocyte-like phenotype.³⁹ In a study by Li et al., rat BM MSCs were cocultured with neonatal rat ventricular myocytes, while separated by a semipermeable membrane. After coculturing, these BMCs were able to contract spontaneously, and expressed cardiac specific proteins, such as sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2), ryanodine receptor 2 (RyR2), cardiac troponin T (cTnT), and cardiac troponin I (cTnI).^{36,40-41}

In a similar study, MSCs were isolated from BM of GFP-transgenic mice and were cocultured with neonatal rat ventricular myocytes. After seven days in coculture, MSCs became α -actin+ and formed gap junctions with native cardiomyocytes. Furthermore, these differentiated cardiomyogenic cells exhibited synchronous contractions with native cardiomyocytes. However, when the MSCs were separated from myocytes in culture by a semipermeable membrane, these cells did not express α -actin, indicating that cell-to-cell interaction is important for MSCs to express the phenotype of native cells.³⁸ An additional study by Rose et al. demonstrated similar results where rat BM MSCs from adult female GFP-transgenic mice were cocultured with male rat embryonic cardiomyocytes.⁴² After five days in coculture, these cells expressed Nkx2.5, α -actin, cTnI, and ANF. Interestingly, these cells were unable to generate action potentials, or provide any evidence of ionic currents similar to native cardiomyocytes. This study demonstrates that while MSCs may take on a cardiomyogenic phenotype, they still retain a part of their stromal cell phenotype.⁴²

2.3.3 In Vivo Studies

Numerous *in vivo* studies have demonstrated the ability of MSCs to engraft within the heart and participate in cardiac repair. In order to track engraftment and distribution of transplanted MSCs, Li and colleagues delivered male rat MSCs into female rats via intra-aortic, intravenous, and intramyocardial injections, either 3 or 28 days after a myocardial infarction was induced.⁴³ At 24 and 48 hours after implantation, a significantly greater number of MSCs was retained in the heart when delivered through direct intramyocardial injection, rather than through other delivery routes.⁴³ In a swine model, Shake et al. transplanted autologous Di-I-labeled MSCs into post-MI hearts by direct injection.⁴⁴ Two weeks later, MSC engraftment was seen in the scar tissue. Additionally, the degree of contractile dysfunction and LV wall thinning was significantly reduced due to MSC transplantation. The MSCs also expressed muscle specific proteins, such as α -actin, tropomyosin, cTnT, myosin heavy chain, and phospholamban.⁴⁴

In order to improve outcomes associated with MSC transplantation therapy, several investigators have used genetically modified MSCs in animal models. In a murine AMI model, Deuse et al. transplanted hepatocyte growth factor (HGF) or VEGF overexpressing MSCs into the infarct, and demonstrated a decrease in infarct size, increase in vascular densities, and better preserved LV function, when compared to normal MSCs.⁴⁵ Similarly, Kim et al. demonstrated that transplantation of hypoxia-inducible VEGF expressing MSCs resulted in attenuation of LV remodeling and a significant increase in myocardial neovascularization.⁴⁶

In a similar approach, Yau et al. used multimodal gene therapy to demonstrate enhanced angiogenesis and LV function.⁴⁷ In a series of experiments, Yau et al. transplanted rat heart infarcts with donor BM MSCs transfected with VEGF, basic fibroblast growth factor (bFGF), and a combination of VEGF+bFGF. Four weeks after transplantation, regional perfusion, vascular densities, and LV ejection fraction (LVEF) were all significantly improved with the transplantation of VEGF- and bFGF- expressing MSCs.⁴⁷ In another similar study conducted by Yau et al., transplantation of VEGF- and IGF- expressing BM MSCs reduced apoptosis, enhanced transplanted cell survival, and improved LV function.⁴⁸

2.3.4 Clinical Trials

The efficacy and safety of cell transplantation therapy has been demonstrated repeatedly by clinical studies. (See **table 1**) One of the first trials to evaluate the safety of BMC transplantation was conducted by Wernet and colleagues.⁴⁹ Autologous mononuclear cells were transplanted in 10 patients during percutaneous transluminal coronary angiography into the infarct-related artery, after standard therapy was administered. A control group received only standard therapy. Three months later, the group that received BMCs experienced improvement in stroke volume, left ventricular end systolic volume (LVESV), contractility, myocardial perfusion, and a decrease in infarct size. Furthermore, no major adverse events were reported due to BMC transplantation, establishing that cell therapy is, in fact, a safe procedure.⁴⁹

The major focus of earlier clinical trials was to establish 1) efficacy and 2) safety of cell transplantation, especially in patients who had an acute MI. In the TOPCARE-AMI trial,

twenty-eight patients with STEMI were randomized to receive either bone marrow-derived or circulating blood-derived progenitor cells (PCs) into the infarct artery after AMI.⁵⁰ Compared to a nonrandomized reference group, four months after cell infusion, the global ejection fraction (EF) was improved and infarct size was decreased in groups that received cell therapy. However, no significant differences were detected in terms of improvement between bone marrow-derived or circulating blood-derived progenitor cells. More importantly, no major adverse events or inflammatory response were observed.⁵⁰

Fernandez-Aviles et al. conducted a study where they investigated the regenerative capacity of BMCs.⁵¹ In the first part of the study, the authors extracted BMCs from 20 patients with reperfused MI and cultured the cells on top of cryoinjured murine heart slices. One week later, the cultured BMCs expressed cardiac proteins and displayed a cardiomyogenic phenotype. In the second part of the study, autologous BMCs were transplanted through intracoronary injection in 20 patients after PCI was performed. At 6 months, compared to the nonrandomized control group, the patients who received BMCs showed a decrease in ESV, and an increase in the thickness of the infarcted wall, improvements in regional and global LV function, and coronary restenosis of only 15%. Furthermore, no major cardiac adverse events were reported.⁵¹

In comparison, one of the first trials to be stopped prematurely due to adverse events was the MAGIC trial by Kang et al.⁵² In this study, 27 patients with AMI were randomized to receive either peripheral blood stem cells PBCs, G-CSF, or were assigned to the control group. At six months, myocardial perfusion and systolic function showed significant improvement in patients who received PBCs. However, the group that received G-CSF

experienced very high rates of coronary stent restenosis, prompting the study to be stopped prematurely.⁵²

Another randomized controlled trial, the BOOST study by Wollert and colleagues investigated the effects of intracoronary BM-MNC infusion on improvement of cardiac function.⁵³ A total of 60 patients with STEMI were randomized to either the control group (with standard treatment) or the group that received standard treatment in conjunction with autologous cell transplantation 4-8 days after PCI. After 6 months, the global LVEF was improved significantly in the group that received the BM-MNC infusion.⁵³ However, at 18 month and 5-year follow-ups, LVEF had equalized between the two groups, suggesting that BM-MNC transplantation accelerated recovery, but did not provide a sustained improvement in EF recovery.⁵⁴⁻⁵⁵

On the other hand, the ASTAMI trial showed no improvement in cardiac function due to cell therapy. The ASTAMI trial conducted by Lunde et al. was a comparatively larger study that recruited 100 patients with STEMI who underwent PCI.⁵⁶ Patients were randomized to receive either autologous BM mononuclear cells through intracoronary injection, whereas control groups received no cells. At 3 months, patients who received BM-MNCs showed an improvement in EF. However, at 6 month and 3 years follow-up, patients showed no improvement in global LVEF, infarct size, & LV end diastolic volume (LVEDV) attributable to BMC injection.⁵⁶⁻⁵⁷ Dimmeler and colleagues compared the BMC isolation protocols of ASTAMI and REPAIR-AMI trials in a murine hindlimb ischemia model, and studied the effects of cell isolation procedures on functional activity.⁵⁸ Dimmeler and colleagues demonstrated that the Lymphoprep cell preparation technique used in the

ASTAMI trial may have resulted in decreased mobility and engraftment potential of the BMC, compared to the cell prep used in the REPAIR-AMI trial.⁵⁸

In contrast, the REPAIR-AMI trial conducted by Dill et al. compared autologous BM-MNC transplantation with placebo after BM aspiration in patients who had PCI after a STEMI.⁵⁹ One year after intracoronary cell infusion, the group that received BM-MNC did not show any statistically significant improvement in global EF, EDV, or ESV. However, patients who had a baseline EF of $\leq 48.9\%$ experienced added benefit of BMC transplantation, as they had significant improvement in EF and a decrease in LVEDV and LVESV.⁵⁹

One of the first trials to evaluate cell therapy in chronic ischemic cardiomyopathy was the phase II randomized controlled FOCUS trial by the Cardiovascular Cell Therapy Research Network.⁶⁰ The patients were randomized to receive either 100 million autologous BMCs or placebo through transendocardial injection. After six months, no significant changes were detected in terms of LVESV, maximum oxygen consumption, and size of defect.⁶⁰ However, post-hoc subgroup analyses demonstrated that LVEF improvement was related to patient age, with younger patients (≤ 63 years) exhibiting an increase in EF compared to lack of effect in older patients, and the extent of EF increase was also directly proportional to the percentage of CD133+ cells in the donor population.⁶⁰

The POSEIDON study by Hare and colleagues also investigated the effects of cell transplantation in ischemic cardiomyopathy.⁶¹ The patients were randomized to receive either 20 million, 100 million, or 200 million allogeneic or autologous MSCs through transendocardial injection. One year later, the allogeneic group had lower number of serious adverse events reported than autologous transplantation, although there was not a statistically

significant difference among these groups. Furthermore, autologous cell transplantation improved maximal exercise oxygen consumption and the 6-minute walk test compared to baseline, whereas allogeneic MSC transplantation decreased LVEDV. However, neither group experienced an improvement in EF. Somewhat counterintuitively, this study showed that the low-dose concentration of cells, i.e., 20 million cells, resulted in the greatest reductions in LV volumes and increased EF. The inverse dose response due to increased cell concentration was attributed to impaired cell retention, survival, and performance.⁶¹

In a different approach, Terzic and colleagues exposed autologous MSCs to a cocktail consisting of TGF- β , FGF2, and other cardiopoietic factors prior to transplanting them in patients with heart failure through endomyocardial injections.⁶²⁻⁶⁴ At six months, LVEF was improved and LVESV was decreased in the group that underwent cell transplantation, compared to the control group. Moreover, the group that received cell therapy showed improvements in a 6-min walk test, quality of life, and physical performance compared to other groups.⁶²⁻⁶⁴

The safety and efficacy of BMC transplantation has been established repeatedly through clinical trials in an acute MI model. However, establishing long-term efficacy and sustained improvement in LV function has presented to be challenging using cell therapy. Therefore, other cell sources, besides BMCs, are being investigated in a clinical setting in both AMI and HF models. Menasche and colleagues conducted a phase I trial in which patients with severe LV dysfunction ($EF \leq 35\%$) received autologous skeletal myoblasts through intracavitary delivery.⁶⁵ At an average follow-up of 10.9 months, the group that received skeletal myoblasts showed a significant improvement in LVEF and systolic thickening, as well as in New York Heart Association functional class.⁶⁵

In the COMPARE-AMI study, Mansour et al. transplanted CD133+ hematopoietic stem cells (HSCs) in patients with STEMI, and demonstrated significantly greater improvement in LVEF in the CD133+ HSCs group compared to placebo group.⁶⁶ Furthermore, the SCIPIO trial showed that autologous c-kit+ cardiac stem cell transplantation in patients with HF improved LVEF and regional EF, decreased infarct size and nonviable LV mass, and increased viable LV mass at both 4 and 12 months after cell infusion.²⁶ Similarly, the CADUCEUS trial by Makkar et al. demonstrated that cardiosphere-derived cell transplantation resulted in decreased scar mass, improvement in viable heart mass, increased regional contractility, and increased regional systolic wall thickening, but failed to improve LVEF, LVESV, and LVEDV.²⁸

While BMC transplantation has been extensively investigated in phase I/II clinical trials, further phase II/III trials, conducted over longer time periods, are needed before cell therapy can be made available in clinics as a regular treatment option for patients with CVD.

Study	Therapy	Follow-up	Outcomes
Strauer et al. ⁴⁹	BM-MNC or standard therapy	3 months	↑ stroke volume, contractility, myocardial perfusion, ↓ infarct size, LVESV
TOPCARE-AMI ⁵⁰	BMPC or CPC or standard therapy	12 months	↑ global EF, ↓ infarct size in cell therapy groups
Fernandez-Aviles et al. ⁵¹	BMC or standard therapy	6 months	↑ thickness of infarcted wall, regional and global EF, ↓ LVESV
Kang et al. MAGIC trial ⁵²	BMC or G-CSF or standard therapy	6 months	↑ myocardial perfusion, LVEF, ↓ LVESV in BMC group
BOOST trial ⁵³	BM-MNC or standard therapy	18 months	↑ LVEF in cell infusion group at 6 months, but not at 18 months or 5 year follow-up
ASTAMI trial ⁵⁶	BM-MNC or standard therapy	12 months	No difference in global LVEF, infarct size, & LVEDV
REPAIR-AMI trial ⁵⁹	BM-MNC or placebo	12 months	No difference in global EF, EDV, or ESV; patients with baseline EF of ≤ 48.9% had ↑ LVEF and ↓ LVESV and LVEDV in BMC group
FOCUS-CCTRN ⁶⁰	BM-MNC or placebo	6 months	No differences in LVESV, O ₂ consumption, and infarct size
POSEIDON ⁶¹	Allogeneic or autologous MSC	12 months	↑ O ₂ consumption, 6-min walk test in autologous cell group; ↓ LVEDV in allogeneic group; no differences in EF
Terzic et al. ⁶²	MSC exposed to cardiopoietic factors or standard therapy	6 months	↑ LVEF, 6-min walk test, quality of life, ↓ LVESV in MSC group
Menasche et al. ⁶⁵	Skeletal myoblasts or standard therapy	Avg. 10.9 months	↑ LVEF, systolic thickening, and NYHA functional class in cell therapy group
COMPARE-AMI ⁶⁶	CD133+ HSC or placebo	12 months	↑ LVEF in cell therapy group
SCIPIO trial ²⁶	c-kit+ CSC or standard therapy	12 months	↑ LVEF, LV viable mass, ↓ infarct size, LV nonviable mass in cell therapy group
CADUCEUS trial ²⁸	CDC or standard therapy	6 months	↑ LV viable mass, regional contractility, regional systolic wall thickening, ↓ scar mass in cell therapy group; no difference in LVEF, LVESV, and LVEDV

Table 1: Summary of selected stem cell clinical trial results.

CHAPTER THREE

MESENCHYMAL STEM CELL TRAFFICKING AND HOMING

3.1 MESENCHYMAL STEM CELL TRAFFICKING

The exact mechanism of mesenchymal stem cell trafficking and mobilization is still being described. It has been hypothesized that the recruitment of mesenchymal stem cells to the site of injury involves a cascade of events, similar to the ones described in EPC and HSC recruitment, including mobilization from bone marrow, rolling of MSCs in the blood vessels, adhesion to endothelial cell surface of the vessels, transendothelial migration, extravasation from the blood vessels, and finally migration along a chemotactic gradient towards the site of injury.⁶⁷ The stem cells may be mobilized from the bone marrow through growth factors, such as G-CSF or PDGF- β , inflammatory cytokines, i.e., high mobility group box-1 (HMGB-1), or other chemoattractants released by the injured tissue, such as stem cell factor (SCF) or stromal derived factor-1 (SDF-1).⁶⁷⁻⁶⁹

The rolling of MSCs in the blood vessels occurs in a manner similar to EPC and HSC migration, and is mediated mostly by cell adhesion molecule P-selectin that is expressed by endothelial cells. The MSCs express a carbohydrate ligand for P-selectin. Like velcro, transitory bonds are formed and broken between selectins and ligands, allowing the MSCs to adhere and roll along the inner lining of the vessel wall. Furthermore, tight adhesion by various adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells and very late antigen-4 (VLA-4) on MSCs, allows MSCs to withstand the shear forces of the blood flow and remain attached to the endothelial lining of the vessels.^{67,70-72}

The process of transendothelial migration and extravasation of MSCs is not yet well understood. It has been hypothesized that the Rho family of GTPases may play a role in

reorganization of MSC cytoskeleton, allowing them to migrate through the endothelium, penetrate the basement membrane, and escape into the interstitium.⁷³ It is in the interstitium that the MSCs follow the chemotactic gradient, due to cytokines such as SCF and SDF-1, and home towards the site of injury or inflammation.⁶⁷

3.2 BIOLOGY OF STEM CELL FACTOR AND C-KIT

The receptor tyrosine kinase, c-kit, along with its ligand SCF, plays an important role in mesenchymal stem cell signaling and engraftment in the infarcted tissue.

3.2.1 Expression of SCF and C-kit

The c-kit receptor is a transmembrane protein that is expressed on mast cells, melanocytes, interstitial cells of Cajal (i.e., telocytes), astrocytes, megakaryocytes, CD56+ natural killer cells, and at least 70% of CD34+ bone marrow cells. It was first identified through mutations on the *W* locus on chromosome 5, whereas its ligand, SCF, was found on the steel (*Sl*) locus. The SCF ligand is a glycosylated, non-covalent homo-dimer that is widely expressed throughout the body by stromal cells, fibroblasts, and endothelial cells.⁷⁴

3.2.2 Isoforms of C-kit

As a result of alternate mRNA splicing, there are 2 isoforms of c-kit that have been identified in mice, and 4 isoforms in humans. The alternate splicing is characterized by

presence or absence of a tetrapeptide sequence (GNNK) in the juxtamembrane region. Additional isoforms result due to presence or absence of a serine residue in the kinase region of the c-kit. While GNNK+ and GNNK- isoforms of c-kit do not differ in their affinity for SCF, GNNK- form of c-kit is the one that is predominantly expressed in most tissues. Moreover, the GNNK- isoform is found to be more highly tyrosine phosphorylated and as a result, is more rapidly internalized. Furthermore, GNNK- isoform has been shown to activate MAPK and ERK more strongly than the GNNK+ form of c-kit. Interestingly, both GNNK+ and GNNK- isoforms have similar ability to activate PI3K and its downstream effector Akt.⁷⁴⁻⁷⁶

In addition, two other isoforms of c-kit have been detected. A soluble extracellular form of c-kit, which is generated by proteolysis of cell surface protein, has been detected in human serum. However, no mRNA that encodes for soluble c-kit has yet been identified, and it is unclear whether it is from GNNK+ or GNNK- isoform of c-kit. Moreover, in culture, soluble c-kit binds to SCF with greater affinity than cell-surface c-kit, and has been implicated in regulating SCF. The other isoform of c-kit is the truncated form (tr-kit) that lacks extracellular and transmembrane domains, and its function has not yet been identified.⁷⁴⁻⁷⁵

3.2.3 Isoforms of SCF

The two isoforms of SCF, soluble and membrane-bound, are a result of alternate mRNA splicing. The inclusion or exclusion of exon 6 produces two transmembrane SCF isoforms, SCF 248 and SCF 220, respectively. The membrane-bound SCF forms dimers,

which is important for their function. The SCF 248 isoform undergoes rapid proteolysis and gives rise to soluble SCF form that has the ability to form dimers in solution. On the other hand, the SCF 220 undergoes slow proteolysis, due to a secondary cleavage site encoded by exon 7, and also gives rise to soluble SCF.⁷⁴⁻⁷⁶

Both membrane-bound and soluble forms of SCF are equally important in activating c-kit. However, the binding of soluble form of SCF only transiently activates c-kit, and results in rapid autophosphorylation and degradation of the receptor. The interaction of membrane-bound SCF to c-kit is more sustained, probably due to anchoring of the ligand to the membrane, which may prevent rapid internalization and degradation of the receptor. Furthermore, membrane-bound SCF binding to c-kit results in prolonged activation of downstream kinases, ERK 1 or 2 and MAPK.⁷⁴⁻⁷⁶

3.2.4 C-kit Binding to SCF

The structure of receptor c-kit is comprised of an extracellular domain consisting of 5 immunoglobulin-like domains and a single transmembrane region. The first three Ig-like domains of the receptor have a complementary shape and charge. Thus, when SCF binds to c-kit, then the first three Ig-like domains of the receptor come together to form a homodimer with another c-kit molecule. As a result, two adjacent c-kit monomers are brought together, and the interactions between Ig-like domains 4 and 5 further stabilize the complex.⁷⁶ The dimerization of the c-kit receptors due to above process leads to activation of its intrinsic tyrosine kinase activity. Hence, the activated c-kit becomes phosphorylated on a number of

tyrosine residues in the juxtamembrane region, followed by autophosphorylation of other tyrosine residues in an orderly manner.⁷⁵⁻⁷⁶

The direct binding of SCF to c-kit activates several downstream pathways, including the PI3K pathway. Located downstream of PI3K is the serine/threonine kinase Akt, which is a molecule that promotes cell survival by phosphorylating Bcl-2-associated death promoter (BAD). BAD is a pro-apoptotic protein of the Bcl-2 family, and upon phosphorylation, it dissociates from the Bcl-2/Bcl-X complex, and loses its pro-apoptotic function. C-kit may also activate Src family of kinases (SFK), which may in turn, activate the ERK 1 or 2, p38, c-Jun N-terminal kinases (JNK), or the MAPK pathways, which have a variety of cellular functions, including cell survival and migration.⁷⁶

3.2.5 Functions of SCF and C-kit

SCF is a chemotactic agent for hematopoietic stem cells, progenitor cells, and MSCs.^{74,77} It mediates cell adhesion through one of the two mechanisms. Firstly, membrane-bound SCF may bind directly to c-kit that is located on HSC or MSC and enable cell attachment.^{74,77} Secondly, the presence of c-kit receptor upregulates the affinity of VLA-4, and other integrins located on the MSCs, for fibronectin on the extracellular matrix, which in turn promotes cell adhesion.⁷⁴

In addition to cell migration, c-kit is important for cardiac stem cell differentiation, recruitment of endothelial progenitor cells during inflammation, and cardiomyocyte terminal differentiation. Besides recruitment, SCF—c-kit mediated signaling has been shown to promote cell survival through Akt-mediated phosphorylation and inactivation of the pro-

apoptotic BAD protein.⁷⁸⁻⁷⁹ Furthermore, release of SCF, along with activation of c-kit and MMP-9, has been shown to be a crucial component of BM progenitor cell mobilization. In fact, murine cardiac infarction/reperfusion model has demonstrated that loss-of-function mutations in either c-kit or SCF causes progression of HF after MI, but the failing phenotype can be rescued by bone marrow transplantation.⁸⁰ In another study by Liu and colleagues, SCF has been shown to contribute to progenitor mediated-cell survival and reduced post-MI injury.⁸¹

Therefore, the role of SCF and c-kit in cell survival and SC recruitment to the site of injury is incontrovertible, and thus makes an appealing target for manipulation in further therapies involving cell transplantation.

3.3 BIOLOGY OF STROMAL-DERIVED FACTOR-1 AND CHEMOKINE RECEPTOR TYPE 4

The chemokine receptor type 4, CXCR4, along with its ligand SDF-1, plays an important role in mesenchymal stem cell signaling and engraftment in the infarcted tissue.

3.3.1 Expression of SDF-1 and CXCR4

The CXCR4 receptor and SDF-1 ligand (CXCL12) belong to the C-X-C family of chemokines, which is expressed in several tissues, including BM, heart, liver, kidney, thymus, spleen, and brain. The distinguishing feature of this family of chemokines is that the N-terminal cysteine residues are separated by one amino acid. They are further subdivided into ELR+ and ELR- categories, depending on the presence or absence of glutamic acid-

leucine-arginine sequence near the N-terminal of the molecule. The SDF-1/CXCL12 ligand belongs to the ELR- family, is expressed on stromal cells, and is capable of recruiting lymphocytes and monocytes. On the other hand, CXCR4 is associated with G protein coupled receptor family, and is expressed on HSCs, MSCs, EPCs, and other progenitor cells.⁸²⁻⁸⁴

3.3.2 Isoforms of CXCR4

Until recently, no known isoforms of CXCR4 existed. However, in a series of experiments by Sloane et al., many different molecular weight isoforms of CXCR4 have been discovered.⁸⁵ The authors used different kinds of cells, including HeLa cells, HEK-293, breast cancer cells, human umbilical vascular endothelial cells (HUVECs), etc., and performed flow cytometry, immunoprecipitation, and Western Blotting to identify different isoforms of CXCR4. Sloane et al. were able to identify different molecular weight isoforms of CXCR, ranging between 45-47 kDa to 90 kDa forms. These molecular weight isoforms were found to be products of N-glycosylation, disulfide formation, tyrosine sulfation, and proteolysis, etc. However, the exact functional differences for each of the CXCR4 isoforms are yet to be determined.⁸⁵

3.3.3 Isoforms of SDF-1

There are two different isoforms of SDF-1 that are encoded by the same gene, but result from alternate splicing. SDF-1 α consists of 3 exons and encodes an 89 amino acid

long protein. In comparison, SDF-1 β is comprised of 4 exons and encodes a protein of 93 amino acids. Another splice variant, SDF-1 γ , has been identified in adult rat brain and lungs, with strongest expression observed in rat hearts. Interestingly, after a myocardial infarction, the expression of SDF-1 γ is unaltered in rat hearts, but the expression of SDF-1 α is greatly increased. In addition to the aforementioned isoforms of SDF-1, three more splice variants of the ligand have been identified in humans, namely, SDF-1 δ , SDF-1 ϵ , and SDF-1 ζ . While the exact roles of these three isoforms have not been defined, they are thought to play a role in cell migration.⁸²

3.3.4 CXCR4 Binding to SDF-1

The hypoxic environment resulting from ischemia causes increased transcription of hypoxia-inducible factor-1 (HIF-1 α), which causes upregulation of SDF-1 α , and promotes recruitment of CXCR4⁺ MSCs to the ischemic site.⁸⁶ The binding of SDF-1 α to CXCR4 stabilizes homo- or hetero-dimerization of the receptor, which results in activation of G protein-coupled receptor. The activation of G α inhibitory protein pathways by CXCR4 initiates several downstream signaling cascades, including inactivation of adenylyl cyclase, activation of PI3K, Akt, MAPK, focal adhesion kinase (FAK) and JAK/STAT (Janus kinase/Signal transducer and activator of transcription) pathways. The initiation of above pathways in MSCs and other PCs induces homing to the site of injury, whereas activation of the same pathways in cardiomyocytes promotes cell survival.⁸²⁻⁸³

Until recently, it was thought that SDF-1 α binds exclusively to CXCR4, but new studies show that it also binds to the CXCR7 receptor. The role of CXCR7 is still unknown, but it is thought to regulate cell migration.⁸²

3.3.5 Functions of SDF-1 and CXCR4

The CXCR4—SDF-1 signaling axis has been shown to activate cell survival signaling pathways. Under hypoxic conditions and myocardial ischemia/reperfusion model, CXCR4—SDF-1 signaling was shown to be cardioprotective through activation of antiapoptotic ERK1/2 and AKT kinases.⁸⁷⁻⁸⁸ Moreover, this receptor-ligand pair plays an important role in SC trafficking. The SDF-1 ligand creates a chemotactic signal to recruit CXCR4+ MSCs to the site of the injury.⁸⁶ The CXCR4+ BM resident MSCs are bound to the niche by SDF-1 expressed by the BM stromal cells. However, the release of SDF-1 by the injury site causes a disruption of the bone marrow SDF-1/CXCR4 signaling, mobilizing BMCs into the peripheral blood. The SDF-1 signaling induces activation of MMP-9, releasing soluble kit ligand (i.e., SCF), which stimulates nitric oxide. This leads to recruitment of CXCR4+ cells to the site of injury.⁸²⁻⁸³

The SDF-1 and CXCR4 ligand-receptor pair plays a crucial role in MSC recruitment to the site of ischemia. In addition, the antiapoptotic function of CXCR4—SDF-1 signaling axis is beneficial to improving survival of the transplanted cells, which is one of the key challenges of cell based therapy. Therefore, this signaling axis could serve as a potential target for therapies involving SC transplantation aimed to improve cardiac regeneration.

CHAPTER FOUR

TRANSMYOCARDIAL REVASCULARIZATION

4.1 PROCEDURE AND MECHANISM OF ACTION

Transmyocardial revascularization (TMR) is a procedure used clinically to treat patients with CAD, who are not candidates for conventional revascularization. The TMR procedure involves creation of 1mm transmural channels in the ischemic myocardium. The patient is anesthetized using a short-acting inhalation agent supplemented with low-dose narcotics, and the distal two-thirds of left ventricle is exposed using a left anterolateral thoracotomy. The laser or needle channels are created in every cm x cm area of the left ventricle.⁸⁹ Currently, there are two laser-based systems that have been approved for performing TMR; it can also be performed using mechanical means through needles. The Holmium:yttrium-aluminum-garnet (Ho:YAG) laser system (Cardiogenesis Corporation, Irvine, CA) uses a 20W pulsed laser to create 1mm channels. The other TMR system is the carbon dioxide (CO₂) laser (PLC Medical Systems, Franklin, MA) that delivers 800W in a single pulse that lasts anywhere from 1 to 99 msec long, and creates 1mm channels. The CO₂ system uses a helium-neon laser for proper positioning.⁸⁹

The two laser systems have been compared side-by-side in a study by Horvath and colleagues.⁹⁰ In a porcine model of chronic myocardial ischemia, TMR was performed using either a CO₂ laser or a Ho:YAG laser. Six weeks later, the hearts that were treated with CO₂ laser showed improvement in ischemic wall thickening, whereas the ones treated with Ho:YAG laser showed no improvement. Furthermore, greater degree of fibrosis was detected in the groups treated with Ho:YAG, compared to CO₂ laser.⁹⁰

In another study by Horvath et al., 25 pigs were randomized to receive TMR treatment by excimer laser, a hot needle, a normothermic needle, an ultrasonic needle, or no

treatment.⁹¹ Six weeks later, hearts that were treated with laser TMR showed significant improvement in cardiac function, and contractility. Furthermore, histological analysis demonstrated a significant increase in ischemic tissue post-treatment. In comparison, mechanical TMR did not improve cardiac function, and instead, demonstrated significant scarring.⁹¹

Early on in the development of TMR, it was thought that TMR worked by creating channels, which would stay patent and allow direct myocardial perfusion, like amphibian hearts. However, it is now evident that these channels do not remain patent for long-term. There are two currently accepted theories of physiological mechanism of action. Initially, TMR causes denervation, which occurs early enough for the patients to walk out of the hospital without angina.⁹² However, this is followed by angiogenesis, which develops over longer period of time.^{89,93}

4.2 PATENCY OF THE TMR CHANNELS

Transmyocardial revascularization was modeled after an amphibian heart. It was originally thought that the channels created by TMR would stay patent, allowing the blood to freely perfuse the myocardium. However, long-term channel patency has been questioned. In a single case study by Cooley et al., heart tissue was obtained from the autopsy a patient who died three months after TMR was performed (of causes unrelated to the operation).⁹³ Hematoxylin and eosin (H&E) staining of the tissue revealed presence of multiple patent channels, although fibrous scar tissue had caused narrowing of the original TMR tract. In

addition, the channels had evidence of endothelialization and also contained red blood cells, suggesting that the channels were functional.⁹³

4.3 CELL THERAPY AND TMR

Transmyocardial revascularization has shown to be effective in providing pain relief to the patients suffering from angina. Even more importantly, it has been shown to induce angiogenesis in the ischemic myocardium. In a sheep infarct model, the hearts were reperfused after the infarction, either by removing the occlusion, or by CO₂ laser TMR.⁹⁴ Thirty days later, the long-term average contractility was improved in the group that underwent TMR, and the area of necrosis was decreased.⁹⁴ This study suggested that TMR alone could improve cardiac function in a heart infarct model.

In another study by Zhang et al., mini-swine were subjected to an acute MI, and after 6 hours were treated with TMR along with heparinized bFGF-incorporating stent.⁹⁵ Compared to non-treatment group, the animals treated with TMR showed increased expression of SDF-1 α , CXCR4, and cardiac stem cell-mediated myocardial regeneration. Furthermore, the TMR group had greater improvement in LVEF than controls.⁹⁵

Woo and colleagues studied the pro-angiogenic effect of TMR in a pig infarct model. Four weeks after the infarct was created, the animals underwent either sham treatment or TMR with a Ho:YAG laser.⁹⁶ Four weeks later, the group that was treated with TMR showed increase in expression of nuclear factor κ B (NF- κ B), angiopoietin-1. Furthermore, TMR resulted in increased vasculogenesis, perfusion, increased myofilament density, contractility, and global hemodynamic function. In addition, TMR enhanced vascular

densities and increased the number of circulating endothelial progenitor cells, which are necessary for vasculogenesis.⁹⁶

Lastly, in an important study by Yau and colleagues, TMR was combined with cell therapy to demonstrate increased transplanted cell survival in a rat infarct model.⁹⁷ Three weeks after an infarct was created, female Lewis rats underwent needle TMR, followed by transplantation of male donor BMCs, BMCs transfected with VEGF, bFGF, and IGF-1 (BMC+VBI), or medium alone. At both 3 and 7 days, the survival of transplanted cells was significantly increased due to TMR, and was greatest in the group that received combined therapy of TMR + BMC + VBI. Seven days later, combined therapy with TMR + BMC + VBI significantly increased LVEF. This study suggested that pretreatment of scar tissue with TMR greatly increases cell survival, angiogenesis, and LV function.⁹⁷ Further studies are needed that investigate the potential of cell therapy in conjunction with TMR.

CHAPTER FIVE

TRANSMYOCARDIAL REVASCULARIZATION AND MESENCHYMAL STEM CELL ENGRAFTMENT

5.1 STUDY RATIONALE

Cell transplantation has been extensively studied as a possible therapeutic option for ischemic heart disease. As discussed before, both modified and unmodified progenitor cells have been utilized, and they have shown promise. However, the effectiveness of cell therapy is highly dependent upon the survival and engraftment of the transplanted progenitor cells. We have reported previously that in rats, transmyocardial revascularization performed prior to transplantation of mesenchymal stem cells into infarcted rat hearts results in significantly greater MSC engraftment and survival.⁹⁷⁻⁹⁸ TMR prior to implantation of MSCs also resulted in greater angiogenesis, increased total capillary densities and arteriolar densities, as well as improved recovery of left ventricular ejection fraction.⁹⁷⁻⁹⁸

Two progenitor cell-signaling axes that may mediate the effect of TMR include the SCF–c-kit axis and the SDF-1–CXCR4 axis.⁹⁵ Our previous data shows that transcription of both SCF and its receptor c-kit was significantly upregulated compared to control hearts, and this upregulation corresponded temporally to the donor MSC engraftment in the infarct zone.⁹⁸ (**Appendix A**) Furthermore, CXCR4 was also upregulated, while levels of its ligand, SDF-1, rose more slowly. The differences in c-kit, CXCR4, and their ligands SCF and SDF-1 between groups and over time suggest that one or perhaps both of these progenitor cell-signaling axes may mediate the observed signaling effect of TMR.

5.2 OBJECTIVES

In this study, our objective was to evaluate the role stem cell factor—c-kit and stromal derived factor-1—CXCR4 signaling axes may play in TMR-enhanced stem cell engraftment. Furthermore, we also wanted to assess the effects of SCF—c-kit and SDF-1—CXCR4 stem cell signaling axes on recovery of ventricular function, after pretreatment of infarcted tissue with TMR.

5.3 HYPOTHESIS

The SCF—c-kit and SDF-1—CXCR4 stem cell signaling axes are **required** for TMR-enhanced engraftment of circulating MSCs and recovery of ventricular function after myocardial infarction.

5.4 EXPERIMENTAL PROTOCOL

A myocardial infarction was created in female rats by ligation of the left anterior descending (LAD) coronary artery. After 3 weeks, 10-channel transmyocardial revascularization (TMR) was performed in the infarct zone using 25 gauge needles, followed by daily intravenous injections of 1 million green fluorescent protein (GFP)-positive male MSCs, with either wild type (WT) or knockdown of c-kit or CXCR4 expression, for 5 days. Control (Ctrl) rats had MSC infusions without TMR (**Figure 1**). The experimental groups were as follows: No TMR—WT, No TMR—c-kit –, No TMR—CXCR4 –, TMR—WT,

TMR—c-kit –, and TMR—CXCR4 – (N=6/group). Knockdown of c-kit and CXCR4 was accomplished via a short hairpin RNA (shRNA)+plasmid, using a lentiviral vector delivery system.

The end points were assessed three days and one week later. Rats were sacrificed and hearts were evaluated for *in vivo* male donor MSC survival by quantitative PCR for sry and GFP, as well as transcriptional expression of SCF and SDF-1 and their receptors c-kit and CXCR4 (N=6/group). The expression of c-kit and CXCR4 *in vitro* was assessed using flow cytometry (N=3). Immunohistochemical staining was performed for GFP to further evaluate *in vivo* donor MSC engraftment (N=6). Vascular densities were evaluated by quantitative histology and LVEF by echocardiography (N=5).

Figure 1: C-kit or CXCR4 knockdown mesenchymal stem cell transplantation with or without transmyocardial revascularization experimental study outline.

CHAPTER SIX

MATERIALS AND METHODS

6.1 ANIMAL CHARACTERISTICS

All experimental procedures were approved by the Animal Care Committee of the University Health Network and were performed according to the *Guide for the Care and Use of Laboratory Animals* by the National Research Council.

Adult female Wistar rats weighing 200–250g (recipients) and male Wistar rats weighing 250–300g (cell donors) were obtained from Charles River Canada (Quebec, QC, Canada).

6.2 LEFT ANTERIOR DESCENDING (LAD) CORONARY ARTERY LIGATIONS

Myocardial infarctions were induced in female rats by ligation of the LAD coronary artery. The rats were first anesthetized in an induction chamber with gauze soaked with ketamine hydrochloride (20mg/kg) and pentobarbital sodium (30 mg/kg). They were then intubated and ventilated with room air supplemented with oxygen and isoflurane (0.5%-2%) by a Harvard ventilator.

After the thoracic area was shaved, it was cleaned with an iodine solution 3 times, and an incision was made in the left lateral thoracic area. The proximal LAD coronary artery was identified and was ligated with a 6-0-propylene suture, until the tissue visually turned pale, indicating ischemia. The incision was closed using silk sutures, and antibiotics (150,000 U/mL penicillin G benzathine and 150,000 U/mL penicillin G procaine) and buprenorphine hydrochloride (0.01 mg/kg) were administered after the operation.

6.3 TRANSMYOCARDIAL REVASCULARIZATION (TMR)

Three weeks after LAD ligation, the rats were randomly assigned to TMR or no TMR groups. TMR was performed in the experimental group by creating 10 transmural channels with a 25-gauge needle in a 10mmx10mm area within the LAD infarct zone. The combined rat mortality for the LAD ligation and TMR procedures was 15-20 percent.

6.4 MESENCHYMAL STEM CELL (MSC) ISOLATION

The MSC population was isolated following the specific protocol outlined by Yau and colleagues.^{47-48,97} The donor GFP+ MSCs were isolated from male Wistar rat femurs and cultured in Iscove's Modified Dulbecco's Medium (IMDM), containing 10% fetal bovine serum (FBS), penicillin G (100 Units/mL) and streptomycin (100µg/mL). Briefly, the MSCs were transferred to a sterile 50mL conical tube, and centrifuged at 600 x relative centrifugal force (*g*) for 5 minutes. The supernatant was removed and cell pellet was re-suspended in 10mL of IMDM solution and cultured in 100mm dishes. The cultured dishes were then incubated at 37°C in humid air supplemented with 5% CO₂ for 4 to 5 days.

Cells were maintained in culture for 7 to 8 passages, splitting plates at 70-80% confluence. Cultures were partially depleted of erythroid progenitor cells by the removal of nonadherent cells with each change of medium every 3 days. No additional fractionation was performed.

6.5 CELL TRANSPLANTATION

The cultured MSCs (wild type or with loss of c-kit or CXCR4 receptor) were washed twice with FBS free IMDM medium. The shRNA+plasmid+lentiviral transduction of the MSCs was performed in a manner so that on each cell injection day, the cells that were transplanted had fresh c-kit or CXCR4 receptor knockdown. Cell numbers were calculated using a hemacytometer and were adjusted to a concentration of 1×10^6 cells/100 μ L cell supernatant with FBS free IMDM medium for injection.

On cell injection days, rats were placed into a restraint box. After sterilizing the tail vein injection site with 70% alcohol, 100 μ L of cell suspension (1×10^6 cells), with 0.3mg of cyclosporine/kg/day were injected slowly over 60 seconds into the tail vein with a 28 gauge needle on a 1/2mL insulin syringe.

6.6 PROCURING THE HEARTS

Three and seven days after the last MSC injection was performed, the rats were sacrificed. The aorta was clamped to prevent systemic blood flow and 0.1mg/kg of heparin was injected into the heart. After five minutes, with the aorta still clamped, 1mL of potassium chloride (KCl), along with 10mL of 0.9% saline, was injected so the heart could be extracted in ventricular diastole. After extraction, the hearts were washed with 20-30mL of phosphate-buffered saline (PBS) solution to remove any residual blood. The left ventricular free wall was weighed and divided into two equal portions, one for quantitative histology and the other for DNA and RNA isolation for quantitative polymerase chain

reaction (PCR) analysis of genes of interest, including the sex-determining region of the Y chromosome (sry). Each section of the LV wall had equal portions of the scar zone (transmural scar), the border zone (partial-thickness scar containing both fibrous tissue and surviving muscle), and a rim of the grossly normal-appearing myocardium surrounding the infarct.

6.7 CXCR4 RECEPTOR KNOCKDOWN

Day 1

Transformation of Plasmid into Bacteria

On day one of the procedure, the shRNA clone against rat CXCR4 receptor (GeneCopoeia catalogue no. RSH050339-LvH1) was packaged into bacterial plasmids, along with reporter gene enhanced GFP (eGFP), forming the CXCR4 shRNA+plasmid complex. The transformation of the bacteria was performed by combining 200ng of CXCR4 shRNA clone with 100µL of chemically competent *E. coli* (GCI-5a Chemically Competent *E. coli* Cells, GeneCopoeia catalogue no. STK200-10) in a vial, followed by incubation on ice for 30 minutes. The *E. coli* cells were heat shock by placing the vial into a 42°C water bath for 45 seconds, and then immediately placing it on ice for 5 minutes. Then 1mL of lysogeny broth (LB) media was added to each tube and mixed gently, followed by incubation for one hour in a 37°C shaker at 200rpm. Finally, the transformation culture was added onto selective LB plates and incubated for 24 hours at 37°C. (See **figure 2**).

Day 2

Selection of Ampicillin Resistant Bacteria

On the next day, the ampicillin resistant bacteria was selected by combining 4mL of lysogeny broth (LB) media, 4μL of ampicillin, and 20μL of previously transformed *E. coli* (from the previous step) in a vial, followed by incubation in a centrifuge at 37°C at 300rpm for 24 hours.

Day 3

Purification of Plasmids

On day two, ampicillin resistant bacteria was removed from the incubator and centrifuged at 4000 rpm for 25 minutes. The resultant pellet was then frozen on ice for 30 minutes, and the supernatant was removed. Following the manufacturer's instructions, the pellet was then processed using the QIAGEN Endofree Plasmid Maxi Kit (catalogue no. 12362), and bacterial plasmids were isolated.

Preparation of CXCR4 shRNA+Plasmid+Lentivirus Complex

Immediately after CXCR4 shRNA encoding plasmid was isolated, the resultant shRNA+plasmid product was then combined with the lentiviral particles to form the CXCR4 shRNA+plasmid+lentivirus complex. For this step, GeneCopoeia Lenti-Pac FIV Expression Packaging Kit (catalogue no. FPK-LvTR-20) was used, and the manufacturer's instructions were followed.

Briefly, 2.5µg of CXCR4 shRNA+plasmid was added to 5µL of Lenti-Pac FIV, and mixed into 200µL of Opti-MEM medium, provided in the kit. In another vial, 15µL of Endo-Fectin Lenti (lentivirus) was added to 200µL of Opti-MEM medium. In a drop wise manner, Endo-Fectin Lenti solution was added to the CXCR4 shRNA+plasmid solution, while vortexing. This mixture was incubated for 25 minutes at room temperature to allow for the two to mix and combine to form the CXCR4 shRNA+Plasmid+lentivirus complex.

Generation of Lentiviral Vectors in HEK 293Ta Packaging Cells

Several plates of human embryonic kidney (HEK) 293Ta cells (Lenti-Pac 293Ta Cell Line, GeneCopoeia catalogue no. CLv-PK-01) were cultured one week prior to the day of transfection. On the day of transfection, HEK 293Ta cells were 80-90% confluent. First, the media was removed and cells were washed with PBS once. Then 10mL of new IMDM was added to the plates, and 315µL of the shRNA+plasmid+lentivirus complex was added and incubated at 37°C for 48 hours.

Day 5

Propagation of Lentiviral Vector Particles

Forty-eight hours after incubation, lentiviral vector particles carrying the CXCR4 shRNA+plasmid were collected along with the medium from HEK 293Ta packaging cells. Medium was removed after centrifuging and fresh cultured HEK 293Ta cells were obtained and lentivirus-binding medium was added. Then 15µL, 30µL, 45µL, or 90µL aliquots of lentiviral vector particles were added to new HEK 293Ta cells and incubated overnight at

37°C to determine the concentration of viral particles required for desired multiplicity of infection (MOI).

Day 6

Transfection Efficiency

The plaque formation unit (PFU) efficiency was evaluated by using fluorescence microscopy to detect eGFP positive HEK 293Ta cells. Transfection efficiency was visually detected to be the highest, approximately 80%, in the plates that were transfected with 90µL aliquot of lentiviral vector particles.

Day 7

Transduction of MSCs

Transduction medium was created using 50mL of IMDM and 2-5µg/mL of Polybrene (Santa Cruz catalogue no. sc-134220), which is a cationic polymer that is used to increase the efficiency of cell transduction. Once the old medium was removed, the MSCs were washed with PBS and the transfection medium was added. Based upon the transfection efficiency, 90µL of lentiviral vector particles carrying the CXCR4 shRNA+plasmid were added to the plates and were incubated at 37°C.

Day 9

Selection of CXCR4 Knockdown Cells Using Puromycin

Forty-eight hours after transduction, 3 μ g/mL of puromycin was administered to the cells. Hence, only the cells with the resistance cassette, which had taken up the CXCR4 receptor knockdown construct, were selected. The antibiotic and medium was replaced again after 8 hours.

Day 10

CXCR4 Knockdown MSC Transplantation

On the day of cell transplantation day, the MSCs were washed with PBS and prepared for injection.

Figure 2: Summary of the CXCR4 receptor knockdown process.

6.8 C-KIT RECEPTOR KNOCKDOWN

Day 1

Transduction of MSCs

Transduction medium was created using 50mL of IMDM and 2µg/1mL of Polybrene (Santa Cruz catalogue no. sc-134220). The MSCs were washed with PBS and the transfection medium was added. Then 20µL of lentiviral vector particles (c-kit shRNA ® Lentiviral Particles, Santa Cruz catalogue no. SC-63363-V) were added to MSC cells, which were incubated for 48 hours at 37°C. (See **figure 3**).

Day 3

Selection of c-kit Knockdown Cells Using Puromycin

After transduction, 3ug/mL of puromycin was administered to the cells. Hence, only the cells with the resistance cassette, which had taken up the c-kit receptor knockdown construct, were selected. The antibiotic and medium was replaced again after 8 hours.

Day 4

c-kit Knockdown MSC Transplantation

On cell transplantation day, the MSCs were washed with PBS and prepared for injection.

Figure 3: Summary of the c-kit receptor knockdown process.

6.9 DNA ISOLATION

DNA was isolated from snap-frozen and powdered female Wistar rat heart specimens, using the QIAGEN Genomic-tip 20/G DNA Isolation Kit (QIAGEN Incorporated, Mississauga, Canada, catalogue no. 10223). The frozen heart sections were ground into a fine powder with liquid nitrogen in a precooled mortar and pestle. Then the ground tissue was combined with 2mL of Buffer G2 (with RNase A) and 0.1mL of the QIAGEN Protease solution and mixed through vortexing. The homogenized tissue was incubated at 50°C for two hours to completely dissolve the particulate matter. A QIAGEN Genomic-tip 20/G was equilibrated in 2mL of Buffer QBT, and the above DNA solution was applied to the Genomic-tip after vortexing for 10 seconds. DNA was then washed 3 times by 1mL of Buffer QC, eluted twice with 1mL of Buffer QF, and then finally precipitated with 1.4mL of isopropanol at room temperature. The centrifuged DNA pellet was isolated and washed in 70% ethanol, centrifuged again, and dissolved in 100µL of tris-EDTA (TE) buffer overnight on a shaker prior to performing real-time PCR.

6.10 QUANTIFICATION OF THE Y CHROMOSOMAL DNA BY REAL-TIME PCR

To quantify the number of Y chromosome-bearing male donor mesenchymal stem cells within the female recipient hearts, a pair of specific primers was designed based on the GeneBank sequence (National Center for Biotechnology Information, Washington, DC) of the rat *sry2* gene (sense 5'-GAGGCACAAGTTGGCTC-AACA-3', antisense 5'-CTCCTGCAAAAAGGGCCTTT-3'). Quantitative real-time PCR was done on the 9700HT

System (Applied Biosystems) using the Master Mix SYBR Green Kit (Applied Biosystems), performing 3-fold serial dilution of the sry2 DNA standards in ddH₂O to generate standards ranging from the sry2 DNA of 1x10⁴ cells to the sry2 DNA of 41 cells.

Briefly, total DNA content was measured spectrophotometrically, and equal amounts of DNA were taken from all samples and were transferred to a 96-well PCR plate. Each assay was performed in duplicate, and 5µL of ddH₂O were assayed as a no-template control. Five microliters of a 5 picomoles per liter (pM) sry2 sense and antisense primer mixture and 10µL of Master SYBR Green Mix were added to each well. The reaction sequence included: stabilization for 5min at 50°C and pre-denaturation for 5min at 95°C, before starting 45 cycles of denaturation for 15s at 95°C, annealing and primer extension for 1min at 60°C, and dissociation for 15s at 95°C, 15s at 60°C, and 15s at 95°C. PCR products were labeled with SYBR Green and detected by a laser detector on the 9700HT system. Real time PCR data were analyzed with SDS 2.3 software (Applied Biosystems).

6.11 RNA ISOLATION

RNA was isolated from snap-frozen and powdered female Wistar rat heart specimens. The frozen heart sections were grinded into a fine powder with liquid nitrogen in a precooled mortar and pestle. The tissue was then homogenized in 1mL of TRIzol Reagent (Life Technologies, catalogue no. 15596-026), and incubated for 5 minutes at room temperature. The homogenized tissue was then combined with 0.2mL of chloroform, incubated at room temperature for another 2 minutes, and centrifuged at 12,000 x g for 15 minutes at 4°C. This resulted in a mixture that existed in a phenol-chloroform phase, an interphase, and a colorless

upper aqueous phase. The colorless phase containing RNA was isolated and combined with 0.5mL of isopropyl alcohol and allowed to precipitate in -20°C for 30 minutes. The precipitate was then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol by vortexing and centrifuging at 7,500 x g for 5 minutes at 4°C. Finally, the pellet was re-dissolved in 25µL of RNase-free water and the concentration was determined spectrophotometrically.

6.12 QUANTIFICATION OF C-KIT, SCF, CXCR4, AND SDF-1 BY REAL-TIME PCR

The expression of SCF and SDF-1 and their receptors c-kit and CXCR4, respectively, was assessed by reverse transcribed real-time PCR. The mRNA for each gene was isolated and purified as described above. Then the mRNA was reverse transcribed to produce complementary DNA (cDNA) for c-kit, SCF, CXCR4, and SDF-1 using Moloney murine leukemia virus (MMLV) retroviral reverse transcriptase and a mixture of oligo(dT)primers. The reaction sequence included synthesis of cDNA at 42°C for 60 minutes and denaturation of enzymes for 5 min at 72°C. Quantitative real-time PCR was then performed using the reverse-transcribed cDNA product for each of the aforementioned genes to quantify their expression. Specific sense and anti-sense primers were designed for c-kit, SCF, CXCR4, and SDF-1 based on the sequences available on GeneBank (National Center for Biotechnology Information, Washington, DC). Quantitative real-time PCR was done on the 9700HT System (Applied Biosystems) using the Master Mix SYBR Green Kit (Applied Biosystems), as described above.

6.13 FLOW CYTOMETRY

Mesenchymal stem cells were first counted using a hemacytometer and then 1×10^6 MSCs were aliquoted into assay tubes (by volume). The cells were then fixed in 2% paraformaldehyde for 10 minutes at 37°C, and washed three times with incubation buffer (0.5% bovine serum albumin in PBS). In each assay tube, 3mL of incubation buffer was added and cells were rinsed by centrifugation. The MSCs were then re-suspended in 100 μ L of incubation buffer and blocked for 10 minutes at room temperature.

MSCs were then incubated for one hour at room temperature with primary antibody for either c-kit (rabbit polyclonal anti-c-kit antibody, Abcam catalogue no. ab5506) or CXCR4 (rabbit polyclonal anti-CXCR4 antibody, Abcam catalogue no. ab2074) at 1:100 dilution. Then the cells were rinsed in incubation buffer by centrifugation and incubated for another hour at room temperature with secondary antibody (for cells with c-kit 1° Ab: goat polyclonal anti-rabbit IgG DyLight® 488, Abcam catalogue no. ab96983, for cells with CXCR4 1° Ab: donkey polyclonal anti-rabbit IgG DyLight® 550, Abcam catalogue no. ab96982) at 1:500 dilution. Finally, MSCs were re-suspended in 0.5mL PBS and analyzed on Becton-Dickinson FACS Canto II flow cytometer (BD Biosciences, San Jose, California).

6.14 IMMUNOHISTOCHEMISTRY

The left ventricular wall was defined as consisting of the scar zone (transmural scar), the border zone (partial-thickness scar containing both fibrous tissue and surviving muscle), and a rim of the grossly normal-appearing myocardium surrounding the infarct. The LV wall

was isolated, weighed, and divided into two equal portions by weight: 50% for RNA isolations, while the other 50% was left for histological analysis (see previous notes about how the heart was divided). The LV tissue sections were fixed in 4% of paraformalin dehydrate, and were embedded in paraffin wax. The paraffin embedded LV tissue was cut into 0.5µm thin slices and placed upon glass slides.

Immunohistochemistry was performed, using the following procedure, to visualize male donor green fluorescent protein (GFP) positive cells that had engrafted in the recipient rat hearts. The tissue was first deparaffinized through a series of following washes: 1) Xylene: 2 x 5 minutes, 2) 100% ethanol: 2 x 5 minutes, 3) 95% ethanol: 2 x 5 minutes, 4) 75% ethanol: 2 x 5 minutes, 5) water: 2 x 5 minutes. In order to perform the antigen retrieval, the slides were soaked in 10mM of sodium citrate buffer, placed in a microwavable vessel, and boiled for 12 minutes at full power, and another 15 minutes at 30% power. The slides were washed with tris-buffered saline (TBS) three times with gentle agitation and then subsequently blocked with a solution of 1% bovine serum albumin (BSA) in TBS for thirty minutes at room temperature.

After TBS washes, the slides were incubated overnight at 4°C with the primary antibody (goat polyclonal anti-GFP antibody, abcam catalogue no. ab6673) at 1:100 dilution in 1% BSA in TBS. Then the slides were rinsed in TBS and incubated for another two hours at room temperature with secondary antibody (donkey anti-goat IgG Alexa Fluor® 488, Invitrogen A11055) at 1:400 dilution in 1% BSA in TBS. Lastly, the slides were incubated with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen catalogue no. D1306) at room temperature for five minutes at 1:2000 dilution in 1% BSA in TBS. Then

the slides were washed with TBS for the final time, soaked with fluorescent mounting medium (Dako catalogue no. S3023), and covered with a glass coverslip.

6.15 VASCULAR DENSITIES

The LV sections allocated for determining vascular densities were immunohistochemically stained with hematoxylin and eosin (Sigma-Aldrich Incorporated) for morphological evaluation of the scar, and then stained for factor VIII or α -smooth muscle actin using horseradish peroxidase (Dako Canada Incorporated, Mississauga, Canada). The total number of vessels at high-power (400X) per 0.2mm² field, as well as the number of vessels with a smooth muscle cell layer, was counted in five randomly selected fields per heart. The mean number of vessels per field was used for analysis.

6.16 LEFT VENTRICULAR FUNCTION

The *in vivo* left ventricular function in rats was assessed using transthoracic echocardiography (ACUSON Sequoia C256; Siemens, Malvern, Pennsylvania), and left ventricular ejection fraction was calculated. The rats were sacrificed either 3 days or 1 week after the last MSC injection was performed. Therefore, the LVEF was evaluated before LAD ligation was performed, after the ligation, and either 3 days or 1 week after the last MSC injection was performed (N=5/group per time point).

Prior to the imaging studies, the rats were anesthetized using ketamine hydrochloride (20mg/kg) and pentobarbital sodium (30mg/kg) in an induction chamber. The rats were then

prepared for echocardiography by shaving the fur from neckline to mid-chest level. Then the rats were placed upon heating pads, intubated, and ventilated with room air supplemented with oxygen and isoflurane (0.5%-2%) by a Harvard ventilator.

The left parasternal images were taken in the right lateral decubitus position using a 15-MHz linear transducer. Two-dimensional mode images in the parasternal short-axis view of the left ventricle and M-mode images at the level of papillary muscles were recorded. LVEDD, LVESD, LV end-diastolic area (LVEDA), and LV end-systolic area (LVESA) were measured. The LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were calculated by using the LVEDD and LVESD and the Teichholz formula.⁹⁹⁻¹⁰¹

$$\textbf{Equation 1. } Volume = \left(\frac{7D^3}{2.4 + D} \right)$$

The standard M-mode echocardiographic equations were used to calculate ejection fraction (EF),

$$\textbf{Equation 2. } EF = \left(\frac{LVEDV - LVESV}{LVEDV} \right) \times 100$$

fractional area shortening (FAS),

$$\textbf{Equation 3. } FAS = \left(\frac{LVEDA - LVESA}{LVEDA} \right) \times 100$$

and fractional shortening (FS).

$$\textbf{Equation 4. } FS = \left(\frac{LVEDD - LVESD}{LVEDD} \right) \times 100$$

For each measurement, three consecutive cardiac cycles were traced and averaged by an experienced examiner in a blinded fashion. All animals were measured at baseline, before treatment, and on the day of sacrifice.

6.17 STATISTICAL ANALYSIS

Data are presented as mean $\pm$ standard error of means, unless otherwise noted. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA). When the ANOVA F-value was significant, a *post hoc* Tukey's multiple range test was used to specify the differences between groups. All p-values are reported to two decimal places with those less than 0.01 reported as $P < 0.01$. The criterion for statistical significance was set *a priori* at $\alpha = 0.05$. All analyses were done using QI Macros Lean Sigma Six Software (Denver, Colorado).

CHAPTER SEVEN

RESULTS

7.1 EFFICIENCY OF THE KNOCKDOWN CONSTRUCT

7.1.1 *In Vitro mRNA Expression of C-kit or CXCR4*

To evaluate the efficiency of the knockdown construct, the expression of c-kit or CXCR4 was knocked down *in vitro* using shRNA+plasmid delivered via lentiviral vectors (N=5/group). The *in vitro* mRNA expression was evaluated using PCR in untreated MSCs, empty cassette group, c-kit shRNA+plasmid+lentivirus group, and CXCR4 shRNA+plasmid+lentivirus group (**Table 2**). The expression of 18s rRNA was assessed to serve as a positive control. The expression of 18s rRNA was not significantly different among all groups at both 1-week (P=0.80) and at 2-week (P=0.82) time points.

At one week, the expression of c-kit was found to be significantly lower in the c-kit and CXCR4 shRNA+plasmid+lentivirus groups, compared to empty cassette group and untreated MSCs (P<0.01). Interestingly, the expression of c-kit was not statistically different between the c-kit shRNA+plasmid+lentivirus group and CXCR4 shRNA+plasmid+lentivirus group (P=0.16).

Even at two weeks, the knockdown construct lowered the expression of c-kit in the c-kit and CXCR4 shRNA+plasmid+lentivirus groups, in contrast to the empty cassette group and untreated MSCs (P<0.01). No statistically significant differences were found in c-kit expression among the c-kit shRNA+plasmid+lentivirus group and CXCR4 shRNA+plasmid+lentivirus group (P=0.38).

Similarly, at one week, the expression of CXCR4 (N=3/group) was significantly lowered in the c-kit and CXCR4 shRNA+plasmid+lentivirus groups, in comparison to the

empty cassette group and untreated MSCs ($P < 0.01$). However, the expression of CXCR4 was not statistically different between the c-kit shRNA+plasmid+lentivirus group and CXCR4 shRNA+plasmid+lentivirus group ($P = 0.50$).

By the end of two weeks, the knockdown construct still lowered the expression of CXCR4 in the c-kit shRNA+plasmid+lentivirus group and CXCR4 shRNA+plasmid+lentivirus group, compared to the empty cassette group and untreated MSCs ($P = 0.04$). Yet again, the expression of CXCR4 was not significantly different between the c-kit shRNA+plasmid+lentivirus group and CXCR4 shRNA+plasmid+lentivirus group ($P = 0.41$). The knockdown construct was effective in significantly reducing the mRNA expression of c-kit and CXCR4 receptors at both 1-week and 2-week time points.

<i>In vitro</i> Gene Expression at 1 Week			
	c-kit	CXCR4	18s rRNA
c-kit shRNA+plasmid+ lentivirus	13.20 ± 2.89X10 ^{8*}	11.60 ± 9.23X10 ^{11*}	120.00 ± 1.18X10 ⁸
CXCR4 shRNA+plasmid+ lentivirus	8.21 ± 1.40X10 ^{8*}	5.88 ± 3.27X10 ^{11*}	121.00 ± 1.12X10 ⁸
Empty cassette	16.60 ± 6.60X10 ¹¹	5.91 ± 2.04X10 ¹⁷	121.00 ± 1.26X10 ⁸
Untreated MSCs	22.50 ± 7.04X10 ¹¹	6.46 ± 2.15X10 ¹⁷	120.00 ± 6.71X10 ⁷
<i>In vitro</i> Gene Expression at 2 Weeks			
	c-kit	CXCR4	18s rRNA
c-kit shRNA+plasmid+ lentivirus	14.5 ± 4.67X10 ^{8*}	7.25 ± 5.83X10 ^{12*}	119.00 ± 7.21X10 ⁷
CXCR4 shRNA+plasmid+ lentivirus	11.9 ± 4.19X10 ^{8*}	9.05 ± 1.54X10 ^{11*}	119.00 ± 3.76X10 ⁷
Empty cassette	11.2 ± 3.83X10 ¹¹	6.24 ± 2.32X10 ¹⁷	119.00 ± 3.83X10 ⁷
Untreated MSCs	7.43 ± 3.41X10 ¹¹	9.24 ± 2.15X10 ¹⁷	120.00 ± 7.80X10 ⁷
<p>Values are represented as mean ± standard error of means. The expression of c-kit and CXCR4 was found to be significantly lower in the c-kit shRNA+plasmid+lentivirus group and CXCR4 shRNA+plasmid+lentivirus group at both 1-week and 2-week time points (P<0.01).</p> <p>Post-hoc P values are reported in comparison to untreated MSC group at each time point.</p> <p>*=P<0.05</p>			

Table 2: *In vitro* mRNA expression of c-kit, CXCR4, and 18s rRNA (housekeeping) genes one and two weeks after knockdown.

7.1.2 In Vitro Cell Surface Expression of C-kit or CXCR4

The cell surface expression of c-kit or CXCR4 was determined using flow cytometry to further evaluate the efficiency of knockdown construct. The number of c-kit positive MSCs were calculated in untreated MSCs group, empty cassette group, and c-kit shRNA+plasmid+lentivirus group at both 1 and 2 weeks after transduction (N=3/group). The same procedure was used for determining CXCR4 knockdown efficiency (N=3/group).

One week after the knockdown procedure was performed, the c-kit shRNA+plasmid+lentivirus construct was significantly able to knockdown the average cell surface expression of c-kit from $13.87 \pm 0.43\%$ of untreated MSCs and $13.43 \pm 1.13\%$ of empty cassette group to $1.63 \pm 0.23\%$ in the c-kit shRNA+plasmid+lentivirus group ($P < 0.01$) (**Figures 4 & 5**). Even at two weeks, the average cell surface expression of c-kit was lowest in the c-kit shRNA+plasmid+lentivirus group, where only $2.00 \pm 0.10\%$ of MSCs expressed the c-kit receptor, as compared to $10.83 \pm 0.94\%$ in untreated MSCs group and $9.70 \pm 0.32\%$ in empty cassette group ($P < 0.01$).

Similarly, one week after the CXCR4 knockdown was performed, the average CXCR4 expression was reduced from $38.97 \pm 5.88\%$ of untreated MSCs and $36.47 \pm 2.84\%$ of empty cassette group to $3.67 \pm 0.43\%$ in the CXCR4 shRNA+plasmid+lentivirus group ($P = 0.03$). Even after two weeks, the knockdown construct was successful in lowering the CXCR4 cell surface expression. Only $5.13 \pm 0.58\%$ of MSCs expressed CXCR4 in shRNA+plasmid+lentivirus group, as compared to $40.17 \pm 0.97\%$ in untreated MSCs group and $40.63 \pm 2.04\%$ in empty cassette group ($P < 0.01$). The knockdown construct significantly reduced cell surface expression of both c-kit and CXCR4 at both 1 and 2 weeks.

Figure 4: The above flow cytometry dot plots show a representative distribution of GFP+ MSCs and c-kit+ MSCs in only one sample per group. On average, the percentage of both GFP+ and c-kit+ MSCs was more than eight fold lower in the c-kit shRNA+plasmid+lentivirus group, as compared to the empty cassette and untreated MSCs at both 1 and 2 weeks time points (N=3/group).

Figure 5: The c-kit shRNA+plasmid+lentivirus construct was significantly able to knockdown the average cell surface expression of c-kit compared to untreated MSCs and empty cassette group at both 1-week and 2-week time points ($P<0.01$) ($N=3/\text{group}$).

Figure 6: These flow cytometry dot plots show a representative distribution of GFP+ MSCs and CXCR4+ MSCs in only one sample per group. On average, the percentage of both GFP+ and CXCR4+ MSCs was approximately 10-fold lower in the CXCR4 shRNA+plasmid+lentivirus group, as compared to the empty cassette and untreated MSCs at both 1 and 2 weeks time points (N=3/group).

Figure 7: The CXCR4 shRNA+plasmid+lentivirus construct was significantly able to knockdown the average cell surface expression of CXCR4 compared to untreated MSCs and empty cassette group at both 1-week and 2-week time points ($P < 0.01$) ($N = 3/\text{group}$).

7.2 *IN VIVO* DONOR MSC ENGRAFTMENT AFTER DELIVERY

7.2.1 *Quantification of Donor MSC Engraftment via mRNA Expression of sry2*

The number of male donor GFP+ MSCs that had engrafted into the female recipient hearts was quantified by real-time PCR for the Y chromosomal gene *sry2* (N=6/group). Three days after the last MSC injection, the number of MSCs that had homed into the infarct was affected by both TMR and donor cell type (TMR, cell type and interaction, all $P<0.01$) (**Figure 8**). Post hoc Tukey's multiple comparisons test demonstrated that compared to no TMR—WT group, the TMR—WT group experienced 2.7 fold engraftment of MSC at three days ($P<0.01$). However, when the c-kit receptor was knocked down on the MSCs that were transplanted, the resultant MSC engraftment was reduced despite TMR. The MSC engraftment was reduced to 0.4 fold in the TMR—c-kit- group, compared to the TMR—WT group ($P<0.01$). Even the group without TMR experienced a reduction in MSC homing, where the engraftment was only 0.5 fold in no TMR—c-kit- in comparison to the no TMR—WT group ($P<0.01$). Similarly, the loss of CXCR4 receptor resulted in decreased MSC engraftment in both TMR and non-TMR groups. The engraftment was 0.3 fold in TMR—CXCR4- group than TMR—WT ($P<0.01$). Likewise, the no TMR—CXCR4- group had 0.4 fold MSC engraftment in comparison to TMR—WT group ($P<0.01$).

The MSC homing and engraftment pattern was consistent even at 1 week (TMR, cell type, both $P<0.05$; interaction, $P=0.37$), where the TMR—WT group had 1.3-fold engraftment than the no TMR—WT group ($P=0.05$) (**Figure 9**). Even at 1 week, reductions similar to 3 days were evident in terms of MSC engraftment. The knockdown of c-kit

receptor resulted in a 0.4 fold MSC engraftment in the TMR—c-kit- group ($P<0.01$) and a 0.3 fold in no TMR—c-kit- group ($P<0.01$) compared to TMR—WT and no TMR—WT groups, respectively. In a similar manner, knockdown of the CXCR4 receptor resulted in a 0.2 fold MSC engraftment in TMR—CXCR4- in contrast to TMR—WT ($P<0.01$). Furthermore, only 0.1 fold MSCs engrafted in the no TMR—CXCR4- group, when compared to no TMR—WT ($P<0.01$). In summary, at both 3-day and 1-week time points, knockdown of either c-kit or CXCR4 receptor reduced MSC engraftment, even without TMR.

Figure 8: Three days after the last MSC injection (N=6/group), the number of MSCs that had homed into the infarct was affected by both TMR and donor cell type (TMR, cell type and interaction, all $P < 0.01$) Male donor MSC engraftment was greater in groups that underwent TMR. However, the knockdown of c-kit or CXCR4 receptor resulted in decreased MSC engraftment despite TMR.

Figure 9: One week after the last MSC injection was performed (N=6/group), the number of MSCs that had homed into the infarct was affected by both TMR and cell type (TMR, cell type, both $P < 0.05$; interaction, $P = 0.37$). Male donor MSC engraftment was still greater in groups that underwent TMR. However, the knockdown of c-kit or CXCR4 receptor resulted in decreased MSC engraftment despite TMR.

7.2.2 *Qualitative Analysis of Donor MSC Engraftment via Immunohistochemistry*

The scar tissue from recipient hearts was immunostained to visualize engraftment of male donor MSCs (N=6/group), using antibodies directed against GFP (green), and DAPI (blue). The tissue was then observed under fluorescence microscopy. At three days, the highest engraftment occurred in the groups that received wild type MSCs, i.e., no TMR—WT and TMR—WT groups (**Figure 10A-B**). Even more importantly, the tissue that was also treated with TMR, i.e., the TMR—WT group (**Figure 10B**), was the one that exhibited the greatest MSC homing amongst all groups. However, the loss of c-kit (**Figure 10C-D**) or CXCR4 (**Figure 10E-F**) receptors resulted in decreased MSC engraftment, with or without TMR.

Even at 1 week, similar MSC homing and engraftment pattern was observed (N=6/group). The no TMR—WT and TMR—WT groups experienced the highest MSC engraftment (**Figure 11A-B**). Consistent with the homing pattern observed at the 3-day time point, the TMR—WT group had greatest MSC engraftment. Furthermore, despite TMR, knockdown of c-kit (**Figure 11C-D**) or CXCR4 (**Figure 11E-F**) receptor critically reduced MSC engraftment.

Figure 10: Pictomicrographs (200X magnification) of scar tissue from 3-day recipient hearts (N=6/group), immunostained to visualize engraftment of male donor MSCs, using antibodies directed against GFP (green), and DAPI (blue). The highest engraftment occurred in the no TMR—WT and TMR—WT groups (A, B). The loss of c-kit (C, D) or CXCR4 (E, F) receptors resulted in decreased MSC engraftment, with or without TMR.

Figure 11: Pictomicrographs (200X magnification) of scar tissue from 1-week recipient hearts (N=6/group), immunostained to visualize engraftment of male donor MSCs, using antibodies directed against GFP (green), and DAPI (blue). The highest engraftment occurred in the no TMR—WT and TMR—WT groups (A, B). The loss of c-kit (C, D) or CXCR4 (E, F) receptors resulted in decreased MSC engraftment, with or without TMR.

7.3 *IN VIVO* mRNA EXPRESSION OF STEM CELL SIGNALING AXES AFTER MSC DELIVERY

7.3.1 *In Vivo mRNA Expression of Stem Cell Factor (SCF)*

The mRNA expression of SCF was quantified by reverse transcribed real-time PCR (N=6/group). At 3 days, the expression of SCF was affected by both TMR and MSC cell type (TMR and cell type $P<0.01$; interaction $P=0.41$) (**Figure 12**). Post hoc Tukey's multiple comparisons test demonstrated the expression of SCF ligand was transiently upregulated in the TMR—WT, but was statistically similar to the no TMR—WT group ($P=0.07$). In the c-kit receptor knockdown groups, the pretreatment with TMR resulted in significantly greater expression of SCF in the TMR—c-kit- compared to no TMR—c-kit- group ($P<0.01$). Similarly, in the CXCR4 receptor knockdown groups, TMR upregulated the expression of SCF in the TMR—CXCR4- compared to the no TMR—CXCR4- group ($P<0.01$).

At 1 week, the expression of SCF dropped across all groups, but was upregulated in groups that received TMR (TMR $P=0.02$; cell type $P=0.47$; interaction $P=0.78$) (**Figure 12**). Post hoc analysis revealed no significant differences between no TMR—WT and no TMR—c-kit- and their TMR counterparts. However, TMR—CXCR4- group had significantly greater expression of SCF when compared to no TMR—CXCR4- ($P=0.02$).

Figure 12: At three days, the mRNA expression of SCF (N=6/group per time point) was significantly upregulated due to TMR and MSC cell type (TMR and cell type $P < 0.01$; interaction $P = 0.41$). At 1 week, the expression of SCF dropped across all groups, but was upregulated in groups that received TMR (TMR $P = 0.02$; cell type $P = 0.47$; interaction $P = 0.78$).

7.3.2 *IN VIVO* mRNA EXPRESSION OF C-KIT

The mRNA expression of c-kit was quantified in infarcted recipient hearts using quantitative PCR (N=6/group). Three days after the last MSC injection was performed, the expression of c-kit was significantly upregulated in the groups that underwent TMR (TMR $P<0.01$; cell type $P=0.26$; interaction $P=0.84$) (**Figure 13**). Post hoc analysis revealed that the differences in c-kit expression between no TMR—WT and no TMR—c-kit- and their TMR counterparts were not statistically significant. However, the expression of c-kit was significantly increased in the TMR—CXCR4- group compared to its non-TMR counterpart ($P=0.018$).

At 1 week, the expression of c-kit decreased across all groups, and was affected only by the type of cells that were transplanted (TMR $P=0.79$; cell type $P=0.02$; interaction $P=0.58$) (**Figure 13**). Furthermore, post hoc analysis revealed no statistically significant difference in c-kit expression among groups.

Figure 13: The mRNA expression of c-kit (N=6/group per time point) was significantly upregulated at three days in the groups that underwent TMR (TMR $P < 0.01$; cell type $P = 0.26$; interaction $P = 0.84$). At 1 week, the expression of c-kit decreased across all groups, and was affected only by the type of cells that were transplanted (TMR $P = 0.79$; cell type $P = 0.02$; interaction $P = 0.58$).

7.3.3 *IN VIVO* mRNA EXPRESSION OF STROMAL-DERIVED FACTOR-1 (SDF-1)

Three days after the last MSC injection, the expression of SDF-1 (N=6/group) was upregulated due to both TMR and cell type (TMR $P<0.01$; cell type $P=0.04$; interaction $P=0.43$) (**Figure 14**). Post hoc analysis revealed that the expression of SDF-1 was significantly increased in the TMR—WT group compared to no TMR—WT group ($P=0.03$). The differences in SDF-1 expression between no TMR—c-kit- and no TMR—CXCR4- and their TMR counterparts were not statistically significant.

At 1 week, the expression of SDF-1 was upregulated due to TMR (TMR $P<0.01$; cell type $P=0.64$; interaction $P=0.69$) (N=6/group) (**Figure 14**). Post hoc Tukey's multiple comparisons test demonstrated that the expression of SDF-1 ligand gradually increased in the TMR—WT compared to the no TMR—WT group ($P=0.04$). Furthermore, in the c-kit receptor knockdown groups, pretreatment with TMR resulted in significantly greater expression of SDF-1 in the TMR—c-kit- compared to no TMR—c-kit- group ($P=0.03$). Similarly, in the CXCR4 receptor knockdown groups, TMR upregulated the expression of SDF-1 in the TMR—CXCR4- compared to the no TMR—CXCR4- group ($P=0.04$).

Figure 14: Three days after the last MSC injection, the expression of SDF-1 (N=6/group per time point) was upregulated due to both TMR and cell type (TMR $P < 0.01$; cell type $P = 0.04$; interaction $P = 0.43$). At 1 week, the expression of SDF-1 was upregulated in the groups that received TMR (TMR $P < 0.01$; cell type $P = 0.64$; interaction $P = 0.69$).

7.3.4 *IN VIVO* mRNA EXPRESSION OF CHEMOKINE RECEPTOR TYPE 4 (CXCR4)

The mRNA expression of CXCR4 (N=6/group) was significantly upregulated at 3 days due to TMR and cell type (TMR $P<0.01$; cell type $P<0.03$; interaction $P=0.44$) (**Figure 15**). Post hoc analysis demonstrated that the expression of CXCR4 was significantly upregulated in TMR—WT group, in comparison to no TMR—WT group ($P=0.02$). TMR significantly upregulated the expression of CXCR4 in TMR—c-kit- compared to no TMR—c-kit- group ($P=0.03$), and in TMR—CXCR4- when compared to no TMR—CXCR4 group ($P<0.01$).

At 1 week, all of the groups experienced a dramatic drop in the expression of CXCR4 (N=6/group), but was still upregulated in the groups that received TMR (TMR $P<0.01$; cell type $P=0.02$; interaction $P=0.37$) (**Figure 15**). Post hoc Tukey's multiple comparisons test demonstrated that the expression of CXCR4 was significantly greater in the TMR—c-kit- group compared to the no TMR—c-kit- group ($P=0.04$). TMR also upregulated the expression of CXCR4 in the TMR—CXCR4- compared to the no TMR—CXCR4- group ($P=0.02$).

Figure 15: The mRNA expression of CXCR4 (N=6/group per time point) was significantly upregulated at 3 days due to TMR and cell type (TMR $P < 0.01$; cell type $P < 0.03$; interaction $P = 0.44$). At 1 week, all of the groups experienced a dramatic drop in the expression of CXCR4, but was still upregulated in the groups that received TMR (TMR $P < 0.01$; cell type $P = 0.02$; interaction $P = 0.37$).

7.4 VASCULAR DENSITIES AFTER MSC DELIVERY

7.4.1 *Capillary Density (Factor VIII)*

The quantitative histological analysis of capillary density was performed by immunostaining for factor VIII in five randomly selected fields per heart (N=5x5/group) (**Figure 16**). At three days, no significant differences were detected in capillary density amongst groups (TMR P=0.52; cell type P=0.54; interaction P=0.97). Even at one week, no significant differences were detected in terms of capillary density (TMR P=0.89; cell type P=0.41; interaction P=0.73). While there was a trend towards increased capillary densities with the addition of TMR in all three cell-type groups, these differences did not reach statistical significance.

7.4.2 *Arteriolar Density (α -SMA)*

The arteriolar density was determined by immunostaining for α -smooth muscle actin in five randomly selected fields per heart (N=5x5/group) (**Figure 17**). Three days after the last MSC injection was performed, no significant differences were detected in arteriolar density among all groups (TMR P=0.71; cell type P=0.64; interaction P=0.92). Even at one week, the arteriolar density was found to be statistically not different among groups (TMR P=0.48; cell type P=0.44; interaction P=0.96).

Figure 16: No significant differences were detected in capillary density (factor VIII) amongst groups at both 3 days (TMR $P=0.52$; cell type $P=0.54$; interaction $P=0.97$) and at 1 week (TMR $P=0.89$; cell type $P=0.41$; interaction $P=0.73$) ($N=5 \times 5/\text{group}$).

Figure 17: No significant differences were detected in α -smooth muscle actin (α -SMA) amongst groups at both 3 days (TMR $P=0.71$; cell type $P=0.64$; interaction $P=0.92$) and at 1 week (TMR $P=0.48$; cell type $P=0.44$; interaction $P=0.96$) ($N=5 \times 5/\text{group}$).

7.5 LEFT VENTRICULAR FUNCTION

The left ventricular function was evaluated by calculating left ventricular ejection fraction using echocardiography. The LVEF was evaluated before LAD ligation was performed, after the ligation, and either 3 days or 1 week after the last MSC injection was performed (N=5/group per time point) (**Figure 18**).

Before the LAD ligation was performed, the LVEF was different due to cell type (TMR $P=0.84$; cell type $P=0.02$; interaction $P=0.99$). However, after LAD ligation, all the groups experienced a similar decline in LVEF. The LVEF after ligation ranged from 67-70% of the baseline and was not statistically different amongst groups (TMR $P=0.58$; cell type $P=0.20$; interaction $P=0.67$). Three days after the last MSC injection, no significant differences were detected in LVEF (TMR $P=0.70$; cell type $P=0.31$; interaction $P=0.94$).

Similarly, in the one-week rats, before LAD ligation, LVEF was different due to cell type (TMR $P=0.75$; cell type $P=0.03$; interaction $P=0.08$). After the ligation was performed, the LVEF ranged from 66-70% of baseline, and was not statistically different amongst groups (TMR $P=0.48$; cell type $P=0.91$; interaction $P=0.46$). One week after the last MSC injection, LVEF was improved only with the combination of WT MSCs and TMR (TMR $P=0.01$; cell type $P<0.01$; interaction $P=0.54$). Post hoc analysis revealed that the TMR—WT group had significantly greater improvement in LVEF compared to no TMR—WT group ($P=0.01$). Knockdown of either c-kit or CXCR4 abrogated this TMR-mediated augmentation of MSC reparative effect.

Figure 18: In the 3-day rats ($N=6/\text{group}$), before the LAD ligation, LVEF was different due to cell type (TMR $P=0.84$; cell type $P=0.02$; interaction $P=0.99$). Three weeks after the LAD ligation was performed, all the groups experienced a similar decline in LVEF (TMR $P=0.58$; cell type $P=0.20$; interaction $P=0.67$). Three days after the last MSC injection, no significant differences were detected in LVEF (TMR $P=0.70$; cell type $P=0.31$; interaction $P=0.94$). In the 1-week rats ($N=6/\text{group}$), before the LAD ligation was performed, LVEF was different due to cell type (TMR $P=0.75$; cell type $P=0.03$; interaction $P=0.08$). All of the groups experienced a similar decline in LVEF (TMR $P=0.48$; cell type $P=0.91$; interaction $P=0.46$) one week after ligation. However, one week after the last MSC injection, LVEF was improved due to TMR and cell type (TMR $P=0.01$; cell type $P<0.01$; interaction $P=0.54$).

CHAPTER EIGHT

DISCUSSION

8.1 SUMMARY

Cell transplantation has been studied extensively as a potential therapy for postinfarction ventricular dysfunction. However, the effectiveness of cell therapy is dependent upon the number of cells that survive and engraft into the infarcted tissue. We have previously shown that pretreatment of ischemic tissue with transmyocardial revascularization improves mesenchymal stem cell survival and engraftment. Moreover, TMR performed prior to MSC transplantation results in significantly increased angiogenesis and recovery of left ventricular function.⁹⁷⁻⁹⁸ We have previously reported that the two progenitor cell-signaling axes that may mediate the effect of TMR include the SCF–c-kit axis and the SDF-1–CXCR4 axis. Our previous data shows that the expression of SCF, its receptor c-kit, as well as SDF-1 and its receptor CXCR4 was upregulated due to TMR, and this upregulation in signaling axes corresponded temporally to the donor MSC engraftment in the infarct zone.⁹⁸

In this study, we evaluated the importance of SCF–c-kit and SDF-1–CXCR4 signaling axes in TMR mediated enhanced engraftment of MSCs in infarcted tissue, as well as improvement in LV function. We hypothesized that the SCF–c-kit and SDF-1–CXCR4 stem cell signaling axes are both required for TMR-enhanced engraftment of circulating MSCs and recovery of ventricular function after myocardial infarction. Therefore, we knocked down the c-kit and CXCR4 receptor expression on male donor MSCs prior to transplantation in female recipient rats, and studied the effects on donor MSC engraftment, expression of signaling axes, vascular densities, and LVEF.

8.2 KNOCKDOWN CONSTRUCT REDUCES *IN VITRO* mRNA AND CELL SURFACE EXPRESSION OF C-KIT AND CXCR4

The efficiency of the knockdown construct was evaluated using PCR for *in vitro* mRNA expression, and flow cytometry was performed for verifying gene knockdown on the protein level. Using our lentiviral vector delivery system, our shRNA+plasmid construct was significantly able to knockdown the cell surface expression of c-kit and CXCR4, at both 1 week and 2 weeks, compared to the untreated MSCs group. In addition, the shRNA+plasmid construct, delivered via lentiviral vectors, significantly knocked down the mRNA expression of c-kit and CXCR4 at both 1-week and 2-week time points, in comparison to the empty cassette and untreated MSC groups. This validated the efficiency of our knockdown constructs for the purpose of our study.

Interestingly, the knockdown of c-kit receptor also reduced mRNA expression of CXCR4 at both 1 and 2 weeks. Similarly, CXCR4 knockdown significantly decreased the mRNA expression of c-kit at both time points. The co-downregulation of c-kit and CXCR4 at the mRNA level is an unexpected and novel finding. While there could be many possible explanations for this behavior, we took great care in optimizing our knockdown protocol to minimize any off-target effects that could be held responsible. The knockdown constructs used for either of the genes were handled and applied separately. Prior to knockdown of the target gene in MSCs, the commercial c-kit or CXCR4 shRNA product was optimized for gene knockdown. For example, the commercial Genecopoeia CXCR4 shRNA kit contained four different shRNA expression constructs with 19-29 base stem sequences corresponding to the target gene. After verification through PCR analysis and Western blotting, one

CXCR4 shRNA sequence was selected that yielded the greatest knockdown of the targeted transcript. By optimizing the shRNA sequence and subsequent transduction efficiency, utilizing replication deficient lentiviral vectors, and by including proper controls for the experiment, we were able to monitor any potential off-target effects.

Furthermore, off-target effects were accounted for using recommendations outlined by Taxman and colleagues.¹⁰² The shRNA design and delivery protocols by Taxman and colleagues recommended the use of Basic Local Alignment Search Tool (BLAST) by the National Center for Biotechnology Information (NCBI) when selecting shRNA to eliminate any target sequences that match with an off-target gene.¹⁰³⁻¹⁰⁴ By performing BLAST search for c-kit and CXCR4, we were able to eliminate any overlap between the genes, which could have contributed to potential off-target effects.

While off-target co-downregulation of c-kit and CXCR4 at mRNA level seems unlikely, it is possible that there are other yet unknown mechanisms that may be responsible for this effect. Some evidence of crosstalk between these two signaling axes has emerged recently. In one study, Luo et al. administered AMD3100 (a CXCR4 antagonist) to rats after an infarct, in order to disrupt the BM SDF-1—CXCR4 axis and mobilize circulating progenitor cells in a manner similar to normal BMC mobilization.¹⁰⁵ Luo et al. demonstrated that administration of AMD3100 enhanced c-kit⁺ PC mobilization to the site of the infarct, suggesting crosstalk between the two signaling axes.¹⁰⁵

In a series of experiments by Ma and colleagues, the authors demonstrated that SDF-1 α and CXCR4 can upregulate the expression of c-kit in c-kit⁺ cardiac progenitor cells, and make c-kit⁻ CPCs express the c-kit receptor.¹⁰⁶ Moreover, Ma and colleagues found that this c-kit expression upregulation due to SDF-1 α —CXCR4 activity can be attributed to inhibition

of DNA methyltransferase (DNMT) activity and demethylation of the c-kit gene.¹⁰⁷ Lastly, the authors concluded that SDF-1 α has the ability to enhance the expression of c-kit, as well as promoting c-kit⁺ CPC proliferation and migration.¹⁰⁷ Taken together, the above studies suggest that c-kit expression and activity may be affected by the SDF-1—CXCR4 signaling axis.

Interestingly, an important study by Cheng et al. showed that SDF-1—CXCR4 signaling in turn may be mediated by c-kit activity.¹⁰⁸ Cheng et al. demonstrated that in c-kit^{W/W⁻V} and c-kit mutant mice, AMD3100 failed to mobilize BM PCs. More importantly, Cheng et al. found that SDF-1—CXCR4 signaling upregulated c-kit phosphorylation in BM MNCs *in vitro*, and that antagonism or genetic deletion of CXCR4 resulted in downregulation of BM c-kit phosphorylation *in vivo*.¹⁰⁸ The evidence from this study suggests that SDF-1—CXCR4 signaling upregulates c-kit activity at the protein level, and that the loss of CXCR4 receptor resulted in decreased c-kit activity.

In comparison to the above literature, we have found that the loss of the CXCR4 receptor also downregulates c-kit expression at the mRNA level, and that loss of c-kit receptor results in decreased CXCR4 expression. While it is a limitation of our study that co-downregulation of CXCR4 and c-kit receptors was analyzed only at the mRNA level, future loss-of-protein studies may further shed light into the crosstalk between these two axes.

8.3 TMR INCREASES DONOR MSC ENGRAFTMENT

The number of male donor GFP⁺ MSCs that had engrafted into the female recipient hearts, at both 3 days and 1 week, was quantified by real-time PCR for *sry2*. Cell numbers

quantified through this methodology may include not only viable transplanted cells, but also cells that may have died during implantation. A similar methodology was employed by Muller-Ehmsen et al. to observe survival and development of neonatal male rat cardiomyocytes transplanted into normal adult female rat hearts.¹⁰⁹ The authors reported 57% transplanted cell survival at 0-1 hour, 24% at 24 hours, 28% at 7 days, 27% at 14 days, 23% at 4 weeks, and lastly, 15% by 12 weeks. More importantly, the authors noted that majority of the cellular DNA quantified through PCR was from surviving cells. After a known quantity of nonviable male cardiomyocytes was injected, 33% of male DNA was detected at 1 hour, 2.2% at 24 hours, and a mere 0.1% of male DNA was detected at 7 days.¹⁰⁹ Therefore, it is evident that contribution of DNA from nonviable cells to sry quantification would be extremely low. Hence, donor MSC engraftment would reflect mostly surviving cells.

Our data demonstrated that MSC engraftment was upregulated due to TMR at 3 days and 1 week across all groups. Interestingly, the group that received wild type MSCs, and did not undergo TMR, experienced a greater overall MSC engraftment at 1 week, as compared to 3 days. There is a possibility that the peak MSC engraftment between TMR and no TMR wild type groups is similar, and that TMR also has a temporal effect, and may make the engraftment happen earlier.

However, loss of either the c-kit or CXCR4 receptor resulted in decreased MSC engraftment in comparison to WT groups. The amount of reduction in MSC engraftment observed between the TMR and no TMR groups with the c-kit knockdown was disproportionate. Moreover, the knockdown of either c-kit or CXCR4 receptor does not completely abolish MSC homing, but it does markedly diminish it. The addition of TMR,

however, to the knockdown groups still results in greater MSC homing compared to no TMR, but at much reduced levels compared to WT. This data suggests that TMR enhances MSC engraftment, but knockdown of either c-kit or CXCR4 receptor attenuates the effect of TMR on donor MSC homing in the infarcted tissue. Furthermore, even without TMR, c-kit or CXCR4 knockdown reduced MSC engraftment.

In addition, immunostaining of the scar tissue from the recipient hearts to visualize engraftment of male donor MSCs, using antibodies directed against GFP (green) and DAPI (blue), revealed similar results. At both 3 days and 1 week, the highest engraftment occurred in the groups that received wild type MSCs in conjunction with TMR. Even more importantly, the tissue that was pre-treated with TMR, i.e., the TMR—WT group, was the one that exhibited the greatest MSC homing amongst all groups. However, the loss of c-kit or CXCR4 receptors critically reduced MSC engraftment, even with TMR.

It is important to note that not only did greater number of MSCs engrafted in the tissue pretreated with TMR, but also that MSCs tended to engraft closer to the TMR channels. Immunostaining of the histology sections from 3 days revealed occasional patent TMR channels at 3 days, but not at 1 week.

Our data is consistent with the previous findings by Woo and colleagues, as they attributed TMR's therapeutic effect to an increase in number of circulating EPCs.⁹⁶ In our study, we found that TMR's beneficial effect on LV function is due to increased engraftment of circulating MSCs (discussed below). Therefore, we hypothesize that part of the clinical therapeutic benefit of TMR might be attributable to TMR-induced transmigration and engraftment of circulating progenitor cells, although the source and number of these cells are yet to be clearly delineated. In addition to that, our group has previously reported an increase

in transplanted BMC survival due to TMR.⁹⁷ In our previous study, we have demonstrated that TMR induces transmigration and significantly increases engraftment of circulating MSCs by upregulating SCF—c-kit and SDF-1—CXCR4 signaling axes.⁹⁸ Consistent with prior findings, our current study demonstrated that TMR enhances engraftment of MSCs, and that both c-kit and CXCR4 receptors play a critical role in TMR-enhanced MSC homing.

8.4 TMR INCREASES EXPRESSION OF SIGNALING CYTOKINES

The *in vivo* mRNA expression of SCF, c-kit, SDF-1, and CXCR4 was quantified by reverse transcribed real-time PCR. At three days, the expression of both c-kit and SCF was upregulated in the groups that underwent TMR. Moreover, the groups that underwent TMR had approximately twice as much expression of both c-kit and SCF, as compared to their non-TMR counterparts. The injury created by TMR may induce overexpression of SCF and other proangiogenic cytokines, such as SDF-1. However, the increased expression of c-kit, compared to WT, despite c-kit knockdown may seem counterintuitive, but is not an unexpected finding. This is because the expression of c-kit was quantified in host scar tissue, which contained not only the c-kit knockdown donor MSCs that were transplanted, but also c-kit⁺ host cells like BM resident c-kit⁺ PCs or cardiac resident CPCs. Therefore, even if we had successfully knockdown 100% of c-kit in donor MSCs, we would still see expression of c-kit in the injured tissue.

At 1 week, however, the expression of both SCF and c-kit dropped dramatically across all groups. This pattern of SCF and c-kit expression from 3 days to 1 week corresponded temporally with the greater MSC engraftment that we had seen at 3 days,

compared to 1 week. A critical pattern that re-emerged was that the expression of c-kit was lower in the no TMR—CXCR4- group than no TMR—WT at 3 days. The co-downregulation of c-kit due to CXCR4 knockdown seems to persist *in vivo*, even after three days post-injection of MSCs with *ex-vivo* knockdown of the CXCR4 receptor. It is important to note that the use of replication deficient lentiviral vectors and stable shRNA prevented any significant leakage of the knockdown construct into the host tissue, and therefore, any unintended infection of non-target cells. Furthermore, the small magnitude of difference between no TMR—WT and no TMR—CXCR4- in terms of c-kit expression suggests that the low c-kit expression may be due to transplanted cells that exhibit co-downregulation. By 1-week time point, however, the expression of c-kit was similar between the no TMR—WT and no TMR—CXCR4- groups. Changes in the expression of cytokines and other paracrine factors in postinfarction environment and loss of viable transplanted cells by the end of 1 week may be reasons as to why CXCR4 receptor knockdown no longer caused co-downregulation of c-kit expression.

The SDF-1—CXCR4 axis exhibited similar pattern of expression as the SCF—c-kit axis. At 3 days, the expression of both SDF-1 and CXCR4 was greater in the groups that received TMR. As was the case with c-kit expression, CXCR4 receptor expression was lower in the no TMR—c-kit- group, compared to no TMR—WT group at 3 days, but not at 1 week. The reduced expression of CXCR4 in the c-kit knockdown group was consistent with the co-downregulation effect we had seen earlier. Moreover, the overall expression of CXCR4 experienced a dramatic drop by the end of 1-week time point, albeit it was significantly greater expression in the TMR groups. On the other hand, all of the groups exhibited an increase in the expression of SDF-1 due to TMR, even by the end of 1 week.

This suggests that TMR may cause an early engraftment, but the continued increase in SDF-1 expression, even by the end of 1-week time point, means that there is a possibility of sustained angiogenic response due to TMR.

In addition, the pattern of SCF and SDF-1 ligand expression is noteworthy. Both of the ligands were upregulated in the TMR—c-kit- and TMR—CXCR4- groups, despite the fact that knockdown was performed in donor MSCs. The upregulation in SCF ligand is comparable to the data presented by Welte and colleagues. In a series of experiments, the authors exposed human umbilical vein endothelial cells (HUVECs) to proinflammatory stimuli, such as gram-negative bacterial strain or IL-1 α .¹¹⁰⁻¹¹¹ Welte and colleagues then demonstrated a downregulation of c-kit and an upregulation of SCF mRNA expression in response to inflammation. The authors proposed that SCF expression is activated in response to inflammatory stimuli, and the downregulation of c-kit receptor may allow SCF to maintain a steady state for hematopoietic homeostasis.¹¹⁰⁻¹¹¹ The results presented by Welte and colleagues suggested a counter-regulatory relationship between a ligand and its receptor, which can be extrapolated to our study. In the present study, we found that the SCF and SDF-1 expression had increased due to TMR in the c-kit and CXCR4 knockdown groups. It can be surmised that the cell-cell interactions between host stromal cells and donor MSCs, in the presence of a TMR injury, may induce an endogenous repair response. This may, in turn, trigger a counter-regulatory increase in the expression of SCF and SDF-1 ligands. However, further studies are needed to fully elucidate the possible counter-regulatory relationship of SCF with c-kit and SDF-1 with CXCR4.

The SCF—c-kit and SDF-1—CXCR4 expression patterns discussed above correspond with the MSC homing and engraftment pattern we have witnessed. The increased

expression of SDF-1 and CXCR4 axis is consistent with the findings of Zhang et al.⁹⁵ In their study, the authors demonstrated an increase in SDF-1 α and CXCR4 expression after treating animals with TMR and implantation of a heparinized bFGF-incorporating stent. Zhang et al. suggested that TMR-enhanced expression of SDF-1 and CXCR4 resulted in recruitment and proliferation of CXCR4+ CSCs, and promoted differentiation into myocardium.⁹⁵ The findings from Zhang et al. provide strong evidence of TMR-induced upregulation of SDF-1—CXCR4 signaling axis.

Similarly, in our previous study we found that TMR increased expression of SCF and c-kit at 3 days, and this expression declines over time by the end of 1-week time point. SDF-1, on the other hand, gradually increases in expression at 3 days, but also continues to increase over time in expression, whereas CXCR4 experiences a rapid increase in expression at 3 days, and a dramatic drop in expression by 1 week. Both SCF—c-kit and SDF-1—CXCR4 were upregulated due to TMR in our previous study, and this finding was replicated in our current study.⁹⁸ Despite knockdown of either axis, TMR significantly upregulated the expression of the SCF—c-kit and SDF-1—CXCR4 signaling.

8.5 EFFECT OF TMR ON VASCULAR DENSITIES

The quantitative histological analysis of capillary density was performed by immunostaining for factor VIII in five randomly selected fields per heart, whereas arteriolar density was analyzed by staining for α -SMA. At both 3-day and 1-week time points, no statistically significant differences were detected among groups in terms of capillary and arteriolar densities. This finding is at odds with the evidence presented by Woo and

colleagues and Cooley et al. of increased vascular densities.^{93,96} The trends observed in the capillary densities suggested that the groups that underwent TMR had greater angiogenesis, compared to the ones that did not receive TMR. This is consistent with our previous data, as we saw greater vascular densities in the groups treated with TMR.⁹⁷⁻⁹⁸ However, due to high variability amongst groups, and possibly due to inadequate sample size, the increase in vascular densities observed in our current study did not reach statistical significance.

8.6 TMR IMPROVES LEFT VENTRICULAR FUNCTION ONLY WHEN SCF—C-KIT AND SDF-1—CXCR4 AXES ARE INTACT

The left ventricular function was evaluated by calculating LVEF using echocardiography in two different sets of rats, one group that was sacrificed 3 days after the last MSC injection, and the other one was sacrificed at 1 week. Prior to ligation both 3 day and 1 week groups of rats exhibited similar baseline LVEF, and both sets of rats experienced a similar decline in LVEF after ligation. However, 1 week after last MSC injection was performed, the groups that underwent TMR experienced a statistically significant improvement in LVEF. Yet, this improvement in LVEF was not evident as early as 3 days. Also, the transplantation of WT MSCs improved LVEF greater than the transplantation of c-kit or CXCR4 knockdown MSCs. More importantly, the group that was pretreated with TMR and received WT MSCs experienced greatest improvement in LVEF compared to other groups. But knockdown of either c-kit or CXCR4 abrogated this TMR-mediated augmentation of MSC reparative effect.

It is also important to note that at 1 week, even the groups without TMR and with knockdown of c-kit or CXCR4 had experienced some extent of improvement in the LVEF, albeit significantly less than the groups with TMR or with WT MSCs. This small improvement in LVEF could be attributed to the recipient's own endogenous repair responses, which may have recruited repair cells and other progenitor cells that were not tracked in our experiments.

While some improvement in LVEF was seen across all groups by 1 week, the TMR-mediated improvement in LVEF could be attributed to enhanced MSC engraftment in the ischemic tissue. The injected MSCs may improve cardiac function through a variety of mechanisms. Firstly, MSCs can transdifferentiate into myofibroblasts, activate resident cardiomyocytes, or stimulate CSCs to differentiate into cardiomyocytes, thus reducing scar size and ventricular wall thinning. As a result of this, the left ventricular end-systolic volume decreases, thus improving LVEF.^{10,11,112} MSCs can also differentiate into VSMCs and SMCs, which participate in cardiac repair through angiogenesis and neovascularization. The formation of *de novo* vessels allow for supply of oxygen and nutrients to the ischemic myocardium and prevent further loss of cardiomyocytes. Moreover, by releasing MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-9, IL-10, and TGF- β , etc., MSCs can influence extracellular matrix remodeling and attenuate adverse cardiac remodeling. Lastly, MSCs may release various factors, such as M-CSF, G-CSF, IGF-1, etc., promoting recruitment and proliferation of additional circulating or other bone-marrow resident progenitor cells to the site of infarct and further contribute to cardiac repair.^{35,112} Thus, TMR-mediated enhanced engraftment of MSCs in the scar may improve cardiac function through a variety of mechanisms.

Furthermore, TMR-mediated improvement in LV function observed in our study was consistent with the previously reported findings. Zhang et al. reported higher LVEF at 6 weeks in the group treated with TMR, along with bFGF incorporated stent implantation, compared to the control group.⁹⁵ Our lab has previously reported greater improvement in LVEF due to TMR in conjunction with transplantation of BMCs transfected with VEGF, bFGF, and IGF-1 at 1 week.⁹⁷ Furthermore, we have reported that TMR alone significantly improves LVEF at 1 week compared to the control group.⁹⁷⁻⁹⁸

As expected, our data suggests that TMR in conjunction with MSC transplantation improves LV function. However, this TMR-mediated reparative effect is highly dependent on the integrity of the SCF—c-kit and SDF-1—CXCR4 signaling axes, and that disruption of either of the axes attenuated the effect of TMR on improvement of LVEF.

8.7 CONCLUSIONS AND CLINICAL IMPLICATIONS

The results from our study suggest that SCF—c-kit and SDF-1—CXCR4 signaling axes must be intact in order for TMR to enhance the recovery of LVEF. In addition, knockdown of either axis dramatically attenuates, but does not eliminate TMR-induced MSC engraftment. The effect of TMR on stem cell signaling and engraftment may explain part of the antianginal effect observed in patients. What we know is that some patients respond better clinically to TMR than others. The reasons for that variability in response are not well understood. Our data suggests that one of the predictors of clinical response may be the integrity of these two stem cell signaling axes and the patients' stem cell reserve. Our data implies that patients with greater stem cell reserve and preserved stem cell signaling axes

may be expected to derive greater benefit from TMR and this may be a useful predictor when evaluating patients for potential TMR therapy.

8.8 STUDY LIMITATIONS

This study has a number of limitations. For accurate quantification of MSC engraftment, we delivered gender mismatched MSCs to the recipient rats. This allowed us to demonstrate the effect of TMR on c-kit or CXCR4 receptor knockdown donor MSCs that were introduced in the recipient blood circulation. To see the effects of disrupting SCF—c-kit and SDF-1—CXCR4 signaling axes on TMR induced mobilization of BM MSCs out of the bone marrow, more complex animal models are needed. These animal models may include chimeric mice with reconstituted sex-mismatched BMCs, or mice with tamoxifen-inducible knockdown/knockout of above transgenes. Furthermore, due to logistic and time restraints, we did not include a c-kit and CXCR4 double receptor knockdown group. While knockdown of one receptor resulted in downregulation of both, we do not know whether a simultaneous use of both knockdown constructs would result in further depression or complete abolition of TMR-induced MSC signaling. Another limitation of our study is that we have only assessed the effects of TMR in conjunction with MSC transplantation on SCF—c-kit and SDF-1—CXCR4 signaling axes, but have not evaluated possible paracrine effects of MSCs on the surrounding host myocardium. It was beyond the scope of our study to evaluate possible pro-inflammatory effect of TMR, or resulting upregulation of inflammatory cytokines.

8.9 FUTURE DIRECTIONS

The results from this study add to the current body of literature dedicated to understanding the function and mechanism of TMR. The increased MSC engraftment and survival, as well as improvement in LVEF, are a testament to TMR-mediated improvement of cardiac function. More complex animal models are needed to further understand TMR-induced recovery of LV function. Furthermore, the effects of TMR must be studied over longer time points to fully elucidate its effectiveness. Only then can we move to clinical studies where TMR could be used in conjunction with cell therapy to improve patient outcomes.

CHAPTER NINE

REFERENCES

9.1 REFERENCES

1. Statistics Canada. Mortality, summary list of causes. 2011; Catalogue number 84F0209X. Available at: <http://www.statcan.gc.ca/pub/84f0209x/84f0209x2008000-eng.pdf>.
2. Public Health Agency of Canada. Tracking heart disease and stroke in Canada. 2009; Catalogue number HP32-3/2009E. Available at: <http://www.phac-aspc.gc.ca/publicat/2009/cvd-avc/toc-tdm-eng.php>.
3. Katz A. Physiology of the Heart. (5th ed.). Philadelphia, PA: Wolters Kluwer--Lippincott Williams & Wilkins; 2011.
4. Thygesen K, Alpert JS, Jaffe AS, et al. Third universal definition of myocardial infarction. *Circulation*. 2012;126(16):2020–2035.
5. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (first of two parts). *NEJM*. 1992;326(4):242–250.
6. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (second of two parts). *NEJM*. 1992;326(5):310–318.
7. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation*. 2005;111(25):3481–3488.
8. Makki N, Brennan TM, Girotra S. Acute coronary syndrome. *J Intensive Care Med*. 2013. doi: 10.1177/0885066613503294
9. Chang J, Nair V, Luk A, Butany J. Pathology of myocardial infarction. *Diagn Histopathol*. 2013;19(1):7–12.

10. Yousef ZR, Redwood SR, Marber MS. Postinfarction left ventricular remodeling: a pathophysiological and therapeutic review. *Cardiovas Drug Ther.* 2000;14:243–252.
11. Sutton MGSJ, Sharpe N. Left ventricular remodeling after myocardial infarction: Pathophysiology and therapy. *Circulation.* 2000;101(25):2981–2988.
12. Boateng S, Sanborn T. Acute myocardial infarction. *Disease-a-Month.* 2013;59(3):83–96.
13. White HD, Chew DP. Acute myocardial infarction. *Lancet.* 2008;372(9638):570–584.
14. Welch TD, Yang EH, Reeder GS, Gersh BJ. Modern management of acute myocardial infarction. *Curr Prob Cardiology.* 2012;37(7):237–310.
15. Jopling C, Sleep E, Raya M, et al. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature.* 2010;464(7288):606–609.
16. Hsieh PCH, Segers VFM, Davis ME, et al. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med.* 2007;13(8):970–974.
17. Laflamme MA, Murry CE. Heart regeneration. *Nature.* 2011;473(7347):326–335.
18. Bergmann O, Bhardwaj RD, Bernard S, et al. Evidence for cardiomyocyte renewal in humans. *Science.* 2009;324(5923):98–102.
19. Kajstura J, Urbanek K, Perl S, et al. Cardiomyogenesis in the adult human heart. *Circ Res.* 2010;107(2):305–315.
20. Mohsin S, Siddiqi S, Collins B, Sussman MA. Empowering adult stem cells for myocardial regeneration. *Circ Res.* 2011;109(12):1415–1428.
21. Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circ Res.* 2012;110(4):624–637.

22. Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol. & Cell. Cardiol.* 2005;39(5):733–742.
23. Yoon CH. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. *Circulation.* 2005;112(11):1618–1627.
24. Hur J, Yoon CH, Kim HS, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasclogenesis. *Arterioscler., Thrombos., & Vasc. Biol.* 2004;24(2):288–293.
25. Leri A, Kajstura J, Anversa P. Role of cardiac stem cells in cardiac pathophysiology: A paradigm shift in human myocardial biology. *Circ Res.* 2011;109(8):941–961.
26. Chugh AR, Beache GM, Loughran JH, et al. Administration of cardiac stem cells in patients with ischemic cardiomyopathy: The SCIPIO Trial: Surgical aspects and interim analysis of myocardial function and viability by magnetic resonance. *Circulation.* 2012;126(11_suppl_1):S54–S64.
27. Koudstaal S, Jansen of Lorkeers SJ, Gaetani R, et al. Concise review: Heart Regeneration and the role of cardiac stem cells. *Stem Cells Transl Med.* 2013;2(6):434–443.
28. Makkar RR, Smith RR, Cheng K, et al. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *Lancet.* 2012;379(9819):895–904.
29. Unno K, Jain M, Liao R. Cardiac side population cells: moving toward the center stage in cardiac regeneration. *Circ Res.* 2012;110(10):1355–1363.

30. Oyama T, Nagai T, Wada H, et al. Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. *J Cell Biol.* 2007;176(3):329–341.
31. Pfister O. CD31- but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res.* 2005;97(1):52–61.
32. Ieda M, Fu J-D, Delgado-Olguin P, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell.* 2010;142(3):375–386.
33. Qian L, Huang Y, Spencer CI, et al. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature.* 2012:1–8.
34. Efe JA, Hilcove S, Kim J, et al. Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nat Cell Bio.* 2011;13(3):215–222.
35. Williams AR, Hare JM. Mesenchymal stem cells: Biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res.* 2011;109(8):923–940.
36. Huang XP, Sun Z, Miyagi Y, et al. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation.* 2010;122(23):2419–2429.
37. Uemura R, Xu M, Ahmad, N, et al. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. *Circ Res.* 2006;98(11):1414–1421.
38. Xu M, Wani, M, Dai, YS, et al. Differentiation of bone marrow stromal cells into the cardiac phenotype requires intercellular communication with myocytes. *Circulation.* 2004;110(17):2658–2665.

39. Xu W, Zhang X, Qian H, et al. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Exper Biol Med*. 2004;229:623–631.
40. Li X, Yu X, Lin Q, et al. Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. *J Mol Cell Cardio*. 2007;42(2):295–303.
41. Edling CE, Hallberg B. c-Kit—A hematopoietic cell essential receptor tyrosine kinase. *Int J Biochem Cell Biol*. 2007;39(11):1995–1998.
42. Rose RA, Jiang H, Wang X, et al. Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. *Stem Cells*. 2008;26(11):2884–2892.
43. Li S-H, Lai TYY, Sun Z, et al. Tracking cardiac engraftment and distribution of implanted bone marrow cells: Comparing intra-aortic, intravenous, and intramyocardial delivery. *JTCVS*. 2009;137(5):1225–1233.e1.
44. Shake JG, Gruber PJ, Baumgartner WA, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg*. 2002;73:1919–1926.
45. Deuse T, Peter C, Fedak PWM, et al. Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. *Circulation*. 2009;120(11_suppl_1):S247–S254.
46. Kim SH, Moon H-H, Kim HA, et al. Hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cells prevent myocardial ischemic injury. *Mol*

- Ther.* 2009;1–10.
47. Yau TM, Kim C, Li G, et al. Enhanced angiogenesis with multimodal cell-based gene therapy. *Ann Thorac Surg.* 2007;83(3):1110–1119.
 48. Yau TM, Kim C, Li G, et al. Maximizing ventricular function with multimodal cell-based gene therapy. *Circulation.* 2005;112(suppl I):I-123–I-128.
 49. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation.* 2002;106(15):1913–1918.
 50. Assmus B, Schächinger V, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation.* 2002;106(24):3009–3017.
 51. Fernandez-Aviles F, San Roman JA, Garcia-Frade J, et al. Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. *Circ Res.* 2004;95(7):742–748.
 52. Kang H-J, Kim H-S, Zhang S-Y, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet.* 2004;363(9411):751–756.
 53. Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet.* 2004;364(9429):141–148.
 54. Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: Eighteen months' follow-up data from the randomized, controlled

- BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation*. 2006;113(10):1287–1294.
55. Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: 5-year follow-up from the randomized-controlled BOOST trial. *Eur Heart J*. 2009;30(24):2978–2984.
 56. Lunde K, Solheim S, Aakhus S, et al. Autologous stem cell transplantation in acute myocardial infarction: The ASTAMI randomized controlled trial. Intracoronary transplantation of autologous mononuclear bone marrow cells, study design and safety aspects. *Scand Cardiovasc J*. 2005;39(3):150–158.
 57. Beitnes JO, Hopp E, Lunde K, et al. Long-term results after intracoronary injection of autologous mononuclear bone marrow cells in acute myocardial infarction: the ASTAMI randomised, controlled study. *Heart*. 2009;95(24):1983–1989.
 58. Seeger FH, Tonn T, Krzossok N, Zeiher AM, Dimmeler S. Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. *Eur Heart J*. 2007;28(6):766–772.
 59. Dill T, Volker S, Rolf A, et al. Intracoronary administration of bone marrow-derived progenitor cells improves left ventricular function in patients at risk for adverse remodeling after acute ST-segment elevation myocardial infarction: Results of the Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction study (REPAIR-AMI) cardiac Magnetic Resonance Imaging substudy. *Am Heart J*. 2009;157(3):541–547.
 60. Perin EC, Willerson JT, Pepine CJ, et al. Effect of transendocardial delivery of

- autologous bone marrow mononuclear cells on functional capacity, left ventricular function, and perfusion in chronic heart failure: The FOCUS-CCTRN trial. *JAMA*. 2012;307(16):1717.
61. Hare JM, Fishman JE, Gerstenblith G, et al. Comparison of allogeneic vs. autologous bone marrow–derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy. *JAMA*. 2012;308(22):2369.
 62. Bartunek J, Behfar A, Dolatabadi D, et al. Cardiopoietic stem cell therapy in heart failure. *J Am Coll Cardiol*. 2013;61(23):2329–2338.
 63. Behfar A, Terzic A. Cardioprotective repair through stem cell-based cardiopoiesis. *J Appl Physiol*. 2007;103(4):1438–1440.
 64. Behfar A, Yamada S, Crespo-Diaz R, et al. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. *JAC*. 2010;56(9):721–734.
 65. Menasché P, Hagege AA, Vilquin J-T, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol*. 2003;41(7):1078–1083.
 66. Mansour S, Roy D-C, Bouchard V, et al. COMPARE-AMI Trial: comparison of intracoronary injection of CD133+ bone marrow stem cells to placebo in patients after acute myocardial infarction and left ventricular dysfunction: Study rationale and design. *J Cardiovasc Trans Res*. 2009;3(2):153–159.
 67. Frenette PS, Subbarao S, Mazo IB, Andrian von UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc. Natl. Acad. Sci*. 1998;95(24):14423–14428.

68. Palumbo R, Bianchi ME. High mobility group box 1 protein, a cue for stem cell recruitment. *Biochemical Pharmacology*. 2004;68(6):1165–1170.
69. Zhang D, Fan G-C, Zhou X, et al. Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. *Journal of Molecular and Cellular Cardiology*. 2008;44(2):281–292.
70. Quesenberry PJ, Becker PS. Stem cell homing: rolling, crawling, and nesting. *PNAS*. 1998;95:15155–15157.
71. Mazo IB, Gutierrez-Ramos J-C, Frenette PS, et al. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *J Exper Med*. 1998;188(3):465–474.
72. Ruster B, Gottig S, Ludwig RJ, et al. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood*. 2006;108(12):3938–3944.
73. Jaganathan BG, Ruester B, Dressel L, et al. Rho inhibition induces migration of mesenchymal stromal cells. *Stem Cells*. 2007;25(8):1966–1974.
74. Ashman LK. The biology of stem cell factor and its receptor c-kit. *Int J Biochem Cell Biol*. 1999;31:1037–1051.
75. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. *Cell. Mol. Life Sci*. 2004;61(19-20):2535–2548.
76. Lennartsson J, Ronnstrand L. Stem cell factor receptor/c-kit: from basic science to clinical implications. *Physiol Rev*. 2013;92(4):1619–1649.
77. Gagari E, Rand MK, Tayari L, et al. Expression of stem cell factor and its receptor, c-kit, in human oral mesenchymal cells. *Eur. J. Oral Sci*. 2006;114(5):409–415.
78. Blume-Jensen P, Janknecht R, Hunter T. The kit receptor promotes cell survival via

- activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. *Curr. Biol.* 1998;8(13):779–782.
79. Carson WE, Haldar S, Baiocchi RA, Croce CM, Caligiuri MA. The c-kit ligand suppresses apoptosis of human natural killer cells through the upregulation of bcl-2. 1994;91:7553–7557.
 80. Fazel SS, Fazel SS, Chen L, et al. Activation of c-kit is necessary for mobilization of reparative bone marrow progenitor cells in response to cardiac injury. *FASEB J.* 2007;22(3):930–940.
 81. Ayach BB, Yoshimitsu M, Dawood F, et al. Stem cell factor receptor induces progenitor and natural killer cell-mediated cardiac survival and repair after myocardial infarction. *Proc. Natl. Acad. Sci. U.S.A.* 2006;103(7):2304–2309.
 82. Ghadge SK, Mühlstedt S, Özcelik C, Bader M. SDF-1 α as a therapeutic stem cell homing factor in myocardial infarction. *Pharmacol Therapeut.* 2011;129(1):97–108.
 83. Kucia M, Reza R, Miekus K, et al. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: Pivotal role of the SDF-1-CXCR4 axis. *Stem Cells.* 2005;23(7):879–894.
 84. Petit I, Jin D, Rafii S. The SDF-1–CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. *Trends Immunol.* 2007;28(7):299–307.
 85. Sloane AJ, Raso V, Dimitrov DS, et al. Marked structural and functional heterogeneity in CXCR4: Separation of HIV-1 and SDF-1 α responses. *Immunol Cell Biol.* 2005;83(2):129–143.
 86. Wang Y, Deng Y, Zhou G-Q. SDF-1 α /CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model.

- 2008;1195:104–112.
87. Hu X, Dai S, Wu WJ, et al. Stromal Cell Derived Factor-1 Confers Protection Against Myocardial Ischemia/Reperfusion Injury: Role of the Cardiac Stromal Cell Derived Factor-1 CXCR4 Axis. *Circulation*. 2007;116(6):654–663.
 88. Saxena A, Fish JE, White MD, et al. Stromal Cell-Derived Factor-1 Is Cardioprotective After Myocardial Infarction. *Circulation*. 2008;117(17):2224–2231.
 89. Allen KB, Kelly J, Borkon AM, et al. Transmyocardial laser revascularization: From randomized trials to clinical Practice: A Review of techniques, evidence-based outcomes, and future directions. *Anesthesiol Clin*. 2008;26(3):501–519.
 90. Estvold SK, Mordini F, Zhou Y, et al. Does laser type impact myocardial function following transmyocardial laser revascularization? *Laser Surg Med*. 2010;42:746–751.
 91. Horvath KA, Belkind N, Wu I, et al. Functional comparison of transmyocardial revascularization by mechanical and laser means. *Ann Thorac Surg*. 2001;72:1997–2002.
 92. Al-Sheikh T, Allen KB, Straka SP, et al. Cardiac sympathetic denervation after transmyocardial laser revascularization. *Circulation*. 1999;100:135–140.
 93. Cooley DA, Frazier OH, Kadipasaoglu KA, et al. Transmyocardial laser revascularization: Anatomical evidence of long-term channel patency. *Tex Heart J J*. 1994;21:220–224.
 94. Horvath KA, Smith WJ, Laurence RG, et al. Recovery and viability of an acute myocardial infarct after transmyocardial laser revascularization. *J Am Coll Cardiol*. 1995;25:258–263.
 95. Zhang G-W, Wen T, Gu T-X, et al. Transmyocardial drilling revascularization

- combined with heparinized bFGF-incorporating stent activates resident cardiac stem cells via SDF-1/CXCR4 axis. *Exper Cell Res*. 2012;318(4):391–399.
96. Atluri P, Panlilio CM, Liao GP, et al. Transmyocardial revascularization to enhance myocardial vasculogenesis and hemodynamic function. *JTCVS*. 2008;135(2):283–291.e1.
 97. Spiegelstein D, Kim C, Zhang Y, et al. Combined transmyocardial revascularization and cell-based angiogenic gene therapy increases transplanted cell survival. *Am J Physiol Heart Circ*. 2007;293(6):H3311–H3316.
 98. Shahzad U, Li G, Zhang Y, Yau TM. Transmyocardial revascularization induces mesenchymal stem cell engraftment in infarcted hearts. *Ann Thorac Surg*. 2012;94(2):556-562.
 99. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determinations: echocardiographic-angiographic correlations in the presence or absence of asynergy. *Am J Cardiol*. 1976;37:7–11.
 100. Folland ED, Parisi AF, Moynihan PF, et al. Assessment of left ventricular ejection fraction and volumes by real- time, two-dimensional echocardiography: A comparison of cineangiographic and radionuclide techniques. *Circulation*. 1979;60(4):760–766.
 101. Wandt B, Bojo L, Tolagen K, Wranne B. Echocardiographic assessment of ejection fraction in left ventricular hypertrophy. *Heart*. 1999;82:192–198.
 102. Moore CB, Guthrie EH, Huang MT-H, Taxman DJ. *Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown*. (Sioud M, ed.). Totowa, NJ: Methods in Molecular Biology; 2010:141–158.
 103. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA

- sequences. *J Comput Biol.* 2000;7(1/2):203–214.
104. Morgulis A, Coulouris G, Raytselis Y, et al. Database indexing for production MegaBLAST searches. *Bioinformatics.* 2008;24(16):1757–1764.
 105. Luo Y, Zhao X, Zhou X, et al. Short-term intermittent administration of CXCR4 antagonist AMD3100 facilitates myocardial repair in experimental myocardial infarction. *Acta Bioch Bioph Sin.* 2013;45(7):561–569.
 106. Chen Z, Pan X, Yao Y, et al. Epigenetic regulation of cardiac progenitor cells marker c-kit by stromal cell derived factor-1 α . *PLoS ONE.* 2013;8(7):e69134.
 107. Chen Z, Pan X, Yao Y, et al. Regulation of c-kit⁺ progenitor cells by stromal cell derived factor-1 α in adult murine heart. *Heart Lung Circ.* 2013;e-pub ahead of print:S1443–9506.
 108. Cheng M, Zhou J, Wu M, et al. CXCR4-mediated bone marrow progenitor cell maintenance and mobilization are modulated by c-kit activity. *Circ Res.* 2010;107(9):1083–1093.
 109. Müller-Ehmsen J, Whittaker P, Kloner RA, et al. Survival and development of neonatal rat cardiomyocytes transplanted into adult Myocardium. *J Mol Cell Cardiol.* 2002;34(2):107–116.
 110. Köenig A, Yakisan E, Reuter M, et al. Differential regulation of stem cell factor mRNA expression in human endothelial cells by bacterial pathogens: an in vitro model of inflammation. *Blood.* 1994;(83):2836–2843.
 111. Köenig A, Corbacoglu S, Ballmaler M, Welte K. Downregulation of c-kit expression in human endothelial cells by inflammatory stimuli. *Blood.* 1997;90:148–155.
 112. Wen Z, Zheng S, Zhou C, Wang J, Wang T. Repair mechanisms of bone marrow

mesenchymal stem cells in myocardial infarction. 2011;15(5):1032–1043.

APPENDIX 1

TRANSMYOCARDIAL REVASCULARIZATION INDUCES MESENCHYMAL STEM CELL ENGRAFTMENT IN INFARCTED HEARTS

Reproduced from Shahzad U, Li G, Zhang Y, Yau TM. Transmyocardial revascularization induces mesenchymal stem cell engraftment in infarcted hearts. *Ann Thorac Surg.* 2012;94(2):556-562, with permission from Elsevier under license no. 3253601340196.

Invited commentary from Kipshidze N. Comment on “transmyocardial revascularization induces mesenchymal stem cell engraftment in infarcted hearts.” *Ann Thorac Surg.* 2012;94(2):562-563, reproduced with permission from Elsevier under license no. 3254220827439.

Transmyocardial Revascularization Induces Mesenchymal Stem Cell Engraftment in Infarcted Hearts

Uswa Shahzad, BHSc, Guangming Li, MD, Yaoguang Zhang, MD, and Terrence M. Yau, MD, MS

Division of Cardiovascular Surgery, Peter Munk Cardiac Centre, University Health Network, Department of Surgery, University of Toronto, Toronto, Ontario, Canada

Background. Transmyocardial revascularization (TMR) prior to mesenchymal stem cell (MSC) transplantation augments repair of infarcted hearts. We evaluated the effects of TMR on homing and engraftment of circulating MSCs and mediators of this effect.

Methods. Three weeks after left anterior descending coronary artery ligation in female rats, 10-channel needle TMR was performed in the infarct, followed by daily intravenous injections of 1 million male MSCs for 5 days. Control rats had MSC infusions without TMR ($n = 16/\text{group}$). Donor MSC survival was evaluated at 3 days and at 1 week by quantitative polymerase chain reaction, as well as expression of stem cell factor (SCF), stromal derived factor-1 (SDF-1), c-kit, and chemokine receptor type 4 (CXCR4).

Results. The MSCs engrafted into the infarct, clustering around TMR channels. The MSC engraftment was greater in TMR hearts at 3 days and at 1 week. Both SCF

($p = 0.03$) and c-kit ($p = 0.01$) were upregulated by TMR at 3 days, but their levels fell at 1 week ($p = 0.3$, $p = 0.5$, respectively). The SDF-1 levels were higher in TMR hearts at both 3 days ($p = 0.04$) and at 1 week ($p = 0.04$). The CXCR4 was upregulated early by TMR ($p = 0.0002$) but levels dropped dramatically at 1 week ($p = 0.045$).

Conclusions. Transmyocardial revascularization induces transmigration and engraftment of circulating MSCs. Post TMR, the transcription of SCF and c-kit is rapid and corresponds temporally to MSC engraftment, while SDF-1 levels rise slowly. The CXCR4 is also transiently upregulated. The TMR-augmented repair of infarcted hearts by stem cell transplantation may be mediated by a novel mechanism: transmigration and engraftment of circulating progenitor cells.

(Ann Thorac Surg 2012;94:556–63)

© 2012 by The Society of Thoracic Surgeons

Cell transplantation has been investigated as a potential therapy for postinfarction ventricular dysfunction. Mesenchymal stem cells (MSCs), one of the most frequently studied cell types, support hematopoiesis by secreting cytokines and growth factors and can inhibit inflammatory responses, reduce apoptosis, prevent fibrosis, and play a role in neovascularization in a paracrine fashion [1]. However, transplantation of unmodified or genetically modified MSCs or other progenitor cells does not normalize the function or perfusion of infarcted hearts [2–4]. An alternative treatment intended to enhance myocardial perfusion is transmyocardial revascularization (TMR), usually using a laser to create channels in ischemic myocardium, that has been associated with relief of otherwise refractory angina [5, 6].

We have previously demonstrated that in a rat infarct model pretreatment of an infarct zone by TMR prior to transplantation of MSCs or MSCs overexpressing vascular endothelial growth factor, basic fibroblast growth factor, and insulin-like growth factor-1 results in signifi-

cantly increased angiogenesis and restoration of ventricular function [7]. However, the mechanism by which TMR improves MSC transplantation is unknown. We hypothesized that one potential mechanism by which TMR exerted its beneficial effect on MSC transplantation could be by signaling to circulating progenitor cells through the stem cell factor (SCF)-c-kit signaling axis or the stromal-derived factor-1 (SDF-1)-chemokine receptor type 4 (CXCR4) axis [8, 9].

The c-kit is a tyrosine kinase receptor expressed on stem cells and mast cells [8, 10]. In response to myocardial injury, c-kit⁺ cells can express markers that are specific to a cardiac lineage [8, 10]. The c-kit plays a role in neovascularogenesis by binding to stromal membrane-bound SCF, a chemotactic agent for hematopoietic stem cells [8]. This results in autophosphorylation and initiation of PI-3 kinase-Akt and the renin-angiotensin system-mitogen-activated protein kinase signaling pathways, which in turn promote progenitor cell adhesion [11]. Similarly, the SDF-1 and CXCR4 signaling pathways modulate angiogenesis by inducing mobilization and homing of progenitor cells [9]. The SDF-1 signaling induces activation of matrix metalloproteinase-9, release of SCF, and stimulation of nitric oxide, which subse-

Accepted for publication March 20, 2012.

Address correspondence to Dr Yau, 4N-470, Toronto General Hospital, 200 Elizabeth St, Toronto, Ontario, Canada M5G2C4; e-mail: terry.yau@uhn.ca.

Abbreviations and Acronyms

Ctrl	= Control
CXCL12	= chemokine ligand 12
CXCR4	= chemokine receptor type 4
ddH ₂ O	= distilled deionized water
DNA	= deoxyribonucleic acid
EF	= ejection fraction
EPC	= endothelial progenitor cell
FAS	= fractional area shortening
FBS	= fetal bovine serum
FS	= fractional shortening
GFP	= green fluorescent protein
IMDM	= Iscove modified Dulbecco medium
IV	= Intravenous
LAD	= left anterior descending
LV	= left ventricle
LVEDA	= left ventricular end-diastolic area
LVEDV	= left ventricular end-diastolic volume
LVESA	= left ventricular end-systolic area
LVESV	= left ventricular end-systolic volume
MAP	= mitogen-activated protein
MMP-9	= matrix metalloproteinase-9
MSC	= mesenchymal stem cell
PCR	= polymerase chain reaction
PI-3	= phosphatidylinositol-3
RNA	= ribonucleic acid
SCF	= stem cell factor
SDF-1	= stromal derived factor-1
sry	= sex-determining region on the Y chromosome
TMR	= transmyocardial revascularization
α-SMA	= alpha-smooth muscle actin

quently leads to recruitment of CXCR4+ proangiogenic cells to the sites of ischemic injury [9].

In the current study we evaluated the effect of TMR prior to MSC transplantation on the homing and engraftment of circulating MSCs, as well as on angiogenesis and ventricular function. Furthermore, we measured the expression of the SCF-c-kit and SDF-1–CXCR4 stem cell signaling pathways in order to elucidate the mechanisms through which TMR enhanced the reparative effect of stem cell transplantation.

Material and Methods

Animals and Experimental Model

All experimental procedures were approved by the Animal Care Committee of the University Health Network and were performed according to the “Guide for the Care and Use of Laboratory Animals” by the National Research Council. Adult female Wistar rats weighing 200 to 250 g (recipients) and male Wistar rats weighing 250 to 300 g (cell donors) were obtained from Charles River Canada (Quebec, QC, Canada).

Myocardial infarctions were induced in female rats by ligation of the left anterior descending (LAD) coronary artery. The rats were anesthetized with ketamine hydrochloride (20 mg/kg) and pentobarbital sodium (30 mg/

kg), intubated, and ventilated with room air supplemented with oxygen and isoflurane (0.5% to 2%) by a Harvard ventilator. Through a left lateral thoracotomy, a 6-0 polypropylene suture was used to ligate the proximal LAD coronary artery. The incision was closed and antibiotics (150,000 U/mL penicillin G benzathine and 150,000 U/mL penicillin G procaine) and buprenorphine hydrochloride (0.01 mg/kg) were administered after the operation.

MSC Isolation

Male donor MSCs were isolated from rat femurs and cultured in Iscove modified Dulbecco medium (IMDM) with 10% fetal bovine serum for 4 to 5 days. Cells were maintained in culture for 7 to 8 passages, splitting plates at 70% to 80% confluence. Cultures were partially depleted of erythroid progenitor cells by the removal of nonadherent cells with each change of medium every 3 days. No additional fractionation was performed. The cells were evaluated by flow cytometry before transplantation.

TMR and Cell Transplantation

Three weeks after infarct creation, rats were randomly assigned to 1 of 2 experimental groups (n = 16 per group). One group underwent mechanical TMR followed by daily intravenous injections of 1 million green fluorescent protein-positive male MSCs for 5 days as described below. The other group served as the control (Ctrl), and received intravenous injections of MSCs without prior TMR. The TMR was performed in the experimental group by creating 10 transmural channels with a 25-gauge needle in a 10 mm × 10 mm area within the LAD infarct zone. The combined rat mortality for the LAD ligation and TMR procedures was 15% to 20%.

Cultured MSCs were washed twice with fetal bovine serum-free IMDM medium, and cell numbers were calculated. A standardized dose of 1 × 10⁶ cells/100 μL circulating MSCs with fetal bovine serum-free IMDM medium was used for the injection as it would allow for potential recruitment to the infarct in a way that would give each rat a similar potential to respond.

On cell injection days, the tail vein injection site was sterilized with 70% alcohol, and 100 μL of cell suspension (1 × 10⁶ cells), with 0.3 mg of cyclosporine/kg per day were injected slowly over 60 seconds into the tail vein with a 28-gauge needle on a ½ mL insulin syringe.

Three and 7 days after the last cell injection, the rats were sacrificed. The hearts were then excised and the left ventricular free wall was divided into 2 equal portions; 1 for quantitative histology and the other for deoxyribonucleic acid (DNA) and ribonucleic acid isolation for quantitative polymerase chain reaction (PCR) analysis of genes of interest, including the sex-determining region of the Y chromosome (sry) (n = 6 for each group at each interval). The left ventricle (LV) was divided into the scar zone (transmural scar), the border zone (partial-thickness scar containing both fibrous tissue and surviving muscle), and a rim of grossly normal appearing myocardium surrounding the infarct. The expression of SCF and

SDF-1 and their receptors c-kit and CXCR4, respectively, was also assessed by PCR ($n = 10$ for each group at each interval).

Quantitative Histology of Vascular Densities

In each group, 50% of each rat heart was subjected to quantitative histologic analysis ($n = 4$ for each group at each interval). Sections were fixed in 4% of paraformaldehyde, stained with hematoxylin and eosin or immunohistochemical staining for factor VIII or alpha-smooth muscle actin. Two blinded observers counted the total number of vessels per high-power field (0.2 mm^2) in 5 randomly selected fields per heart. The mean number of vessels per field was used for analysis.

Quantitation of Y Chromosomal DNA by Real-Time PCR

The DNA was isolated from snap-frozen and powdered heart specimens, as described previously [12–14]. To accurately quantitate the number of Y chromosome-bearing male donor cells within the female recipient hearts, a pair of specific primers was designed based on the GeneBank sequence (National Center for Biotechnology Information, Washington, DC) of the rat *sry2* gene (sense 5'-GAGGCACAAGTTGGCTC-AACA-3', antisense 5'-CTCCTGCAAAAAGGGCCTTT-3'). Quantitative PCR was performed on the 9700HT System (Applied Biosystems, Carlsbad, CA) using the Master Mix SYBR Green Kit (Applied Biosystems), utilizing threefold serial dilution of the *sry2* DNA standards in distilled deionized water to generate standards ranging from the *sry2* DNA of 1×10^4 cells to the *sry2* DNA of 41 cells. Total DNA content was measured spectrophotometrically, and equal amounts of DNA were taken from all samples and were transferred to a 96-well PCR plate. Each assay was performed in duplicate and 5 μL of distilled deionized water was assayed as a no-template control. Five microliters of a 5 pmol *sry2* sense and antisense primer mixture and 10 μL of Master SYBR Green Mix (Applied Biosystems) were added to each well. The reaction sequence included the following: stabilization for 5 minutes at 50°C and pre-denaturation for 5 minutes at 95°C before 45 cycles of denaturation for 15 seconds at 95°C ; annealing and primer extension for 1 minute at 60°C ; and dissociation for 15 seconds at 95°C , 15 seconds at 60°C , and 15 seconds at 95°C . The PCR products were labeled with SYBR Green and detected by a laser detector on the 9700HT system. Real-time PCR data were analyzed with SDS 2.3 software (Applied Biosystems).

Left Ventricular Function

The LV function was evaluated in vivo in rats by transthoracic echocardiography (ACUSON Sequoia C256; Siemens, Malvern, PA), as described previously [15]. Left parasternal images were taken in the right lateral decubitus position with the use of a 15-MHz linear transducer. Two-dimensional mode images in the parasternal long-axis view of the LV and M-mode images at the level of papillary muscles were recorded. The LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV

end-diastolic area (LVEDA), and LV end-systolic area (LVESA) were measured. Ejection fraction (EF), fractional area shortening (FAS), and fractional shortening (FS) were calculated with standard M-mode echocardiographic equations ($\text{EF} = \text{LVEDV} - \text{LVESV} / \text{LVEDV} \times 100$; $\text{FAS} = \text{LVEDA} - \text{LVESA} / \text{LVEDA} \times 100$; $\text{FS} = \text{LVEDD} - \text{LVESD} / \text{LVEDD} \times 100$). For each measurement, 3 consecutive cardiac cycles were traced and averaged by an experienced examiner in a blinded fashion. All animals were measured at baseline, before treatment, and 1 day before sacrifice ($n = 10$ in each group at each interval).

Statistical Analysis

Data are presented as means $\pm$ standard deviation. Continuous data were analyzed by one-way analysis of variance.

Results

Histology

Immunohistochemical staining of myocardial sections with an antibody directed against the green fluorescent protein expressed by the donor MSC, and with a secondary antibody linked to horseradish peroxidase, revealed donor cells clustered around the TMR needle tract in the TMR hearts (Fig 1A). In the control group, donor cells were also visible but were fewer in number and not as closely clustered (Fig 1B).

Donor MSC Homing to the Infarcted Heart

Male donor MSC engraftment in the infarcted female recipient hearts was assessed in the scar tissue, border zone, and normal myocardium surrounding the border zone, and was compared with control hearts. On day 3, male MSC engraftment in both the scar ($p = 0.0002$) and the border zone ($p = 0.005$) was significantly greater in TMR hearts than in the control hearts ($p = 0.01$), with a fourfold greater number of donor MSCs in the scar in TMR hearts (Fig 2A). These differences between groups persisted at 1 week (scar, $p = 0.04$; border, $p = 0.03$; normal, $p = 0.4$), but were not as markedly pronounced (Fig 2B; Table 1).

Expression of SCF, SDF-1, c-kit, and CXCR4

Expression of SCF, SDF-1, and their receptors c-kit and CXCR4 was assessed 3 days and 1 week after the last MSC injection. Three days after the last MSC injection, expression of SCF was significantly higher in TMR hearts than controls ($p = 0.03$). However, levels fell at 1 week and were similar between groups ($p = 0.3$). Expression of c-kit, the receptor for SCF, followed the same pattern exhibited by its ligand (3 days, $p = 0.01$; 1 week, $p = 0.5$) (Table 2).

At 3 days, SDF-1 levels were significantly higher in TMR hearts than controls ($p = 0.04$). The SDF-1 levels continued to increase over the next few days, and at 1

Fig 1. Photomicrograph (200 $\times$) of (A) a section of a transmyocardial revascularization (TMR) heart and a (B) control heart. Nuclei of native cardiomyocytes are indicated with red arrows. Green fluorescence protein-expressing donor mesenchymal stem cells (MSCs) are stained brown and are indicated with green arrows. In panel (A), white arrows indicate a residual needle TMR tract. In the (A) TMR heart, donor MSCs are visible, clustered around the TMR needle tract. In the (B) control heart, donor MSCs are apparent but appear fewer in number and were not clustered together.

week, the levels of SDF-1 were significantly greater than levels in controls hearts ($p = 0.04$). In contrast, transcription of the CXCR4 receptor followed an opposite trend,

with early upregulation by TMR (at 3 days, $p = 0.0002$ vs Ctrl) but a dramatic drop in CXCR4 transcription by 1 week ($p = 0.045$ vs Ctrl) (Table 2).

Fig 2. Male donor mesenchymal stem cells (MSC) engraftment was markedly greater in transmyocardial revascularization (TMR) hearts than in control rats at both 3 days (panel A) and 1 week (panel B). Differences between groups were most pronounced at 3 days.

Table 1. The Number of Donor Mesenchymal Stem Cells per mg of Scar Zone, Border Zone, and Normal Zone Tissue, After Tail Vein Injections

Zone	Control Group	TMR Group	p Value
At 3 days			
Scar zone	125.8 ± 71.9	520.2 ± 153.4	0.0002
Border zone	324.6 ± 117.4	657.8 ± 191.9	0.005
Normal zone	91.4 ± 85.8	290.5 ± 135.2	0.01
At 7 days			
Scar zone	301.8 ± 91.3	411.4 ± 62.2	0.04
Border zone	364.9 ± 96.3	510.8 ± 103.8	0.03
Normal zone	244.3 ± 47.6	276.6 ± 70.9	0.4

TMR = transmyocardial revascularization.

Left Ventricular Function

After LAD ligation, LVEF was reduced from 0.84 ± 0.02 ($p = 0.7$) at baseline to 0.57 ± 0.03 ($p = 0.97$), with no differences between the TMR and Ctrl groups. Three days after the last MSC injection, LVEF was essentially unchanged, at 0.58 ± 0.07 for both the TMR and Ctrl groups ($p = 0.7$). However, 1 week after MSC delivery, LVEF was significantly greater in the TMR rats than the controls (TMR 0.71 ± 0.03 , Ctrl 0.65 ± 0.05 , $p = 0.01$).

Vascular Densities

At day 3, total vascular density (all factor VIII-positive structures in the border zone) was greater in the TMR hearts (TMR 5.7 ± 1.7 , Ctrl 3.8 ± 1.6 vessels/ 0.2 mm^2 , $p = 0.03$), with no significant differences in the scar zone (TMR 4.3 ± 2.0 , Ctrl 3.2 ± 1.0 vessels/ 0.2 mm^2 , $p = 0.2$) and normal tissue (TMR 1.8 ± 1.7 , Ctrl 1.9 ± 0.7 vessels/ 0.2 mm^2 , $p = 0.3$). Vascular densities increased in both groups between the 3-day and 1 week time points but remained significantly higher in the TMR group (scar: TMR 7.0 ± 2.0 , Ctrl 5.1 ± 1.6 vessels/ 0.2 mm^2 , $p = 0.001$; border zone: TMR 9.7 ± 1.7 , Ctrl 6.2 ± 1.7 vessels/ 0.2 mm^2 , $p = 0.0007$; normal zone: TMR 3.3 ± 1.0 , Ctrl 3.3 ± 0.9 vessels/ 0.2 mm^2 , $p = 0.8$).

Arteriolar density (α -SMA positive vessels) was higher in the border zone of the TMR hearts (TMR 4.4 ± 1.1 , Ctrl 3.2 ± 1.0 vessels/ 0.2 mm^2 , $p = 0.03$) at 3 days when compared with the scar zone (TMR 3.3 ± 1.3 , Ctrl 2.8 ± 1.4 vessels/ 0.2 mm^2 , $p = 0.2$) and normal zone (TMR 0.9 ± 0.6 , Ctrl 0.8 ± 0.7 vessels/ 0.2 mm^2 , $p = 0.8$). At 1 week, arteriolar density was still higher in the TMR group (scar: TMR 5.4 ± 1.3 , Ctrl 4.4 ± 1.0 vessels/ 0.2 mm^2 , $p = 0.006$; border zone: TMR 7.1 ± 1.1 , Ctrl 5.7 ± 1.1 vessels/ 0.2 mm^2 , $p = 0.002$; normal zone: TMR 1.5 ± 0.6 , Ctrl 1.4 ± 0.6 vessels/ 0.2 mm^2 , $p = 0.5$) (Fig 3A,B).

Comment

Laser transmyocardial revascularization has been employed clinically for the relief of angina refractory to conventional revascularization [5, 6]. After initial controversy over the mechanisms through which TMR exerted its antianginal effect [6], it appears now that denervation may be responsible for the near-immediate symptomatic relief, while angiogenesis in response to the laser injury may account for longer term freedom from angina and potentially for improved late survival compared with patients not receiving TMR [5, 16, 17]. In addition to these effects, we have reported previously that in rats, needle TMR performed prior to LAD ligation results in significantly increased angiogenesis and recovery of LV function after transplantation of MSCs [7]. While this benefit was associated with increased survival of the donor MSC, the mechanisms through which TMR enhanced the reparative effect of stem cell transplantation are unknown. We hypothesized that TMR acted at least in part by harnessing an endogenous myocardial repair response by inducing the transmigration and engraftment of normally bone marrow-resident MSC.

Consistent with the results of our previous study, TMR prior to intravenous MSC delivery resulted in greater angiogenesis, manifested in increased total vascular densities and arteriolar densities, as well as greater recovery of LV function by various echocardiographically assessed

Table 2. The Gene Expression of SCF, c-kit, SDF-1, and CXCR4 in TMR and Control Groups at 3 Days and 7 Days After the Last MSC Injection

Gene Expression Post MSC Transplantation (molecular copies/ μg total RNA $\pm$ SD)			
Transgene	Control Group	TMR Group	p Value
At 3 days			
SCF	$2.70 \times 10^9 \pm 1.80 \times 10^8$	$5.70 \times 10^9 \pm 2.20 \times 10^8$	0.03
c-kit	$2.80 \times 10^9 \pm 9.90 \times 10^8$	$4.60 \times 10^9 \pm 1.8 \times 10^9$	0.01
SDF-1	$1.10 \times 10^9 \pm 2.80 \times 10^8$	$1.90 \times 10^9 \pm 8.50 \times 10^8$	0.04
CXCR4	$2.40 \times 10^{10} \pm 4.00 \times 10^{10}$	$9.40 \times 10^{10} \pm 2.70 \times 10^{10}$	0.0002
At 7 days			
SCF	$2.10 \times 10^9 \pm 1.50 \times 10^8$	$3.00 \times 10^9 \pm 1.40 \times 10^8$	0.3
c-kit	$3.30 \times 10^9 \pm 1.20 \times 10^9$	$2.90 \times 10^9 \pm 1.40 \times 10^9$	0.5
SDF-1	$1.44 \times 10^9 \pm 3.95 \times 10^8$	$2.43 \times 10^9 \pm 9.19 \times 10^8$	0.04
CXCR4	$4.50 \times 10^9 \pm 5.30 \times 10^9$	$1.10 \times 10^{10} \pm 8.40 \times 10^9$	0.045

CXCR4 = chemokine receptor type 4; MSC = mesenchymal stem cell; derived factor-1; TMR = transmyocardial revascularization.

RNA = ribonucleic acid; SCF = stem cell factor; SDF-1 = stromal

Fig 3. Photomicrograph (200 $\times$) of a section of (A) a transmyocardial revascularization (TMR) heart and (B) a control heart stained brown for alpha-smooth muscle actin positive vessels. (A) The TMR hearts had greater arteriolar density than control hearts.

parameters including LVEF [7]. More importantly, in the current study, TMR pretreatment resulted in almost quadrupling the number of MSCs that engrafted in the scar zone of the infarct at 3 days. The MSC engraftment in the border zone was approximately doubled, and in the rim of grossly normal-appearing myocardium just adjacent to the border zone was approximately tripled. The spatial extent of donor cell engraftment indicates that TMR induced homing of MSC to the entire field of injury. To our knowledge, this is the first report to show that TMR can potentially signal circulating MSCs to transmigrate to, and engraft in, the zone of injury. Whether TMR can induce MSCs not already in the circulation, but residing in their normal bone marrow niche, to transmigrate and engraft remains to be determined in future studies.

In contrast, TMR has been shown to induce engraftment of circulating endothelial progenitor cells (EPCs). Atluri and colleagues [18] found that TMR increases mobilization of EPCs to a porcine myocardial infarction. This EPC response likely contributes to the angiogenic effect of TMR. Fazel and colleagues [10] have also reported that transplanted MSCs mobilized EPCs to the cell-transplanted infarct and promoted angiogenesis. In these studies, circulating EPCs would be exposed to the infarct zone and only local signaling would be required to induce myocardial engraftment. If TMR can signal normally bone marrow-resident progenitor cells such as MSCs, then a remote signaling mechanism must be at work.

Two progenitor cell-signaling axes that may mediate the effect of TMR observed in our study include the SCF–c-kit axis and the SDF-1–CXCR4 axis [8, 9]. We evaluated the transcriptional regulation of both the circulating cytokines and cell surface receptors for both of these signaling axes to try to correlate the observed TMR effect.

Transcription of both SCF and its receptor c-kit was significantly upregulated by 3 days after the last MSC injection, with an approximate doubling of messenger ribonucleic acid transcript levels compared with control hearts. At the 3-day interval, donor MSC engraftment in the scar and border zone was quadrupled and doubled, respectively, indicating that the magnitude of effect on MSC was at least as great as the magnitude of upregulation of this potential signaling axis.

Over the interval from 3 days to 1 week, transcription

of SCF fell by almost 50%, and that of c-kit fell by approximately 35%, with little change in c-kit and SCF levels in the control rats. The decline in SCF and c-kit transcription over time, therefore, appears specific to the TMR effect. At 1 week, donor MSC numbers in the TMR hearts had declined slightly from their 3-day values but remained approximately 35% greater than values in controls. These time-related differences were due to an increase in MSC numbers in the scar of control hearts and a slight reduction in donor cell numbers in the border zone of the TMR group.

The changes in SDF-1 were quite different. At both 3 days and 1 week, SDF-1 levels were approximately doubled in TMR hearts compared with controls but there was little change over time in either group. In contrast, transcription of its receptor, CXCR4, was dramatically upregulated at 3 days with levels more than triple those in controls. The CXCR4 upregulation was short-lived, however, as levels fell by approximately eightfold and fivefold, respectively, in the TMR and control rats from 3 days to 1 week. At 1 week, CXCR4 levels in the TMR rats remained more than double those of the controls.

The differences in c-kit, CXCR4, and their ligands SCF and SDF-1 between groups and over time indicate that both of these progenitor cell-signaling axes may mediate the observed signaling effect of TMR. While the temporal association and magnitude of effect are consistent with a causative effect rather than epiphenomena, further studies inducing loss of specific ligand or receptor function will be necessary to precisely delineate which of these axes is involved and in which manner at which time.

This study has a number of limitations. For logistical purposes, we delivered sex-mismatched MSCs to the study animals by intravenous injection. This permitted highly accurate quantitation of donor MSC engraftment in the myocardium and showed that a normally marrow-resident progenitor cell population could be recruited to the heart by TMR. To definitively show MSC mobilization out of the marrow, these experiments would need to be repeated in chimeric mice in which the marrow has been reconstituted with sex-mismatched marrow cells. However, performing TMR in a myocardial infarction in mice is technically challenging and is associated with high periprocedural mortality. For this reason, we elected to use a rat infarct model. We also evaluated modulation of signaling axis components at the transcriptional level but did not assess translational or protein levels, includ-

ing segregation of membrane-bound versus soluble forms of SCF. We did not evaluate the expression of proangiogenic factors; instead, we chose to look at the changes in vascular density. Moreover, we did not include a second control group in which only TMR was performed and no MSCs were transplanted.

In conclusion, TMR has a novel mechanism of action; inducing homing and engraftment of circulating MSCs into a myocardial infarction. It also enhances angiogenesis and improves recovery of LV function compared with controls. These effects of TMR appear to be mediated by transcriptional upregulation of components of both the c-kit-SCF and CXCR4-SDF-1 progenitor cell signaling axes. Further loss-of-function studies are ongoing to clarify the specific mediators that are required for TMR to exert this effect.

Terrence M. Yau holds the Angelo & Lorenza DeGasperi Chair in Cardiovascular Surgery Research.

References

1. Rose RA, Jiang H, Wang X, et al. Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. *Stem Cells* 2008;26:2884–92.
2. Deuse T, Peter C, Fedak PW, et al. Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. *Circulation* 2009;120(11 suppl):S247–54.
3. Yau, TM, Fung K, Weisel RD, Fujii T, Mickle DAG, Li R. Enhanced myocardial angiogenesis by gene transfer with transplanted cells. *Circulation* 2001;104(12 suppl 1):I218–22.
4. Kim SH, Moon H, Kim HA, Hwang K, Lee M, Choi D. Hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cells prevent myocardial ischemic injury. *Mol Ther* 2011;19:741–50.
5. Horvath KA, Smith WJ, Laurence RG, Schoen FJ, Appleyard RF, Cohn LH. Recovery and viability of an acute myocardial infarct after transmyocardial laser revascularization. *J Am Coll Cardiol* 1995;25:258–63.
6. Pratali S, Chiamonti F, Milano A, Bortolotti U. Transmyocardial laser revascularization 12 years later. *Interact Cardiovasc Thorac Surg* 2010;11:480–1.
7. Spiegelstein D, Kim C, Zhang Y, et al. Combined transmyocardial revascularization and cell-based angiogenic gene therapy increases transplanted cell survival. *Am J Physiol Heart Circ Physiol* 2007;293:H3311–6.
8. Fransioli J, Bailey B, Gude NA, et al. Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. *Stem Cells* 2008;26:1315–24.
9. Kucia M, Reza R, Miekus K, et al. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1—CXCR4 axis. *Stem Cells* 2005;23:879–94.
10. Fazel S, Chen L, Weisel RD, et al. Cell transplantation preserves cardiac function after infarction by infarct stabilization: Augmentation by stem cell factor. *J Thorac Cardiovasc Surg* 2005;130:1310.
11. Gagli E, Rand MK, Tayari L, et al. Expression of stem cell factor and its receptor, c-kit in human oral mesenchymal cells. *Eur J Oral Sci* 2006;114:409–15.
12. Kakinoki R, Bishop AT, Tu YK, Matsui N. Detection of the proliferated donor cells in bone grafts in rats, using a PCR for a Y-chromosome specific gene. *J Orthop Sci* 2002;7:252–7.
13. Yau TM, Kim C, Li G, Zhang Y, Weisel RD, Li RK. Maximizing ventricular function with multimodal cell-based gene therapy. *Circulation* 2005;112(suppl 9):I123–8.
14. Yau TM, Kim C, Ng D, et al. Increasing transplanted cell survival with cell-based angiogenic gene therapy. *Ann Thorac Surg* 2005;80:1779–86.
15. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. *Circulation* 1978;58:1072–83.
16. Beek J, van der Sloot JAP, Huikeshoven M, et al. Cardiac denervation after clinical transmyocardial laser revascularization: Short-term and long-term iodine 123-labeled meta-iodobenzylguanide scintigraphic evidence. *J Thorac Cardiovasc Surg* 2004;127:517–24.
17. Al-Sheikh T, Allen KB, Straka SP, et al. Cardiac sympathetic denervation after transmyocardial laser revascularization. *Circulation* 1999;100:135–40.
18. Atluri P, Panlilio CM, Liao GP, et al. Transmyocardial revascularization to enhance myocardial vasculogenesis and hemodynamic function. *J Thorac Cardiovasc Surg* 2008;135:283–91.

INVITED COMMENTARY

Cell transplantation for treating cardiac disease represents one intriguing approach for developing new therapeutic strategies for the treatment of end-stage heart disease. Today a significant number of patients are not optimal candidates for current standard forms of therapy, including surgical revascularization and interventional procedures, because of the extent and/or anatomy of disease. Promotion of angiogenesis by means of cell therapy may be one of the most promising approaches for the treatment of these patients.

Evidence from several preclinical and clinical studies suggests that myocardial function is improved after cell transplantation using several different cell types to reconstruct damaged myocardium and/or coronary vessels and to induce angiogenesis. These studies showed that cell transplantation was associated with smaller infarcts,

prevented ventricular dilatation and remodeling, and improved the ventricular systolic function in some of these studies.

At the same time, transmyocardial revascularization (TMR/TMLR) by means of needles and later with a laser beam also showed promise in the early 1980s, and several preclinical and clinical studies were published showing positive results [1–3]. The mechanism of action was initially thought to be direct perfusion of the myocardium with the left ventricular blood via endocardial channels (duplicating reptilian circulation). However, additional studies showed early closure of these channels, and histopathologic studies demonstrated the absence of true communication between the channels and the endocardial cavity. It is, however, now accepted that laser irradiation may stimulate angiogenesis. TMR now can be

performed percutaneously by use of the less invasive catheter-based approach: percutaneous transmyocardial laser revascularization. Unfortunately, the results of large studies [4–6] tempered the initial enthusiasm surrounding myocardial laser therapy. The results were very similar for the treatment and control arms, representing a large placebo effect.

In the current issue, Shahzad and colleagues [7] examine the effect of mesenchymal stem cell transplantation in combination with TMR in a rat model of myocardial infarction. They demonstrate that male mesenchymal stem cells given to female rats were engrafted around the TMR channels, and the recipients were markedly more avid than those animals that did not receive TMR. The authors postulate that this increased engraftment was related to TMR-induced increases in stromal derived factor-1 and other chemokines.

This original experimental work addresses the effects and mechanisms responsible for combining TMR and stem cell therapies. Interestingly, the authors took more a therapeutic approach, using serial daily injections of mesenchymal stem cells. The rationale for this approach is that a series of injections can be accomplished in a simple and feasible manner and would provide an adequate supply of circulating cells to damaged myocardium and enhance the possible angiogenic effect of TMR.

Despite certain limitations of the study, it continues to suggest that a combination of cell therapy and TMR may enhance angiogenesis and improve impaired left ventricular function in patients with end-stage heart disease.

Nicholas Kipshidze, MD

New York Cardiovascular Research
2638 21st St
New York, NY 11102
e-mail: nkipshidze@lenoxhill.net

References

1. Schofield PM, Sharples LD, Caine N, et al. Transmyocardial laser revascularisation in patients with refractory angina: a randomized controlled trial. *Lancet* 1999;353:519–24.
2. Allen KB, Dowling RD, Fudge TL, et al. Comparison of transmyocardial revascularization with medical therapy in patients with refractory angina. *N Engl J Med* 1999;341:1029–36.
3. Burkhoff D, Schmidt S, Schulman SP, et al. Transmyocardial laser revascularization compared with continued medical therapy for treatment of refractory angina pectoris: a prospective randomised trial. ATLANTIC Investigators. *Angina Treatments—Lasers and Normal Therapies in Comparison*. *Lancet* 1999;354:885–90.
4. Oesterle SN, Sanborn TA, Ali N, et al. Percutaneous transmyocardial laser revascularisation for severe angina: the PACIFIC randomised trial. Potential class improvement from intramyocardial channels. *Lancet* 2000;356:1705–10.
5. Whitlow PL, DeMaio SJ, Perin EC, et al. One-year results of percutaneous myocardial revascularization for refractory angina pectoris. *Am J Cardiol* 2003;91:1342–6.
6. Leon M. DIRECT trial: late breaking trials. Presented at Transcatheter Therapeutics, October 20, 2000, Washington, DC.
7. Shahzad U, Li G, Zhang Y, Yau TM. Transmyocardial revascularization induces mesenchymal stem cell engraftment in infarcted hearts. *Ann Thorac Surg* 2012;94:556–63.