April 2013

Improving Cardiac Repair by Stem Cell Factor Post-Myocardial Infarction

Fuli Xiang
The University of Western Ontario

Supervisor
Dr Qingping Feng
The University of Western Ontario

Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

© Fuli Xiang 2013

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Circulatory and Respiratory Physiology Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/1204
IMPROVING CARDIAC REPAIR BY STEM CELL FACTOR
POST-MYOCARDIAL INFARCTION

(Thesis format: Integrated Article)

by

Fu-Li Xiang

Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Fu-Li Xiang 2013
Abstract

Myocardial infarction (MI) occurs due to the complete occlusion of a coronary artery and is a leading cause of death, and a huge social and economic burden worldwide. Despite advances in treatment, patients still have a poor prognosis and there is a pressing need for strategies to improve cardiac repair post-MI. Stem cell factor (SCF), expressed by many cells and tissues including the myocardium, binds to its receptor, *c-kit*, and regulates the activity of *c-kit*-expressing cells. SCF has two isoforms: a soluble (S-SCF), and a membrane-associated isoform (M-SCF). M-SCF is more potent in supporting cell adhesion and survival. To date, little is known about the role of M-SCF in MI. This thesis investigated how M-SCF affects cardiac repair post-MI and the related mechanisms. To study the effects of M-SCF in cardiac repair post-MI, we generated a novel mouse model of inducible cardiac specific over-expression of membrane-associated human SCF (hSCF). I utilized mouse models of MI and ischemia/reperfusion (I/R) induced through permanent and reversible ligation of the left coronary artery, respectively. Using these models, I investigated the effects of hSCF on cardiac function, cardiac repair, and related cellular and molecular mechanisms. I demonstrated for the first time that cardiomyocyte-specific overexpression of hSCF improves myocardial function and animal survival post-MI. These beneficial effects appear to have resulted from increases in *c-kit*+ stem cell retention, neovascularization, and decreases in myocardial apoptosis and cardiac remodeling. I also examined the cardioprotective effects of M-SCF in acute myocardial I/R injury. Overexpression of hSCF decreased myocardial apoptosis and infarct size via Akt-dependent signaling. Furthermore, I found that hSCF overexpression increases CD45/c-kit+ cell activation following acute I/R through SCF/c-kit signaling. Finally, I examined the role of M-SCF in epicardial activation post-MI. I found
that overexpression of hSCF increased epicardial activation, enhanced epicardium-derived cell (EPDC) proliferation and migration via SCF/c-kit signaling, and promoted arteriogenesis post-MI. In conclusion, M-SCF improves cardiac repair and function through inhibiting myocardial apoptosis and cardiac remodeling, and by promoting neovascularization via c-kit+ stem cell recruitment and EPDC activation and migration.

**Keywords:** Stem cell factor; Myocardial infarction; Cardiac remodeling ; Endothelial progenitor cells; Angiogenesis; Epicardium-derived cells ; Cardiac stem cells
Co-Authorship Statement

The studies outlined in Chapters 2-4 were performed by Fu-Li Xiang in the laboratory of Dr. Qingping Feng, with the assistance of co-authors as listed below.

Dr. Qingping Feng contributed to experimental design, data interpretation, and manuscript preparation for all experiments. Dr. P. Chidiac, Dr P. Zhu and Dr J. Robbins contributed to manuscript preparation for Chapter 2. Additionally, Dr. Sharon Lu designed and created the hSCF/tTA mice and assisted with the troubleshooting in all the experiments. Mrs. Murong Liu coordinated the animal breeding and genotyping.

Chapter 2: Dr. L Hammoud performed the RT-PCR and western blot analysis of hSCF expression (Figure 2.1).

Chapter 3: Yin Liu assisted with the fluorescent imaging (Figure 3.5 and 3.7).

Chapter 4: Yin Liu performed the isolation of fetal hearts (Figure 4.3-4.5).
Acknowledgments

I would like to thank my supervisor and mentor Dr. Qingping Feng for his help, support, and guidance throughout my Ph.D. studies. Dr. Feng is one of the most hard-working people I have ever met in my life and his commitment to excellence has made him an excellent researcher and a highly respected professor. His passion for research and craving for knowledge inspires me all the time. It is one of the best decisions I made in my life to work in his lab. He creates a very positive environment in the lab that fosters academic learning, creativity, and the career development for future cardiovascular investigators.

To the Feng laboratory, I could not have asked for a more stimulating and supportive environment to work. I would like to particularly thank Dr. Sharon Lu. She is a wonderful teacher and a good friend. To all other members of the Feng lab you have enriched my experience as a graduate student and made my stay in Feng lab enjoyable. To my advisory committee Dr. Peter Chidiac, Dr. David Hess and Dr. Douglas Jones: as a group you always provided unique perspectives and valuable suggestions on my studies and helped strengthen them. On an individual level Dr. Chidiac was the most approachable and lovely person in the department, Dr. Hess was a constant source of support and was very helpful in terms of unique suggestions that solidified my studies. Dr. Jones, was a model of professionalism and his guidance with regards to my career will always be appreciated.

To Heart and Stroke Foundation of Canada, the doctoral research award gave me financial support for my PhD studies, and to attend and present in international conferences.

To my family and all my friends who have been there for me, providing me with emotional and intellectual support, I could not have done this without you!
I also would like to acknowledge the Lawson Health Research Institute, and the Heart Failure Group at Western for providing me with a very stimulating and supportive environment that helped me to converse scientifically and become a skilled presenter. Additionally, I truly enjoyed my experience TAing for Physiology 3130y. Tom, Jamie and Chris were all wonderful people to work with.

Finally, I would like to thank Dr. Shin-Ichi Nishikawa, RIKEN Center for Developmental Biology, Kobe, Japan for providing the ACK2 antibody.
# Table of Contents

Abstract ........................................................................................................................................ ii

Co-Authorship Statement ........................................................................................................ iv

Acknowledgments ................................................................................................................ v

Table of Contents ................................................................................................................ vii

List of Tables ........................................................................................................................ xii

List of Figures ........................................................................................................................ xiii

List of Appendices ................................................................................................................ xvi

List of Abbreviations, Symbols and Nomenclature ............................................................... xvii

1  CHAPTER 1 .......................................................................................................................... 1

1.1 ISCHEMIC HEART DISEASE (IHD) ............................................................................. 1

  1.1.1 Physiology of the Heart ......................................................................................... 1

  1.1.2 Myocardial Infarction (MI) .................................................................................. 2

  1.1.3 Heart Failure (HF) ............................................................................................. 3

  1.1.4 Current Treatment for MI and HF ...................................................................... 4

1.2 NEW APPROACHES TO TREAT IHD ....................................................................... 5

  1.2.1 Inhibition of Myocardial Apoptosis ................................................................... 6

  1.2.2 Activation of Akt Signaling .............................................................................. 7

  1.2.3 Improvement of Cardiac Repair post-MI ......................................................... 10

1.3 STEM CELLS IN CARDIAC REPAIR POST-MI ....................................................... 14

  1.3.1 Bone Marrow-derived Stem Cells (BMSC) ....................................................... 15

  1.3.2 Cardiac Stem Cells (CSCs) .............................................................................. 19

  1.3.3 Epicardium-derived Cells (EPDCs) ................................................................. 22

1.4 STEM CELL FACTOR (SCF) ..................................................................................... 25

  1.4.1 SCF Biology ................................................................................................... 25
1.4.2 SCF receptor c-kit ................................................................. 30
1.4.3 Potential Clinical Applications of SCF .................................. 31
1.4.4 SCF and IHD .................................................................... 32
1.5 OVERALL HYPOTHESIS AND AIMS ................................. 32
1.6 REFERENCES .................................................................... 38

2 Chapter 2 ............................................................................. 68
2.1 CHAPTER SUMMARY .......................................................... 69
2.2 INTRODUCTION .................................................................. 70
2.3 METHODS ......................................................................... 71
  2.3.1 Generation of hSCF/tTA Double Transgenic Mice ........... 71
  2.3.2 Determination of mRNA Expression by Real-time RT-PCR .. 72
  2.3.3 Animal Procedures .......................................................... 73
  2.3.4 Hemodynamic Measurements ......................................... 73
  2.3.5 Histology ...................................................................... 74
  2.3.6 Caspase-3 Activity and Cytoplasmic Histone-Associated DNA Fragments ......... 75
  2.3.7 In vivo Matrigel Angiogenesis .......................................... 75
  2.3.8 Statistical Analysis .......................................................... 75
2.4 RESULTS ............................................................................ 75
  2.4.1 Generation and Characterization of the Conditional Cardiac-specific hSCF/tTA Double Transgenic Mice ................. 75
  2.4.2 Post-MI Stem Cell Factor Expression ......................... 78
  2.4.3 Survival and Cardiac Function Post-MI .......................... 82
  2.4.4 Myocardial Apoptosis 5 days Post-MI .......................... 89
  2.4.5 Stem Cell Recruitment and Growth Factors Release Post-MI .......... 89
  2.4.6 LV Hypertrophy 30 days Post-MI ................................. 96
  2.4.7 Capillary Density post-MI .............................................. 104
2.4.8  In Vivo Myocardial Angiogenesis ........................................................... 109
2.5  DISCUSSION .................................................................................................. 109
2.6  REFERENCES ............................................................................................... 116
3  CHAPTER 3 ........................................................................................................... 120
  3.1  CHAPTER SUMMARY ................................................................................ 121
  3.2  INTRODUCTION ........................................................................................ 122
  3.3  METHODS ................................................................................................... 123
      3.3.1 Animals ............................................................................................... 123
      3.3.2 Myocardial I/R ................................................................................... 124
      3.3.3 Measurement of Infarct Size ............................................................... 124
      3.3.4 Determination of Myocardial Apoptosis .......................................... 125
      3.3.5 Western Blot Analysis ....................................................................... 126
      3.3.6 Growth Factor mRNA Expression ................................................... 126
      3.3.7 Immunofluorescent Staining for Stem Cells ..................................... 127
      3.3.8 Isolation and Culture of $c$-kit$^+$ CSCs .......................................... 127
      3.3.9 Flow Cytometry .................................................................................. 128
      3.3.10 Toluidine Blue Staining for Mast Cells ........................................... 128
      3.3.11 Statistical Analysis ........................................................................... 128
  3.4  Results .......................................................................................................... 129
      3.4.1 Overexpression of hSCF Reduces Infarct Size and Myocardial Apoptosis after I/R .......................................................... 129
      3.4.2 Akt Signalling Contributes to hSCF Overexpression-induced Cardiac Protection after I/R .......................................................... 129
      3.4.3 Overexpression of hSCF Increases $c$-kit$^+$ Cells in the Myocardium after I/R ........................................................................... 134
      3.4.4 Overexpression of hSCF Enhances Protective Growth Factor Expression after I/R .......................................................... 141
3.4.5 Sub-population of c-kit+ Cells in Hearts with hSCF Overexpression after I/R ........................................................................................................... 144

3.5 DISCUSSION ........................................................................................................ 147

3.6 REFERENCES .................................................................................................. 154

4 CHAPTER 4 .............................................................................................................. 160

4.1 CHAPTER SUMMARY ........................................................................................ 161

4.2 INTRODUCTION ................................................................................................ 162

4.3 METHODS ......................................................................................................... 163

4.3.1 Animals ........................................................................................................ 163

4.3.2 MI Mouse Model .......................................................................................... 164

4.3.3 EPDC Culture .............................................................................................. 165

4.3.4 Neonatal Cardiomyocyte Culture ............................................................. 165

4.3.5 Transwell Migration Assay ......................................................................... 166

4.3.6 Real-time RT-PCR ...................................................................................... 166

4.3.7 Immunohistochemistry ................................................................................ 167

4.3.8 Western Blot Analysis ................................................................................ 168

4.3.9 Statistical Analysis ....................................................................................... 168

4.4 Results ................................................................................................................ 168

4.4.1 Epicardial Activation 3 Days post-MI ......................................................... 168

4.4.2 Arteriolar Density in the Peri-infarct Area 5 Days post-MI ....................... 171

4.4.3 Outgrowth of Cultured EPDCs ................................................................... 174

4.4.4 Migration of Cultured EPDCs .................................................................... 181

4.4.5 Lineage Tracing of Wt1+ EPDCs ................................................................. 181

4.5 DISCUSSION ..................................................................................................... 191

4.6 REFERENCES: ................................................................................................. 198

5 CHAPTER 5 .............................................................................................................. 202
5.1 SUMMARY OF MAJOR FINDINGS................................................................. 202
5.2 STUDY LIMITATIONS .............................................................................. 206
  5.2.1 Inducible Cardiac-specific hSCF Overexpressing Transgenic Mice ..... 206
  5.2.2 Use of Mouse Model to Simulate Human Disease Conditions......... 209
  5.2.3 Contributions of Soluble SCF........................................................... 211
  5.2.4 Use of Pharmacological Inhibitors and Neutralizing Antibody to Delineate
        Signaling Pathways............................................................................ 211
5.3 SUGGESTIONS FOR FUTURE RESEARCH.......................................... 216
5.4 CLINICAL APPLICATION......................................................................... 219
5.5 CONCLUSIONS......................................................................................... 220
5.6 REFERENCES ........................................................................................... 221
APPENDIX................................................................................................... 225
Curriculum Vitae .......................................................................................... 227
List of Tables

Table 2.1 General information of control and hSCF/tTA mice subjected to sham or MI surgeries .................................................................................................................................. 79

Table 2.2 Hemodynamic parameters of WT and hSCF/tTA mice 5 days post-MI ............... 85

Table 2.3 Hemodynamic parameters of control and hSCF/tTA mice 30 days post-MI ....... 86

Table 2.4 Number of mast cells in the left ventricular myocardium 5 and 30 days after sham or MI surgeries ........................................................................................................ 97
List of Figures

Figure 1.1 Cardioprotective PI3K/Akt signaling pathways in ischemia/reperfusion (I/R). ..... 8

Figure 1.2 Potential cell sources for cardiogenesis post-MI............................................. 16

Figure 1.3 Illustration of soluble (S-SCF) and membrane-associated SCF (M-SCF). .......... 28

Figure 1.4 Proposed cellular mechanisms by which membrane-associated SCF (M-SCF) regulates cardiac function post-MI. ................................................................. 34

Figure 2.1 Generation and characterization of the inducible hSCF/tTA transgenic mouse. .. 76

Figure 2.2 Mouse SCF expression in the heart post-MI. .................................................. 80

Figure 2.3 Survival and LV function 30 days post-MI....................................................... 83

Figure 2.4 Cardiac function 5 days post-MI. .................................................................. 87

Figure 2.5 Myocardial apoptosis in the peri-infarct area 5 days post-MI......................... 90

Figure 2.6 c-kit+ and c-kit+/VEGFR2+ cells retained in the peri-infarct area 5 days post-MI. 92

Figure 2.7 c-kit+ and c-kit+/VEGFR2+ cells retained in the peri-infarct area 30 days post-MI. ......................................................................................................................... 94

Figure 2.8 Mast cell staining using toluidine blue in heart tissue sections from WT and hSCF/tTA mice. ........................................................................................................... 98

Figure 2.9 Growth factor expression in the peri-infarct area 5 days post-MI.................... 100

Figure 2.10 LV hypertrophy 30 days post-MI or sham operations................................. 102

Figure 2.11 Myocardial capillary density 5 days post-MI................................................. 105

Figure 2.12 Myocardial capillary density 30 days post-MI............................................. 107

Figure 2.13 Myocardial angiogenesis WT and hSCF/tTA mice....................................... 110
Figure 3.1 Effects of hSCF overexpression on infarct size after I/R. ................................. 130
Figure 3.2 Myocardial apoptosis in peri-infarct area after I/R. ........................................ 132
Figure 3.3 Myocardial Akt phosphorylation in WT and hSCF/tTA mice after I/R......... 135
Figure 3.4 Myocardial eNOS phosphorylation in WT and hSCF/tTA mice after I/R.......... 137
Figure 3.5 $c$-kit$^+$ cells in WT and hSCF/tTA hearts. ...................................................... 139
Figure 3.6 Cardioprotective cytokine expression in WT and hSCF/tTA mice after I/R. ..... 142
Figure 3.7 Sub-population of $c$-kit$^+$ cells in the myocardium after I/R. ...................... 145
Figure 3.8 Overexpression of hSCF in $c$-kit$^+$ CSCs in vitro. ........................................ 148
Figure 4.1 Epicardial Wt1 expression 3 days post-MI. ...................................................... 169
Figure 4.2 Arteriolar density in the peri-infarct area 5 days post-MI. ............................ 172
Figure 4.3 In vitro culture of EPDCs ............................................................................... 175
Figure 4.4 Outgrowth, proliferation and growth factor expression in cultured EPDCs. .... 177
Figure 4.5 Expression of hSCF protein in adenovirus treated EPDCs. ......................... 179
Figure 4.6 Transwell EPDC migration. ............................................................................. 182
Figure 4.7 Lineage tracing of Wt1$^+$ cell derived EPDCs 5 days post-MI...................... 185
Figure 4.8 Expression of hSCF protein in adenovirus treated hearts. ......................... 187
Figure 4.9 In vitro lineage tracing of Wt1$^+$ cell derived EPDCs. ................................. 189
Figure 4.10 Fate-mapping of Wt1$^+$ cell derived EPDCs post-MI. ............................... 192
Figure 5.1 Summarized cellular mechanisms by which M-SCF improves cardiac function and
animal survival post-MI. .................................................................................................. 207
Figure 5.2 Expression of hSCF in WT and hSCF/tTA mice. ............................................ 212
Figure 5.3 Expression of soluble and membrane-associated hSCF in the serum of WT and hSCF/tTA mice.
List of Appendices

Appendix A: UWO Animal use sub-committee protocol approvals.......................... 225
List of Abbreviations, Symbols and Nomenclature

ACE: angiotensin-converting-enzyme
Ad-GFP: adenoviral green fluorescent protein construct
Ad-hSCF: adenoviral membrane-associated human stem cell factor construct
Ad-LacZ: adenoviral LacZ construct
BAD: B cell lymphoma (Bcl)-xi/Bcl-2–associated death promoter
bFGF: basic fibroblast growth factor
BMP2: bone morphogenetic protein-2
BMSCs: Bone marrow-derived stem cells
CDCs: cardiosphere-derived cells
CSCs: cardiac stem cells
CSPCs: cardiac side population cells
DAB: diaminobenzidine tetrahydrochloride
DOX: doxycycline
E: embryonic day
ELISA: enzyme-linked immunosorbent assay
EMT: epithelial-mesenchymal transition
eNOS: endothelial nitric oxide synthase
EPCs: endothelial progenitor cells
EPDCs: epicardium-derived cells
EPO: erythropoietin
ERK: extracellular signal-regulated kinase
G-CSF: granulocyte-colony stimulating factor
GFP: green fluorescent protein
GSK-3β: glycogen synthase kinase 3β
HF: heart failure
HGF: hepatocyte growth factor
hPGK: human phosphoglycerate kinase
hSCF: membrane-associated human stem cell factor
HSCs: hematopoietic stem cells
I/R: ischemia and reperfusion
IGF-1: insulin-like growth factor 1
IHD: ischemic heart disease
IP: intraperitoneal
JAK/STAT: Janus kinase/signal transducers and activators of transcription
LV: left ventricle
LVEF: left ventricle ejection fraction
LVW/BW: left ventricle to body weight ratio
MAP: mean artery pressure
MHC: myosin heavy chain
MI: myocardial infarction
MMLV: moloney murine leukemia virus
MNCs: mononuclear cells
MOI: multiplicity of infection
M-SCF: membrane-associated stem cell factor
MSCs: multipotent mesenchymal stromal cells
mTmG: membrane-Tomato/membrane-Green
mTOR: mammalian target of rapamycin

NF-κB: nuclear factor kappa B

P0: postnatal day 0

PAMP: preload adjusted maximum power

PDK1: phosphoinositide-dependent kinase 1

PI3K: phosphoinositide-3-kinase

PKC: protein kinase C

PLC-γ: phospholipase-γ

PMA: phorbol 12-myristate 13-acetate

RISK: reperfusion injury salvage kinase

SCF: stem cell factor

SH2: Src homology 2

S-SCF: soluble stem cell factor

SW: stroke work

TGFβ: transforming growth factor β

tTA: tetracycline activator

TTC: triphenyltetrazolium chloride

VEGF: vascular endothelial growth factor

VEGFR2: vascular endothelial growth factor receptor 2

vWF: von Willebrand factor

WT: wild type

Wt 1: Wilms tumor 1

α-SMA: α-smooth muscle actin

β1AR: β1 adrenergic receptor
*, † and ‡ are used for denoting statistical significance.

μ was used for micro, α was used for alpha, and β was used for beta.
CHAPTER 1

Literature Review

1.1 ISCHEMIC HEART DISEASE (IHD)

IHD is a condition in which the heart muscle is damaged or works inefficiently due to an absence or relative deficiency of its blood supply. Most often caused by atherosclerosis, the clinical manifestation of IHD includes angina pectoris, acute myocardial infarction, chronic ischemic heart disease, and sudden death. IHD is the leading cause of death and a financial burden worldwide. For example, in the United States, IHD resulted in 445,687 deaths (about one of every five deaths) in 2005 and was reported to place a direct and indirect cost of $165.4 billion in 2009. Despite optimal treatment with existing drugs, the prognosis of IHD remains poor. Heart transplantation is the last but an effective option for end-stage IHD patients. The five-year survival rate of heart transplantation has achieved 70% in the United States. However, the limited donors for heart transplantation are not meeting the need of IHD patients. Thus, novel and effective treatments for IHD are in high demand.

1.1.1 Physiology of the Heart

The main function of the heart is to pump blood to meet the nutritional and metabolic requirements of the body. The muscular walls of the heart consist of three layers. The endocardium is the inner layer, consisting of endothelium and connective tissue, which covers the chambers and valves. The myocardium contains cardiomyocytes, fibroblasts, elastin and connective tissue, and the endomysium, which contains the
capillaries that supply the heart with oxygen. The outer layer of the heart wall is the epicardium, consisting of mesothelium and connective tissues.

The heart pumps blood through two series circulations by coordinated actions of the atria and ventricles. Oxygenated blood from the lungs is received by the left atrium and is subsequently pumped into the aorta (systemic circulation) by the left ventricle (LV). The blood flows through arteries and capillaries to all tissues in the body to supply them with oxygen and nutrients. The deoxygenated blood returns to the right atrium of the heart from veins and is then pumped into the pulmonary circulation via the right ventricle to be re-oxygenated in the lungs. The heart generates continuous and sufficient blood circulation and can, by demand, increases its output. The coronary circulation is critical to maintain heart muscle function. Insufficient coronary perfusion caused by coronary artery narrowing or constriction results in cardiac ischemia leading to impaired cardiac function.

### 1.1.2 Myocardial Infarction (MI)

Acute MI is an event which typically presents with a sudden onset of severe chest discomfort associated with elevations of ST segment on an electrocardiogram and increased enzyme levels in the blood such as creatine kinases, indicating cardiomyocyte death (heart muscle damage). MI is often due to the formation of thrombus (clot) in a coronary artery, which obstructs blood flow to the area of the myocardium supplied by the artery. Acute MI results in cardiomyocyte death starting within a few minutes after an ischemic attack. The loss of functional cardiomyocytes accompanied by inflammation in the first few days triggers a cascade of events resulting in the expansion of the infarcted area and early ventricular remodeling. At the same time, stem cells are activated
and recruited to the infarcted region.\textsuperscript{6} Following acute MI, re-establishing coronary blood flow by thrombolysis or primary angioplasty is widely used to salvage viable myocardium. However, the oxidative stress and abrupt metabolic changes immediately after reperfusion have been shown to initiate further cellular injury.\textsuperscript{7,8} Healing of the infarcted myocardium leads to scar formation and ventricular remodeling, which is characterized by ventricular enlargement, fibrosis and hypertrophic growth of the peri-infarct and non-infarct myocardium. The remodeled LV causes cardiac dysfunction, clinical heart failure and increased mortality.\textsuperscript{5,9}

1.1.3 Heart Failure (HF)

HF is a clinical syndrome characterized by impaired cardiac function and altered structure, leading to dyspnea and fatigue at rest or with exertion.\textsuperscript{10} Patients who survive a MI are at an increased risk of developing HF. The extent of LV systolic and diastolic dysfunction is associated with clinical symptoms and cardiovascular mortality. HF can be caused by the continuous abnormal loading conditions post-MI as a result of the infarct scar formation. The LV remodeling is a dynamic process that is driven by a number of factors.\textsuperscript{11} During cardiac remodeling post-MI, the LV becomes dilated, hypertrophic, and undergoes morphological and pathophysiological alterations. Pathologically, there is extensive fibrosis as well as disarray of abnormally structured myocardial cells. Furthermore, apoptosis becomes more active, resulting in additional loss of myocardial cells.\textsuperscript{12} It is the combination of these pathological changes that alters the structure of the heart and impairs cardiac function.
1.1.4 Current Treatment for MI and HF

After an acute MI, patients often undergo revascularization in order to regain adequate blood supply to the myocardium. The two most widely employed treatments are angioplasty, in which the blocked artery is mechanically opened, and bypass surgery, in which vessels from elsewhere in the body are attached to bypass the blocked artery and re-establish blood flow. Also, MI patients receive pharmacological therapy to relieve angina, prevent thrombus propagation and to stabilize the “vulnerable” plaque. Antianginal drugs, which affect the balance between oxygen supply and demand, include nitroglycerin, β-adrenergic blockers, and calcium channel blockers. To reduce thrombus formation, anti-platelet and antithrombotic treatments are also recommended, unless contraindicated. Antiplatelet agents include aspirin, thienopyridines, and glycoprotein IIb/IIIa inhibitors. Commonly used antithrombotic agents include unfractionated heparin, low-molecular-weight heparin, fondaparinux, and bivalirudin. Furthermore, statin therapy promotes plaque stabilization and restores endothelial function, and has been shown to reduce the incidence of myocardial infarction, recurrent ischemia and mortality.

HF is a chronic disease that requires lifelong management including pharmacological therapy and lifestyle modification. Angiotensin-converting-enzyme (ACE) inhibitors are now considered a first-choice in HF drug therapy. ACE inhibitors limit the formation of angiotensin II resulting in vessel dilation as well as the prevention of cardiac hypertrophy, fibrosis and decreased afterload and preload of the heart. Angiotensin II receptor blockers, which have similar effects, can be used as an alternative for patient who are unable to tolerate ACE inhibitors. Diuretics are prescribed for patients who have volume overload in the body and swelling of the tissues. Digoxin increases the
force of cardiac contractions and relieves heart failure symptoms, especially when the patient is not responding to ACE inhibitors and diuretics. Beta-blockers are prescribed to reduce heart rate and lower blood pressure. They also inhibit ventricular remodeling and reduce the risk of arrhythmias and sudden death. Lifestyle modifications include losing weight, quitting smoking, avoiding alcohol and caffeine, eating a low-saturated-fat low-sodium diet, exercising and reducing stress, all of which have been shown to alleviate symptoms, slow disease progression and improve quality of life.

In spite of significant progress in clinical treatment, the prognosis of HF remains poor. At present there is no cure for patients with end-stage HF except heart transplantation. Drug treatment can attenuate myocardial remodeling and improve survival, but does not reduce the size of the scar. Unfortunately, angioplasty or bypass surgery does not reverse the damage induced by MI. LV assist devices can prolong patient lives only for a few years. On the other hand, although the survival rate after heart transplantation is excellent, the source of transplantable donor hearts is scarce. Therefore, there is a need for developing new and effective therapies to treat patients with HF post-MI. Approaches to limit cardiomyocyte loss, rebuild the coronary microvascular network and impede cardiac remodeling have been widely investigated to improve cardiac function post-MI.

### 1.2 NEW APPROACHES TO TREAT IHD

The fundamental pathophysiological changes post-MI include cardiac cell death, pro-inflammatory response, dynamic change of extracellular matrix, cardiac fibrosis, cardiac neovascularization, cardiac remodeling and very limited regeneration of cardiac myocytes, which are potential therapeutic targets for developing new treatments for IHD.
Approaches to decrease myocardial cell death, enhance cardiac regeneration and impair cardiac remodeling have shown promising beneficial outcomes in animal models.

1.2.1 Inhibition of Myocardial Apoptosis

An extensive loss of cardiomyocytes is associated with IHD. During acute MI, cell death is seen at the earliest onset of the disease.\textsuperscript{18} It is widely accepted that both necrotic and apoptotic cell death are involved in the loss of cardiomyocytes in MI and during ischemia/reperfusion (I/R) injury.\textsuperscript{19,20} In human acute MI, ischemic regions of the heart contain between 0.8\% and 12\% apoptotic cardiac myocytes compared to approximate 0.005\% apoptotic cells in non-ischemic regions.\textsuperscript{21} Apoptosis is a finely regulated mode of cell death initiated by an intracellular program,\textsuperscript{22} which results in a loss of cells without inflammation. Apoptotic cell death is of great interest to the medical community because it can be effectively manipulated by pharmacological interventions.\textsuperscript{20,23} Inhibition of apoptotic cell death has beneficial effects in HF. For example, excessive stimulation of the $\beta_1$ adrenergic receptor ($\beta_1$AR), such as that seen during HF, is known to induce myocardial apoptosis.\textsuperscript{24} Beneficial effects of $\beta$ receptor blockade in patients with HF may partially be due to an inhibition of $\beta_1$AR-induced apoptosis.\textsuperscript{25} A recent study by Chandrashekhar et al. demonstrated that inhibition of apoptosis improves cardiac function of rats after MI.\textsuperscript{26} In this study, reduction of apoptosis via caspase-3 inhibition was associated with improved systolic function, the preservation of myocardial contractile proteins and the attenuation of ventricular remodeling. A return of blood flow after ischemia through reperfusion of the tissue can also cause “reperfusion injury”. Reperfusion is known to accelerate cardiomyocyte apoptosis following myocardial ischemia.\textsuperscript{27} Interventions which suppress myocardial
apoptosis during I/R injury have been demonstrated to decrease infarct size and improve cardiac function. Additionally, activation of pro-survival kinases including Akt has been shown to provide significant anti-apoptotic effects in MI and I/R.

### 1.2.2 Activation of Akt Signaling

Injury due to myocardial I/R results in loss of functional cardiomyocytes and inflammatory responses which lead to stress signaling and oxidative damage. Since signaling molecules and pathways are potential therapeutic targets, it is important to unravel the relevant signaling pathways involved in cardiac injury. Numerous studies on acute cardio-protective signaling pathways activated by pre- and post-conditioning have shown that phosphoinositide-3-kinase (PI3K)/Akt, glycogen synthase kinase-3β (GSK-3β), endothelial nitric oxide synthase (eNOS), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) are involved. Collectively, these pro-survival pathways are known as the reperfusion injury salvage kinase (RISK) pathway. The ability of Akt to phosphorylate multiple substrates positions it as a central regulator in the RISK pathway. Akt is a 57-kD protein-serine/threonine kinase. The activation of Akt requires both translocation and phosphorylation. Akt is recruited to the plasma membrane by PIP3, the phosphorylation product of PI3K, and phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) to achieve full activation. The substrates of Akt include eNOS, GSK-3β, Bcl-xl/Bcl-2–associated death promoter (BAD), caspase-9, Ikkα, mammalian target of rapamycin (mTOR) and forkhead transcription factor (Figure 1.1). Studies from our lab demonstrated that activation of Akt by erythropoietin (EPO) treatment reduces infarct
Figure 1.1 Cardioprotective PI3K/Akt signaling pathways in ischemia/reperfusion (I/R).

In response to I/R injury, increased myocardial expression of cardioprotective growth factors activate PI3K/Akt signaling pathways. Through phosphorylation by PI3K, Akt either inhibits or activates its downstream substrates in the cytoplasm and nucleus to prevent apoptosis and promote cell survival. In the cytoplasm, Akt phosphorylates BAD and caspase-9 to directly inhibit apoptosis. Phosphorylation of eNOS by Akt results in enhanced cell survival. In the nucleus, Akt inhibits forkhead transcription factors and activates nuclear factor κB (NF-κB) to induce expression of anti-apoptotic factors and protective growth factors.
size and protects against myocardial apoptosis after I/R by phosphorylation of eNOS via the PI3K/Akt signaling pathway.\textsuperscript{34} Akt can also directly inhibit caspase-mediated cell death through the phosphorylation of BAD, releasing Bcl-2 family members to maintain mitochondrial permeability \textsuperscript{43} and the phosphorylation of caspase-9.\textsuperscript{44} Additionally, Akt can protect against cardiomyocyte apoptosis by regulating gene expression of Glut-1, vascular endothelial growth factor (VEGF), Bcl-2 and forkhead transcription factors.\textsuperscript{45} Furthermore, Akt plays a critical role in endothelial homeostasis and angiogenesis by maintaining endothelial cell viability, regulating migration and promoting the homing of endothelial progenitor cells (EPCs) in the ischemic heart after I/R.\textsuperscript{46,47}

1.2.3 Improvement of Cardiac Repair post-MI

The endogenous healing process post-MI is manifested by increased matrix synthesis, fibroblast proliferation and the release of pro-fibrotic cytokines, resulting in scar formation. The infarct size, which is critical to disease progression and prognosis, is determined by the area of coronary artery occlusion and dynamic changes during the remodeling process.\textsuperscript{48} The ideal cardiac repair would include the complete regeneration of the myocardium and neovascularization, which has been observed in Zebra fish,\textsuperscript{49} the adult newt,\textsuperscript{50} and the neonatal mouse.\textsuperscript{51} However, the ability to regenerate myocardial tissue follow injury is diminished in the adult mammals.\textsuperscript{51}

1.2.3.1 Neovascularization post-MI

Effective cardiac repair post-MI most likely depends on the blood supply in the border zone and the infarcted area, which not only supports the surviving cardiomyocytes, but also promotes the establishment of myofibroblast-rich granulation
tissues. Neovascularization can be achieved by a number of different mechanisms including sprouting of capillaries from pre-existing endothelial cells (angiogenesis), in situ formation of blood vessels from EPCs or angioblasts (vasculogenesis) and by increasing in the size and caliber of pre-existing arteriolar collaterals (arteriogenesis). Tissue hypoxia stimulates neovascularization by up-regulating pro-angiogenic factors partially via the transcription factor, hypoxia inducible factor. Pro-angiogenic growth factors such as VEGF and basic fibroblast growth factor (bFGF) are increased in the ischemic myocardium. VEGF stimulates endothelial cell proliferation and EPC mobilization from the bone marrow, and contributes to both angiogenesis and vasculogenesis. bFGF is a potent mitogen of endothelial cells and acts as a chemotactic factor promoting endothelial cell migration during angiogenesis. Studies have shown that intracoronary artery administration of VEGF and bFGF post-MI in animal models enhances vessel collateral formation and improves cardiac function. Despite a potential risk of growth and destabilization of atherosclerotic plaques associated with pro-angiogenic factor treatment, protein-based angiogenic clinical trials in IHD using VEGF or bFGF have been carried out worldwide. The results of recombinant human VEGF or bFGF delivery to patients with coronary artery disease were disappointing, showing no improvement compared to placebo. However, beneficial clinical outcomes were reported in some of the clinical trials using adenoviral VEGF gene therapy. Thus, further development of gene delivery techniques and optimization of therapeutic indications are required to translate the promising outcomes obtained from animal studies using pro-angiogenic growth factors into clinical success.
An alternate approach for angiogenic therapy is stem cell treatment including stem cell transplantation or manipulating stem cell recruitment and retention. Since the early 2000’s, efforts to repair the infarcted heart with newly generated cardiomyocytes from bone marrow-derived stem cells have evolved and boosted intensive worldwide research on stem cell therapy post-MI. Although initial animal studies claimed cardiomyocyte trans-differentiation from bone marrow stem cells post-MI, many later studies were not able to repeat this observation. The incidence of cardiomyocyte trans-differentiation from bone marrow stem cells, if it occurs at all, is far too low to effectively repair the infarcted heart. The current understanding is that the protective effects observed in most bone marrow-derived stem cell treatments post-MI in animal studies are mediated by the paracrine effects leading to decreased apoptosis, increased angiogenesis or vasculogenesis, modulation of local inflammatory milieu and decreased cardiac remodeling. Mesenchymal stem cells also have the ability to differentiate into perivascular cells that stabilize nascent blood vessels and respond to vasoactive stimuli. Thus, the enhanced recruitment of different types of stem cells and progenitors may work together to create a more functional microcirculation network, leading to more efficient cardiac repair post-MI. However, despite the success in enhancing cardiac repair post-MI in animal models, treatment with bone marrow or peripheral blood derived stem cells had only minor or no functional improvements in clinical trials. The unsatisfactory results from these clinical trials have revealed many critical questions of stem cell therapy in MI. For example, which stem cell type has the best therapeutic effects? How might it be possible to retain functional stem cells in the infarcted heart? Answers to these questions may help to improve stem cell therapy in patients with MI.
1.2.3.2 Endogenous Cardiomyocyte Regeneration post-MI

The general belief that the heart is a completely post-mitotic organ has been challenged by the recent evidence, which suggests the possibility of cardiomyocyte regeneration in cardiac homeostasis and pathology. Myocyte death occurs in the normal heart and the frequency of cell death increases with IHD. In the rat heart, it has been estimated that approximately 94,200 myocytes die every 24 hours under normal physiological condition, which in turn would be expected to lead to the loss of all cardiomyocytes within 5 months. In order to maintain normal heart homeostasis, a balance between cell death and regeneration must be achieved. Recently 2 independent studies have measured cardiomyocyte DNA synthesis in human hearts based on retrospective $^{14}$C birth dating of cells and incorporation of radiosensitizer iododeoxyuridine, respectively. Although the measured myocyte turnover rates differ between them, both studies have provided direct evidence of the regeneration of cardiomyocytes and the plasticity of the adult human heart. Furthermore, the observation of the host Y-chromosome in sex-mismatched female donor hearts strongly suggests the regeneration of myocytes and vascular structures from the host. Despite the intense debate on the cellular sources of newly generated myocytes, it is now widely accepted that the adult heart has some, albeit limited, endogenous repair ability. A recent study using genetic fate mapping to distinguish cardiomyocyte renewal from pre-existing cardiomyocytes or non-cardiomyocyte sources has revealed the contribution of non-cardiomyocyte cells in myocyte renewal in the remote area post-MI in mice. However, the stem/progenitor cell source of the regenerated myocytes and the underlying mechanisms regulating myocardial renewal remains to be investigated.
1.3 STEM CELLS IN CARDIAC REPAIR POST-MI

Stem cells are defined by their unique properties: self-renewal capacity and the potential to differentiate into one or more specialized cell types. Stem cells have the ability to undergo asymmetric and symmetric cell division. Asymmetric cell division gives rise to two daughter cells that have different fates, one maintaining the stem cell population and the other generating differentiated cells. Stem cells can be classified into 3 major categories: embryonic, induced pluripotent and adult stem cells. Both embryonic and induced pluripotent stem cells are pluripotent and can generate any cell type in the body. However, they present a high risk of teratoma formation, which makes them unacceptable for clinical treatment at this time. Interestingly, small populations of clonogenic, self-renewing, and multipotent cells have been found in different adult tissues including bone marrow, vascular walls, adipose tissues, skeletal muscles, heart and brain as well as the epithelium of the lung, liver, pancreas, digestive tract, skin, limbus, retina, breast, ovaries, prostate and testis. These multipotent stem cells, referred to as adult stem cells, have the potential to differentiate into mature cell lineages in tissues from which they originate and contribute to postnatal organ homeostasis. Furthermore, bone marrow-derived stem/progenitor cells may also migrate to distant peripheral sites after intense injury and contribute to tissue repair. Adult stem cells reside in a specific cellular niche, which ensures a balance between the maintenance of the stem cell pool and the production of daughter progenitor cells engaged in lineage differentiation. The in vivo stimulation of adult stem cells or the use of their ex vivo expanded and differentiated progenies is emerging as a promising approach for cell replacement-based therapies. Interestingly, several types of adult stem cells, including
bone marrow-derived stem cells, resident cardiac stem cells and epicardium-derived cells have shown cardiac myogenic and vasculogenic potential, which make them favorable candidates for cardiac repair post-MI.\textsuperscript{77,94} (Figure 1.2)

1.3.1 Bone Marrow-derived Stem Cells (BMSC)

Bone marrow is a rich and readily accessible source of adult stem cells including hematopoietic stem cells (HSCs), multipotent mesenchymal stromal cells (MSCs) and EPCs. The potential of HSCs to give rise to myogenic lineages has been intensively investigated. \textit{In vitro}, HSCs have been shown to transdifferentiate into cardiac myogenic cells in co-cultures with neonatal cardiomyocytes,\textsuperscript{95} or by treatment with VEGF and bFGF\textsuperscript{96} or transforming growth factor β (TGFβ).\textsuperscript{97} However, cardiac regeneration by stem cell transplantation using Lin\textsuperscript{-}c-kit\textsuperscript{+} HSCs post-MI remains highly controversial. Evidence of the cardiac regeneration potential of BMSCs \textit{in vivo} was initially determined by gender mismatch heart transplantation studies in which Y chromosome cardiomyocytes and endothelial cells were detected at a very low level in male recipients receiving female donor hearts.\textsuperscript{83,98} In 2001, Orlic \textit{et al.} reported a substantial engraftment of the transplanted Lin\textsuperscript{-}c-kit\textsuperscript{+} HSCs post-MI and significant regeneration of cardiomyocytes and vascular structure from the HSCs in mice.\textsuperscript{67} However, 3 subsequent studies in 2004 failed to observe any cardiomyocyte regeneration from the transplanted HSCs using similar experimental approaches.\textsuperscript{69,99,100} Nevertheless, the beneficial effects of transplanted Lin\textsuperscript{-}c-kit\textsuperscript{+} HSCs on cardiac function have been confirmed by a number of studies.\textsuperscript{101-103} The improved cardiac function in such cases may mainly result from
Figure 1.2 Potential cell sources for cardiogenesis post-MI.

Cell therapy using bone marrow-derived stem cells (BMSCs), cardiac stem cells (CSCs) and epicardium-derived cells (EPDCs) has been shown to inhibit cardiomyocyte cell death and cardiac remodeling, and promote regeneration of blood vessels and cardiomyocytes post-MI.
trophic factors released from the HSCs, which are referred to as paracrine effects.\textsuperscript{104}

MSCs were originally identified as a small subpopulation of bone marrow cells which have the ability to differentiate into tissue types of mesodermal origin, such as chondrocytes, adipocytes and osteocytes.\textsuperscript{105} MSCs have been successfully isolated from various non-marrow tissues such as umbilical cord blood\textsuperscript{106} and adipose tissue.\textsuperscript{107} DNA demethylating agents, such as 5-azacytidine, have been shown to induce MSC transdifferentiation into cardiomyocytes \textit{in vitro}.\textsuperscript{108,109} Cell therapy using MSCs has been well-documented to improve cardiac performance and promote neovascularization, which are now considered mostly due to the paracrine effects instead of cardiomyocyte regeneration.\textsuperscript{110,111} There are several advantages of using MSCs in cell therapy in IHD compared to HSCs. First, they can be easily cultured and expanded \textit{in vitro} to the large population needed for transplantation. Second, they can be engineered \textit{in vitro} to achieve specific characteristics. Third, they have been shown to contribute to both myocardial and vascular repair post-MI. Last, they have immunological privilege by regulating immune cell activity, which makes MSCs a potential candidate for allogeneic treatment.\textsuperscript{112}

To date, the meta-analysis of numerous clinical trials applying autologous BMSCs to IHD patients has confirmed the safety of this treatment and revealed a moderate improvement in physiological and anatomical cardiac parameters above and beyond conventional therapy.\textsuperscript{113,114} However, the 2 recent trials studying the effects of bone marrow-derived mononuclear cells (MNCs) in acute MI (TIME)\textsuperscript{76} or bone marrow derived MSCs in chronic MI (CCTRN)\textsuperscript{75} showed no improvement in cardiac performance. Although better characterization and understanding of different subpopulations of BMSCs, and optimal time and conditions for their transplantation may
be needed in order to achieve the best outcome for BMSCs stem cell therapy in IHD patients. The lack of benefit of bone marrow-derived MNCs therapy suggests that it is not an optimal stem cell type for IHD treatment.

Endothelial progenitor cells (EPCs) are a rare population of cells that circulate in the blood and have the ability to differentiate into endothelial cells and promote neovascularization. Bone marrow is the major source of adult EPCs. EPCs share many markers with HSCs, which makes it difficult to identify EPCs strictly by specific marker expression. The most widely used markers for EPCs include c-kit, CD34, CD133 and vascular endothelial growth factor receptor 2 (VEGFR2). In vitro culture of mononuclear bone marrow cells yields 2 types of EPCs: early outgrowth and late outgrowth EPCs. The early outgrowth EPCs have been shown to be of hematopoietic origin and potently induce angiogenesis without incorporating in blood vessels, while the late outgrowth EPCs are considered real EPCs which can be clonally expanded and give rise to endothelial cells. During neovascularization, EPCs are released from bone marrow to the peripheral circulation and mobilize to the target tissue. Mobilization of EPCs after cardiac ischemia is also observed in patients after acute MI. Animal studies have demonstrated that EPC transplantation or treatment with agents that enhance EPC mobilization and recruitment post-MI improves cardiac function and reduces cardiac remodeling via angiogenesis and vasculogenesis.

1.3.2 Cardiac Stem Cells (CSCs)

The adult heart has traditionally been considered as a post-mitotic organ with limited ability to regenerate. Recent studies have challenged this dogma and shown that resident cardiac stem cells exist in the adult heart. Although the number of CSCs is
low in the adult heart, they are committed to generate cardiomyocytes and their numbers can be expanded \textit{ex vivo} for therapeutic purposes.\textsuperscript{66} Different approaches are employed to identify the resident CSCs, including detection of specific surface markers such as \textit{c-kit}, and assessment of their unique physiological properties such as the ability to form cardiospheres in culture or to efflux Hoechst 33342 dye (side population progenitors).

CSCs expressing \textit{c-kit} have been intensively studied. They are found in niches in the normal heart at a frequency of 1 out of approximately 30,000 cells.\textsuperscript{122,124-126} In culture, \textit{c-kit}$^+$ CSCs are multi-potent, giving rise to cardiomyocytes, smooth muscle cells and endothelial cells.\textsuperscript{122} Moreover, transplantation of \textit{c-kit}$^+$ CSCs into the infarcted myocardium induces cardiomyocyte regeneration post-MI.\textsuperscript{124,127} However, negative results were reported in studies using cell fate-mapping strategies. In this regard, no evidence of cardiomyocyte differentiation from \textit{c-kit}$^+$ cells has been found in the adult mouse heart.\textsuperscript{128,129} Despite this controversy, a phase I clinical trial has been initiated to study the safety and feasibility of autologous \textit{c-kit}$^+$ CSCs in patients undergoing coronary bypass surgery (ClinicalTrials.gov Identifier: NCT00474461). Cells were isolated and cultured from each patient’s right atrial appendage, followed by immunomagnetic sorting with anti-\textit{c-kit} antibody-coated microbeads.\textsuperscript{130} Significant improvement in LV ejection fraction (41.2\% vs 27.5\%) and viable cardiac tissue mass was reported 12 months after \textit{c-kit}$^+$ CSC infusion in 20 patients,\textsuperscript{130} which so far is the best clinical outcome achieved in all stem cell therapy trials in coronary heart disease. However, global percentage change of ventricle infarct size, which may indicate the myocardial regeneration, were not reported.\textsuperscript{130}
Cardiosphere-derived cells (CDCs) are also CSCs identified through their ability to migrate from cultured cardiac tissues and form floating spheres. CDCs are isolated from minimally invasive percutaneous endomyocardial biopsies from the adult human heart \(^{131}\) and the myocardium from other species (mice, rats and pigs).\(^{132,133}\) The CDCs are a mixture of \(c-kit^+\) cells and supporting endothelial and mesenchymal cells that can be amplified in culture and grown to millions in a few weeks.\(^{133}\) Intracoronary delivery of CDCs has been shown to improve cardiac repair and function in animal MI models.\(^{131,134}\) A phase I clinical trial was carried out to study the safety and efficacy of CDCs in patients with a recent MI (ClinicalTrials.gov Identifier: NCT00893360). Reduction in scar mass, increased viable heart mass and regional contractility was observed in 17 patients with MI, 6 months after CDCs treatment, while no significant changes in ejection fraction, end-diastolic and end-systolic volume were found.\(^{135}\) Cell therapies with autologous \(c-kit^+\) CSCs or CDCs after MI have been shown to be safe and beneficial, and warrant phase 2 studies to test their efficacy.

Several other populations of CSCs are also under investigation. Stem cell antigen 1 (\(Sca-1\)) positive CSCs express early cardiac transcription factors including GATA4 and MEF2. When treated with 5-azacytidine or oxytocin, \(Sca1^+\) CSCs can differentiate into spontaneously beating cardiomyocytes \textit{in vitro}.\(^{136}\) Intravenous administrations of \(Sca1^+\) CSCs to mice following myocardial ischemia/reperfusion showed 50% fusion with host cardiomyocytes.\(^{137}\) Moreover, transplantation of \(Sca-1^+/CD31^-\) CSCs in the infarcted mouse heart improved cardiac function and angiogenesis through paracrine effects.\(^{138}\) Further studies are needed to determine the clonogenic and self-renewal potential of \(Sca-1^+\) CSCs and their capacity in cardiomyocyte differentiation. Another population of
multipotent CSCs marked by the LIM-homeodomain transcription factor islet-1 (Isl-1) has been shown to give rise to cardiomyocytes, endothelial and smooth muscle cells both \emph{in vitro} and \emph{in vivo}. The Isl-1$^+$ CSCs are mainly located in the outflow tract and right atrium of fetal or very young postnatal hearts and are negative for \emph{c-kit} and \emph{Sca-1}.\footnote{139} The fact that Isl-1$^+$ CSCs exist in very low numbers in the adult heart limits their therapeutic potential. Furthermore, the property of cardiac progenitor cells to expel toxic molecules through an ATP-binding cassette transporter\footnote{140} is also employed to define a pool of CSCs. They are termed cardiac side population cells (CSPs) because after exposure to the DNA-binding dye, Hoechst 33342, they can clear the fluorochrome and appear on the side of the flow cytometry profile.\footnote{141} CSPs are positive for \emph{Sca-1} and negative for \emph{c-kit}.\footnote{142} CD31$^-$/Sca-1$^+$ CSPs form colonies \emph{in vitro} and differentiate into cardiomyocytes.\footnote{142,143} When transplanted into animals with MI, CSPs can migrate to the injured myocardium and differentiate into cardiomyocyte- and endothelial-like cells.\footnote{144,145}

To date, whether the resident CSCs identified by different methods represent distinct populations of stem/progenitor cells or they are actually the same line of stem cells at different stage of maturation remains unknown. Exploitation of CSCs in stem cell therapy for IHD is certainly an exciting and promising strategy. However, further investigations of the endogenous regulatory mechanisms of CSCs survival, proliferation and differentiation under physiological or pathological conditions are definitely needed before stem cell therapy can be safely and effectively applied to IHD patients.

\subsubsection{1.3.3 Epicardium-derived Cells (EPDCs)}

During embryonic cardiomorphogenesis, cells from the proepicardial organ migrate to the myocardial surface after looping of the primitive heart tube to form the
epicardium. A fraction of these cells undergo epithelial to mesenchymal transition (EMT) and invade the myocardium to give rise to coronary smooth muscle cells, subendocardial and atrioventricular cushion mesenchymal cells and adventitial, perivascular and cardiac interstitial fibroblasts. These cells are referred to as EPDCs. Besides their structural contribution, an important modulatory role of EDPCs in the formation of the compact myocardium and coronary vessels, and differentiation of Purkinje fibers has been proposed. During embryonic heart development, Wilms tumor 1 (Wt1) protein is expressed in EPDCs and is involved in the regulation of EMT. However, expression of Wt1 is lost in fully differentiated EPDCs. Recently, the adult epicardium, which has been considered to be terminally differentiated, was found to be a potential source of vascular progenitors after appropriate stimulation. Riley et al. demonstrated a significant outgrowth from adult mouse epicardial explants after thymosin β4 stimulation. These EPDCs were able to migrate and differentiate into fibroblasts, smooth muscle and endothelial cells. Moreover, upregulation of cardiomyocyte prokineticin receptor-1signaling has been shown to be able to trigger adult mouse EPDCs migration and differentiation into endothelial and smooth muscle cells. Similar findings were observed in adult human EPDCs which differentiated into smooth muscle cells after treatment with TGF-β or bone morphogenetic protein-2 (BMP-2). Interestingly, expression of the cardiac marker genes GATA4 and cardiac troponin T was also detected in cultured human adult EPDCs. The ability of the adult epicardium to restore multi-potency makes adult EPDCs a novel promising therapeutic candidate in ischemic heart disease.
Several studies have investigated the potential of adult EPDCs in cardiac repair post-MI. Transplantation of human adult EPDCs into the ischemic myocardium preserved cardiac function and attenuated ventricular remodeling via enhanced vascularization in mice. The engrafted EPDCs were found to express endothelial marker von Willebrand factor (vWF) and the smooth muscle marker α-smooth muscle actin (α-SMA) 2 weeks post-MI, suggesting neovascularization. However, the transplanted EPDCs did not express cardiomyocyte markers such as atrial natriuretic peptide, sarcomeric myosin or cardiac troponin I. Furthermore, c-kit+ and CD34+ cells were detected in adult mouse epicardium and the number of epicardial c-kit+ cells significantly increased post-MI. After fresh isolation, these cells expressed cardiac transcription factors and acquired an endothelial phenotype in vitro. Although, in that study, the authors found increased c-kit+/GATA4+, c-kit+/vWF+ and c-kit+/smooth muscle actin+ cells in the subepicardial space 3 days post-MI, there was no direct proof that these cells were EPDCs. Interestingly, some of the EPDCs traced by lentiviral green fluorescent protein (GFP) were found to express α-sarcomeric actin (a cardiac muscle actin) post-MI, but the authors did not quantify the incidence of this transdifferentiation. Following MI, the adult epicardium is reactivated and may have the potential to repair the infarcted myocardium. The ability of the adult epicardium to regain progenitor potential is defined as epicardial activation. Thus, understanding the role of the epicardium and factors that regulate its activation will be critical to developing therapies to enhance endogenous cardiac repair post-MI.
1.4 STEM CELL FACTOR (SCF)

SCF, also known as Steel factor, mast cell growth factor, or c-kit ligand, binds to c-kit and promotes survival, proliferation, mobilization, and adhesion of all c-kit-expressing cells. c-kit is a tyrosine kinase receptor expressed in stem cells and progenitors including BMSCs, CSCs and some EPDCs. while SCF is ubiquitously expressed as a glycosylated transmembrane protein widely throughout the body by stromal cells, fibroblasts, endothelial cells and the myocardium. Alternative mRNA splicing leads to two isoforms of SCF: soluble (S-SCF) and membrane-associated (M-SCF), which differ in the absence or presence of a particular proteolytic cleavage site encoded by exon 6. S- and M-SCF have distinct but overlapping roles. M-SCF is the dominant isoform in vivo and induces more persistence and long-term support of target cell survival via juxtacrine, but may be less effective in stimulating growth.

1.4.1 SCF Biology

In 1990, SCF was initially found to be a growth factor that can stimulate both primitive and mature hematopoietic progenitor cells. Then it was identified to be the product of Steel (Sl) gene in mouse chromosome 12, between 12q14.3 and 12qter in human and the ligand for the c-kit tyrosine kinase receptor. The protein sequence is highly conserved, and there is more than 80% amino acid homology between mouse and human. Mutation of Sl locus results in alterations of coat color, sterility, anemia and lack of mast cells due to impaired development of hematopoietic cells, germ cells and neural crest derivatives such as melanocytes caused by the altered microenvironment. Sl/Sl homozygotes are deficient in germ cells, devoid of coat pigment and die perinatally from macrocytic anemia. SCF has a major role in hematopoiesis, spermatogenesis and
melanogenesis, and works in synergy with other growth factors. *In vitro*, SCF treatment induces survival, proliferation and migration of HSCs, melanoblasts and primordial germ cells.\(^\text{173}\) *In vivo*, recombinant S-SCF elicits a multilineage response in peripheral counts and increases bone marrow cell mass, which protects against lethal irradiation by accelerating hematopoietic recovery.\(^\text{174-176}\)

S- and M-SCF result from pre-translational processing of primary SCF mRNA transcripts. The full-length mouse SCF consists of 248 amino acids (SCF\(^\text{248}\)) with an extracellular domain, a membrane-spanning region and a short cytoplasmic tail. Proteolytic cleavage at Ala 165 releases a 164-amino-acid biological active growth factor, S-SCF. Another mRNA transcript produced by alternative splicing leads to the production of the 220 amino acid M-SCF (SCF\(^\text{220}\)), which lacks the main cleavage site Ala 165 encoded by exon 6. Compared to full-length SCF\(^\text{248}\), the release of S-SCF from SCF\(^\text{220}\) is less efficient in COS cells\(^\text{168}\) and mouse stromal cells.\(^\text{177}\) As suggested by protease inhibitor profiles and sequence analysis, a distinct protease on a secondary cleavage site may be involved in producing the S-SCF from M-SCF.\(^\text{168,178}\) To this end, Majumdar et al identified the second cleavage site in mouse M-SCF encoded by exon 7.\(^\text{177}\) Interestingly, a species specific difference of the secondary proteolytic cleavage was demonstrated in M-SCF, showing that 95% human M-SCF remained membrane-bounded while 20% of mouse M-SCF was cleaved into S-SCF after glycosidase overnight digestion *in vitro*.\(^\text{177}\) The protease involved in natural shedding of S-SCF remains unknown, but it may be a metalloprotease.\(^\text{179}\) *In vivo*, metalloprotease-9 has been shown to be important in S-SCF release from HSCs, but the cleavage domain was not identified.\(^\text{180}\) Activation of protein kinase C (PKC) signaling by phorbol 12-myristate 13-
acetate (PMA) is able to increase the shedding in COS-1 cells transfected with mouse M-SCF. The biological activity of the S-SCF released from M-SCF is similar to that of the proteolytic product of the full length SCF.

The expression patterns of full length SCF and M-SCF are also different. Full length SCF is expressed widely in most organs and tissues while M-SCF is expressed in a tissue-specific manner. It can be easily detected in heart, lung, spleen, bone marrow and testis but is barely seen in brain. This suggests that M-SCF may play a different role from the S-SCF in different organs. In this regard, mice with the Sl/Sld mutation (deletion of the SCF trans-membrane and cytoplasmic domain) exhibit abnormal phenotypes including anemia, sterility and white spotting. When M-SCF was expressed in Sl/Sld mice the anemia, bone marrow hypocellularity and runting were improved. On the other hand, M-SCF may substitute for full-length SCF in most situations except for mast cell production and regeneration of hematopoietic tissue after radiation. The distinct but overlapping effects of the 2 isoforms are based on their natural characteristics (Figure 1.3). S-SCF is a diffusible factor that acts on target cells over short or long distances (endocrine and paracrine). The normal human serum level of S-SCF is about 3.3 ng/mL and can be altered by different disease conditions such as cancer and Alzheimer’s disease. Moreover, S-SCF may also be associated with or sequestered in the extracellular matrix and thus function in a manner similar to M-SCF. M-SCF mainly mediates cell-cell interactions and adhesion, during which it may be able to act at a sustained high concentration as a localized signal to target cells (juxtacrine). On the other hand, the intercellular signaling induced by S-SCF may be relatively low and variable.
Figure 1.3 Illustration of soluble (S-SCF) and membrane-associated SCF (M-SCF).

S-SCF is released from the full-length SCF after proteolytic cleavage at the main cleavage site encoded by exon 6. It can act on c-kit expressing cells nearby (paracrine) or at long distant (endocrine). M-SCF mainly remains membrane bounded and is involved in adhesion and activation of c-kit expressing cells by cell-cell interaction (juxtacrine). Full length SCF can also have juxtacrine effects before the S-SCF is released.
Thus, it is not surprising that M-SCF provides greater survival and proliferation signals compared to the soluble isoform.\textsuperscript{181,186} It also appears that the 2 isoforms of SCF have different signaling pathways for their biological activities. For example, although both S-SCF and M-SCF can induce proliferation of erythroid progenitors via activation of p38 and ERK pathways, M-SCF-induced signaling is more sensitive to ERK inhibition while p38 inhibition works better on S-SCF.\textsuperscript{186} Thus, through expression of different SCF isoforms, stromal cells regulate the activity, survival, proliferation, migration and adhesion of local \textit{c-kit} expressing cells as well as those in remote organs and tissues.

\subsection*{1.4.2 SCF receptor \textit{c-kit}}

The \textit{c-kit} protein is a 145 KD glycoprotein encoded at the white spotting (\textit{W}) locus on murine chromosome 5 \textsuperscript{187} and human chromosome 4q11-q12.\textsuperscript{188} It is mainly expressed on hematopoietic stem cells,\textsuperscript{173} prostate stem cells \textsuperscript{189} and tumors.\textsuperscript{190} Recent studies also showed that it is a marker for various adult stem cells such as CSCs.\textsuperscript{122} In addition, mast cells, melanocytes in the skin, and interstitial cells of Cajal in the digestive tract, human aortic and umbilical vein endothelial cells also express \textit{c-kit}.\textsuperscript{191-193} \textit{c-kit} is a type III tyrosine kinase receptor characterized by an extracellular binding domain for SCF, a hydrophobic transmembrane region and an intracellular region with ATP binding, and phosphotransferase domain separated by a kinase insert.\textsuperscript{194} SCF forms a non-covalent homodimer that binds to 2 \textit{c-kit} monomers and promotes \textit{c-kit} dimerization, which results in autophosphorylation of \textit{c-kit} cytoplasmic domain at tyrosine residues.\textsuperscript{195,196} The phosphotyrosine residues serve as docking sites for signal transduction molecules containing Src homology 2 (SH2) and other phosphotyrosine binding domains.\textsuperscript{194} There are multiple signaling pathways that can be activated by SCF/\textit{c-kit} including MAP
kinase, PI3 kinase, phospholipase-γ (PLC-γ), Src and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways, all of which contribute to the biological effects of SCF. SCF/c-kit signaling can be inhibited by blockage of SCF/c-kit binding via neutralizing antibody, ACK2, or inhibition of c-kit receptor activation by AG 1296.

1.4.3 Potential Clinical Applications of SCF

Recombinant human SCF is currently used to mobilize BMSCs for collection of HSCs for hematopoietic reconstitution and to prevent chemotherapy-induced anemia and thrombocytopenia. SCF has also been indicated as a drug target for cancer treatment. Analysis of serum SCF levels is recommended to aid the diagnosis of many diseases including pancreatic cancer, gastrointerstitial stromal tumor, primary uveal melanoma, acute renal allograft rejection, delayed and slow graft function and Alzheimer’s disease.

The biological function of SCF in various diseases has also been widely investigated. SCF/c-kit signaling has been shown to have an anti-apoptotic role in acute kidney injury, diabetic neuropathy and steroid associated bone damage. Furthermore, SCF can regulate endothelial cell and EPC function, leading to neovascularization in tissue repair. In synergy with granulocyte-colony stimulating factor (G-CSF), SCF is able to induce liver regeneration, reduce β-amyloid deposits in a mouse model of Alzheimer’s disease and enhance neuronal lineage commitment of neural stem cells. The role of SCF in IHD will be discussed in the following section.
1.4.4 SCF and IHD

The role of granulocyte colony-stimulating factor (G-CSF) and S-SCF in MI was first studied by Orlic et al, showing enhanced BMSCs mobilization and recruitment to the infarcted heart and improved cardiac function in mice post-MI.\(^{68}\) Those beneficial effects resulted from decreased myocardial apoptosis,\(^{218}\) reduced arrhythmia,\(^{219}\) increased neovascularization\(^{220}\) and impaired cardiac remodeling.\(^{221}\) However, Ohtsuka et al found SCF treatment alone failed to induce cardiac protective effects post-MI.\(^{220}\) Myocardial expression of SCF is decreased post-MI. Augmentation of SCF expression through transplanted MSCs has been shown to increase angiogenesis and cardiac function.\(^{222}\) Moreover, cardiac transplantation of MSCs transfected with a full length mouse SCF cDNA plasmid, which express both S- and M-SCF, enhanced EPC recruitment and myocardial angiogenesis for about 2 weeks post-MI. However, there was no change in animal survival among all treatment groups.\(^{222}\) This is not surprising as more than 95% of the transplanted MSCs are cleared within 7 days following transplantation into the myocardium.\(^{223}\) Intra-myocardial administration of SCF sustainably mobilized exogenously administered Lin\(^{-}\)c-kit\(^{+}\) bone marrow-derived cells from the circulation homing to the infarcted myocardium up to 72 hours post-MI, however, cardiac function was not investigated in this study.\(^{224}\) The molecular and cellular mechanisms by which SCF regulates cardiac function post-MI are not fully understood.

1.5 OVERALL HYPOTHESIS AND AIMS

IHD, a leading cause of death, is responsible for social and financial burden worldwide.\(^{1}\) Recently, treatment with S-SCF in combination with G-CSF was found to improve cardiac repair and survival after MI in mice.\(^{220}\) Furthermore, peri-infarct
injections of SCF increased recruitment of BMSCs to the infarcted heart. Although numerous animal studies using stem cell therapy showed significant protection post-MI, only minor improvements have been observed in most clinical trials, while two recent trials showed no significant benefit at all. Thus, a better understanding of the pathophysiology of different types of stem cells in cardiac repair post-MI is needed.

SCF is expressed as a glycosylated trans-membrane protein by many cells and tissues, including the myocardium. SCF binds to its receptor c-kit and plays a critical role in cell survival, proliferation, mobilization, and adhesion. c-kit positive adult stem cells including HSCs, EPCs and CSCs have been demonstrated to be potential candidates for cardiac repair post-MI, which implies a possible regulatory role of SCF in MI. M-SCF is more potent than S-SCF in supporting cell survival and proliferation. In adult mice lacking M-SCF, cardiac morphology and function at basal conditions were normal, but significantly higher mortality and worse cardiac function were observed post-MI. To study the role of M-SCF in cardiac repair post-MI, we generated a novel mouse model of inducible cardiac specific over-expression of hSCF. The aim of this thesis was to study the effects of M-SCF on cardiac function post-MI and to investigate the underlying cellular mechanisms especially the role of M-SCF in regulation of c-kit+ stem/progenitor cell activity in the ischemic heart. Our overall hypothesis was that cardiac-specific overexpression of hSCF protects the heart via inhibition of myocardial apoptosis and enhancement of neovascularization and stem cell activity post-MI.
Figure 1.4 Proposed cellular mechanisms by which membrane-associated SCF (MSCF) regulates cardiac function post-MI.

Interaction of SCF with c-kit receptors improves cardiac repair post-MI by enhancing circulating stem cell and resident stem cell activities.
M-SCF

Circulating $c$-$kit^+$ stem cells

Resident $c$-$kit^+$ stem cells in the myocardium and epicardium

Retention, proliferation, migration, differentiation and paracrine factors release

Apoptosis $\downarrow$ Neovascularization $\uparrow$ Hypertrophy $\downarrow$

Cardiac function
Animal survival
In order to induce cardiac-specific hSCF overexpression, a hSCF transgenic mouse was generated with the hSCF transgene expression under the control of a tetracycline-responsive element containing the α-MHC promoter. The hSCF mice were then crossed with cardiac-specific tetracycline activator (tTA) mice to generate hSCF/tTA double transgenic mice. The hSCF/tTA transgenic mice specifically express the hSCF in the heart. These transgenic mice were healthy and fertile with no cardiac abnormalities. Furthermore, myocardial expression of hSCF in hSCF/tTA mice can be abrogated by treatment with doxycycline (DOX). Thus, the specific effects of hSCF can be tested in the absence and presence of DOX to turn on and off hSCF expression in the heart.

The first aim of this thesis was to determine the effects of hSCF on cardiac function and survival post-MI. Thus, in Chapter 2, I hypothesized that cardiomyocyte-specific over-expression of hSCF improves myocardial function and survival post-MI. We found that the protective effects of hSCF post-MI were due to increased myocardial EPC population, decreased apoptosis and improved cardiac remodeling. The significant cardiac protection by hSCF at 5 and 30 days post-MI led to our query on the effects of hSCF in acute cardiac injury. Suggested by the observation that hSCF decreased myocardial apoptosis 5 days post-MI, the second aim was to determine the potential of hSCF to limit the infarct size after acute I/R. Moreover, Akt phosphorylation was increased in hSCF over-expressing myocardium 5 days post-MI. Thus, in Chapter 3 I hypothesized that cardiomyocyte-specific over-expression of hSCF decreases infarct size via Akt signaling after acute I/R. Interestingly, our results showed that decreased infarct size in hSCF over-expressing mice after acute I/R resulted from increased CD45^+ ckit^+ cell retention and CD45^− ckit^+ CSCs activation in the myocardium. Besides EPCs and
CSCs, recent studies have shown that EPDCs are another potential resource of cardiovascular progenitors.\cite{230} Thus, the third aim of my thesis was to investigate the role of hSCF in EPDC activation, migration into the sub-epicardium and contribution to cardiac repair post-MI.\cite{161} So I hypothesized in Chapter 4 that cardiac specific over-expression of hSCF promotes epicardial activation. We found that hSCF overexpression increased EPDC activation, proliferation and mobilization which contribute to myocardial neovascularization post-MI.

In summary, the overriding aim of this thesis was to examine the cellular and molecular mechanisms that mediate the cardio-protective and beneficial effects of hSCF post-MI in mice. To accomplish this goal, experiments utilized both in vivo models of cardiac I/R and MI, and in vitro cultures of cardiomyocytes and EPDCs. The effects of hSCF on cardiac function and animal survival 30 days post-MI, changes in infarct size after acute I/R, and its impact on adult stem cell retention, activation, migration and differentiation were examined.
1.6 REFERENCES


93. Kindler V. Postnatal stem cell survival: does the niche, a rare harbor where to resist the ebb tide of differentiation, also provide lineage-specific instructions? *J Leukoc Biol.* 2005;78:836-44.


110. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. Marrow-derived stromal cells express genes encoding a broad spectrum of
arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res.* 2004;94:678-85.

111. Gneechi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J.* 2006;20:661-669.


131. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E. Regenerative potential of cardiosphere-derived cells


169. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR, et al. Identification, purification, and


2 Chapter 2

Cardiomyocyte Specific Overexpression of Human Stem Cell Factor Improves Cardiac Function and Survival post-Myocardial Infarction in Mice

A version of this chapter has been published in Circulation 2009,120:1065-1074.


“Cardiomyocyte-Specific Overexpression of Human Stem Cell Factor Improves Cardiac Function and Survival After Myocardial Infarction in Mice.”
2.1 CHAPTER SUMMARY

S-SCF has been shown to mobilize bone marrow stem cells and improve cardiac repair post-MI. However, the effect of M-SCF on cardiac remodeling post-MI is not known. This chapter investigated the effects of cardiomyocyte specific overexpression of membrane-associated hSCF on cardiac function post-MI. A novel mouse model with tetracycline-inducible and cardiac-specific overexpression of membrane-associated hSCF was generated. MI was induced by left main coronary artery ligation. This chapter illustrates that cardiac-specific overexpression of membrane-associated hSCF improved 30-day mortality and cardiac function post-MI. Moreover, increased angiogenesis and decreased myocardial apoptosis and remodeling were observed. Importantly, the EPC population was increased in hSCF overexpressing myocardium post-MI. Furthermore, hSCF overexpression promoted de novo angiogenesis as assessed by matrigel implantation into the LV myocardium. This data demonstrate that cardiomyocyte specific overexpression of hSCF improves myocardial function and survival post-MI. These beneficial effects of hSCF may result from increases in EPC retention, neovascularization, and decreases in myocardial apoptosis and cardiac remodeling.
2.2 INTRODUCTION

MI is responsible for about one third of heart failure cases and causes approximately 2 million deaths per year worldwide.\textsuperscript{1} MI leads to scar formation and subsequent ventricular remodeling, which is characterized by infarct expansion, progressive fibrous replacement of myocardium, hypertrophic growth of the non-infarct myocardium and LV dilatation. Cardiac remodeling contributes to development of HF post-MI.\textsuperscript{2} Despite optimal pharmacological treatment, the prognosis of heart failure remains poor.\textsuperscript{3}

SCF, also known as Steel factor, mast cell growth factor, or c-kit ligand, binds to its receptor c-kit and promotes survival, proliferation, mobilization, and adhesion of all c-kit expressing cells, which includes HSCs, EPCs,\textsuperscript{4,5} and CSCs.\textsuperscript{6} SCF is expressed as a glycosylated transmembrane protein by many cells and tissues including stromal cells, fibroblasts, endothelium, and myocardium.\textsuperscript{7,8} Alternative splicing leads to 2 isoforms of SCF, soluble (S-SCF) and membrane-associated (M-SCF) isoforms, which differ in the absence or presence of a proteolytic cleavage site encoded by exon 6. S-SCF and M-SCF have distinct but overlapping roles. M-SCF, the dominant isoform in vivo, induces a more persistent receptor activation and is more effective at promoting long-term support of target cell survival.\textsuperscript{9,10} Transgene expression of M-SCF in Steel-dickie (sld) mutant mice resulted in a significant relief from anemia and bone marrow hypoplasia.\textsuperscript{11} In contrast, overexpression of S-SCF transgene had no effect on red blood cell production but corrected the myeloid progenitor cell deficiency seen in these mutants.\textsuperscript{11} Recently, treatment with S-SCF in combination with G-CSF improved cardiac repair and survival post-MI while S-SCF treatment alone did not.\textsuperscript{12} Since M-SCF has a much different
biological profile compared to S-SCF, it is possible that cardiac specific overexpression of M-SCF may promote cardiac repair mechanisms post-MI.

We hypothesized that inducible cardiomyocyte specific overexpression of human M-SCF (hSCF) could improve cardiac repair, myocardial function and survival post-MI. To test this hypothesis, we generated a novel transgenic mouse that overexpresses membrane-associated hSCF in cardiomyocytes under the control of a Tet-off system.\textsuperscript{13} We demonstrated that cardiomyocyte-specific hSCF overexpression increased myocardial neovascularization, decreased ventricular remodelling, and increased survival post-MI. Our study suggests a novel therapeutic potential of hSCF in the treatment of heart failure post-MI.

2.3 METHODS

2.3.1 Generation of hSCF/tTA Double Transgenic Mice

A new line of tetracycline-inducible cardiac-specific overexpressing membrane-associated hSCF mice was generated as previously described.\textsuperscript{13} Briefly, membrane-associated hSCF cDNA (accession #: NM_003994) was inserted into the inducible α-myosin heavy chain (MHC) promoter expression vector 1 to permit DOX-regulated expression in combination with a cardiac-specific tTA-expressing transgene. The fragment containing tetracycline operon, α-MHC promoter and hSCF was injected into the fertilized oocytes, which were then transferred into the oviduct of a pseudo-pregnant female mouse. hSCF transgenic founders were verified by Southern blot analysis. Transgenic line 1 mice were used in the present study. The hSCF mice were crossed with mice created previously that carry tTA driven by α-MHC promoter \textsuperscript{13} to produce wild type (WT), tTA, hSCF, and hSCF/tTA mice. Genotypes were identified by PCR using
genomic DNA from tail biopsies. The following primer pairs were used: tTA, forward, AGC GCA TTA GAG CTG CTT AAT GAG GTC, reverse, GTC GTA ATA ATG GCG GCA TAC TAT C; hSCF, forward, CAA CTG CAG GTC GAC CTG TTT GTG CTG GAT CGC AGC, reverse, CCC AAG CTT GAA GCA AAC ATG AAC TGT TACC. To turn off hSCF expression, the hSCF/tTA mice were treated with 0.2 mg/ml DOX in their drinking water for 2 weeks prior to and continued for 5 or 30 days after sham or MI surgery.

2.3.2 Determination of mRNA Expression by Real-time RT-PCR

Total RNA was extracted from heart tissue using Trizol and cDNA was synthesized using moloney murine leukemia virus (MMLV) reverse transcriptase as previously described. Real-time PCR was conducted using SYBR Green PCR Master Mix as per the manufacturer’s instructions (Eurogentec, CA). 28S rRNA was used as a loading control. The primer sequences were as follows: hSCF upstream 5’TCA TTC AAG AGC CCA GAA CC3’ and downstream 5’CAG ATG CCA CTA CAA AGT CC3’ and 28S rRNA upstream 5’ TTG AAA ATC CGG GGG AGA G 3’ and downstream 5’ ACA TTG TTC CAA CAT GCC AG 3’. Samples were amplified for 35 cycles using MJ Research Opticon Real-time PCR machine. For quantification of the growth factors mRNA level in the heart, the following primer pairs were used: VEGF-A, forward, GAT TGA GAC CCT GGT GGA CAT C, reverse, TCT CCT ATG TGC TG G CTT TGG T; insulin growth factor (IGF-1), forward, CTG CTT GCT CAC CTT CA C, reverse, ATG CTG GAG CCA TAG CCT GT; bFGF, forward, CAA GGG AGT GTG TGC CAA CC, reverse, TGC CCA GTT CGT TTC AGT GC; mouse SCF, forward, CGG GAT GGA TGT TTT GCC TA, reverse, CTT CGG TGC GTT TTC TTC CA. The levels of
mRNA expression in relation to 28S were determined using a comparative threshold cycle (Ct) method as we previously described.\textsuperscript{14}

2.3.3 Animal Procedures

Animals in this study were handled in accordance with the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. After mice were anaesthetized with an IP injection of a mixture of ketamine (50 mg/kg) and xylazine (12.5 mg/kg), myocardial infarction was induced by surgical occlusion of the left main coronary artery through a left anterolateral approach as we previously described.\textsuperscript{15} Both male and female mice at the age between 2-6 months of age were randomly selected to undergo coronary artery ligation or sham surgery (same procedure without occlusion of the left main coronary artery). The investigator was blinded to the genotype of the mice during surgeries.

Experiments were performed 5 or 30 days after surgery. Survival was monitored and the LV to body weight ratio was recorded. Infarct size was measured after animals were sacrificed and was expressed as a fraction of the total cross-sectional endocardial circumference of the LV as we described previously.\textsuperscript{15}

2.3.4 Hemodynamic Measurements

Mice were anaesthetized as described in the previous section. A Millar pressure-conductance catheter (Model SPR-839, Size 1.4F) was inserted into the right carotid artery and advanced into the LV. After stabilization for 10 minutes, the signal was recorded continuously and later analyzed with a cardiac pressure-volume analysis
program (PVAN 3.2; Millar Instruments, TX). Mice were then sacrificed and hearts were fixed in 4% paraformaldehyde or stored at -80°C for further analysis.

2.3.5 Histology

To measure myocyte cell size, cardiac tissue sections were stained with hematoxylin/eosin and photographed using a digital camera attached to a microscope (Observer D1, Zeiss) at a magnification of 400x. Myocyte shortest cross sectional diameters at nuclei level were assessed by the AxioVision software. To study stem cell recruitment, tissue sections were stained with rabbit anti-mouse c-kit (1:200, Santa Cruz Biotechnology, CA) and rabbit anti-mouse VEGFR2 primary antibody (1:200, Abcam, MA) followed by goat anti-rabbit fluorescent secondary antibody. Nuclei were stained with Hoechst 33342. Ten to 15 fields of the peri-infarct area (200-300 µm adjacent to the infarct) were examined for each heart using a fluorescent microscope (Observer D1, Zeiss) at 630x magnification to quantify the c-kit positive and c-kit/VEGFR2 double positive cells. Images were taken using a laser confocal microscope (LSM 510 Meta, Zeiss). To analyze capillary density, tissue sections were stained with biotinylated lectin I (1:100, Vector Laboratory, CA) followed by Vectastain Elite ABC peroxidise kit with diaminobenzidine tetrahydrochloride (DAB) as a chromogen, and counterstained by hematoxylin. Ten to 14 images were taken in the peri-infarct area of each heart using a digital camera attached to a microscope (400x, Observer D1, Zeiss). To quantify the mast cells in the heart, cardiac tissue sections were stained with 0.02% toluidine blue in 0.25% acetic acid (pH 2.0-2.5). Two to 4 heart sections of each mouse were evaluated.
2.3.6 Caspase-3 Activity and Cytoplasmic Histone-Associated DNA Fragments

Caspase-3 activity and cytoplasmic histone-associated DNA fragments were measured using caspase-3 cellular activity assay kit (BIOMOL, Plymouth Meeting, PA) and cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche, Mississauga, ON), respectively as we previously described.\(^{20}\)

2.3.7 In vivo Matrigel Angiogenesis

In vivo angiogenesis was assessed by myocardial matrigel implantation as described.\(^{21}\) Three days after matrigel implantation, heart tissues were collected and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Results are expressed as the percentage of the vessel-like area to the total matrigel area.

2.3.8 Statistical Analysis

Data are expressed as the mean ± SEM. One- or two-way ANOVA followed by Bonferroni test was performed for multiple group comparisons. Unpaired Student’s t test was used to detect significance between two groups. Kaplan and Meier survival curves were analyzed by Log-rank (Mantel-Cox) test. Differences were considered significant at the level of \(P<0.05\).

2.4 RESULTS

2.4.1 Generation and Characterization of the Conditional Cardiac-specific hSCF/tTA Double Transgenic Mice

hSCF mice were generated with the hSCF transgene expression under the control of a tetracycline-responsive element containing the \(\alpha\)-MHC promoter (Figure 2.1A). The hSCF transgenic mice were then crossed with cardiac-specific tTA mice to generate the
Figure 2.1 Generation and characterization of the inducible hSCF/tTA transgenic mouse.

A, Illustration of the hSCF transgenic constructs used to create the hSCF transgenic mice. B, Expression of membrane-associated hSCF mRNA was detected in the heart, but not in other organs of hSCF/tTA mice in the absence of DOX. C, Cardiac expression of membrane-associated hSCF mRNA in hSCF/tTA mice after treatment with DOX for 5, 10 and 14 days. D, Protein expression of human SCF in heart tissue was measured by western blotting. Human SCF protein was exclusively expressed in hSCF/tTA-DOX mice. E, Endogenous mouse SCF mRNA expression determined by real-time PCR was not significantly different among WT, hSCF/tTA-DOX and hSCF/tTA+DOX mice. Data are mean ± SEM, n=5-7 per group. One-way ANOVA followed by Bonferroni test.
A

5500 bp

TetO
α-MHC promoter

834 bp

hSCF

600 bp

hGH poly A

B

200 bp

100 bp

200 bp

100 bp

Marker Heart Liver Kidney Lung Skin Skeletal Muscle

hSCF

28S

C

200 bp

100 bp

200 bp

100 bp

Marker WT -Dox +Dox +Dox +Dox 5D 10D 14D hSCF/tTA

hSCF

28S

D

hSCF

α-Actinin

30kDa

100kDa

WT -DOX +DOX 14D hSCF/tTA

E

Mouse SCF/28S ratio

WT -DOX +DOX hSCF/tTA
inducible cardiac-specific hSCF/tTA double transgenic mice. The resulting offspring were genotyped by PCR using both tTA and hSCF primers. Cardiac specific expression of hSCF was verified by Real-time-PCR. Expression of hSCF was detected in the heart tissue but not in liver, kidney, skin, lung or skeletal muscle (Figure 2.1B). The inducible expression of hSCF in cardiomyocytes was verified using both RT-PCR and western blot techniques. Results showed a high level of hSCF mRNA and protein expression in the hearts of hSCF/tTA mice in the absence of DOX. After 10-14 days of continuous DOX administration, the expression of hSCF in hSCF/tTA mice was abrogated in both mRNA and protein levels (Figure 2.1C and D). Transgene expression did not affect endogenous mouse SCF expression as myocardial mouse SCF mRNA levels analyzed using real-time RT-PCR were similar among WT, hSCF/tTA-DOX and hSCF/tTA+DOX mice (Figure 2.1E). Furthermore, there were no coat color changes in any of the hSCF/tTA mice.

2.4.2 Post-MI Stem Cell Factor Expression

A total of 87 hSCF/tTA mice and 105 littermates (tTA, hSCF and WT) were subjected to coronary artery ligation or sham operation. General characteristics of these animals are shown in Table 2.1. There were no statistical differences in age, sex or body weight between hSCF/tTA and littermates within sham or MI groups (P=n.s.). Protein expression of mouse SCF post-MI was studied by western blot analysis in WT mice. Myocardial mouse SCF protein levels were significantly lower at 5 days (P<0.05, Figure 2.2A) but not at 30 days (P=n.s, Figure 2.2B) post-MI compared to sham group. The expression of hSCF mRNA as determined by real-time RT-PCR in hSCF/tTA mice was unchanged in the peri-infarct area at 5 days (Figure 2.2C) and 30 days (Figure 2.2D).
Table 2.1 General information of control and hSCF/tTA mice subjected to sham or MI surgeries

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham Controls</th>
<th>hSCF/tTA Controls</th>
<th>MI Controls</th>
<th>hSCF/tTA Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>24</td>
<td>21</td>
<td>81</td>
<td>66</td>
</tr>
<tr>
<td>Age at surgery, days</td>
<td>85±4</td>
<td>83±6</td>
<td>83±3</td>
<td>86±3</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>24/0</td>
<td>21/0</td>
<td>13/68</td>
<td>10/56</td>
</tr>
<tr>
<td>Body Weight, g</td>
<td>27.8±0.9</td>
<td>26.0±0.8</td>
<td>26.6±0.6</td>
<td>26.5±0.6</td>
</tr>
<tr>
<td>Infarct Size (% of LV)</td>
<td>-</td>
<td>-</td>
<td>38.6±0.8</td>
<td>38.5±0.7</td>
</tr>
</tbody>
</table>

Data are mean ± SEM and analyzed by two-way ANOVA with Bonferroni test for age, sex and body weight. Infarct size was compared by Student’s t test. There was no statistical difference between control and hSCF/tTA mice in any of the parameters listed between the sham or MI group. Controls include WT, tTA and hSCF mice. Total number of animals includes all 5- and 30-day groups as well as DOX treatment groups.
Figure 2.2 Mouse SCF expression in the heart post-MI.

Myocardial mouse SCF protein levels at 5 days (A) and 30 days (B) post-MI were measured by western blot analysis in WT mice. Compared to sham group, mouse SCF protein levels were significantly lower at 5 but not 30 days post-MI. Human SCF mRNA expression was detected in hSCF/tTA mice at 5 days (C) and 30 days (D) post-MI by real-time RT-PCR. There was no significant change in cardiac hSCF expression in hSCF/tTA mice post-MI. Data are mean ± SEM, n=4-5 per group. One-way ANOVA followed by Bonferroni test, *P<0.05 vs Sham.
post-MI compared to sham.

2.4.3 Survival and Cardiac Function Post-MI

Post-operation survival was monitored for 30 days in WT and hSCF/tTA sham groups, and in WT and hSCF/tTA MI groups (n=13, 11, 37 and 34, respectively). Survival of sham animals was 100%. MI resulted in significantly lower survival compared to sham groups ($P<0.05$). However, the 30-day survival of hSCF/tTA MI mice was significantly higher compared to WT mice (85% vs. 54%, $P<0.05$, Figure 2.3A).

To evaluate the effect of cardiac-specific hSCF expression on cardiac function, LV hemodynamic parameters were analysed by a pressure-volume analysis system 5 and 30 days post-MI. There was no significant difference in heart rate, mean arterial pressure, or cardiac output between hSCF/tTA and littermate controls (Table 2.2 and 2.3). Five days post-MI, cardiac function of WT MI mice as determined by LV $+dP/dt$, $-dP/dt$ and preload adjusted maximum power (PAMP) was significantly lower as compared to sham groups ($P<0.05$, Figure 2.4). In the absence of DOX, which expresses hSCF, LV $+dP/dt$, $-dP/dt$ and PAMP were preserved in hSCF/tTA MI mice (hSCF/tTA MI vs. WT MI, $P<0.05$, Figure 2.4). The preservation of cardiac function was completely blocked by DOX treatment, which turned off hSCF expression (hSCF/tTA+DOX MI vs. hSCF/tTA MI, $P<0.05$, Figure 2.4). Thirty days post-surgery, LV $+dP/dt$, $-dP/dt$ and ejection fraction were significantly lower in WT, tTA and hSCF MI groups compared to WT sham ($P<0.05$, Figure 2.3B). Overexpression of hSCF preserved cardiac function (hSCF/tTA MI vs. WT, tTA and hSCF MI groups, $P<0.05$, Figure 2.3B). Furthermore, LV end diastolic volume was significantly higher in WT, tTA and hSCF MI groups compared to WT sham, but function was preserved in hSCF/tTA MI mice compared to
Figure 2.3 Survival and LV function 30 days post-MI.

A, Mortality was significantly lower in the hSCF/tTA MI group compared with WT MI (Log-rank test). B, Changes in LV dP/dt (change of pressures over time), ejection fraction (LVEF) and LV end diastolic volume 30 days post-MI. Both systolic and diastolic functions were significantly improved in hSCF/tTA MI mice, which were abrogated by DOX treatment. Data are mean ± SEM, n=5-8 per group. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs sham groups; † P<0.05 vs WT MI; ‡ P<0.05 vs hSCF/tTA MI.
Table 2.2 Hemodynamic parameters of WT and hSCF/tTA mice 5 days post-MI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>hSCF/tTA</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>362±26</td>
<td>354±20</td>
<td>0.82</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>93±6</td>
<td>90±8</td>
<td>0.88</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>23±4</td>
<td>40±3</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>SW, mW</td>
<td>171±35</td>
<td>441±111</td>
<td>0.07</td>
</tr>
<tr>
<td>CO, µL/min</td>
<td>2087±293</td>
<td>3089±628</td>
<td>0.20</td>
</tr>
<tr>
<td>PAMP, mW/mL²</td>
<td>31±9</td>
<td>75±10</td>
<td><strong>0.008</strong></td>
</tr>
</tbody>
</table>

Data are mean ± SEM and analyzed by unpaired Student’s t test. MAP, mean arterial pressure; LVEF, left ventricle ejection fraction; SW, stroke work; CO, cardiac output; PAMP, preload adjusted maximum power.
Table 2.3 Hemodynamic parameters of control and hSCF/tTA mice 30 days post-MI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>tTA</th>
<th>hSCF</th>
<th>hSCF/tTA</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>326±25</td>
<td>323±12</td>
<td>311±29</td>
<td>318±21</td>
<td>0.97</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>87±5</td>
<td>83±8</td>
<td>76±7</td>
<td>85±5</td>
<td>0.63</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>31±7</td>
<td>32±5</td>
<td>28±7</td>
<td>58±7</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>SW, mW</td>
<td>438±219</td>
<td>509±150</td>
<td>409±114</td>
<td>700±298</td>
<td>0.77</td>
</tr>
<tr>
<td>CO, µL/min</td>
<td>4052±1302</td>
<td>3895±744</td>
<td>3635±1134</td>
<td>4027±867</td>
<td>0.99</td>
</tr>
<tr>
<td>PAMP, mW/mL²</td>
<td>24±4</td>
<td>38±15</td>
<td>46±25</td>
<td>212±45</td>
<td><strong>0.0003</strong></td>
</tr>
</tbody>
</table>

Data are mean ± SEM and analyzed by one-way ANOVA followed by Bonferroni test. *P* values are comparisons between WT and hSCF/tTA mice. Abbreviations are the same as in Table 2.
Figure 2.4 Cardiac function 5 days post-MI.

hSCF/tTA MI group had preserved LV function compared to WT MI. The improvement was abrogated in hSCF/tTA+DOX group. No difference in heart rate was found between groups. Data are mean ± SEM, n=5-6 per group. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs sham groups; † P<0.05 vs WT MI; ‡ P<0.05 vs hSCF/tTA MI.
other MI groups \((P<0.05, \text{Figure 2.3B})\). However, the preserved cardiac function in hSCF/tTA mice 30 days post-MI was abrogated by DOX treatment (\text{Figure 2.3B}).

### 2.4.4 Myocardial Apoptosis 5 days Post-MI

To investigate mechanisms responsible for preserved cardiac function by cardiac-specific hSCF overexpression, myocardial apoptosis in the peri-infarct area was studied 5 days post-MI. Myocardial apoptosis was determined by both caspase-3 activity and cell death ELISA. Both caspase-3 activity and cytosolic DNA fragments were significantly higher in the WT MI group compared to the sham groups \((P<0.05)\) while these indices were significantly lower in the hSCF/tTA MI mice compared with the WT MI group \((P<0.05, \text{Figure 2.5A and 2.5B})\). The lower in apoptosis was abrogated in the hSCF/tTA+DOX MI group in which the expression of hSCF was turned off \((P<0.05, \text{Figure 2.5A and 2.5B})\).

### 2.4.5 Stem Cell Recruitment and Growth Factors Release Post-MI

Stem cell recruitment to the peri-infarct area of the myocardium was evaluated by \(c\text{-kit}\) and \(c\text{-kit}/\text{VEGFR2}\) double staining. Since VEGFR2 and \(c\text{-kit}\) are well-recognized markers for EPCs, cells positive for \(c\text{-kit}/\text{VEGFR2}\) are likely EPCs. Representative fluorescent photomicrographs at 5 and 30 days post-MI are shown in \text{Figure 2.6A and Figure 2.7A}. Quantitative analysis showed that there were significantly more \(c\text{-kit}^{+}\) and \(c\text{-kit}^{+}/\text{VEGFR2}^{+}\) cells retained in the peri-infarct area in hSCF/tTA compared to WT mice 5 days post-MI \((P<0.05, \text{Figure 2.6B})\). Treatment with DOX which turns off hSCF expression abrogated the recruitment of \(c\text{-kit}^{+}\) and \(c\text{-kit}^{+}/\text{VEGFR2}^{+}\) cells \((P<0.05, \text{Figure 2.6B})\). Interestingly, 80\% of the cells were \(c\text{-kit}^{+}/\text{VEGFR2}^{+}\), indicating that majority of
Expression of hSCF in hSCF/tTA-DOX MI mice had significantly lower apoptosis as measured by caspase-3 activity (A) and cell death ELISA (B). These effects were abrogated by turning off the expression of hSCF in hSCF/tTA+DOX MI mice. Data are mean ± SEM, n=5-8. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs Sham; † P<0.05 vs WT MI; ‡ P<0.05 vs hSCF/tTA MI.

Figure 2.5 Myocardial apoptosis in the peri-infarct area 5 days post-MI.
Figure 2.6 *c-kit*⁺ and *c-kit*⁺/VEGFR2⁺ cells retained in the peri-infarct area 5 days post-MI.

A, Representative confocal images of Hoechst (blue, nuclei), c-kit (red) and VEGFR2 (green) staining. Panel 3 in A shows a capillary positive for c-kit (*) and a capillary positive for both c-kit and VEGFR2 (**) in hSCF/tTA mice. Arrows, positive signals; arrowheads, red blood cells. B, Quantitative analysis of *c-kit*⁺ and *c-kit*⁺/VEGFR2⁺ cells in the peri-infarct area. Data are mean ± SEM, n=5-8. One-way ANOVA followed by Bonferroni test: *P<0.05 vs WT MI, † P<0.05 vs hSCF/tTA-DOX MI.
Figure 2.7 \( c-kit^+ \) and \( c-kit^+/\text{VEGFR2}^+ \) cells retained in the peri-infarct area 30 days post-MI.

A, Representative confocal images of Hoechst (blue, nuclei), \( c-kit \) (red) and VEGFR2 (green) staining; B, Quantitative analysis of \( c-kit^+ \) and \( c-kit^+/\text{VEGFR2}^+ \) cells in the peri-infarct area, Data are mean ± SEM, n=5. Unpaired Student’s t test: * P<0.05 vs WT.
A

Hoechst  c-kit  VEGFR2  Merge

WT  30D MI

hSCF/tTA  30D MI

B

[Graph showing c-kit+ cells/mm² and c-kit+/VEGFR2+ cells/mm² for WT and hSCF/tTA groups]
the stem cells were EPCs. Furthermore, capillaries in the peri-infarct area were positive for \textit{c-kit} and VEGFR2 in the hSCF/tTA mice (\textbf{Figure 2.6A}, panel 3), suggesting incorporation of EPCs into the newly formed vessels. At 30 days post-MI, myocardial stem cell density in the peri-infarct area was at similar low levels in both WT and hSCF/tTA mice (\textbf{Figure 2.7B}).

Mast cells also express \textit{c-kit}. To analyze myocardial mast cell density, heart sections were stained using toluidine blue. Consistent with previous studies, there were very few mast cells in sham or infarct myocardium in WT mice \textsuperscript{19} and no significant difference in mast cells was observed in hSCF/tTA mice in either 5 or 30 days post-MI (\textbf{Table 2.4} and \textbf{Figure 2.8}). Thus, the influence of mast cells on \textit{c-kit}\textsuperscript{+} stem cell analysis in the infarct myocardium is negligible in the present study. Previous studies have implicated growth factors including VEGF-A, IGF-1 and bFGF in cardiac repair post-MI.\textsuperscript{22} To determine expression of these growth factors, real-time RT-PCR was employed. The mRNA levels of VEGF-A, IGF-1 and bFGF in the peri-infarct area 5 days post-MI were significantly higher in hSCF/tTA compared to WT mice \textit{(P<0.05, Figure 2.9)}.

\textbf{2.4.6 LV Hypertrophy 30 days Post-MI}

The LV weight to body weight ratio, a measure of LV hypertrophy, was similar between sham groups, but was significantly higher in WT MI mice compared to WT sham \textit{(P<0.05, Figure 2.10A)}. However, the ratio in hSCF/tTA MI mice was significantly lower when compared with WT MI mice \textit{(P<0.05, Figure 2.10A)}. MI-induced LV hypertrophy was further studied at the cellular level by histological analysis. Representative photomicrographs of hematoxylin and eosin stained sections are shown in \textbf{Figure 2.10B}. Myocyte transverse diameters were assessed using the minimum cross-
Table 2.4 Number of mast cells in the left ventricular myocardium 5 and 30 days after sham or MI surgeries.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mouse genotype</th>
<th>Sub-epicardium</th>
<th>Non-infarct</th>
<th>Peri-infarct</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-day sham</td>
<td>WT</td>
<td>1.7±0.8</td>
<td>3.1±1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>hSCF/tTA</td>
<td>2±1.1</td>
<td>2.6±1.2</td>
<td>-</td>
</tr>
<tr>
<td>5-day MI</td>
<td>WT</td>
<td>2.3±1.0</td>
<td>0.4±0.2</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td></td>
<td>hSCF/tTA</td>
<td>0.7±0.4</td>
<td>0.2±0.2</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>30-day sham</td>
<td>WT</td>
<td>1.4±0.4</td>
<td>1.9±0.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>hSCF/tTA</td>
<td>0.9±0.9</td>
<td>1.0±0.7</td>
<td>-</td>
</tr>
<tr>
<td>30-day MI</td>
<td>WT</td>
<td>2.3±0.9</td>
<td>1.1±0.8</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td></td>
<td>hSCF/tTA</td>
<td>2.3±0.1</td>
<td>0.0±0.0</td>
<td>0.7±0.5</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of mast cells per heart section from 4 animals per group. Two-way ANOVA followed by Bonferroni test. There was no statistical difference between any groups.
Figure 2.8 Mast cell staining using toluidine blue in heart tissue sections from WT and hSCF/tTA mice.

Mice were subjected to sham or myocardial infarction (MI) for 5 or 30 days. Arrows indicate mast cells. Infarct area is labeled.
Figure 2.9 Growth factor expression in the peri-infarct area 5 days post-MI.

Myocardial VEGF-A, IGF-1 and bFGF mRNA were determined by real-time RT-PCR 5 days post-MI. Data are mean ± SEM, n=6. Unpaired Student’s t test: * $P<0.05$ vs WT.
Figure 2.10 LV hypertrophy 30 days post-MI or sham operations.

A, LV to body weight ratios. B, Representative photomicrographs of hematoxylin and eosin stained sections showing the cross-sections of cardiomyocytes. C, Quantitative analysis of myocyte diameters in WT and hSCF/tTA mice in the presence or absence of DOX. Data are mean ± SEM, n=5-11; Two-way ANOVA followed by Bonferroni test: *P<0.05 vs sham groups; † P<0.05 vs WT MI.
sectional diameter at the nuclear level to reduce the effects of myocyte orientation on cell size measurements. However, the minimum cross-sectional diameter was similar between sham groups but was significantly higher in the WT MI compared to WT sham (P<0.05, Figure 2.10C). However, the hSCF/tTA MI mice showed significantly smaller myocyte cross-sectional diameters compared to those of WT MI mice (P<0.05, Figure 2.10C). Furthermore, these effects were abrogated when hSCF expression was turned off by DOX treatment (Figure 2.10A and 2.10C).

2.4.7  Capillary Density post-MI

To quantify myocardial capillary density, lectin-I was employed to specifically stain endothelial cells. Representative photomicrographs of lectin-I staining are presented in Figure 2.11A (5 days post-MI) and Figure 2.12A (30 days post-MI). In mice 5 days post-surgery, myocardial capillary density was similar between WT and hSCF/tTA sham mice (Figure 2.11B). The capillary density in the peri-infarct area of the MI group was significantly lower compared to the sham groups (P<0.05, Figure 2.11B). When hSCF expression was activated in the hSCF/tTA mice in absence of DOX, capillary density was significantly higher (hSCF/tTA MI vs. WT MI, P<0.05, Figure 2.11B). However, this was abrogated by DOX treatment in hSCF/tTA mice (hSCF/tTA+DOX MI vs. hSCF/tTA MI, P<0.05, Figure 2.11B). Capillary density was also assessed 30 days post-surgery. Myocardial capillary density was similar between WT and hSCF/tTA sham mice (Figure 2.12B). Capillary density in the peri-infarct area of the MI groups was significantly lower compared to the respective sham groups (P<0.05, Figure 2.12B). Overexpression of hSCF had significantly higher capillary density (hSCF/tTA MI vs. WT MI, P<0.05,
Figure 2.11 Myocardial capillary density 5 days post-MI.

Capillaries were identified by endothelial specific lectin-I staining in WT, hSCF/tTA and hSCF/tTA+DOX mice. A, Representative photomicrographs of lectin-I stained sections from each experiment group, showing individual capillaries (brown staining). Nuclei were stained by hematoxylin. B, Quantitative analysis of capillary density. Data are mean ± SEM, n=5-6 per group. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs sham; † P<0.05 vs WT MI; ‡ P<0.05 vs hSCF/tTA MI.
Figure 2.12 Myocardial capillary density 30 days post-MI.

Capillaries were identified by endothelial specific lectin-I staining in WT, hSCF/tTA and hSCF/tTA+DOX mice. A, Representative photomicrographs of lectin-I stained sections from each experiment group, showing individual capillaries (brown staining). Nuclei were stained by hematoxylin. B, Quantitative analysis of capillary density. Data are mean ± SEM, n=5-6 per group. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs sham; † P<0.05 vs WT MI; ‡ P<0.05 vs hSCF/tTA MI.
Figure 2.12B). DOX treatment abrogated the higher capillary density in hSCF/tTA mice (Figure 2.12B).

2.4.8 In Vivo Myocardial Angiogenesis

The ability of the hSCF expressed in the cardiomyocytes to promote angiogenesis was investigated in vivo by implanting matrigel into the LV myocardium for 3 days. As shown in Figure 2.13A, the matrigel was surrounded by inflammatory cells and could be easily identified in the myocardium. The newly formed vessels that penetrated into the gel plug showed aneurysm-like structures inside the gel. The area of capillaries and aneurysm-like structures penetrating the matrigel plug was quantified in relation to the total matrigel area. The percentage of vessel-like areas was significantly higher in hSCF/tTA compared to those of WT mice (P<0.05, Figure 2.13B).

2.5 DISCUSSION

The present study demonstrates for the first time that cardiomyocyte-specific expression of membrane-associated hSCF preserves survival and cardiac function post-MI. The beneficial effects of transgene expression are associated with lower myocardial apoptosis, higher recruitment of stem cells to the infarcted myocardium, enhanced myocardial neovascularization and lower ventricular remodeling. The preserved cardiac function and myocardial angiogenesis are a result of hSCF overexpression since treatment with DOX, which turns off hSCF expression, abrogates these effects post-MI. Our study suggests that hSCF may have therapeutic potential in the treatment of heart failure post-MI.
Figure 2.13 Myocardial angiogenesis WT and hSCF/tTA mice.

Matrigel (M) was implanted into the LV myocardium for 3 days. A. Representative images of H/E stained heart sections. The lumens and the aneurysm-like structures are the newly formed vessels, which have grown into the matrigel. Arrows indicate epicardium. B. Quantitative analysis of angiogenesis. The area of capillary-like structures in relation to the total matrigel area was quantified and expressed as percent of vessel-like area. Data are mean ± SEM from 6 mice per group. Unpaired Student’s t test: *P<0.05 vs WT group.
c-kit signaling can promote cardiac repair post-MI. Administration of c-kit+ stem cells, whether using intravenous injection or local delivery into the infarcted myocardium post-MI, improves cardiac function, enhances angiogenesis and impairs cardiac remodeling.\textsuperscript{23,24} On the other hand, c-kit deficient (W/W\textsuperscript{v}) mice exhibit dilated cardiomyopathy post-MI. After replacement with normal WT bone marrow, the cardiomyopathic phenotype of W/W\textsuperscript{v} mice is rescued.\textsuperscript{25,26} However, the W/W\textsuperscript{v} mice have defects in hematopoietic stem cell function, abnormal islet beta-cell development and impaired glucose tolerance.\textsuperscript{27,28} These abnormalities also likely contribute to the impaired cardiac repair post-MI. As the ligand of c-kit, soluble SCF is increased in the bone marrow after myocardial ischemia-reperfusion injury, leading to EPC mobilization and improvement in myocardial neovascularization and cardiac function.\textsuperscript{29} Furthermore, peri-infarct injections of soluble SCF increased c-kit+ stem cell recruitment to the infarcted heart following intravenous administration of bone marrow derived stem cells.\textsuperscript{30} Although this effect lasted for 72 hours, there was no functional benefit observed.

Following MI, circulating c-kit+ bone marrow stem cells and EPCs are increased.\textsuperscript{31-33} However, stem cell attractants SDF-1 and SCF as well as pro-angiogenic genes such as VEGF-A and VEGFR2 are decreased in the infarct region post-MI.\textsuperscript{8} The stem/progenitor cells mobilized into the peripheral blood may not be recruited in sufficient numbers or retained in the infarcted myocardium to participate in cardiac repair because of decreased myocardial expression of stem cell attractants such as SCF.\textsuperscript{8}

In order to increase the recruitment and retention of stem/progenitor cells in the infarcted myocardium and to improve cardiac repair post-MI, a cardiomyocyte-specific membrane associated hSCF overexpressing mouse was generated in the present study.
Our model differs from the model reported by Kapur et al. in which overexpression of hSCF under the control of human phosphoglycerate kinase (hPGK) promoter inhibited normal SCF function in mice. These mice displayed forehead blaze with white spots over the cervical region and the belly as a result of melanocyte deficiency, and their endogenous SCF expression was diminished. The major difference between this mouse model and our transgenic mice is that their hPGK promoter directed hSCF expression in many organs including the skin, thymus, spleen, brain, bone marrow, and testes during embryonic development while our mouse model is cardiac specific and hSCF was expressed only in the heart postnatally. Furthermore, our mice did not display any coat color deficiencies, and importantly, endogenous mouse SCF expression was not altered. Thus, we did not see any functional antagonism of hSCF in our model.

Our data suggest that cardiac specific overexpression of hSCF may promote retention of \textit{c-kit}^+ cells including EPCs in the infarcted myocardium, which remains to be verified in future studies. Importantly, these cells participate in the angiogenic process as they were incorporated into the newly formed vessels in the infarcted myocardium. In addition, \textit{c-kit} signaling can increase cell survival and stem cell function. Consistent with this hypothesis, myocardial capillary density was higher in the hSCF overexpressing mice post-MI. However, the relative contribution of enhanced cell survival versus angiogenesis to the effect of hSCF overexpression leading to higher capillary density is not clear. Furthermore, although the absolute number of stem cells retained in the infarcted myocardium is only doubled with hSCF overexpression, it is possible that the preserved survival and functional capacity associated with these stem cells could result in the increased release of soluble factors, causing beneficial paracrine actions on the
myocardium. In support of this paracrine hypothesis, expression of several candidate mediators such as VEGF-A, bFGF, and IGF-1 was higher in the myocardium overexpressing membrane associated hSCF post-MI. Indeed, hSCF overexpression resulted in lower myocardial apoptosis and cardiac hypertrophy, leading to preserved cardiac function and survival post-MI.

The effects of SCF on cardiac function have also been studied using cardiac transplantation of MSC transfected with a full length mouse SCF cDNA plasmid expressing both S- and M-SCF. Transplantation of MSCs containing SCF plasmids enhanced EPC recruitment and myocardial angiogenesis post-MI for about 2 weeks. However, there was no change in animal survival among all treatment groups. Furthermore, cardiac function determined at 4 weeks post-MI was not significantly improved by SCF transfection compared to the MSC group. This is not surprising as more than 95% of the transplanted MSCs are cleared within 7 days following transplantation into the myocardium. In the present study, in order to study the long-term effects of SCF on the infarcted myocardium, we chose to express the membrane-associated isoform of hSCF specifically in the heart. Thus hSCF expression was stable and sustained over the entire study period. hSCF expressed on the membrane of cardiomyocytes serves as a chemoattractant for circulating stem cells to migrate to, and stay in the myocardium. Furthermore, Tet-off system was employed, allowing for reversible cardiac expression of hSCF. Treatment with doxycycline turned off hSCF expression and reversed the beneficial effects of hSCF in mice with MI. Our data showed that cardiomyocyte specific hSCF overexpression resulted in angiogenesis, higher capillary density, less myocardial apoptosis, and less LV hypertrophy post-MI. These
beneficial effects led to significantly preserved in cardiac function and survival in hSCF/tTA mice post-MI, and are likely due to the enhanced recruitment and retention of EPCs to the infarct myocardium and thus improved cardiac repair. Cardiac regeneration via transdifferentiation from bone marrow stem cells *in vivo* post-MI is still debatable.\textsuperscript{23} Whether hSCF overexpression promotes myocardial regeneration from resident cardiac stem cells requires further investigation.
2.6 REFERENCES


22. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 2006;20:661-669.


CHAPTER 3

Cardiomyocyte-Specific Overexpression of Human Stem Cell Factor Protects against Myocardial Ischemia and Reperfusion Injury

A version of this chapter was accepted by *International Journal of Cardiology*, 2013:

**Xiang F**, Lu X, Liu Y, Feng Q

“Cardiomyocyte-Specific Overexpression of Human Stem Cell Factor Protects against Myocardial Ischemia and Reperfusion Injury.”
3.1 CHAPTER SUMMARY

In Chapter 2, I demonstrated that cardiomyocyte-specific overexpression of hSCF preserves cardiac function and animal survival post-MI via enhanced retention and differentiation of EPCs. However, whether hSCF overexpression protects the heart from acute cardiac injury is not known. In this chapter, I investigated the effects of cardiomyocyte-specific overexpression of hSCF on acute myocardial I/R (45 min/3 h) injury and related signaling mechanisms. I found that infarct size and myocardial apoptosis after I/R were diminished by hSCF overexpression. These cardioprotective effects were related to increased PI3K/Akt/eNOS signaling pathways associated with increased myocardial insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) expression. Furthermore, enhanced retention of circulating $c$-$kit^+$ cells and activation of resident cardiac stem cells (CSCs) contribute to the beneficial effects observed in hSCF overexpressing mice. Thus, cardiomyocyte-specific overexpression of hSCF protects the heart from acute I/R injury. The cardioprotective effects of hSCF overexpression are mediated by increased $c$-$kit^+$ progenitors, enhanced growth factor expression and activation of the PI3K/Akt signaling pathway.
3.2 INTRODUCTION

IHD is the leading cause of death worldwide. Following acute MI, re-establishing coronary blood flow by thrombolysis or angioplasty is used to rescue the ischemic myocardium. However, reperfusion can also induce myocyte death by activation of deleterious signalling cascades. Therapeutic strategies for cardiac protection from I/R injury are needed. Studies on pre- and post-conditioning have shown that I/R injury initiates several pro-survival kinase cascades including the PI3K/Akt signaling pathway. A constitutively active Akt mutant (myr-Akt) has been shown to decrease myocardial apoptosis, infarct size and preserve cardiac function in rats after I/R. Akt activation leads to phosphorylation of multiple protein targets, including eNOS, which promote cell survival. Moreover, the beneficial effects of growth factors, including VEGF, IGF-1 and HGF, and pharmacological treatments such as statins and EPO on ischemic injury mainly result from Akt activation.

SCF is a glycosylated trans-membrane protein widely expressed by many cells and tissues including stromal cells, fibroblasts, endothelium, and myocardium. It plays a critical role in the survival, proliferation, mobilization, and adhesion of all c-kit expressing cells including hematopoietic stem cells, EPCs, and CSCs. Alternative splicing of SCF leads to 2 isoforms: soluble (S-SCF) and membrane-associated (M-SCF) isoforms. M-SCF, the dominant isoform in vivo, is able to induce more persistent receptor activation and is more effective at promoting long-term support of target cell survival. Binding of c-kit with SCF through autocrine or paracrine actions leads to oligomerization and auto-phosphorylation of the receptor which activates multiple downstream signalling pathways including PI3K/Akt. The PI3K/Akt pathway activated by SCF is responsible
for c-kit+ cell proliferation, differentiation, adhesion, secretion, survival, and actin cytoskeletal reorganization.\textsuperscript{11,16,17}

The previous chapter has demonstrated that cardiomyocyte-specific overexpression of hSCF improves cardiac function and animal survival post-MI in mice. The beneficial effects are due to increased c-kit+ cell recruitment, enhanced growth factor expression, decreased myocardial apoptosis, increased angiogenesis and reduced cardiac remodeling. However, whether hSCF overexpression protects the heart from acute I/R injury remains to be determined. This is an important question as limiting myocardial ischemic injury is critical to preserve functional myocardium and prevent subsequent progression to heart failure. In this chapter, we hypothesized that inducible cardiomyocyte-specific overexpression of hSCF protects the heart from I/R injury. To test this hypothesis, a transgenic mouse that overexpresses hSCF in cardiomyocytes under the control of a Tet-off system was employed.\textsuperscript{18} We demonstrated that cardiomyocyte-specific hSCF overexpression protects the heart from I/R injury via increased c-kit+ cell activation, enhanced growth factor expression and Akt activation.

3.3 METHODS

3.3.1 Animals

Animals in this study were handled in accordance with the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and animal handling was approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. The conditional cardiac-specific hSCF overexpressing hSCF/tTA transgenic mice were generated using an \(\alpha\)-MHC promoter as described in \textbf{Chapter 2}. The first generation of hSCF/tTA mice
was from a C57BL6/CBA background and backcrossed to C57BL/6 for 16 generations prior to the present study. In total, 87 adult male hSCF/tTA and 95 WT mice (3-4 months old) were used.

### 3.3.2 Myocardial I/R

Induction of myocardial I/R was performed as previously described. Mice were anesthetized with an intraperitoneal (IP) injection of a mixture of ketamine (50 mg/kg) and xylazine (12.5 mg/kg) and then intubated and artificially ventilated with a respirator (SAR-830, CWE, Ardmore, PA., USA). The heart was exposed by a left intercostal thoracotomy. The pericardium was opened, and the left main coronary artery was occluded for 45 minutes by positioning a suture (8-0) around it secured with PE-10 tubing. The tubing was then removed and the suture was loosened to allow reperfusion for 3 hours. The lungs were hyperinflated using positive end-expiratory pressures (3 cm H$_2$O), and the thorax was closed. To inhibit Akt signaling, animals were treated with a PI3 kinase inhibitor LY294002 (Sigma, 7.5 mg/kg body weight, IP) 15 minutes before reperfusion. To inhibit c-kit signalling, mice were treated with a neutralizing antibody ACK2 (100 µg, IP, eBioscience, San Diego, CA, USA) 15 minutes before ischemia. To inhibit HGF receptor (c-Met) signaling, mice were treated with a c-Met inhibitor Crizotinib (15 mg/kg body weight, IP, Selleckchem, Houston, TX, USA) 15 minutes before ischemia. To turn off hSCF overexpression, the hSCF/tTA mice were treated daily with DOX (0.2 mg/ml in drinking water) starting 2 weeks before surgeries.

### 3.3.3 Measurement of Infarct Size

Infarct size was calculated by infarct area to ischemic area ratio normalized by tissue weight. Briefly, the coronary artery was re-ligated after 3 hour reperfusion. Evans
blue dye solution (1% in PBS) was perfused through the coronary arteries via the cannulated aorta to distinguish ischemic and non-ischemic areas of the heart. Hearts were cut into four transverse slices from the apex to the base and incubated with 1.5% triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO, USA) in PBS for 30 min at room temperature. Sections were weighed and photographed. The non-ischemic area, area at risk, and infarct area were measured using SigmaScan Pro.

### 3.3.4 Determination of Myocardial Apoptosis

Caspase-3 activity and cytoplasmic histone-associated DNA fragments were measured using a caspase-3 cellular activity assay kit (BIOMOL, Plymouth Meeting, PA) and cell death detection ELISA (Roche, Mississauga, ON), respectively. Briefly, heart tissues from the ischemic area was removed and homogenized. After a 5-minute centrifugation at 12,000 x g at 4°C, supernatants were collected and protein concentrations were measured. To assess caspase-3 activity, supernatants from each heart containing 200 µg of protein were loaded into a 96-well plate and incubated at 37°C for 16 hours in the presence of the caspase-3 substrate, Ac-DEVD-AMC (20 µM), or Ac-DEVD-AMC, plus the inhibitor, Ac-DEVD-CHO (4 µM). Fluorescence intensity (excitation at 360 nm and emission at 460 nm) was measured using a Spectra-Max M5 micro-plate reader (Molecular Devices, Sunnyvale, CA, USA). Data were expressed as amount of AMC substrate cleaved per 100 µg of protein. To assess cytoplasmic histone-associated DNA fragments, supernatants containing 20 µg of protein were incubated in an anti-histone antibody coated 96-well plates for 90 minutes. The sample solutions were removed and horseradish peroxidise-conjugated anti-DNA antibody was added and incubated for 90 minutes. The color was developed by adding peroxidase substrate
(ABTS) and detected using a Spectra-Max micro-plate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 405 nm.

### 3.3.5 Western Blot Analysis

Myocardial HGF and IGF-1 expression, and phosphorylated/total Akt and eNOS in peri-infarct area of the myocardium were measured by western blot analysis. Briefly, 40-60 µg of protein were separated by 10-15% SDS-PAGE gel and transferred to nitrocellulose membranes, and blots were probed with antibodies against Akt (1:1000, ABM, Vancouver, BC, Canada), phosphorylated Akt (Ser473, 1:1000, ABM, Vancouver, BC, Canada), eNOS (1:500, Cell Signaling, Danvers, MA, USA), phosphorylated eNOS (1:500, Cell Signaling, Danvers, MA, USA), α-actinin (1:2500, Sigma, Oakville, ON, Canada), HGF (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IGF-1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or GAPDH (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then washed and probed with horseradish peroxidase conjugated secondary antibodies (1:3000, Bio-Rad, Hercules, CA, USA), and detected by using an ECL detection method.

### 3.3.6 Growth Factor mRNA Expression

Total mRNA was isolated from LV heart tissue with TRIzol reagent (Invitrogen, Grand Island, NY, USA). cDNA was synthesized using MMLV reverse transcriptase and random primers. Real-time PCR was conducted using SYBR Green PCR Master Mix (Eurogentec, San Diego, CA, USA). 28S rRNA was used as a loading control. The oligonucleotide primer sequences were as follows: IGF-1, forward, CTG CTT GCT CAC CTT CAC CA, reverse, ATG CTG GAG CCA TAG CCT GT. HGF, forward, GTC AGC ACC ATC AAG GCA AG, reverse, TCC ACGACC AGG AAC AAT GA.
Samples were amplified for 35 cycles using an Eppendorf Mastercycler Real-Time PCR machine (Eppendorf, Hamburg, Germany). The mRNA Levels were determined by the relative expression compared with that of 28S rRNA.

### 3.3.7 Immunofluorescent Staining for Stem Cells

Three hours after I/R, the heart was perfused with 10% potassium chloride to induce diastolic cardiac arrest followed by saline and 4% paraformaldehyde perfusion for 15 minutes. After a 1 hour incubation in 30% sucrose, hearts were embedded in OCT and cut into 5 µm sagittal sections. The sections were stained with primary antibodies (c-kit, 1:200, eBioscience, San Diego, CA, USA; Ki67, 1:200, NeoMarkers, Fremont, CA, USA) at 4°C for overnight followed by fluorescent secondary antibody at room temperature for 1 hour. CD45 was stained by FITC-conjugated anti-CD45 (1:100, eBioscience, San Diego, CA, USA) at 4°C for overnight. Nuclei were stained with Hoechst 33342. The number of c-kit⁺, Ki67⁺, CD45⁺/c-kit⁺ and CD45⁻/c-kit⁺ cells per sagittal heart section was quantified in a blind manner using a fluorescent microscope (Observer D1, Carl Zeiss Canada, Toronto, Canada). Images were taken using a laser confocal microscope (LSM 510 Meta, Carl Zeiss Canada, Toronto, Canada).

### 3.3.8 Isolation and Culture of c-kit⁺ CSCs

$c$-$kit^{+}$ CSCs were isolated using a previous protocol \cite{24,25} with modifications. Briefly, hearts from WT mice were isolated and perfused with cold PBS. LV was minced into small pieces and mild digested with 0.05% Trypsin-EDTA at 37°C for 15 minutes. LV pieces were then transferred to fibronectin coated dish and incubated in complete explant medium (IMDM, 15% fetal bovine serum, 1% penicillin–streptomycin, 1% L-glutamine, 0.1 mM 2-mercaptoethanol) for 2-3 weeks. $C$-$kit^{+}$ CSCs were isolated by $c$-$kit$
antibody (Santa Cruz, CA, USA) conjugated magnetic beads (Invitrogen, CA, USA) from cells mild digested (0.05% Trypsin-EDTA, room temperature, <3 minutes) from the incubated LV tissues and then incubated in CSC culture medium (DMEM/F-12, 3.5% FBS, 1% penicillin–streptomycin, 1% L-glutamine, 0.1 mM 2-mercaptoethanol, 80 ng/mL bFGF and 25 ng/mL EGF) for further experiments. To investigate the effects of hSCF overexpression on growth factor expression, c-kit+ CSCs were seeded at density of 2x10^4/well of 24-well plates and treated with adenoviral membrane-associate hSCF or LacZ constructs for 24 hours. ACK2 (20 µg/mL) was used to block c-kit signaling.

### 3.3.9 Flow Cytometry

The expression of CD45 in isolated CSCs was analyzed using a FACSCalibur (BD Biosciences, Mississauga, ON, Canada). After c-kit+ CSCs were pulled-down by c-kit antibody (Santa Cruz, CA, USA) conjugated magnetic beads, the c-kit+ and c-kit− cells were stained with fluorescein isothiocyanate-conjugated monoclonal antibody against mouse CD45 (eBIOSCIENCE, San Diego, CA, USA). The percentage of CD45+ cells in c-kit+ and c-kit− cells was determined.

### 3.3.10 Toluidine Blue Staining for Mast Cells

To quantify mast cells in the heart, cardiac tissue sections were stained with 0.05% toluidine blue (Sigma, St. Louis, MO, USA) in 1% sodium chloride for 2 minutes. The granules of mast cells show violet as we described in previous chapter.

### 3.3.11 Statistical Analysis

Data are expressed as the mean ± SEM. Two-way ANOVA followed by Bonferroni test was used for multiple group comparisons. Unpaired Student t-test was
used for two group comparisons. Differences were considered statistically significant at the level of $P<0.05$.

3.4 Results

3.4.1 Overexpression of hSCF Reduces Infarct Size and Myocardial Apoptosis after I/R

Infarct sizes were measured in WT and hSCF/tTA mice. Representative images of heart sections following Evans blue and TTC staining are shown in Figure 3.1A. Forty-five minutes of ischemia followed by 3 hours of reperfusion resulted in an infarct size of 54% in WT mice. However, the infarct size of hSCF/tTA mice was significantly smaller (39%) compared to WT ($P<0.05$, Figure 3.1B). Accordingly, myocardial apoptosis measured by caspase-3 activity and cell death ELISA in the infarct border zone was significantly lower in hSCF/tTA mice compared to WT ($P<0.05$, Figure 3.2). To further verify that the protective effects were due to hSCF overexpression, hSCF/tTA mice were treated with DOX for 2 weeks to turn off the cardiac hSCF expression and then subjected to I/R. DOX treatment led to an infarct size of 52% in hSCF/tTA mice (Figure 3.1B). Myocardial apoptosis in DOX-treated hSCF/tTA mice was also increased to similar levels as the WT groups (Figure 3.2).

3.4.2 Akt Signalling Contributes to hSCF Overexpression-induced Cardiac Protection after I/R

To evaluate the molecular mechanism responsible for the effects of cardiac-specific hSCF overexpression, the PI3K/Akt signaling pathway was studied. Significantly
Figure 3.1 Effects of hSCF overexpression on infarct size after I/R.

Myocardial ischemia was induced by occlusion of the coronary artery for 45 minutes followed by 3 hours of reperfusion. Overexpression of hSCF significantly reduced infarct size in hSCF/tTA compared to WT mice. Turning off the hSCF expression by DOX treatment or inhibition of PI3 kinase by LY294002 abrogated the infarct size decrease in hSCF/tTA mice. A: Representative TTC stained heart sections after I/R from each corresponding group. B: Infarct size expressed as percent of the weight of the infarct to the area at risk. C: Area at risk after I/R. Data are mean ± SEM, n=5-7 per group. Two-way ANOVA followed by Bonferroni test: *$P<0.05$ vs I/R; † $P<0.05$ vs WT.
**Figure 3.2 Myocardial apoptosis in peri-infarct area after I/R.**

Overexpression of hSCF in hSCF/tTA mice showed significantly less apoptosis as measured by caspase-3 activity (A) and cell death ELISA (B). These effects were abrogated by turning off the expression of hSCF by DOX treatment or blocking PI3K by LY294002 treatment in hSCF/tTA mice. Data are mean ± SEM, n=5. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs I/R; †P<0.05 vs WT.
A

Caspase-3 Activity Cleaved AMC (pmol/100μg protein)

WT
hSCF/tTA

I/R I/R+DOX I/R+LY294002

B

Cytosolic DNA Fragments (A405nm)

I/R I/R+DOX I/R+LY294002

† *
higher levels of Akt phosphorylation was observed in hSCF/tTA mice after I/R compared to WT (P<0.05, Figure 3.3A), which was abrogated by DOX treatment. Moreover, PI3K inhibitor LY294002 abrogated the difference of Akt phosphorylation between hSCF/tTA and WT mice after I/R. Interestingly, LY294002 treatment at the dose of 7.5mg/kg induced significant impairment of Akt phosphorylation (P<0.05, Figure 3.3B) and resulted in significantly higher levels of myocardial apoptosis (Figure 3.2) and larger infarct size (Figure 3.1B), suggesting an essential role of PI3K/Akt signaling in cell survival after I/R. As one of the important downstream signaling molecules of Akt signaling, eNOS activation was also measured. Similarly, higher levels of eNOS phosphorylation were found in hSCF/tTA mice after I/R compared to WT (Figure 3.4).

### 3.4.3 Overexpression of hSCF Increases c-kit⁺ Cells in the Myocardium after I/R

*c-kit* is the SCF receptor and a surface marker for progenitor/stem cells. Accordingly, *c-kit* immuno-fluorescent staining was performed to analyze the number of *c-kit⁺* cells in the heart. A representative image of *c-kit⁺* cells in the myocardium is shown in Figure 3.5A. The 2 *c-kit⁺* cells with large circular nuclei and a thin rim of cytoplasm are located in the interstitial space of the myocardium. In WT sham hearts, the number of *c-kit⁺* cells was about 10 cells per heart section while the number was doubled in hSCF/tTA sham hearts (P<0.05, Figure 3.5B). After I/R injury, the number of *c-kit⁺* cells was significantly higher in both WT and hSCF/tTA mice while there were significantly more *c-kit⁺* cells in hSCF/tTA compared to WT hearts (P<0.05, Figure 3.5B). Consistent with these findings, *c-kit* mRNA levels were significantly upregulated in both WT and
Figure 3.3 Myocardial Akt phosphorylation in WT and hSCF/tTA mice after I/R.

A: Akt phosphorylation was significantly increased in hSCF/tTA compared to WT mice. DOX treatment abolished this increase in hSCF/tTA mice. B: Inhibition of PI3K significantly lowered Akt phosphorylation both in WT and hSCF/tTA mice. Data are mean ± SEM, n=6 per group. Two-way ANOVA followed by Bonferroni test: *$P<0.05$ vs I/R groups; †$P<0.01$ vs WT.
Figure 3.4 Myocardial eNOS phosphorylation in WT and hSCF/tTA mice after I/R.

A: eNOS phosphorylation was significantly higher in the hSCF/tTA mice compared to WT. DOX treatment abolished this increase in hSCF/tTA mice. B: Inhibition of PI3K with LY294002 significantly impaired eNOS phosphorylation both in WT and hSCF/tTA mice. Data are mean ± SEM, n=6 per group. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs I/R groups; † P<0.01 vs WT.
Figure 3.5 c-kit⁺ cells in WT and hSCF/tTA hearts.

A: A representative image of c-kit⁺ cells in the myocardium. Hoechst (blue, nuclei), c-kit (red) and α-actinin (green). B: Quantitative analysis of c-kit⁺ cells in heart sections. The population of c-kit⁺ cells was significantly higher in both WT and hSCF/tTA hearts after I/R. However, there were significantly more c-kit⁺ cells in hSCF/tTA compared to WT mice after I/R, which was abrogated by blocking c-kit signalling using ACK2. C: Myocardial c-kit mRNA expression was significantly higher in hSCF/tTA compared to WT mice after I/R. Blockade of c-kit signalling by ACK2 abrogated this effect. D: Quantitative analysis of Ki67⁺ cells in the heart of WT and hSCF/tTA mice showed no difference between the two groups (unpaired Student t-test, P=n.s). E: Blockade of c-kit signalling by ACK2 or Crizotinib treatment abolished the reduction of infarct size in hSCF/tTA mice after I/R. Note: The same I/R groups from Figure 3.1B were used in this graph. Data are mean ± SEM, n=5-7. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs Sham, † P<0.05 vs WT.
hSCF/tTA mice after I/R. Furthermore, myocardial c-kit mRNA levels were significantly higher in hSCF/tTA compared to WT mice after I/R (**Figure 3.5C**). However, cell proliferation in the myocardium after I/R assessed by Ki67 staining did not show any significant difference between WT and hSCF/tTA mice (**Figure 3.5D**). Finally, c-kit neutralizing antibody ACK2 was used to further investigate the role of c-kit signalling in the cardioprotection of hSCF overexpression after I/R. Inhibition of c-kit function by ACK2 not only abrogated increases in the number of c-kit+ cells in the heart (**Figure 3.5B**) but also abolished the reduction of infarct size in hSCF/tTA mice after I/R ($P<0.05$, **Figure 3.5E**).

### 3.4.4 Overexpression of hSCF Enhances Protective Growth Factor Expression after I/R

Previous studies have implicated growth factors including HGF and IGF-1 in promoting cardiomyocyte survival after I/R.\(^7,26\) Production of these growth factors by stem cells supports the paracrine effects of stem cell therapy in ischemic heart disease.\(^27,28\) To determine the expression levels of these growth factors, real-time RT-PCR and western blot analysis was employed. HGF and IGF-1 mRNA and protein expression was significantly increased after I/R in both WT and hSCF/tTA mice ($P<0.05$, **Figure 3.6**). However, significantly higher HGF and IGF-1 expression were observed in hSCF/tTA compared to WT hearts ($P<0.05$, **Figure 3.6**). Blocking c-kit signaling by ACK2 abolished the enhanced HGF and IGF-1 expression in the hSCF/tTA heart after I/R ($P<0.05$, **Figure 3.6**). To further demonstrate the role of HGF in cardioprotection by hSCF overexpression, an inhibitor of HGF receptor crizotinib was used. Treatment with
Figure 3.6 Cardioprotective cytokine expression in WT and hSCF/tTA mice after I/R.

Expression of HGF and IGF-1 mRNA was detected by real-time RT-PCR. After I/R, HGF and IGF-1 expression was significantly upregulated both in WT and hSCF/tTA mice. However, a significantly higher level of HGF and IGF-1 expression was found in hSCF/tTA mice compared to WT. This increase was abrogated by ACK2 treatment. Data are mean ± SEM, n=6; Two-way ANOVA followed by Bonferroni test: *P<0.05 vs Sham; † P<0.05 vs WT.
crizotinib (15 mg/kg, IP) abolished the reduction in infarct size after I/R in hSCF/tTA mice with no significant effect in WT mice (Figure 3.5E).

3.4.5 Sub-population of c-kit$^+$ Cells in Hearts with hSCF Overexpression after I/R

c-kit is a reliable marker for cardiac progenitors and circulating stem cells.$^{13,29}$ Moreover, hematopoietic mast cells (CD45$^+$) are also positive for c-kit. In order to further investigate the source of increased c-kit$^+$ cells in hSCF/tTA hearts after I/R, CD45 was used to distinguish the c-kit$^+$ cell sub-populations. Representative images of CD45$^+$c-kit$^+$ and CD45$^-$c-kit$^+$ cells are shown in Figure 3.7A. CD45$^+$c-kit$^+$ cells are identified as cardiac stem cells capable of cardiogenesis.$^{12,13}$ Our data show that in sham and I/R groups, the numbers of CD45$^-$c-kit$^+$ cells in hSCF/tTA myocardium were significantly higher compared to WT ($P<0.05$, Figure 3.7B). Moreover, an endogenous increase in CSCs after I/R was observed in the peri-and non-infarct area of WT myocardium ($P<0.05$, Figure 3.7C), which was further enhanced in hSCF/tTA mice ($P<0.05$, Figure 3.7C). On the other hand, a significantly higher populations of CD45$^+$c-kit$^+$ cells were found in both hSCF/tTA and WT hearts after I/R compared to sham, while there were more CD45$^+$ c-kit$^+$ cells retained in the hSCF/tTA compared to WT myocardium ($P<0.05$, Figure 3.7D). Interestingly, the number of mast cells determined by toluidine blue staining after I/R was similar between WT and hSCF/tTA hearts, suggesting that mast cells do not contribute to the increases of c-kit$^+$ cell population induced by hSCF overexpression (Figure 3.7E).
**Figure 3.7 Sub-population of \( c\text{-kit}^+ \) cells in the myocardium after I/R.**

A: A representative image of CD45\(^+\)\( c\text{-kit}^+ \) cells and CD45\(^-\)\( c\text{-kit}^+ \) cells in the myocardium. Hoechst (blue, nuclei), \( c\text{-kit} \) (red) and CD45 (green). B: Quantitative analysis shows there were significantly more CD45\(^-\)\( c\text{-kit}^+ \) cells in hSCF/tTA compared to WT hearts in sham and I/R groups. C: In peri-and non-infarct area, more CD45\(^-\)\( c\text{-kit}^+ \) cells were observed in both WT and hSCF/tTA mice after I/R compared to sham groups while the population of CD45\(^-\)\( c\text{-kit}^+ \) cells was higher in hSCF/tTA mice compared to WT after I/R. D: CD45\(^+\)\( c\text{-kit}^+ \) cell number was significantly higher in hSCF/tTA mice compared to WT after I/R. E: Quantification of mast cells detected by toluidine blue showed no significant difference between WT and hSCF/tTA mice. Two-way ANOVA followed by Bonferroni test: \(* P < 0.05\) vs Sham; \(† P < 0.05\) vs WT.
3.4.6 Overexpression of hSCF enhances CSC proliferation and HGF expression in vitro

In order to further investigate the effects of hSCF overexpression on CSC activity, c-kit+ CSCs were isolated and cultured in vitro. Flow cytometry analysis showed the isolated c-kit+ CSCs were negative for CD45 (Figure 3.8A). HGF mRNA expression was also higher in the Ad-hSCF group compared to the Ad-LacZ (Figure 3.8B). No difference in IGF-1 expression was found between groups (Figure 3.8C).

3.5 DISCUSSION

The present study demonstrates that cardiomyocyte-specific overexpression of hSCF reduces myocardial apoptosis and infarct size after acute myocardial I/R. When hSCF expression was turned off by DOX treatment, these effects were abrogated, which clearly implies that overexpression of hSCF is responsible for the cardiac protection in I/R. Our novel findings are: 1) hSCF overexpression reduces myocardial apoptosis and infarct size via Akt-PI3K signaling pathway. 2) SCF/c-kit signaling is responsible for the reduced infarct size and enhanced growth factor (IGF-1 and HGF) expression in the hSCF overexpressing myocardium after I/R, and 3) overexpression of hSCF increases endogenous CSCs activation after I/R. Our study suggests that hSCF may have therapeutic potential in the treatment of ischemic heart disease.

In acute myocardial I/R, the lack of blood supply during ischemia and the oxidative stress followed by abrupt metabolic changes immediately after reperfusion leads to severe cellular injuries. It is widely accepted that both necrosis and apoptotic
Figure 3.8 Overexpression of hSCF in c-kit⁺ CSCs in vitro.

A: Flow cytometry analysis showed no CD45 expression in c-kit⁺ CSCs. B: HGF mRNA expression was enhanced in hSCF overexpressing CSCs, which was abrogated by ACK2 treatment. C: No difference of IGF-1 mRNA expression was observed between groups. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs Sham; † P<0.05 vs WT.
cell death contribute to infarct size development and cell loss during I/R.\textsuperscript{31} Apoptosis differs from necrosis in that it is a finely regulated process initiated by the cell itself.\textsuperscript{31} Interventions that suppress myocardial apoptosis during I/R decrease infarct size and improve cardiac function.\textsuperscript{32,33} In the present study, we found that cardiac-specific overexpression of hSCF decreased infarct size and myocardial apoptosis after I/R. Moreover, the fact that reduction in infarct size was completely abolished when hSCF overexpression was turned off further confirmed the beneficial effects are due to hSCF overexpression in the myocardium. The signaling pathway responsible for the improvement was further investigated. As the major component of the reperfusion injury salvage kinase pathway (RISK),\textsuperscript{3} PI3K/Akt/eNOS signaling was increased in hSCF overexpressing hearts after I/R. Blocking PI3K-Akt signaling by LY294002 abrogated Akt/eNOS activation and increased myocardial apoptosis and infarct size, which clearly demonstrate that PI3K/Akt/eNOS signaling is responsible for the cardiac protection in hearts with hSCF overexpression after I/R.

The activation of RISK pathway can be induced by cardiac protective growth factors during I/R.\textsuperscript{7} In this regard, exogenous administration of IGF-1\textsuperscript{34} and HGF\textsuperscript{26,35,36} has been shown to decrease myocardial apoptosis and infarct size in acute I/R injury. As an endogenous response, the heart is also able to produce cardioprotective growth factors after ischemia. In our study, acute I/R induced HGF and IGF-1 expression in the WT myocardium, which is consistent with previous studies in the porcine I/R model.\textsuperscript{37} This endogenous protective mechanism in acute I/R by releasing beneficial growth factors is further enhanced by hSCF overexpression. Inhibition of HGF receptor signaling abrogated the improved infarct size in hSCF overexpressing mice after I/R, suggesting
that HGF contributes to the protective effects of hSCF overexpression. More importantly, our study provided convincing evidence that SCF/c-kit signaling plays a critical role in this process as the higher expression of HGF and IGF-1 was abolished by ACK2 treatment in mice with hSCF overexpression.

It is now recognized that mechanisms of cardiac protection from endogenous stem/progenitor cell recruitment and/or exogenous stem cell delivery may include paracrine effects, which result from the secretion of various cytokines, chemokines and growth factors, and possibly by their direct differentiation into cardiomyocytes and vascular cells. Despite the significant beneficial effects observed in animal models, clinical trials using bone marrow stem cells only showed minor improvement in MI patients. The key issues to be resolved include choosing the optimal stem cell type, safe and efficient delivery method and maximizing cell retention and engraftment. Mobilization of hematopoietic and endothelial progenitors to the peripheral blood occurs within a few hours after acute MI, acting as an endogenous response to tissue damage. Our efforts have been focused on enhancement of the endogenous recruitment to and retention of the stem/progenitor cells in the ischemic myocardium. The previous chapter showed hSCF overexpression increases EPC recruitment and retention in myocardium 5 days post-MI. In this chapter, a significant increase of c-kit+ cell numbers in the WT myocardium after acute I/R compared to that of sham mice was observed, which is an endogenous response to myocardial ischemia. By overexpressing hSCF in cardiomyocytes, we showed for the first time that the c-kit+ cell population was further increased after acute I/R compared to WT. Furthermore, when the SCF/c-kit signaling
was blocked by ACK2, the increase of c-kit+ cells induced by hSCF overexpression after I/R was abrogated, illustrating a critical role of SCF/c-kit interaction in this process.

The c-kit+ cells in the adult heart have 2 sub-populations, CD45c-kit+ and CD45+c-kit+ cells. The CD45c-kit+ cells are endogenous CSCs which are able to differentiate into cardiomyocytes. The increase of CSCs is observed several days after acute MI and contributes to myocardial repair. In the present study we showed that the number of CSCs was increased in the upper half of the heart, which was mainly non-infarct area of the myocardium after I/R, and was further enhanced by hSCF overexpression. The increase in c-kit+ cell population induced by hSCF overexpression is possibly due to cell migration and recruitment from CSC niches or the circulation since cell proliferation assessed by Ki67 staining did not show a significant difference between WT and hSCF overexpressing mice after I/R. Interestingly, under basal conditions, more CSCs were found in hSCF overexpression myocardium compared to the WT, which suggests a pivotal role of membrane-associated SCF in maintaining a CSC pool in the myocardium of the adult heart. Despite increases in number, CSCs in the hSCF overexpression heart remain quiescent until an ischemic insult occurs, which is confirmed by the finding that the expression of HGF and IGF-1 is not increased in hSCF overexpressing mice under basal conditions. The effects of hSCF on CSC activity were further studied in isolated c-kit+ CSCs in vitro. Overexpression of hSCF enhanced HGF expression in CSCs via c-kit signaling.

On the other hand, our study also showed significantly more CD45+c-kit+ cells in the heart after I/R. Moreover, hSCF overexpression resulted in a 1.7 fold higher CD45+c-kit+ cells compared to WT mice after I/R. The CD45+c-kit+ cells are of hematopoietic
lineage which includes mast cells, circulating hematopoietic stem cells and endothelial progenitors. We first confirmed that the increase of CD45\(^+\)c-kit\(^+\) cells was not due to mast cells recruitment through toluidine blue staining. No significant difference in the number of mast cells was observed between WT and hSCF overexpressing mice after I/R. Thus, the higher number of CD45\(^+\)c-kit\(^+\) cells suggests hSCF overexpression promotes recruitment of circulating progenitor cells after I/R. These data are consistent with previous findings from our lab and others that interaction of membrane-associated SCF and c-kit enhances circulating c-kit\(^+\) progenitor recruitment.

In conclusion, our data show that cardiomyocyte-specific hSCF overexpression reduces infarct size and myocardial apoptosis after acute I/R. These beneficial effects are likely due to enhanced activation of PI3K/Akt/eNOS signaling and production of cardioprotective growth factors induced by increased population of c-kit\(^+\) progenitors in the ischemic myocardium. The capability of M-SCF to provide cardioprotection in both acute I/R and chronic heart failure makes it a promising candidate as a new treatment for ischemic heart disease.
3.6 REFERENCES


27. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J*. 2006;20:661-669.


4 CHAPTER 4

Cardiac-Specific Overexpression of Human Stem Cell Factor Promotes Epicardial Activation and Arteriogenesis after Myocardial Infarction

A version of this chapter has been submitted to *Circulation Heart Failure*:

**Xiang F**, Lu X, Liu Y, Feng Q

“Cardiac-Specific Overexpression of Human Stem Cell Factor Promotes Epicardial Activation and Arteriogenesis after Myocardial Infarction.”
4.1 CHAPTER SUMMARY

In Chapters 2 and 3, I showed that cardiomyocyte-specific overexpression of hSCF protects the heart from ischemic injury via regulating the activities of c-kit$^+$ stem/progenitor cells, such as EPCs and CSCs. Recent studies have shown that the adult epicardium is also a potential source of cardiac progenitors following MI. In this chapter, I investigated the effects of cardiomyocyte-specific overexpression of hSCF on epicardial activation post-MI. I found that hSCF overexpression promoted epicardial activation and arteriogenesis post-MI. These cardioprotective effects were related to increased EPDC production, proliferation and migration. Furthermore, lineage tracing of Wt1 EPDCs showed convincing evidence that EPDCs were activated and able to migrate into the infarcted myocardium post-MI, which was further enhanced by hSCF overexpression. In conclusion, cardiomyocyte-specific overexpression of hSCF promotes epicardial activation, EPDC production, proliferation and migration, which contributes to arteriogenesis post-MI.
4.2 INTRODUCTION

Cardiovascular disease remains a leading cause of death worldwide.\textsuperscript{1} In the past decade, adult stem cell therapy has emerged as a promising new strategy for ischemic heart disease.\textsuperscript{2} To this end, both cardiac and extra-cardiac progenitor cells have been demonstrated to improve cardiac function after myocardial infarction (MI).\textsuperscript{2} The epicardium-derived cells (EPDCs) are cardiac progenitors that contribute to coronary artery formation during embryonic heart development.\textsuperscript{3-5} Recent studies have shown that transplantation of adult EPDCs into the ischemic myocardium in mice preserves cardiac function and attenuates ventricular remodeling via enhancement of neovascularization, suggesting their therapeutic potential in cardiac repair post-MI.\textsuperscript{6}

During early heart morphogenesis, cells from the proepicardial organ migrate to the looping heart tube to form the epicardium.\textsuperscript{7} A fraction of these cells undergoes epithelial to mesenchymal transition (EMT), invades into the myocardium and becomes EPDCs, which give rise to smooth muscle cells, perivascular and interstitial fibroblasts, and form coronary arteries with contributions from the endocardium and sinus venosus.\textsuperscript{8-11} Wilms tumor-1 (Wt1) is expressed in the EPDCs and promotes EMT\textsuperscript{12} while its expression is turned off in fully differentiated EPDCs.\textsuperscript{13-15} The adult epicardium is inactive under normal physiological conditions, but can be re-activated and produces EPDCs after MI. Some of the EPDCs also express \textit{c-kit}.\textsuperscript{16,17} However, the number of endogenously generated EPDCs is not sufficient for adequate cardiac repair post-MI. Measures that promote epicardial activation and EPDC production may enhance cardiac repair post-MI.
Stem cell factor (SCF) binds to c-kit and promotes survival, proliferation, mobilization, and adhesion of all c-kit-expressing cells.\textsuperscript{18} Alternative splicing leads to two isoforms of SCF, the soluble and membrane-associated isoforms, which differ in the absence or presence of a proteolytic cleavage site encoded by exon 6.\textsuperscript{19} The membrane-associated SCF is the dominant isoform \textit{in vivo} and induces more persistent and long-term support of target cell survival. However, the effects of SCF on epicardial activation post-MI are not known. In the present study, we hypothesized that inducible cardiomyocyte-specific overexpression of membrane-associated human stem cell factor (hSCF) promotes epicardial activation and EPDC production post-MI. To test this hypothesis, mice with cardiomyocyte-specific overexpression of hSCF were subjected to coronary artery ligation to induce MI. Epicardial Wt1 expression, myocardial growth factor levels and arteriole density were studied. Furthermore, cell fate of Wt1 expressing epicardial cells post-MI was determined using lineage tracing. Our results demonstrated that cardiomyocyte-specific overexpression of hSCF enhances epicardial activation and arteriogenesis post-MI.

4.3 METHODS

4.3.1 Animals

Animals in this study were handled in accordance with the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and animal handling was approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. The conditional cardiac-specific hSCF overexpressing hSCF/tTA transgenic mice were generated using α-
MHC promoter as described in Chapter 2. In the doxycycline (DOX) group, mice were treated with DOX (0.2 mg/ml in drinking water) to turn off hSCF overexpression starting from 2 weeks before the surgeries. The mTmG (membrane-Tomato/membrane-Green) targeted-Rosa26 mice (stock #7576) and Wt1\textsuperscript{CreER} mice (Stock # 010912) were purchased from Jackson Laboratory and bred to generate ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice. Tamoxifen (40 mg/kg, i.p.) treatment was performed for 5 days before surgery to trace the lineage of Wt1\textsuperscript{+} cell derived EPDCs in the myocardium post-MI. To inhibit \textit{c-kit} function, mice were injected intraperitoneally with 100 µg \textit{c-kit} neutralizing antibody ACK2 (a gift from Dr. Shin-Ichi Nishikawa, Kyoto University, Kyoto, Japan) per day post-MI.\textsuperscript{20,21} In total, 68 WT, 68 hSCF/tTA and 24 ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice were used for \textit{in vivo} experiments. Each EPDC culture was from 12-16 hearts of P0 or E13.5 mice.

### 4.3.2 MI Mouse Model

MI was induced by occlusion of the left main coronary artery. Briefly, mice were anesthetized with a ketamine (50 mg/kg) and xylazine (12.5 mg/kg) cocktail (i.m.), intubated and artificially ventilated. The adequacy of anesthesia was monitored by absence of withdrawal reflex to tail pinch. Subsequently, a left intercostal thoracotomy was performed and the left coronary artery was surgically ligated. To achieve \textit{in vivo} adenoviral infection, $10^7$ transducing units of adenoviral vector expressing recombinant hSCF (Ad-hSCF) or LacZ (Ad-LacZ) in 5µL saline was injected into 3-5 sites of the peri-infarct myocardium immediately after coronary artery ligation. The infection efficiency was confirmed by western blot analysis.
4.3.3 EPDC Culture

EPDCs were cultured as previously described.\textsuperscript{22,23} Hearts were removed from postnatal day 0 (P0) or embryonic day 13.5 (E13.5) mice, cut into pieces, rinsed in PBS to remove excess blood and plated onto 1% gelatin-coated culture dish (epicardial side facing down) in DMEM containing GlutaMax\textsuperscript{TM}, 4.5 g/L glucose, 1% penicillin and streptomycin, and 15% fetal calf serum for 6-9 days. EPDCs which had migrated from the heart explants were utilized for further experiments. First passage EPDCs were employed in EPDC proliferation experiments. Each well of a 24-well plate was seeded with $10^4$ EPDCs. Ad-hSCF or Ad-GFP was added at a concentration of 10 MOI (multiplicity of infection). After 24 hours, cells were trypsinized and counted by NucleoCounter (New Brunswick Scientific, Enfield, CT, USA).

4.3.4 Neonatal Cardiomyocyte Culture

P0 mice were sacrificed by cervical dislocation without anesthesia. Hearts were isolated, cardiomyocytes were obtained and cultured in 24 well plates according to methods we previously described.\textsuperscript{24,25} Overexpression of hSCF in WT neonatal cardiomyocytes was accomplished through the adenoviral infection of Ad-hSCF. Viral vectors containing LacZ served as controls. Cells were infected with adenoviruses at an MOI of 10 and incubated for 2 hours in the presence of 200\textmu l of M199 with 2% FCS. Following the 2 hour incubation, 800\textmu l of 10% FCS M199 was applied. Cells were incubated for an additional 48 h and utilized for further experiments.
4.3.5 Transwell Migration Assay

A transwell system was employed to investigate EPDC migration. Neonatal cardiomyocytes infected with Ad-hSCF or Ad-LacZ were cultured in the basal compartment. First passage GFP$^+$ EPDCs ($5 \times 10^4$) were subcultured in transwell cell culture inserts (8-µm diameter pores, Becton Dickinson Labware, Franklin Lakes, NJ, USA). After incubation for 24 hour at 37$^\circ$C and 5% CO$_2$, the cells on upper layer of the insert membrane were completely removed using a cotton swab. GFP$^+$ EPDCs which had migrated to the other side of membrane were imaged by a fluorescent microscope (Observer D1, Carl Zeiss Canada, Toronto, Canada) and quantified.

4.3.6 Real-time RT-PCR

Total mRNA was isolated with TRIzol reagent (Invitrogen, Grand Island, NY, USA) as previously described in Chapter 2. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers. Real-time PCR was conducted using SYBR Green PCR Master Mix (Eurogentec, San Diego, CA, USA). 28S rRNA was used as a loading control. The oligonucleotide primer sequences were as follows: TGF-β1, forward, CAC TGC TTC CCG AAT GTC TG, reverse, GCC CGA AGC GGA CTA CTA TG; soluble mouse SCF (include exon 6), forward, GCT TTG CTT TTG GAG CCT TA reverse, TGA AAT TCT CTC TCT TTC TGT T; VEGF-A, forward, GAT TGA GAC CCT GGT GGA CAT C, reverse, TCT CCT ATG TGC TGG CTT TGG T; bFGF, forward, CAA GGG AGT GTG TGC CAA CC, reverse, TGC CCA GTT CGT TTC AGT GC. Samples were amplified for 35 cycles using an Eppendorf Mastercycler Real-Time PCR machine (Eppendorf, Hamburg, Germany). The mRNA levels in relation to 28S rRNA were determined using a comparative Ct method.
4.3.7 Immunohistochemistry

Paraffin sections of the heart were deparaffinized, hydrated and rinsed in PBS. Antigen retrieval was performed by microwaving the slides in 10mM sodium citrate buffer (PH6.0) for 10 minutes. After serum blocking, slides were incubated with primary antibodies followed by biotinylated secondary antibody. Color development was carried out after incubation using an ABC kit (Vector Laboratory, Burlingame, CA, USA) and then developed with DAB solution. Nuclei were counterstained with hematoxylin. Epicardial Wt1+ cells were quantified and these data are presented as cell number per mm of epicardium. Frozen tissue sections and cover glasses with cells adhering on the surface were fixed in 4% paraformaldehyde. Endogenous fluorescence was bleached by incubation in 0.3% H2O2 overnight in a UV box.12 Primary antibodies and fluorescent second antibodies were then applied. For fluorescent visualization, nuclei were stained with Hoechst reagent. Representative images were taken using a fluorescent microscope (Observer D1, Carl Zeiss Canada, Toronto, Canada). Primary antibody used includes: Wilms tumor 1 (Wt1, 1:300, Calbiochem, San Diego, CA, USA), phosphorylated Smad2 (p-Smad2, 1:200, Cell Signaling Technology, Danvers, MA, USA), smooth muscle actin (SMA, 1:1000, Sigma, St Louis, MO, USA), green fluorescent protein (GFP, 1:2000, Abcam, Cambridge, MA, USA), epicardin (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-kit (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Quantification of epicardial Wt1+ cells was presented by epicardial Wt1+ cell number divided by the length of epicardium.
4.3.8 Western Blot Analysis

Expression of hSCF after Ad-hSCF adenoviral infection was measured by western blot analysis. Briefly, 40 µg of protein were separated by 12% SDS-PAGE gel and transferred to nitrocellulose membranes, and blots were probed with antibodies against hSCF (1:500, Abcam, Cambridge, MA, USA) or α-actinin (1:2500, Sigma, St Louis, MO, USA). Blots were then washed and probed with horseradish peroxidase conjugated secondary antibodies (1:3000, Bio-Rad, Hercules, CA, USA), and detected by using an ECL detection method.

4.3.9 Statistical Analysis

Data are expressed as the mean ± SEM. Two-way ANOVA followed by Bonferroni test was performed for multiple group comparisons. Unpaired Student t-test was used for two group comparisons. Differences were considered statistically significant at the level of \( P<0.05 \).

4.4 Results

4.4.1 Epicardial Activation 3 Days post-MI

WT and hSCF/tTA mice were subjected to either coronary artery ligation to induce MI or sham surgery. Three days after surgery, animals were sacrificed and heart sections were immunostained for Wt1, a specific marker for EPDC activation (Figure 4.1A). Wt1\(^+\) cells in the epicardium were quantified. Few if any Wt1\(^+\) cells were found in the epicardium of sham groups. Three days post-MI, Wt1\(^+\) cells in the epicardium of the infarct area (including peri-infarct area) were increased in both WT and hSCF/tTA mice. Because Wt1 expression in EPDCs is turned off shortly after EMT, \(^26\) it is not surprising
Figure 4.1 Epicardial Wt1 expression 3 days post-MI.

(A) Representative images for Wt1 staining. a: Wt1 positive cells show dark brown nuclear staining. Arrow heads point to Wt1 positive cell in the epicardium. (B) Quantification of Wt1 positive cells in the epicardium. In the epicardium of infarct and peri-infarct area, significantly more epicardial Wt1+ cells were observed in hSCF/tTA compared to WT mice 3 days post-MI. This was abrogated by DOX and ACK2 treatment. In the non-infarct area, epicardial activation was also detected. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs WT. (C) Overexpression of hSCF showed significantly higher TGFβ mRNA expression in hSCF/tTA mice compared to WT post-MI. This was abrogated by ACK2 treatment. (D) Representative images of Wt1 and p-Smad2 staining in epicardium of WT and hSCF/tTA mice 3 days post-MI. White dash line labels the boundary of the epicardium. Smad2 activation was observed in all the epicardial Wt1+ cells. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs Sham, † P<0.05 vs WT. Data are mean ± SEM, n=5-7
that very few Wt1+ cells were observed in the myocardium. However, the population of epicardial Wt1+ cells in hSCF/tTA mice was almost doubled in the infarct area compared to WT (P<0.05, Figure 4.1B). Interestingly, treatment with either DOX to turn off hSCF expression or ACK2 to block the SCF/c-kit signaling, abrogated this response. The number of epicardial Wt1+ cells was similar in the non-infarct area between hSCF/tTA and WT mice.

TGFβ1 is a key regulator of the EMT process. To assess its expression, TGFβ1 mRNA levels in the epicardium and subepicardial area were determined by real-time RT-PCR. Consistent with previous studies,27,28 we found expression of TGFβ1 mRNA was significantly higher post-MI compared to sham groups (P<0.05, Figure 4.1C). Our data further showed that TGFβ1 mRNA levels of epicardial and subepicardial tissues were significantly higher in the peri-infarct area of hSCF/tTA compared to WT mice, and the response was abolished by either DOX or ACK2 treatment (P<0.05, Figure 4.1C). As expected, in the Wt1+ epicardial cells 3 days post-MI, Smad2 was also activated indicating the activation of TGFβ1 signaling in those cells (Figure 4.1D).

4.4.2 Arteriolar Density in the Peri-infarct Area 5 Days post-MI

EPDCs are essential for coronary artery formation during embryonic development.26 To investigate the effects of enhanced epicardial activation on coronary artery formation post-MI, SMA staining was employed to stain smooth muscle cells and the number of arterioles (between 15-150 µm) in the peri-infarct area was quantified. Representative photomicrographs of SMA staining are presented in Figure 4.2A. Each arrow points to an arteriole. Five days post-MI, arteriole density in the peri-infarct area was significantly higher in hSCF/tTA compared to WT mice (P<0.05, Figure 4.2B).
Figure 4.2 Arteriolar density in the peri-infarct area 5 days post-MI.

(A) Representative images of α-SMA staining. Arterioles were identified as small vessels surrounded by SMA positive cells. Arrows point at arterioles. (B) Quantification of arteriole density. Number of arterioles was significantly higher in the peri-infarct area of hSCF/tTA compared to WT mice 5 days post-MI. This increase was abrogated by DOX and ACK2 treatment. Data are mean ± SEM, n=5. Two-way ANOVA followed by Bonferroni test: *P<0.05 vs WT.
Furthermore, the response was abrogated by DOX or ACK2 treatment (P<0.05, Figure 4.2B), suggesting SCF/c-kit signaling promotes myocardial arteriogenesis post-MI.

### 4.4.3 Outgrowth of Cultured EPDCs

EPDCs can grow out from P0 or E13.5 heart explant cultures and form a monolayer of cobblestone shaped cells (Figure 4.3A). The purity of EPDCs was confirmed by detecting EPDC specific marker expression (Figure 4.3B). To study the effects of SCF on the outgrowth of EPDCs, heart tissue sections from P0 WT mice were cultured and randomly divided into 2 treatment groups: control or recombinant mouse SCF (100 ng/mL). Six days after culturing, outgrowth of EPDCs from the heart tissue was observed (Figure 4.4A). Treatment with recombinant mouse SCF resulted in a significantly more robust EPDC outgrowth compared to controls (P<0.05, Figure 4.4B).

**Proliferation and Pro-angiogenic Growth Factor Expression of Cultured EPDCs**

To assess EPDC proliferation, E13.5 mice were obtained through timed breeding. Cultured EPDCs isolated from E13.5 and P0 hearts of WT mice were treated with Ad-hSCF. The expression of hSCF protein after Ad-hSCF infection was confirmed by western blot analysis (Figure 4.5). After 24 hours of treatment, EPDC cell numbers were significantly higher in the Ad-hSCF group compared to Ad-GFP control group (P<0.05, Figure 4.4C and D). Treatment with ACK2 showed significantly lower EPDC cell numbers in both Ad-hSCF and Ad-GFP groups (P<0.05, Figure 4.4C and D).

To investigate pro-angiogenic growth factor expression, cultured EPDCs from P0 hearts of WT mice were treated with Ad-GFP or Ad-hSCF for 24 hours. VEGF-A and bFGF mRNA levels were significantly higher in the Ad-hSCF group compared to Ad-
Figure 4.3 *In vitro* culture of EPDCs.

(A): Six days after heart explant culture, a cobblestone shaped cell monolayer was observed surrounding the heart explants. (B): Cobblestone shaped cells outgrown from the heart explants were 100% positive for epicardin (nuclear: blue, epicardin: red) and Wt1 (nuclear: blue, Wt1: red). Some EPDCs were also positive for *c-kit* (nuclear: blue, *c-kit*: red).
Figure 4.4 Outgrowth, proliferation and growth factor expression in cultured EPDCs.

(A) Representative images showing EPDC outgrowth from the P0 heart explants (H). Arrows indicate the EPDC migration distance from the heart explants. (B) Recombinant human stem cell factor treatment (100 ng/ml) significantly enhanced EPDC outgrowth compared to control (n=7 per group). Unpaired Student’s t test: *P<0.05 vs Control. (C and D) Overexpression of hSCF by Ad-hSCF infection significantly enhanced proliferation of E13.5 (C) and P0 (D) EPDCs compared to the Ad-GFP control group. ACK2 treatment significantly impaired EPDC proliferation in both groups. (E and F) VEGF-A and bFGF mRNA levels in P0 EPDCs detected by real-time RT-PCR were significantly higher in Ad-hSCF treatment compared to Ad-GFP. This effect was abolished by ACK2. Data are mean ± SEM, n=4. One-way ANOVA followed by Bonferroni test: *P<0.05 vs Ad-GFP, † P<0.05 vs Ad-hSCF.
Figure 4.5 Expression of hSCF protein in adenovirus treated EPDCs.

Cultured EPDCs treated with Ad-hSCF or Ad-LacZ were subjected to western blot analysis. Expression of hSCF protein was observed only in Ad-hSCF infected EPDCs.
<table>
<thead>
<tr>
<th></th>
<th>E13.5 EPDC</th>
<th>P0 EPDC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad-LacZ</td>
<td>Ad-hSCF</td>
</tr>
<tr>
<td>hSCF</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>α-actinin</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
GFP control group ($P<0.05$, **Figure 4.4E and F**). Treatment with ACK2 abrogated this effect (**Figure 4.4E and F**).

### 4.4.4 Migration of Cultured EPDCs

A transwell assay was employed to study the effect of hSCF overexpression on EPDC migration. EPDCs were isolated from GFP transgenic mice and seeded on the upper inserts with 8 µm pores. Neonatal cardiomyocytes infected with either Ad-LacZ or Ad-hSCF were seeded on the lower chamber. Cells were allowed to migrate for 24 hours and the number of GFP$^+$ EPDCs which had migrated through the insert membrane were detected using fluorescence microscopy. As shown in **Figure 4.6A**, the green cells are GFP$^+$ EPDCs migrated through the membrane. Quantitative analysis showed that Ad-hSCF significantly enhanced EPDC migration compared to Ad-LacZ ($P<0.05$, **Figure 4.6B**).

Furthermore, inhibition of c-kit signaling by ACK2 significantly attenuated EPDC migration in both Ad-LacZ and Ad-hSCF treatment groups ($P<0.05$, **Figure 4.6B**). The data implies that EPDC migration is enhanced by chemokine release from the Ad-hSCF treated cardiomyocytes in the lower chamber. This effect is likely mediated by autocrine SCF production as soluble mouse SCF expression was significantly higher in hSCF overexpressing cardiomyocytes and lower with ACK2 treatment ($P<0.05$, **Figure 4.6C**), suggesting SCF/c-kit signaling is involved in EPDC migration.

### 4.4.5 Lineage Tracing of Wt1$^+$ EPDCs

EPDC activation and migration into the myocardium post-MI was further investigated by lineage tracing of Wt1$^+$ EPDCs. Cre activation was induced by tamoxifen
Figure 4.6 Transwell EPDC migration.

(A) Representative images of GFP\(^+\) EPDCs which had migrated through the membrane. (B) Quantification of GFP\(^+\) EPDCs which had migrated through the membrane. Number of GFP\(^+\) EPDCs which had migrated to the lower surface of the membrane was significantly higher in Ad-hSCF treated group compared to the Ad-LacZ. The migration was significantly inhibited by ACK2 treatment. (C) Expression level of soluble mouse SCF mRNA was significantly higher in Ad-hSCF treated neonatal cardiomyocytes compared to the Ad-LacZ group. The response was inhibited by ACK2. Data are mean ± SEM, n=3. One-way ANOVA followed by Bonferroni test: *\(P<0.05\) vs Ad-LacZ, † \(P<0.05\) vs Ad-hSCF
injection for 5 days before MI in ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice (Figure 4.7A). Fluorescent staining of GFP and Wt1 confirmed the success of the inducible lineage tracing system. Five days post-MI, 70-80% of the Wt1\textsuperscript{+} cells expressed GFP while all GFP\textsuperscript{+} cells in the epicardium were Wt1\textsuperscript{+}. Myocardial overexpression of hSCF in ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice was induced by intra-myocardial injection of Ad-hSCF in the peri-infarct area immediately following coronary artery ligation. The expression of hSCF protein in the peri-infarct area 5 days post-MI was detected by western blot (Figure 4.8). GFP\textsuperscript{+} cells representing EPDCs derived from Wt1 expressing cells were found in the epicardium 5 days post-MI (Figure 4.7B), which is consistent with our findings in Figure 4.1A and B. Importantly, GFP\textsuperscript{+} cells were also observed in the infarcted myocardium (Figure 4.7C), providing direct evidence that Wt1\textsuperscript{+} EPDCs were activated and migrated into the infarct area. Furthermore, treatment with Ad-hSCF induced significantly more GFP\textsuperscript{+} cells in the epicardium and infarcted myocardium compared to Ad-LacZ ($P<0.05$, Figure 4.7D and E), which was abrogated by ACK2. Additionally, higher myocardial expression of TGF\textbeta, a key factor in promoting EMT, was detected in Ad-hSCF group compared to Ad-LacZ and this effect was also abrogated by ACK2 treatment (Figure 4.7F).

EPDCs play a critical role in coronary artery formation during embryonic heart development mainly by differentiating into vascular cells.\textsuperscript{26} To demonstrate the ability of EPDCs to differentiate, epicardial cells were cultured from hearts of ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice at P0. Treatment with tamoxifen (200 nM) in the cultured ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} EPDCs showed Cre activation resulting in GFP expression in 60-70% of Wt1\textsuperscript{+} EPDCs (Figure 4.9A). The Wt1\textsuperscript{+} EPDCs were also found to have differentiated into SMA\textsuperscript{+} cells (Figure 4.9B). So our next aim was to determine the lineage of Wt1 expressing cells in
Figure 4.7 Lineage tracing of Wt1⁺ cell derived EPDCs 5 days post-MI.

(A) Experimental outline for genetic fate mapping in the adult heart using ROSA<sup>mA-TmG</sup>;Wt1<sup>CreER</sup> mice. (B) Representative images of GFP staining in the epicardium 5 days post-MI. (C) Representative images of GFP staining of the infarct area 5 days post-MI. (D and E) Quantification of GFP⁺ cells in the epicardium (D) and infarcted myocardium (E). Significantly more Wt1⁺ cell derived EPDCs were found in the epicardium and infarct area in the Ad-hSCF treated compared to the Ad-LacZ group 5 days post-MI. ACK2 treatment abolished the increase of GFP⁺ cells in the heart. (F) TGFβ mRNA expression determined by real-time RT-PCR. In vivo Ad-hSCF treatment in the myocardium induced significantly more TGFβ mRNA expression post-MI. This was abrogated by ACK2. Data are mean ± SEM, n=6. Two-way ANOVA followed by Bonferroni test: *<i>P</i><0.05 vs Ad-LacZ.
Figure 4.8 Expression of hSCF protein in adenovirus treated hearts.

Heart tissues from ROSA<sup>mTmG</sup>;Wt<sup>1CreER</sup> mice injected with Ad-hSCF or Ad-LacZ were subjected to western blot analysis. Expression of hSCF protein was observed only in Ad-hSCF injected hearts.
Figure 4.9 *In vitro* lineage tracing of Wt1+ cell derived EPDCs.

(A): Representative images of EPDCs from P0 ROSA<sup>mTmG</sup>;Wt1<sup>CreER</sup> or ROSA<sup>mTmG</sup> mice treated with tamoxifen (200 nM). GFP<sup>+</sup> cells (green) were observed only in EPDCs from the ROSA<sup>mTmG</sup>;Wt1<sup>CreER</sup> hearts. (B): Representative images of passage 1 EPDCs from ROSA<sup>mTmG</sup>;Wt1<sup>CreER</sup> hearts stained with GFP and SMA (nuclear: blue, SMA: red, GFP: green)
the infarct area post-MI in ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice after tamoxifen treatment. As shown in Figure 4.10A, some of the GFP\textsuperscript{+} cells in the infarct area were also positive for SMA, a marker for smooth muscle cells and myofibroblasts, indicating that the Wt1\textsuperscript{+} EPDCs differentiate into smooth muscle cells or myofibroblasts. Furthermore, treatment with Ad-hSCF induced significantly higher numbers of GFP\textsuperscript{+} and SMA\textsuperscript{+} cells (Figure 4.10B and C). However, the percentage of SMA\textsuperscript{+} cells in relation to the total number of GFP\textsuperscript{+} cells (~34\%) in the infarcted myocardium was similar between Ad-LacZ and Ad-hSCF treated groups (Figure 4.10D), suggesting Ad-hSCF treatment does not affect EPDC differentiation. Additionally, no SMA\textsuperscript{+} cells from the Wt1\textsuperscript{+} cell lineage were detected in any vessels in the peri-infarct area.

4.5 DISCUSSION

The present study demonstrates for the first time that cardiomyocyte-specific expression of membrane-associated hSCF promotes epicardial activation post-MI. The hSCF transgene expression increases epicardial Wt1\textsuperscript{+} EPDCs and arteriogenesis post-MI. These effects are specific to hSCF as they are abrogated upon treatment with DOX, which turns off hSCF expression. More importantly, through Wt1 lineage tracing, we showed that hSCF overexpression enhances EPDC activation and migration into the infarct area post-MI through the \textit{c-kit} signaling pathway. Furthermore, hSCF stimulates EPDCs to express VEGF and bFGF, which are important growth factors in arteriogenesis. Our study suggests that hSCF overexpression enhances epicardial activation, EPDC production, and arteriogenesis post-MI.
Figure 4.10 Fate-mapping of Wt1\(^+\) cell derived EPDCs post-MI.

(A) Representative images of GFP and SMA staining of Wt1-derived EPDCs in the infarct area. The GFP\(^+\) cells are also positive for SMA (arrows). (B) Quantification of the SMA\(^+\)/GFP\(^+\) cells in the infarcted myocardium. The number of SMA\(^+\)/GFP\(^+\) cells was higher in Ad-hSCF treated compared to the Ad-LacZ infarcted myocardium. (C) Quantification of the GFP\(^+\) cells in the infarcted myocardium. The number of GFP\(^+\) cells was increased with Ad-hSCF compared to Ad-LacZ treatment. (D) Ratio of SMA\(^+\)/GFP\(^+\) cells. The ratio of SMA\(^+\)/GFP\(^+\) cells was not significantly different between Ad-hSCF and Ad-LacZ treatment. Data are mean ± SEM, n=6. Unpaired student t test: *P<0.05 vs Ad-LacZ.
The adult epicardium can be re-activated and express Wt1 post-MI.\textsuperscript{29} In present study, epicardial activation as determined by Wt1 positivity was observed 3 days post-MI, which is consistent with previous studies.\textsuperscript{29-31} Our novel finding is that overexpression of hSCF significantly enhanced the epicardial activation post-MI, and the response was abrogated by inhibiting \textit{c-kit} receptor function, suggesting an important role of SCF/\textit{c-kit} signaling in epicardial activation. TGF\textbeta has been shown to promote EMT in both embryonic and adult EPDCs.\textsuperscript{32} A significant increase of TGF\textbeta mRNA expression was found in the hSCF overexpressing myocardium post-MI and the effect was attenuated by DOX treatment to turn off hSCF expression or ACK2 treatment to inhibit \textit{c-kit} receptor function. Furthermore, nuclear Smad2 phosphorylation was observed in epicardial Wt1\textsuperscript{+} cells, suggesting activation of TGF\textbeta signaling in Wt1\textsuperscript{+} epicardial cells post-MI.

The embryonic epicardium is active with Wt1\textsuperscript{+} expression and contributes to coronary artery formation.\textsuperscript{4,33} We therefore hypothesize that enhanced epicardial activation by cardiac specific overexpression of hSCF increases arteriogenesis post-MI. To test this hypothesis, myocardial arteriole density was determined. Our data showed that hSCF overexpression significantly increased arteriole density in the peri-infarct area, suggesting a potential contribution of epicardial activation to arteriogenesis post-MI. In order to further investigate the effect of SCF on EPDC activity, overexpression of hSCF was induced in cultured EPDCs. Our data showed that the ability of cultured EPDCs to proliferate and migrate was significantly enhanced by hSCF overexpression via \textit{c-kit} signaling. Moreover, expression of VEGF-A and bFGF, two well-known pro-angiogenic
growth factors involved in arteriogenesis,\textsuperscript{34,35} was up-regulated through SCF/c-kit pathway suggesting an enhanced paracrine effect of EPDCs.

In order to trace cell fate of the activated epicardium \textit{in vivo}, a ROSA\textsuperscript{mTmG};\textsuperscript{Wt1}\textsuperscript{CreER} mouse was generated in which Cre recombinase expression is inducible by tamoxifen treatment, and \textsuperscript{Wt1} expression cells and their progeny will be \textsuperscript{EGFP}. Myocardial hSCF overexpression was achieved by intra-myocardial injection of adenoviral hSCF constructs. Consistent with what we found in the hSCF/tTA mice, overexpression of hSCF by adenoviral infection enhanced epicardial activation post-MI in ROSA\textsuperscript{mTmG};\textsuperscript{Wt1}\textsuperscript{CreER} mice as shown by a significantly higher number of \textsuperscript{EGFP}+ epicardial cells. A hallmark of activated EPDCs is their ability to undergo EMT and migrate into the myocardium.\textsuperscript{17} Zhou et al. also investigated the migration of EPDCs using the same lineage tracing system. However, the \textsuperscript{EGFP}+ EPDCs remained in the epicardial region and did not migrate into the myocardium.\textsuperscript{29} This is not surprising as their study was specifically designed to trace \textsuperscript{Wt1}+ epicardial cells before the onset of MI. In the present study we used a tamoxifen treatment protocol to turn on Cre recombinase activity at the peak of epicardial \textsuperscript{Wt1} expression, and were able to see \textsuperscript{EGFP}+ cells in the infarcted myocardium 5 days post-MI. More importantly, hSCF overexpression significantly increased the number of \textsuperscript{EGFP}+ cells migrated into the myocardium. A significant increase of TGF\textbeta mRNA expression was also observed post-MI in the myocardium of Ad-hSCF treated ROSA\textsuperscript{mTmG};\textsuperscript{Wt1}\textsuperscript{CreER} mice. Furthermore, increases in EPDC migration and TGF\textbeta expression were abrogated by inhibition of \textit{c-kit} signaling. Together, these data show activation of SCF/c-kit signaling promotes EMT of
Wt1+ EPDCs and their migration into the infarcted myocardium via upregulation of TGFβ.

Following EMT and migration into the myocardium during embryonic heart development, EPDCs differentiate into several cellular lineages including cardiomyocytes, endothelial cells, smooth muscle cells and interstitial fibroblasts.\textsuperscript{3,4,26} Although the adult epicardium can be re-activated post-MI, cell fate of EPDCs remains to be determined. Through lineage tracing of Wt1+ EPDCs, we showed that the total number of EPDCs and their SMA\textsuperscript{+} derivatives were significantly increased by hSCF overexpression. However, their commitment to smooth muscle and/or myofibroblast lineages 5 days post-MI was not altered by hSCF overexpression (Figure. 4.10D). The SMA\textsuperscript{+} cell lineage was mainly found in the infarct area while arteriogenesis was predominantly in the peri-infarct area. The fact that not a single artery is derived from the EPDC lineage suggests that the increased arteriogenesis by hSCF overexpression is mainly due to paracrine effects. Of interest, lineage commitment of EPDCs post-MI may be partially modified. To this end, Smart et al. successfully induced 0.6% of Wt1+ EPDCs differentiation into structurally coupled cardiomyocytes using thymosin β4.\textsuperscript{30} However, this observation was not reproduced by Zhou et al.\textsuperscript{36} In the present study, we did not observe any effects of hSCF overexpression on cardiomyocyte generation from the EPDCs.

In summary, the present study showed that cardiac specific hSCF overexpression promotes epicardial activation, EPDC production, growth factor expression and arteriogenesis post-MI. These effects may contribute to a significant improvement in cardiac function and survival in hSCF/tTA mice post-MI as demonstrated in Chapter 2.
Our study provides novel insight in EPDC activation post-MI and suggests that hSCF may have therapeutic potential in promoting cardiac repair post-MI.
4.6 REFERENCES:


adult epicardial cells is regulated by TGFbeta-signaling and WT1. *Basic Res Cardiol.* 2011;106:829-47.


5 \hspace{1cm} \textbf{CHAPTER 5}

5.1 \hspace{1cm} \textbf{SUMMARY OF MAJOR FINDINGS}

The overall objective of this thesis was to investigate the regulatory role of M-SCF in cardiac function and repair post-MI. Specifically, I studied the effects of M-SCF on cardioprotection and myocardial repair post-MI via regulating the activities of \textit{c-kit} expressing stem cells in the heart. To achieve this goal, a novel cardiac specific hSCF overexpressing mouse was generated. Experimental approaches included \textit{in vitro} cell culture and \textit{in vivo} animal studies as well as the use of pharmacological inhibitors and adenoviral constructs.

In \textbf{Chapter 2}, I investigated the effects of cardiac-specific overexpression of hSCF on cardiac function and survival post-MI in mice. I demonstrated for the first time that hSCF overexpression promoted animal survival 30 days post-MI, which is accompanied by a significant preservation of cardiac performance. The underlying protective mechanisms include increases in angiogenesis and decreases in myocardial apoptosis and cardiac remodeling in the transgenic mice compared to WT. Furthermore, increased \textit{c-kit} \textsuperscript{+} stem/progenitor cell number post-MI were observed in the myocardium, which is associated with the beneficial outcomes. Interestingly, cells expressing EPC markers were found to differentiate into small vessels in hSCF overexpressing myocardium, which suggests that M-SCF contributes to vasculogenesis post-MI. When hSCF expression was turned off by DOX treatment, all the beneficial effects were abrogated. The data further support the notion that M-SCF is responsible for the myocardial protection observed in the hSCF overexpressing mice. The cardioprotective
effects of M-SCF were recently confirmed by Higuchi et al, using lentiviral infection to overexpress mouse M-SCF in the myocardium. They showed improved survival and cardiac performance in M-SCF overexpressing mice, which is consistent with our findings.

Loss of functional cardiomyocytes and myocardial injury within a few hours after ischemia or I/R produce pathological changes in the heart which may ultimately lead to heart failure. Thus, in Chapter 3, I investigated the role of M-SCF in acute myocardial I/R injury and related signaling pathways. I demonstrated that cardiac-specific overexpression of hSCF reduces infarct size after acute I/R via inhibition of myocardial apoptosis. Among the signaling pathways involved in protecting cardiomyocytes from apoptosis after I/R, Akt has been found to play a critical role in our previous studies. In agreement with those observations, I further demonstrated that the PI3K/Akt/eNOS signaling pathway is responsible for the anti-apoptotic effect of hSCF overexpression in the heart after acute I/R. Although many studies have shown improvements in infarct size and myocardial function by enhancing circulating stem cell recruitment and/or activation of endogenous CSCs, it remains unclear how such changes affect myocardial cell death within the first few hours after I/R. To this end, I provided novel evidence that the myocardial \( c\text{-}kit^+ \) cell population was increased within 4 hours after I/R in the WT heart, while a further significant increase of \( c\text{-}kit^+ \) cells was observed in mice with cardiac specific hSCF overexpression. The increase of \( c\text{-}kit^+ \) cells was accompanied by higher levels of myocardial expression of HGF and IGF-1, both of which are cardioprotective growth factors well-recognized in anti-apoptotic signaling pathways. As expected, blockade of SCF/\( c\text{-}kit \) signaling abrogated the beneficial effects of hSCF overexpression.
in acute I/R including lower infarct size, and higher protective growth factor expression and c-kit+ cell numbers. The subpopulations of c-kit+ cells were further determined by CD45 expression. The number of CD45+/c-kit+ CSCs was increased in response to acute I/R in peri- and non-infarct area while a further increase of these cells was observed in hSCF overexpressing hearts. Consistent with findings in Chapter 2, retention of circulating CD45+c-kit+ cells was also enhanced in hSCF overexpressing myocardium after acute I/R.

Using adenoviral constructs to overexpress M-SCF in the heart, a recent study demonstrated that infarct size was decreased 2 weeks post-MI in mice. Increased myocardial c-kit+ cell population associated with M-SCF overexpression 1 week post-MI was also reported. Although the authors concluded that there was increased activation of endogenous CSCs in the myocardium treated with the M-SCF recombinant adenovirus post-MI, no clear markers were employed to distinguish circulating stem cells and CSCs.

Finally, I determined the effect of M-SCF on CSC activity in vitro by inducing M-SCF overexpression in isolated cardiac c-kit+ progenitor cells through adenoviral infection. Enhanced proliferation and HGF expression was found in M-SCF overexpressing CSCs. Therefore, my study provided the first direct evidence that endogenous CSCs are activated shortly after I/R and M-SCF can enhance their activation. Taken together, in Chapter 3, I demonstrated that cardiac specific overexpression of hSCF reduces infarct size and myocardial apoptosis via the PI3K/Akt/eNOS signaling pathway through the enhanced paracrine effects from the retained circulating c-kit+ stem cells and activation of endogenous CSCs.
In Chapter 4, I investigated the role of M-SCF on EPDCs in cardiac repair post-MI. Embryonic EPDCs are vascular and myocyte progenitors that regulate vascular cell and cardiomyocyte generation by direct differentiation as well as paracrine effects.\textsuperscript{10,11} The therapeutic potential of adult EPDCs is implied by recent studies showing that these cells can be reactivated post-MI \textsuperscript{12} and that transplantation of human EPDCs into the infarcted myocardium improves cardiac function post-MI.\textsuperscript{13} Limana et al.\textsuperscript{12} reported that a subset of EPDCs post-MI were $c$-$kit^+$ and could give rise to both myocardial and vascular cells. Thus, I hypothesized that cardiac specific overexpression of hSCF promotes epicardial activation post-MI. Using \textit{in vitro} EPDCs in cultures and \textit{in vivo} lineage tracing models, I demonstrated for the first time the effects of M-SCF on EPDC activation and arteriogenesis post-MI. In comparison to results observed with WT mice, cardiac specific M-SCF overexpression promoted EPDC activation and arteriogenesis post-MI. I further investigated the fate of Wt1$^+$ EPDCs post-MI using a lineage tracing model. Overexpression of M-SCF by adenoviral infection enhanced Wt1$^+$ EPDC activation and migration to the infarcted myocardium. In agreement with observations from other studies,\textsuperscript{14,15} one third of the Wt1$^+$ EPDCs which had migrated into the infarcted myocardium and differentiated into SMA positive cells. However, no integration of Wt1$^+$ EPDCs in arterioles in the peri-infarct area was found, suggesting that the increased arteriogenesis in the peri-infarct area of hSCF overexpressing myocardium may result from paracrine effects of EPDCs instead of direct differentiation. This is supported by a recent study demonstrating the importance of paracrine factors in EPDCs-induced cardioprotection.\textsuperscript{14} Moreover, \textit{in vitro}, M-SCF overexpression enhanced EPDC proliferation, migration and pro-arteriogenesis growth factor release, which was
abolished by blocking SCF/c-kit signaling. Additionally, although EPDCs contribute to myocardigenesis during embryogenesis, no cardiomyocyte regeneration from Wt1^+ EPDCs in the adult heart was observed in my study, as well as by others.\textsuperscript{14}

In summary, I have demonstrated the beneficial effects of cardiac specific M-SCF overexpression in regulating activities of circulating c-kit^+ stem/progenitor cells, CSCs and EPDCs, which leads to cardioprotection in acute I/R injury and chronic MI. My studies may provide important information for developing novel therapeutic strategies for the treatment of MI. Overexpression of myocardial M-SCF not only enhances endogenous protection and repair mechanisms but may also increase the efficacy of stem cell therapy post-MI. A summary of the findings is depicted in Figure 5.1.

5.2 STUDY LIMITATIONS

5.2.1 Inducible Cardiac-specific hSCF Overexpressing Transgenic Mice

In a report published in 1996, Majumdar et al described a line of transgenic C3H/HeJ mice with the membrane associated hSCF gene under the control of the hPGK promoter.\textsuperscript{16} The homozygous hSCF transgenic mice displayed a coat color deficiency as well as abnormal thymocyte development. The phenotypes were claimed to result from hSCF interference with c-kit signaling and altered endogenous mouse M-SCF expression. However, no \textit{in vivo} data or direct evidence of hSCF inhibiting mouse SCF/c-kit signaling in melanocytes was shown. Although the relatively low binding affinity
Figure 5.1 Summarized cellular mechanisms by which M-SCF improves cardiac function and animal survival post-MI.

The interaction of SCF with *c-kit* receptors improves cardiac repair post-MI by enhanced EPC retention and activation of CSCs and EPDCs, leading to increased neovascularization, and decreased myocardial apoptosis and cardiac hypertrophy mainly through paracrine effects.
(Kd>700 nM) of soluble hSCF to mouse c-kit expressed on Chinese hamster ovary cells was demonstrated a decade ago,\textsuperscript{17} no study has been performed to demonstrate the binding affinity of membrane-associated isoform of hSCF to mouse c-kit receptor. Despite the difference in binding affinity between species, soluble hSCF has been shown to be able to induce biological effects on mouse c-kit\textsuperscript{+} cells.\textsuperscript{16} Moreover, the normal fertile and hematopoietic system in the hSCF transgenic mice suggested that the hSCF could interact with mouse c-kit and induce biological function signaling.\textsuperscript{16}

In this thesis, inducible cardiac specific hSCF overexpressing mice were created by expressing the hSCF gene under the α-MHC promoter. MHC is the major component of the heart’s contractile apparatus encoded by the α- or β-MHC gene.\textsuperscript{18} In the mouse, during gestation, β-MHC is expressed in ventricles but silenced after birth,\textsuperscript{19} while α-MHC starts to be expressed in ventricles after birth and through adulthood. By using the α-MHC promoter to induce the expression of hSCF transgene, we successfully generated cardiac specific hSCF overexpressing mice without affecting embryonic development. Thus, in our cardiac specific hSCF overexpressing mice, coat color, heart morphology and cardiac function are normal. Furthermore, in our hSCF overexpressing mice, the expression of endogenous mouse SCF was not affected under either sham or MI conditions. However, I did find that after overexpression of hSCF, the expression of mouse soluble SCF was upregulated in cultured neonatal cardiomyocytes, which likely contributed to enhanced EPDC transwell migration \textit{in vitro}.

5.2.2 Use of Mouse Model to Simulate Human Disease Conditions

This thesis mainly utilized \textit{in vivo} experimental mouse models to simulate human heart disease. The use of mouse models to simulate human disease is necessary in the
evaluation of basic pathophysiological changes and potential therapeutics. Advantages for using animal models include: the control of experimental conditions, genetic manipulations (knockout or overexpression), and the ease access to tissues, which is difficult or impossible in humans. Furthermore, over 99% of all mouse genes have human homologues. Thus, insights gained from genetically modified mice may be applied to develop therapeutics for human disease. In this thesis, a novel inducible cardiac specific hSCF overexpressing mouse was generated. The reasons we chose the human M-SCF gene to create the transgenic mice are as follows. First, the effects of human M-SCF on MI can be studied. Second, it is easier to verify transgene expression in the heart. Third, human M-SCF remains more membrane-bound than mouse M-SCF. Last, although the binding affinity of human SCF to mouse c-kit is not the same as mouse SCF, their biological effects are similar as demonstrated in previous studies.

In Chapters 2 and 4, I used a mouse MI model in which the left main coronary artery was permanently ligated and evaluations were performed at 3-5 and 30 days post-MI to simulate early and late stages of human heart failure, respectively. Similar to observations in human MI, our mouse model of MI showed myocardial infarct formation, cardiac dysfunction, and increased mortality. By utilizing inducible overexpression of the hSCF gene in the heart, I was able to study the effects of M-SCF post-MI and elucidate the related mechanisms. In Chapter 3, a murine model of cardiac I/R originally described by Michael et al. was performed by ligation of the left main coronary artery to generate ischemia for 45 minutes followed by untying the artery to return blood flow for 3 hours. In humans, myocardial I/R is associated with significant cell death resulting in formation of infarction. In my study, myocardial I/R in mice were also associated
with increased myocardial apoptosis and infarct formation. However, the direct effects of M-SCF in the human heart remain to be studied in patients with MI.

### 5.2.3 Contributions of Soluble SCF

Although in my thesis, the SCF overexpressed in mice is a membrane-associated isoform (Figure 5.2), the existence of a secondary proteinase cleavage site may also lead to a slow release of soluble SCF, which may possibly contribute to the cardioprotection observed in the M-SCF overexpression mice post-MI. It is well-known that soluble SCF enhances stem cell mobilization from the bone marrow to the peripheral circulation. However, we were not able to detect any soluble human SCF in the serum of hSCF overexpressing mice (Figure 5.3). Moreover, a previous study using lentivirus to overexpress mouse M-SCF in the heart did not show any changes in CD34+ or c-kit+ cells in the peripheral circulation post-MI. Consistent with these data, a recent report using adenovirus to overexpress M-SCF in the myocardium also failed to showed any changes in c-kit+ cells in the bone marrow and peripheral blood post-MI. These studies suggest that the beneficial effects of myocardial M-SCF overexpression post-MI in vivo are not likely due to the release of S-SCF into the circulation from M-SCF.

### 5.2.4 Use of Pharmacological Inhibitors and Neutralizing Antibody to Delineate Signaling Pathways

In Chapter 3 of this thesis, I used the PI3K inhibitor LY294002 to blocking the PI3K/Akt signaling pathway. LY294002 is a morpholine derivative of quercetin. It can selectively and completely abolish PI3K activity with an IC50 of 1.4 µM. Inhibition of other downstream signaling pathways of PI3K is an obvious limitation of using LY294002. Additionally, Akt can be activated independent of PI3K. In my studies
**Figure 5.2 Expression of hSCF in WT and hSCF/tTA mice.**

Fluorescent images showed the expression of hSCF protein. Blue: nuclei; red: hSCF. The hSCF protein was observed on the plasma membrane of cardiomyocytes from hSCF/tTA mice. DOX treatment turned off the expression of hSCF in hSCF/tTA mice.
Figure 5.3 Soluble hSCF is not detectable in the serum of WT and hSCF/tTA mice.

Expression of hSCF protein was detected by western analysis. Expression of soluble hSCF was not detectable in the serum of WT and hSCF/tTA mice. Hearts from hSCF/tTA mice were used as positive control. Each lane represents a sample from a separate animal.
however, LY294002 inhibited more than 50% of myocardial Akt activation, demonstrating its efficiency in blocking Akt signaling.

Crizotinib, a small molecule inhibitor of HGF receptor, c-Met, was also used in Chapter 3. It has been shown to potently inhibit c-Met phosphorylation (IC50=11nM), HGF/c-Met signaling and exhibit an antitumor effect in tumor models at well-tolerated doses in vivo. One of the limitations of using crizotinib is that it can also inhibit anaplastic lymphoma kinase and c-ros oncogene 1.

In Chapters 3 and 4, c-kit neutralizing antibody ACK2 was employed to block SCF/c-kit signaling. ACK2 reacts with c-kit and inhibits the binding of SCF to c-kit. Therefore, this antibody serves as a specific inhibitor to c-kit. The dose I chose for in vivo injection is equivalent to the studies performed by Dr. Nishikawa and colleagues who initially produced this antibody.

5.3 SUGGESTIONS FOR FUTURE RESEARCH

The novelty of this thesis is the demonstration that myocardial M-SCF overexpression protects the heart from MI and I/R injury by regulating the activity of several types of stem/progenitor cells. However, how M-SCF regulates those stem/progenitor cells and whether M-SCF may induce myocardial regeneration warrant further investigation.

In Chapter 2, I observed that M-SCF overexpression increased the retention and differentiation of cells expressing EPC markers. The ability of M-SCF to enhance EPC adhesion was also reported by Dentelli et al. who showed that M-SCF expression in human microvascular endothelial cells induced during inflammation was necessary for c-
EPC recruitment via the interaction of M-SCF and c-kit. Soluble SCF has been established to directly enhance the neovascularization potential of EPCs \textit{in vivo} and \textit{in vitro}.\textsuperscript{29} However, how M-SCF directly regulates EPC activity is still unclear. The regenerated small vessels from EPCs in the infarcted myocardium provide evidence that M-SCF may directly affect the vasculogenic potential of EPCs. It will be interesting to further investigate the role of M-SCF in EPC activity under physiological and different pathological conditions such as inflammation or hyperglycemia.

In Chapter 3, the observation that the population of CSCs is higher in the hSCF overexpressing myocardium under basal conditions provides a reasonable rationale to further study the effects of M-SCF on changes in the CSC population over time from birth to adulthood. Myocardial c-kit\textsuperscript{+} cells have been shown to be dramatically reduced 7 days after birth and remain scarce through the adulthood.\textsuperscript{30} Future studies are necessary to address the following issues. For example, does cardiac specific M-SCF overexpression maintain a higher CSC pool after birth and in the aging myocardium? How does M-SCF regulate the activities of CSCs under basal conditions and in response to ischemic injuries? Additionally, the neonatal mouse heart has been shown to have a transient regeneration capacity after partial LV apex resection (~15\% of LV myocardium).\textsuperscript{31} By lineage mapping of \(\alpha\)-MHC the authors concluded that the regenerated myocardium results mainly from the proliferation of the preexisting myocytes. In my opinion, this conclusion remains questionable based on 2 reasons. First, the half-life of tamoxifen is about 5-7 days, which gives at least a 5-day window to label all cells expressing \(\alpha\)-MHC including the differentiated CSCs. Second, the physiological changes of \(\alpha\)-MHC expression in cardiomyocytes 1 week after birth\textsuperscript{19} make lineage
mapping of α-MHC a sub-optimal method to study the cell source of neonatal myocardial regeneration. Instead, an inducible c-kit lineage tracing system may provide more accurate information on the regeneration potency of CSCs. Thus, the role of M-SCF in neonatal CSC proliferation and differentiation, and also whether M-SCF reserves some of the regenerative potential of the heart after birth, would be interesting topics for further investigations.

The effects of M-SCF on adult EPDC activation, proliferation and migration were demonstrated in Chapter 4. However, whether M-SCF affects embryonic EPDC activity is not known. During embryonic heart development, EPDCs contribute to the formation of coronary arteries. Our recent studies have revealed that eNOS deficiency impairs embryonic coronary artery development via decreased activity of EPDCs. Malformation of coronary arteries renders the adult heart more susceptible to ischemic injury during increased demand for oxygen in conditions such as intense exercise. Consistent with this notion, patients with hypoplastic coronary artery disease die from sudden death mainly due to acute MI. In Chapter 3, I also showed that eNOS is a downstream target of SCF/c-kit activation via PI3K/Akt signaling. Thus, the hypothesis that M-SCF may regulate EPDC activity and promote embryonic coronary artery development can be tested in future studies. Additionally, Chapter 4 showed that M-SCF increases the number of activated EPDCs which migrate into the myocardium and differentiate into SMA+ cells post-MI. During the early infarct healing process, SMA+ myofibroblasts have beneficial effects against cardiac rupture through the production of collagen and extracellular matrix to form an infarct scar. The contribution of EPDCs to the formation
of a strong granulated scar to avoid cardiac rupture post-MI and the role of M-SCF in this process would be another interesting area of future research.

Finally, activities and function of stem/progenitor cells have been shown to be impaired in other pathological conditions in the heart such as diabetic cardiomyopathy and hypertension-induced cardiac hypertrophy. Whether cardiac-specific overexpression of M-SCF enhances cardiac stem/progenitor cell activity and improves myocardial function and animal survival in these disease conditions remains to be investigated.

5.4 CLINICAL APPLICATION

Over the past decade, there has been advancement in transduction efficiency and safety of viral vectors for cardiovascular gene therapy. This powerful technology has opened a window for applying newly discovered potential therapeutic targets to treat IHD. I have shown the dramatic effects of M-SCF in cardiac repair post-MI using transgenic mice. How to translate these observations in animal models into clinical practice remains challenging but presumably feasible. Successful delivery of the M-SCF gene into the myocardium via adenovirus in mice has been achieved in my study as well as by others. Sun et al showed that sustained expression of myocardial M-SCF expression was achieved for a full year after the delivery of the M-SCF gene via lentivirus in neonatal mice. Moreover, the beneficial effects of M-SCF post-MI were also observed in aged mice. Given the growing evidence of M-SCF cardioprotective effects in animal models, there is ostensible therapeutic value for M-SCF gene therapy in patients with IHD.
5.5 CONCLUSIONS

This thesis provides the first evidence that cardiac specific overexpression of M-SCF offers cardiac protection and improves cardiac repair post-MI. While further investigation is required to determine the therapeutic potential of M-SCF in the treatment of IHD, it is clear that cardiac-specific overexpression of M-SCF is beneficial post-MI. Furthermore, this work has provided valuable insight into the mechanisms by which M-SCF increases retention and differentiation of EPCs (Chapter 2), enhances proliferation and paracrine effects of CSCs (Chapter 3) and promotes activation, proliferation and migration of EPDCs (Chapter 4). Overall my doctoral research has broadened our understanding of M-SCF in the regulation of stem/progenitor cell activity and cardiac function in MI, and may contribute to the development of therapeutics for the treatment of IHD.
5.6 REFERENCES


10. Lie-Venema H, van den Akker NM, Bax NA, Winter EM, Maas S, Kekarainen T, Hoeben RC, deRuiter MC, Poelmann RE, Gittenberger-de Groot AC. Origin, fate,


APPENDIX

Appendix A: UWO Animal use sub-committee protocol approvals
Qingping Feng - eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::5

From: [Redacted]
To: [Redacted]
Date: 03/01/2013 2:56 PM
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::5
CC: [Redacted]

Western

2007-011-03::5:

**AUP Number:** 2007-011-03  
**AUP Title:** Modulation of Myocardial Function in Myocardial Infarction, Sepsis, anemia and Diabetes

**Approval Date:** 11/24/2011

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-011-03 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

**REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee
Curriculum Vitae

Fu-Li (Lily) Xiang

Education and Training:

PhD in Physiology 2008-2013
Western University, Canada

Supervisor: Dr. Qingping Feng

Research Fellow, Department of Medicine 2005-2008
Western University, Canada

Supervisor: Dr. Qingping Feng

MSc, Transplant Immunology 2003-2005
Tongji Medical School,
Huazhong University of Science and Technology, China

Supervisor: Dr. Zhonghua Chen

Pediatric Otolaryngologist 2002-2003
Wuhan Children’s Hospital, China

Resident 2000-2002
Wuhan Children’s Hospital, China

Bachelor Degree of Medicine, Pediatric Medicine (MD) 1995-2000
Tongji Medical School,
Huazhong University of Science and Technology, China

Distinctions and Honors:

Heart and Stroke Foundation of Canada Doctoral Research Award 2010-2013
First Prize for poster presentation on Physiology and Pharmacology Research Day,
Western University 2012

Second Prize in Western University 3MT Competition
http://www.youtube.com/watch?v=dWUmNO0C4tc 2012

Chinese Government Award for Outstanding Self-Financed Students Abroad 2011
GTA academic scholarship, Western University 2011
First Prize for poster presentation on Physiology and Pharmacology Research Day,
Western University 2011

Gordon Mogenson Award, Department of physiology and pharmacology,
Western University 2010
First Prize for poster presentation on Physiology and Pharmacology Research Day,
Western University 2010
Second Prize for oral presentation on Lawson Research Day,
Lawson Health Research Institute, London, Ontario 2009
First Prize for poster presentation on Moffat Research Day,
Schulich School of Medicine and Dentistry, Western University 2009
Second Prize for oral presentation on Lawson Research Day,
Lawson Health Research Institute, London, Ontario 2008
Excellent Graduate Student Scholarship, Tongji Medical School,
Wuhan, China 2003-2005

Teaching Experience
Teaching Assistant
Third Year Physiology Lab Course 3130y, Western University, 2008-2012
Publications in Peer-reviewed Journals (English)


