

**CARDIOPROTECTION:
EFFECTS OF INCREASED LEVELS OF
FIBROBLAST GROWTH FACTOR-2 IN THE HEART**

BY

SARAH K. JIMENEZ

**A thesis submitted to the Faculty of Graduate Studies
of the University of Manitoba
in partial fulfilment of the requirements
for the degree of**

DOCTOR OF PHILOSOPHY

**Department of Physiology
University of Manitoba
Winnipeg, Manitoba
CANADA**

Copyright © 2011

ABSTRACT

High mortality rates from cardiovascular disease underscore the need for improved therapies. Thus, it is important to further our understanding of factors and mechanisms promoting cardiac protection and repair.

Fibroblast growth factor-2 (FGF-2), administered to the heart before or during injury exerts beneficial effects such as cytoprotection and angiogenesis. However, the effects of a chronic elevation in endogenous FGF-2 on recovery/remodeling after ischemic injury are not known. **My hypothesis was that chronic elevation in endogenous FGF-2 expression (in FGF-2 overexpressing transgenic mice) exerts beneficial effects such as improved function after isoproterenol-induced injury *in vivo*.**

The first study showed that treatment with the β -adrenergic agonist isoproterenol resulted in exaggerated levels of cellular infiltration and myocardial disarray in transgenic FGF-2 versus non-transgenic mouse myocardium. This was suggestive of increased cardiac injury in transgenic FGF-2 mice. Inhibition of T cells using the immunosuppressants cyclosporine A or antibodies against CD3 ϵ attenuated cellular infiltration in transgenic FGF-2 mice, to levels comparable to those of non-transgenic mice, suggesting a T lymphocyte-mediated effect. Overall morphological data suggested that chronic FGF-2 elevation might have created an adverse outcome after cardiac injury.

In a follow-up study the effect of chronic FGF-2 elevation on cardiac function was

examined, as measured by tissue Doppler imaging (TDI), after isoproterenol administration. FGF-2 overexpressing mice displayed improved cardiac function compared to controls, after isoproterenol, both acutely (24 h) and in a sustained fashion (2-4 weeks). The FGF-2 associated functional improvement at 2-4 weeks was attenuated following immunosuppression with cyclosporine A, but not treatment with anti-CD3 ϵ antibodies. The FGF-2-associated functional improvement may be partially attributed to a cyclosporine A-sensitive (but anti-CD3-insensitive) infiltrating cell population. Thus cellular infiltration, in response to elevated FGF-2, may have a net beneficial effect.

In a third study, non-transgenic mice were put through a brief swimming protocol (exercise) prior to isoproterenol. This acute bout of exercise resulted in significant improvement in TDI function, compared to control groups, measured at 24 hours up to 4 weeks post-isoproterenol.

In conclusion, increased endogenous cardiac FGF-2 expression, or an acute bout of exercise, exert sustained beneficial effects against isoproterenol-induced cardiac injury.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to everyone who made this thesis possible.

First and foremost, to my supervisors, Drs. Peter Cattini and Vetta Kardami, who have been two of my biggest supporters since my Master's studies. It has been an absolute honour to have been part of your labs through the years. Words cannot express how grateful I am for all your advice (science-related or otherwise), and for your unwavering guidance and patience, especially during the times when other aspects of life had presented some minor setbacks. You have been nothing but wonderful mentors, and I really cannot thank you enough.

To my committee members, Drs. Janice Dodd, Grant Hatch, and Thomas Netticadan, thank you for all your support and advice. Your guidance through the years has been an integral part of my growth as a scientist. Thank you also to my external thesis examiner, Dr. Yassemi Capetanaki, and Dr. Dan Gietz for serving as the thesis defense chair.

This thesis project could also not have been possible without certain collaborations. Work by Dr. Davinder Jassal and technician Tielan Fang were essential for the functional assessment portion of the thesis; they are wonderful teachers and collaborators. Drs. Carmen Morales and Yijun Fang, pathologists, were important in identifying the cell types of interest in our model of injury. Successful staining of immune cells was optimized with the help of Dr. Salomon, and their (confocal) imaging could not have

been possible without the help of John Rutherford – who made staring into a microscope for hours on end much more enjoyable. Other collaborators from studies that were not included in the thesis include: Dr. Aaron Marshall, Dr. Kennedy Makondo, Dr. Pawan Singal, and Dr. Tom Thomas. To all the collaborators – many thanks!

To Gail McIndless and Judy Olfert, you were always there to help, even on my oftentimes last-minute requirements. Thank you for being exceptionally patient and supportive, and I am truly thankful for every support you have given me.

The many lab members of the Cattini and Kardami laboratories, past and present, have taught me so much. Marge, Robert, Yan, Barb, Jiang, Xitong, Shunyan, Pat, Shirley, Mark, Farah, Karen, David, Madhu, Wattamon, Yangyang, Alina, Xin Ma, Jon-Jon, Hana, and Jane: you helped mold me into the scientist and person I have become, and are truly like family to me. Thank you for sharing with me your technical expertise, for helping with the numerous troubleshooting I needed to do for “unsuccessful” experiments, for all the moral support through the ups and downs of lab and life. You have created wonderful work environments to come to every day, and as such, I will always think fondly of my days in both laboratories.

I also cannot thank certain people enough for going above and beyond to help me with my thesis work, especially after I had started working from California. Farah, thank you; you have been a wonderful mentor and friend right from the start and I really am grateful for everything you have done. Marge, Robert, Barb, and Wattamon – it would have been

impossible to finish my writing (thesis and papers) if it hadn't been for your patience in being my link to Winnipeg (and for listening to my rants); thank you so very much.

I also would like to acknowledge past mentors who helped shaped my career path. To my high school science and math teachers, Mr. Turnbull and Mr. Wood, and to the late Dr. Halvorson (Department of Microbiology, UofM), your enthusiasm was contagious! Thank you for everything you have taught me, and for helping lead me down this journey.

To all my family (Jimenez, Andres, and MacInnes) and friends, thank you. You have kept me sane through this process. ☺ Really, your support has meant a great deal to me and I could not have done this thesis without you in my life.

To my parents: this thesis, and the life I am living, absolutely could not have been possible without you and your multitudes of sacrifices. Thank you for giving up your comfortable life in the Philippines to move to an unknown place to give me the chance to pursue a life that would not have been possible otherwise. Your sacrifices and support are appreciated more than you know. Thank you for everything.

And last, but not least, to my husband John: it is really difficult to put my gratitude into words without sounding like a total cliché. You have been my biggest supporter, my strength in times of weakness, and my courage in times of doubt (and we both know

those times were many ☺). I guess I can just say in all honesty that, I feel truly blessed to have you in my life. Thank you for everything.

For my parents,

who have sacrificed much to give me better opportunity;

and for my husband John,

who has always been there for me and with me, win or lose.

TABLE OF CONTENTS

ABSTRACT.....	II
ACKNOWLEDGEMENTS	IV
TABLE OF CONTENTS	1
LIST OF FIGURES	5
LIST OF TABLES	7
LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED	8
CHAPTER I.....	9
INTRODUCTION	9
A. BACKGROUND.....	9
B. ISCHEMIC HEART DISEASE AND HEART FAILURE.....	11
B.1. Atherosclerosis.....	12
B.2. Occlusion of blood vessels supplying the heart.....	13
B.3. Ischemic heart disease.....	13
B.4. Myocardial infarction and subsequent repair	14
B.5. Heart failure.....	15
C. RISK FACTORS FOR CARDIOVASCULAR DISEASE	17
C.1. Aging	17
C.2. Gender.....	17
C.3. Genetic predispositions.....	18
C.4. Diabetes, Hypertension, Obesity	19
C.5. Exercise.....	21
C.6. Stress	21
C.7. Tobacco and/or cigarette smoking	22
D. CAVEAT	23
CHAPTER II	24
RODENT MODELS OF MYOCARDIAL INJURY	24
A. CORONARY LIGATION SURGERY	24
B. ISOPROTERENOL MODEL OF INJURY.....	25
B.1. Proposed mechanisms for isoproterenol-induced myocardial injury	27
CHAPTER III.....	29
MYOCARDIAL INJURY AND REPAIR.....	29
A. INTRODUCTION.....	29
B. MYOCARDIAL INJURY	29
C. CARDIAC RESPONSE TO INJURY	32
C.1. Inflammatory response and myocardial repair.....	32
C.2. Infiltrating cells	33
C.3. Stages of myocardial repair.....	40
C.4. Cardiac regeneration	45
D. CARDIOPROTECTION	46
D.1. Ischemic preconditioning.....	46
D.2. Preconditioning stimulated by naturally-produced substances	48
CHAPTER IV.....	50

FIBROBLAST GROWTH FACTOR-2.....	50
A. FGF-2 AND THE FGF FAMILY	50
B. FGF-2 EXPRESSION AND FUNCTION	51
C. FGF-2 STRUCTURE AND TISSUE FUNCTION	52
C.1. FGF-2 gene structure.....	53
C.2. FGF-2 protein structure and subcellular distribution	55
D. FGF-2 RELEASE	56
E. FIBROBLAST GROWTH FACTOR RECEPTORS	57
F. FGF-2 RELEASE AND SIGNALING IN THE HEART/CARDIAC MYOCYTE.....	59
G. EFFECTS OF FGF-2 ON CELL PROLIFERATION	62
H. FGF-2 AND CARDIOPROTECTION	63
I. RESEARCH OBJECTIVES.....	67
CHAPTER V	69
MATERIALS AND METHODS.....	69
A. ETHICS APPROVAL.....	69
B. TRANSGENIC MOUSE MODELS	69
B.1. FGF-2 transgenic mouse line	69
C. ASSESSMENT OF TRANSGENIC ANIMALS	69
C.1. Genotyping by polymerase chain reaction (PCR).....	69
C.2. Detection of RNA by Reverse Transcription (RT) and Quantitative PCR (qRT-PCR)	70
C.3. Protein Immunoblotting	71
D. IN VIVO MODELS OF INJURY	72
D.1. High-dose isoproterenol administration.....	72
E. EXERCISE.....	74
F. ASSESSMENT OF CARDIAC FUNCTION	75
F.1. Echocardiography	75
G. ASSESSMENT OF MYOCARDIAL INJURY IN VIVO AND EFFECTS OF TREATMENT.....	76
G.1. Heart weight – to – body weight ratio	76
G.2. Morphology	76
G.3. Identification of infiltrating cells.....	78
H. STATISTICAL ANALYSIS	80
CHAPTER VI.....	82
RESULTS	82
A. EFFECTS OF INCREASED ENDOGENOUS FGF-2 ON MYOCARDIAL INJURY IN VIVO... 82	
A.1. FGF-2 transgenic mice have increased FGF-2 RNA and protein in their hearts compared with non-transgenic counterparts.....	82
A.2. In vivo model of cardiac injury: high dose isoproterenol treatment induces ischemic damage in the mouse myocardium	85
A.3. Increased heart weight-to-body weight ratio was observed in FGF-2 transgenic mice 28 days after isoproterenol administration	88
A.4. Increased myocardial infiltration and disarray were observed in FGF-2 transgenic mice after isoproterenol-induced myocardial injury	90
A.5. Quantitation of areas of cellular infiltration show significantly increased affected myocardium in FGF-2 transgenic mice compared with non-transgenic mice after myocardial injury in vivo.....	94
A.6. Pathological assessment of the myocardium after isoproterenol-induced myocardial injury revealed a significant increase in infiltrating cells in FGF-2 transgenic mouse hearts	97
A.7. CD4-positive cells co-stain for FGFR-1 within the areas of infiltration after isoproterenol-induced myocardial injury	103
A.8. Inhibition of T cells by immunosuppression using cyclosporine A or anti-CD3ε antibodies decreases cellular infiltrate and myocardial disarray in FGF-2 transgenic and non-transgenic mouse hearts after myocardial injury.....	105
CHAPTER VII	110

RESULTS	110
B. CARDIAC FUNCTION IN FGF-2 TRANSGENIC MICE AFTER MYOCARDIAL INJURY IN VIVO.....	110
<i>B.1. Assessment of left ventricular function in isoproterenol-induced myocardial injury in transgenic mice overexpressing FGF-2 by tissue Doppler imaging.....</i>	<i>110</i>
<i>B.2. Heterozygous FGF-2 transgenic mice have increased FGF-2 RNA and protein in their hearts compared with non-transgenic counterparts.....</i>	<i>111</i>
<i>B.3. High-dose isoproterenol treatment induces infarct-like histological changes in both FGF-2 transgenic and non-transgenic mice</i>	<i>115</i>
<i>B.4. Tissue Doppler imaging predicts improved outcome in FGF-2 transgenic mice after isoproterenol-induced myocardial injury</i>	<i>117</i>
<i>B.5. Pre-treatment of mice with cyclosporine A reduces functional recovery in FGF-2 transgenic mice after isoproterenol-induced myocardial injury</i>	<i>125</i>
<i>B.6. Treatment with anti-CD3 antibodies has no effect on cardiac function of FGF-2 transgenic mice after isoproterenol-induced myocardial injury</i>	<i>130</i>
CHAPTER VIII	136
RESULTS	136
C. EFFECTS OF PRE-INJURY ACUTE EXERCISE ON CARDIAC FUNCTION AFTER INJURY	136
<i>C.1. Acute swimming significantly improves tissue Doppler imaging parameters after isoproterenol-induced myocardial injury</i>	<i>136</i>
<i>C.2. Chronic swimming significantly increases heart weight:body weight ratios in non-transgenic CD-1 mice</i>	<i>141</i>
CHAPTER IX.....	144
DISCUSSION.....	144
A. DISCUSSION OF OBSERVATIONS PRESENTED IN CHAPTER VI: EFFECTS OF INCREASED ENDOGENOUS FGF-2 ON MYOCARDIAL INJURY IN VIVO	144
<i>A.1. Summary of findings</i>	<i>144</i>
<i>A.2. Cellular infiltration after isoproterenol-induced myocardial injury.....</i>	<i>145</i>
<i>A.3. Increased cellular infiltration with increased FGF-2 availability.....</i>	<i>146</i>
<i>A.4. T lymphocytes and the myocardial infiltrate.....</i>	<i>147</i>
<i>A.5. T lymphocytes and the recruitment of the myocardial infiltrate.....</i>	<i>149</i>
<i>A.6. Immunosuppression and the myocardial infiltrate.....</i>	<i>150</i>
<i>A.7. FGF-2 and cardiac hypertrophy.....</i>	<i>151</i>
<i>A.8. Cellular infiltration and late-stage remodeling of the myocardium.....</i>	<i>153</i>
CHAPTER X	156
DISCUSSION.....	156
B. DISCUSSION OF OBSERVATIONS PRESENTED IN CHAPTER VII: CARDIAC FUNCTION IN FGF-2 TRANSGENIC MICE AFTER MYOCARDIAL INJURY IN VIVO.....	156
<i>B.1. Summary of findings</i>	<i>156</i>
<i>B.2. Cyclosporine A-sensitive component of FGF-2-induced beneficial effects</i>	<i>157</i>
<i>B.3. T lymphocytes in myocardial injury and repair.....</i>	<i>159</i>
<i>B.4. Cellular infiltrate and myocardial repair</i>	<i>160</i>
<i>B.5. Cyclosporine A-insensitive component of the beneficial effect of FGF-2 on recovery as assessed by tissue Doppler imaging.....</i>	<i>161</i>
<i>B.6. The isoproterenol model of injury and its functional assessment.....</i>	<i>162</i>
CHAPTER XI.....	165
DISCUSSION.....	165

C. DISCUSSION OF OBSERVATIONS PRESENTED IN CHAPTER VIII: EFFECT OF PRE-INJURY ACUTE EXERCISE ON CARDIAC FUNCTION AFTER INJURY	165
C.1. <i>Acute exercise-induced cardioprotection</i>	165
C.2. <i>The swimming protocol</i>	166
CHAPTER XII	168
DISCUSSION	168
A. FGF-2-INDUCED CARDIOPROTECTION: WHAT COULD BE HAPPENING?	168
A.1. <i>FGF-2-induced cardioprotection</i>	168
A.2. <i>Increased endogenous FGF-2-induced cardioprotection: conclusions and new hypotheses</i>	170
CHAPTER XIII	174
FUTURE DIRECTIONS	174
A. RATIONALE.....	174
B. CARDIOPROTECTION FROM INCREASED ENDOGENOUS FGF-2 LEVELS.....	174
C. EXERCISE-INDUCED CARDIOPROTECTION	178
D. FINAL THOUGHTS / RELEVANCE	180
CHAPTER XIV	182
REFERENCES	182

LIST OF FIGURES

FIGURE 1. A BRIEF FLOW CHART SHOWING THE GENERAL PROGRESSION OF CARDIAC DISEASE.	16
FIGURE 2. THE STAGES OF MYOCARDIAL HEALING.	44
FIGURE 3. DOWNSTREAM EFFECTS OF FGF-2 BINDING TO FGFR-1.	61
FIGURE 4. INCREASED FGF-2 mRNA AND PROTEIN LEVELS IN THE HOMOZYGOUS FGF-2 TRANSGENIC MICE.	84
FIGURE 5. THE CHARACTERISTICS OF ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	87
FIGURE 6. INCREASED HEART WEIGHT-TO-BODY WEIGHT RATIO WAS OBSERVED IN FGF-2 TRANSGENIC MICE 28 DAYS AFTER HIGH DOSE ISOPROTERENOL	89
FIGURE 7. TRANSGENIC MOUSE HEARTS HAVE INCREASED “LESION”-LIKE/CELLULAR INFILTRATION REGIONS IN COMPARISON WITH NON-TRANSGENIC ANIMALS AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	92
FIGURE 8. TRANSGENIC MOUSE HEARTS HAD INCREASED REGIONS OF SCARRING 6 WEEKS AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	93
FIGURE 9. QUANTITATION OF “LESION”-LIKE/CELLULAR INFILTRATION REGIONS IN THE ISOPROTERENOL-INJURED MYOCARDIUM.	95
FIGURE 10. QUANTITATION REVEALED MORE, AND SIGNIFICANTLY LARGER, “LESION”-LIKE/CELLULAR INFILTRATION AREAS IN TRANSGENIC MOUSE HEARTS COMPARED WITH NON-TRANSGENIC MICE AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	96
FIGURE 11. INCREASED INFILTRATING CELLS CORRELATES WITH NUMBER OF DAMAGED CARDIAC MYOCYTES IN FGF-2 TRANSGENIC MICE	100
FIGURE 12. INITIAL ASSESSMENT OF CELLULAR INFILTRATE OF HEARTS FROM MICE GIVEN HIGH-DOSE ISOPROTERENOL REVEALED THE PRESENCE OF IMMUNE CELLS.	101
FIGURE 13. ASSESSMENT OF THE CELLULAR INFILTRATE OF HEARTS FROM MICE GIVEN HIGH-DOSE ISOPROTERENOL REVEALED CD3-POSITIVE CELLS.	102
FIGURE 14. CELLS CO-EXPRESSING THE T CELL MARKER CD4 AND THE FGF-2 RECEPTOR FGFR-1 ARE IDENTIFIED WITHIN THE MYOCARDIAL INFILTRATE.	104
FIGURE 15. HAEMATOXYLIN AND EOSIN-STAINED SECTIONS OF HEARTS TREATED WITH IMMUNOSUPPRESSIVE AGENTS SHOW DECREASED CELLULAR INFILTRATION.	107
FIGURE 16. EFFECT OF IMMUNOSUPPRESSION ON THE DEGREE OF CELLULAR INFILTRATION AND “LESION”-LIKE AREAS.	108
FIGURE 17. HETEROZYGOUS FGF-2 TRANSGENIC MICE HAVE INCREASED CARDIAC FGF-2 RNA LEVELS COMPARED TO NON-TRANSGENIC MICE.	113
FIGURE 18. FGF-2 PROTEIN LEVELS ARE SIGNIFICANTLY HIGHER IN THE HETEROZYGOTE FGF-2 TRANSGENIC MOUSE HEART WHEN COMPARED TO WILD TYPE CD-1 MICE.	114
FIGURE 19. HETEROZYGOUS FGF-2 TRANSGENIC MICE ALSO HAVE GREATER CELLULAR INFILTRATION THAN THEIR NON-TRANSGENIC COUNTERPARTS.	116
FIGURE 20. NO DIFFERENCE IN EJECTION FRACTION WAS OBSERVED AFTER ISOPROTERENOL-ADMINISTRATION AS MEASURED BY M-MODE ECHOCARDIOGRAPHY.	120
FIGURE 21. NO DIFFERENCE IN FRACTIONAL SHORTENING WAS OBSERVED AFTER ISOPROTERENOL-ADMINISTRATION AS MEASURED BY M MODE ECHOCARDIOGRAPHY.	121
FIGURE 22. A SIGNIFICANT DECLINE IN CARDIAC FUNCTION AS ASSESSED BY OF TISSUE DOPPLER IMAGING WAS OBSERVED 24 HOURS AFTER HIGH-DOSE ISOPROTERENOL ADMINISTRATION.	122
FIGURE 23. REPRESENTATIVE TISSUE DOPPLER TRACINGS OF FGF-2 TRANSGENIC AND NON-TRANSGENIC MOUSE HEARTS.	123
FIGURE 24. MICE OVEREXPRESSING FGF-2 SHOW IMPROVED RECOVERY OF MYOCYTE FUNCTION AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	124
FIGURE 25. MICE OVEREXPRESSING FGF-2 CONTINUE TO DISPLAY IMPROVED RECOVERY OF ENDOCARDIAL VELOCITY AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY IN THE PRESENCE OF CYCLOSPORINE A (IMMUNOSUPPRESSIVE) TREATMENT	127
FIGURE 26. CYCLOSPORINE A DECREASES CARDIAC FUNCTION IN TRANSGENIC MICE OVEREXPRESSING FGF-2 AND SUBJECTED TO ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	128

FIGURE 27. CYCLOSPORINE A DOES NOT AFFECT TISSUE DOPPLER INDICES AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY IN NON-TRANSGENIC MICE	129
FIGURE 28. MICE OVEREXPRESSING FGF-2 CONTINUE TO DISPLAY IMPROVED RECOVERY OF ENDOCARDIAL VELOCITY AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY IN THE PRESENCE OF IMMUNOSUPPRESSIVE TREATMENT USING ANTIBODIES AGAINST CD3 ϵ	132
FIGURE 29. ANTI-CD3 ϵ ANTIBODY TREATMENT HAS NO SIGNIFICANT EFFECT ON CARDIAC FUNCTION IN TRANSGENIC MICE OVEREXPRESSING FGF-2 AND SUBJECTED TO ISOPROTERENOL-INDUCED MYOCARDIAL INJURY.	133
FIGURE 30. ANTI-CD3 ϵ ANTIBODY TREATMENT A DOES NOT AFFECT TISSUE DOPPLER INDICES AFTER ISOPROTERENOL-INDUCED MYOCARDIAL INJURY IN NON-TRANSGENIC MICE.....	134
FIGURE 31. IMAGE OF MICE SWIMMING IN A WATER BATH.	138
FIGURE 32. LEFT VENTRICULAR ENDOCARDIAL VELOCITY VALUES PREDICT SIGNIFICANT IMPROVEMENT IN CARDIAC FUNCTION IN THE EXERCISE GROUP UP TO 4 WEEKS POST-INJURY.	139
FIGURE 33. STRAIN RATE PREDICT SIGNIFICANT IMPROVEMENT IN CARDIAC FUNCTION IN THE EXERCISE GROUP UP TO 4 WEEKS POST-INJURY.	140
FIGURE 34. CHRONIC SWIMMING SIGNIFICANTLY INCREASED HEART WEIGHT-TO-BODY WEIGHT RATIOS OF MICE.	142

LIST OF TABLES

TABLE 1. OLIGONUCLEOTIDE SEQUENCES USED	81
TABLE 2. M-MODE ECHOCARDIOGRAPHY VALUES OF FGF-2 TRANSGENIC AND NON-TRANSGENIC MICE BEFORE AND AFTER HIGH-DOSE ISOPROTERENOL ADMINISTRATION	119

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED

IMAGES FROM JTA MEIJ, F SHEIKH, SK JIMENEZ, PW NICKERSON, E KARDAMI, AND PA CATTINI.
EXACERBATION OF MYOCARDIAL INJURY IN TRANSGENIC MICE OVEREXPRESSING FGF-2 IS T CELL
DEPENDENT. *AM J PHYSIOL HEART CIRC PHYSIOL* 2002 282: H547-H555.

FIGURE 6.....	89
FIGURE 10.....	96
FIGURE 11.....	100
FIGURE 12.....	101
FIGURE 13.....	102
FIGURE 14.....	104
FIGURE 16.....	108

CHAPTER I

INTRODUCTION

A. BACKGROUND

Cardiac disease claims over 7 million people annually and is a major killer worldwide [World Health Organization. The top 10 causes of death., 2008]. It is the second leading cause of death in Canada [Statistics Canada. Table 102-0563 - Leading causes of death, total population, by sex, Canada, provinces and territories, annual., 2005] but remains the number one killer in all industrialized nations combined [World Health Organization. The top 10 causes of death., 2008]. The World Health Organization predicts that by the year 2030, 23.6 million people will die annually of cardiovascular disease. A majority of deaths due to cardiovascular disease will likely occur following prolonged disease progression, with increasing patient discomfort and requirement of support systems. With a large percentage of the population rapidly increasing in age, cardiovascular disease will become a greater burden not only to the patient and their family, but also on Canada's health care spending. Although many therapies and strategies are currently in place to try to minimize the effects of cardiovascular disease on the patient, these are costly and may not be available to everyone. Judging by the numbers of deaths annually, it is clear that current therapies are not adequate to significantly deter cardiovascular disease mortality. Thus, it is important to increase our understanding of the mechanisms underlying cardiac health and disease, as well as cardiac protection from, and repair after, injury.

Death from cardiovascular disease can occur acutely due to massive cardiac injury, or can result following progressive injury and maladaptive remodeling when the heart can no longer sustain its workload. Most deaths by cardiovascular disease occur in the adult and aging populations, although congenital and hereditary heart diseases can also result in early mortality. Cardiac injury in the adult heart is accompanied by functional tissue loss, which is practically irreversible because of limited innate regenerative potential. Rather, the heart overcomes tissue loss by “repair” consisting of scar-formation. After tissue loss, the remaining viable myocardium compensates by a remodeling process that includes an increase in myocyte size by hypertrophy, accompanied by reactive fibrosis. Beyond a certain potential for functional compensation, the heart transitions to a decompensated state and heart failure.

Interestingly, an endogenous protein with potent cytoprotective and cardioprotective properties, fibroblast growth factor 2 (FGF-2), has been shown to exert beneficial effects at the different stages of the cardiac injury-repair response. This thesis focuses on further understanding the relationship between FGF-2 and the cardiac response to injury so that, potentially, this factor can be used to treat or prevent heart disease.

In the introductory section, a brief background will be provided on the processes of ischemic heart disease, myocardial injury and repair, heart failure, experimental

models of cardiovascular injury, the biology of FGF-2 in the heart, and the rationale for the presented studies.

B. ISCHEMIC HEART DISEASE AND HEART FAILURE

Ischemic heart disease, myocardial infarction and the ensuing heart failure represent a major component of cardiovascular disease. A generalized schematic of how ischemic cardiac disease develops is presented in Figure 1 (modified from Concise Pathology 3rd edition; www.accessmedicine.com). The chart has been divided into sections by dashed lines to show stages that (1) lead to cardiac injury, (2) occur after injury but remains at a stage in which recovery is still possible, and (3) stages where death becomes inevitable. Clearly, intervention at stages 1 and 2 are essential. At the point when the stage advances from (1) to (2) (yellow dashed line, Figure 1), strategies of prevention and cardioprotection can circumvent or at least limit damage; while within stage (2) (before red dashed line, Figure 1), stimulation of repair and potentially regeneration are important.

Many different factors can lead to the development of ischemic cardiac disease, as shown in Figure 1. Here, the stages that lead to cardiac injury (usually myocardial infarction) and the events that occur afterwards are briefly described.

B.1. Atherosclerosis

Atherosclerosis is the most common root cause for ischemia and myocardial infarction in humans; it is estimated that approximately 50% of all deaths from cardiovascular disease can be attributed to some association with atheroma formation [Lloyd-Jones *et al*, 2009]. Atherosclerosis manifests itself in the formation of a calcified plaque in arteries, and can elicit the most damage when its development occurs in the arteries supplying the heart and the brain. Atherosclerotic disease also includes plaque formation of grafted veins, organs, or those resulting from restenosis of vessels that have undergone angioplasty and stenting [Ashley and Niebauer, 2004; Fuster *et al*, 2008]. Atherosclerotic plaques likely begin with the formation of “fatty streaks”, the first visible lesions on the artery (resembling fat, although not made up of adipose tissue), composed of macrophages and other cell types, which tend to become more prone to the uptake of lipids and cholesterol from the bloodstream. Macrophages begin ingesting lipids and cholesterol in the intimal layers of the arteries, forming what are called “foam cells” (they resemble foam under the microscope due to the fat deposits). Atheroma development appears to involve the migration and turnover of the cells of the blood vessel (endothelial cells, smooth muscle cells) as well as those of the immune system. Progression of plaque formation has also been shown to involve the accumulation of oxidized cholesterol and other lipids within the transformed cells, eventually forming a hard, calcified plaque leading to a decreased diameter of the artery lumen [Sherwood, 1993]. Complex processes involving multiple cell types (macrophages, smooth muscle cells, endothelial cells, and other members of the immune system) contribute to the enlargement of the

plaque, which with continued lipid accumulation, results in the disorganization of the endothelial cells lining the vessel lumen, consequently causing its decreased stability.

B.2. Occlusion of blood vessels supplying the heart

Blockage of critical arteries supplying the heart tissue with blood can result from either near or total occlusion of the vessel by either a thrombus or an embolus formation. A thrombus is a blood clot of platelets and other immune cells activated at the area of atherosclerotic plaque; this mass of cells can cause occlusion of the offending vessel. On the other hand, an embolus is a thrombus that has detached from the vessel wall, which continues to move with the blood flow until it reaches a vessel area too narrow, where it can cause an occlusion. The narrowing of the coronary vessels (blood vessels which supply the myocardium with blood) may also stimulate several critical events in myocardial injury: (1) blood flow changes causing increased shear stress, subsequently leading to more susceptible endothelial lining for formation of more thrombi, and (2) myocardial ischemia, whereby tissues receive insufficient oxygen and nutrients.

B.3. Ischemic heart disease

Ischemic heart disease (myocardial ischemia) is the condition in which cardiac tissue demand for oxygen exceeds supply. Inadequate oxygen (hypoxia) is minimally tolerated by cardiac myocytes, as contraction requires considerable amounts of ATP acquired mainly through aerobic fatty acid metabolism [Fuster *et al*, 2008; Opie, 2004].

Anaerobic metabolism through glycolysis allows survival of cardiac myocytes during brief episodes of ischemia, however, prolonged ischemic events result in irreversible damage [Webster *et al*, 1993]. Chronic ischemia, when mild, can lead to angina pectoris, or a state in which a patient begins to feel chest discomfort. Severe acute ischemia (myocardial infarction=heart attack) is usually accompanied by myocardial tissue loss. There is a direct relationship between extent of initial myocardial infarction and severity of prognosis [Pfeffer *et al*, 1984; Wilhelmsson *et al*, 1981].

B.4. Myocardial infarction and subsequent repair

Survivable infarcts are characterized by recovery with fibrotic scar formation. Myocardial infarction leads to immune cell infiltration and remodelling with deposition of a collagen-rich fibrous scar (Figure 2). To compensate for tissue loss and increased workload, the remaining viable myocardium undergoes an increase in cardiomyocyte mass and size (hypertrophy). Mechanisms governing cardiac and cardiomyocyte hypertrophy continue to be the subject of intense investigation by many groups, because cardiac hypertrophy is a very important predictor and determinant of heart failure [Artham *et al*, 2009]. The initial reparative scar formation is beneficial for keeping the integrity of the ventricle wall and thus preventing cardiac rupture. Compensatory hypertrophy sustains the function of the cardiac pump. Eventually, however, the continuous activation of non-myocytes/fibroblasts (reactive fibrosis), prolongation/exaggeration of the hypertrophic response by cardiomyocytes, as well as

additional cardiomyocyte cell death, create a vicious cycle that leads to heart failure [Takano *et al*, 2003].

B.5. Heart failure

Heart failure is the clinical syndrome in which a patient's heart cannot cope with the workload required to adequately perfuse all tissues of the body. This can stem from the death of cardiac myocytes that result in disorganized conduction patterns (arrhythmias) preventing the proper synchronized contractions of the heart. Heart failure can also result from the progression of cardiac remodeling from a compensatory, adaptive state to a decompensated maladaptive state. The events characterizing this transition are incompletely understood, and as such, the point at which cardiac hypertrophy progresses from a compensatory to a decompensated maladaptive state remains difficult to predict. It is important to note that constant pressure or volume overload of the heart, without an acute myocardial infarction event, can also lead to heart failure. The mechanisms of heart failure are complex and multi-factorial. For a detailed discussion of how heart failure develops and progresses the reader is directed to Braunwald's Heart Disease [Braunwald, 2008].

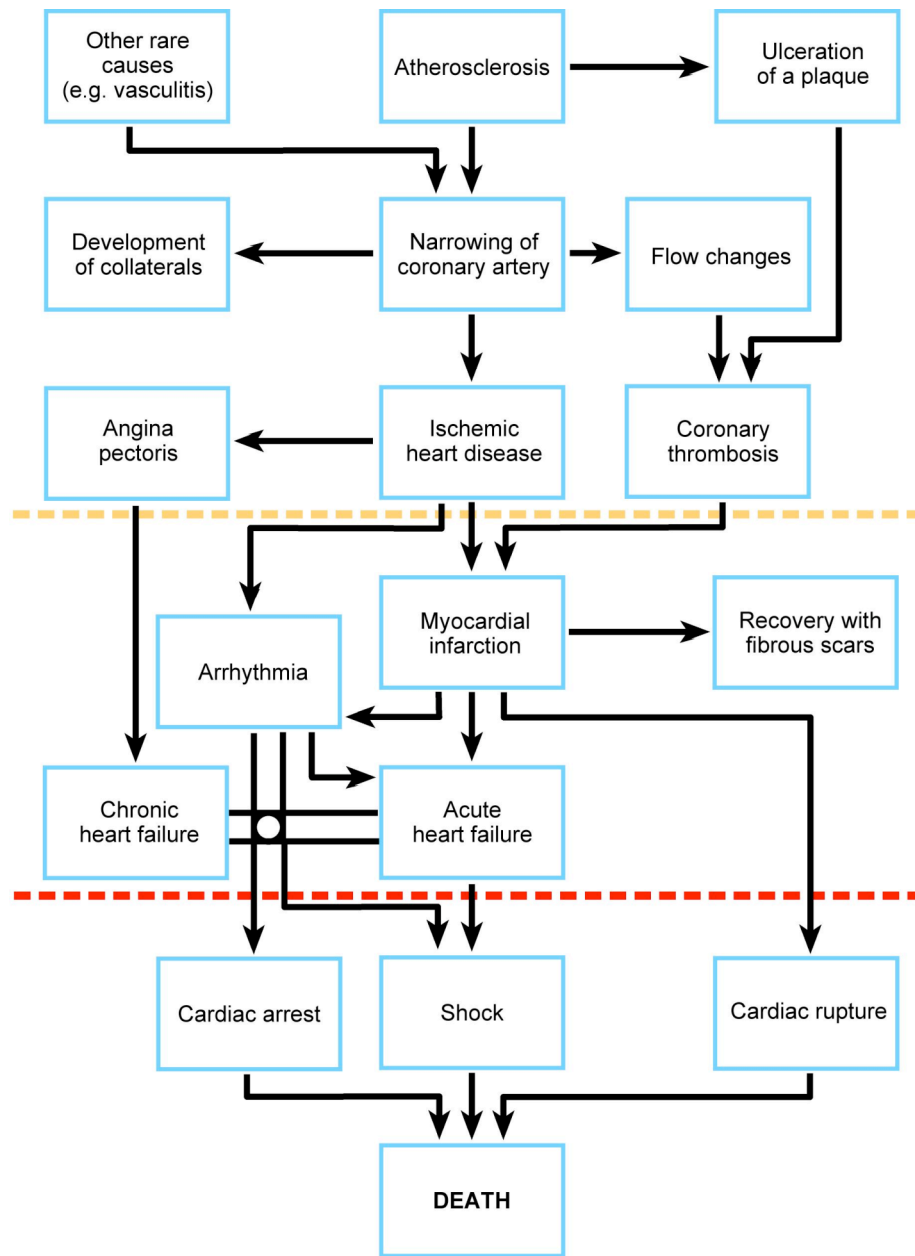


Figure 1. A brief flow chart showing the general progression of cardiac disease.

The boxes above the yellow dashed line describe the events that occur prior to irreversible cardiac damage. The transition at the yellow line indicates occurrence of cell death leading to irreversible cardiac damage, usually myocardial infarction. The boxes below the yellow dashed lines are events resulting from the injury, where positive remodeling is still possible. Events below the red dashed line describe events where death becomes inevitable. Based on a figure by P Chandrasoma and CR Taylor, Concise Pathology 3rd edition: <http://www.accessmedicine.com>.

C. RISK FACTORS FOR CARDIOVASCULAR DISEASE

Several factors are known to increase the risk for cardiovascular disease, with many factors that can be controlled. Some of the major risk factors that cannot be controlled include increasing age, gender (males), and genetic predisposition to certain clinical conditions. Additionally, various clinical conditions increase the risk for the development of cardiovascular disease including diabetes, obesity, and hypertension. Risk factors that stem from lifestyle choices include excessive tobacco consumption, lack of physical activity, not maintaining a 'healthy' body mass index, as well as dietary choices (leading to high blood cholesterol). Other factors that may contribute to risk of heart disease include stress and excessive alcohol consumption [Klatsky, 2009; Scherrer *et al*, 2010].

C.1. Aging

There is a direct relationship between age and risk for cardiovascular disease [Shim, 2010]. Contributing to this condition is the increased damage to tissues, for example, those resulting from telomere shortening due to repetitive cell division [Huzen *et al*, 2010]. Such events contribute to increased likelihood of developing disease conditions such as diabetes and hypertension which in turn increases the risk for other debilitating diseases often associated with aging, including cardiovascular disease [Huzen *et al*, 2010].

C.2. Gender

It is thought that cardiovascular disease claims more men than women [YY Tan *et al*, 2010]. This disparity is more pronounced pre-menopause; after menopause, female deaths from cardiovascular disease increase to levels closer to males [Atsma *et al*, 2006; YY Tan *et al*, 2010]. This observation has then led to the idea that estrogen plays a protective effect on the heart. However, recent studies have shown that hormone replacement therapies do not decrease risk for cardiovascular disease [Penckofer *et al*, 2003]. Another possible reason for the increased risk in men lies in the differences in cardiac development. Ventricular mass is different between males and females even as children [de Simone *et al*, 1995]. Additionally, as males tend to grow taller and generally larger than females, cardiac workload is also increased, presenting higher potential for the development of maladaptive diseases.

C.3. Genetic predispositions

Although members of the same family are likely to participate in similar lifestyles, studies of twins have shown a genetic component to increased cardiovascular risk outside of behavioral influences [Gluba *et al*, 2009; Su *et al*, 2010]. Certain mutations and polymorphisms have been shown to be able to increase the likelihood of a person developing cardiovascular disease. Some of these genes include those that can affect lipid and cholesterol formation and metabolism, the renin-angiotensin system, and the expression and function of adrenergic receptors (Recently reviewed in [Gluba *et al*, 2009]). Such genetic mutations leading to increased risk show clearly that the maintenance of cardiovascular health depends on the maintenance of a delicate balance of several different systems in the body functioning properly.

C.4. Diabetes, Hypertension, Obesity

Conditions and diseases that can be loosely classified under the category of “metabolic diseases” contribute to increased risk of cardiovascular disease [Mehta, 2010]. The underlying reasons for the increased risk for cardiovascular disease overlap greatly in obesity, hypertension, and diabetes, although each may have distinctly different root causes.

Diabetes is the disease whereby the patient has high concentrations of sugar in their blood [Sherwood, 1993]. This condition stems from inadequate insulin production or from deficiencies in the insulin pathway, affecting glucose uptake. Any condition that affects the body’s metabolic processes increases the risk for cardiovascular disease; diabetes mellitus is no exception. Complications from diabetes can contribute to diminished cardiac function, as well as increase the risk for myocardial injury, for example, from the increased likelihood of developing other conditions such as hypertension [Mehta, 2010].

Hypertension is the condition whereby blood pressure is chronically increased [Sherwood, 1993]. This can result from genetics, and can be caused and/or exacerbated by the maintenance of poor dietary choices. Its effects include the dysregulation of vascular control, through alterations in multiple players including the renin-angiotensin system and the natriuretic peptides. Hypertension also increases the risk for cardiovascular disease as increased blood pressure causes increased workload for the

heart, which can result in compensatory cardiac hypertrophy. In addition, it can stimulate and/or exacerbate atherosclerosis: the increase in pressure against the blood vessels increases turbulence which, in turn, can disturb pre-existing atherosclerosis, resulting in further development or the disturbance of unstable plaques [Insull, 2009]. This condition's causes and effects greatly overlap with those of diabetes and obesity; contributors to its development include keeping a sedentary lifestyle as well as poor dietary choices (in the case of hypertension, excess intake of anti-diuretics such as salt) [Fuster *et al*, 2008; Mathieu *et al*, 2010; Mehta, 2010].

Obesity is defined as the condition of having a body mass index ≥ 30 [Lenz *et al*, 2009]. As with the other metabolic conditions mentioned previously, this condition results commonly from unchecked eating habits as well as the maintenance of an inactive lifestyle. The effects of excess visceral fat deposition results in the alteration of key regulators of metabolism including insulin resistance [Mathieu *et al*, 2010]. Additionally, increased body weight can increase vascular resistance thereby resulting in hypertension [Mehta, 2010].

The development of metabolic diseases result in changes in gene expression, lipid metabolism, as well as changes in inflammatory biology [Mathieu *et al*, 2010; Mehta, 2010]. Obesity, hypertension, and diabetes all fall under the category of “metabolic diseases” as they are conditions that usually result from poor dietary and lifestyle (level of activity) choices. Thus, patients usually present clinically with two or more of these

conditions. These risk factors for cardiovascular disease can clearly be “controlled” by limiting the progression of these conditions with improved lifestyle choices.

C.5. Exercise

Increased physical activity is known to be beneficial not just for one’s cardiovascular health, but for maintaining a healthy lifestyle in general. Some possible mechanisms for how exercise contributes to health include the maintenance of a healthy weight, improved glucose metabolism, and increased angiogenesis [Breen *et al*, 1996; Marini *et al*, 2008; Olfert *et al*, 2001]. Exercise decreases the risk for developing obesity, hypertension, and diabetes, consequently lowering the risk for cardiovascular disease. Other possible benefits of exercise in cardiovascular health have also been proposed, including direct effects on the cardiac myocytes, by upregulation of protective antioxidants, or direct effects on ion channels [Adlam *et al*, 2005; Demirel *et al*, 2001; Powers *et al*, 2008]. Although the exact mechanism for protection by exercise has not been fully elucidated, some proposed mechanisms will be discussed in later chapters. Nevertheless, there remains no question that exercise is beneficial to one’s health, cardiovascular or otherwise.

C.6. Stress

Increased stress has also been shown to increase the risk of cardiovascular disease. In addition to suspected stress-related myocardial events (may be stimulated by catecholamine/adrenergic stimulation), stress has been documented to stimulate changes

in the expression profiles of several genes including the pro-hypertrophic early growth response transcription factor (Egr-1) and the vasoconstricting peptide endothelin-1 (Reviewed in [Kloner, 2006]). Upregulation of these genes have been demonstrated in hypertrophied hearts, and may contribute to the increased risk of cardiovascular events [Khachigian, 2006; Zou *et al*, 2002].

C.7. Tobacco and/or cigarette smoking

Smoking has long been established to significantly increase the risk of cardiovascular disease [Capewell *et al*, 2010; Prasad *et al*, 2009]. Its effects are multifactorial, resulting from the multitude of chemicals found in cigarette smoke, as well as other effects including stimulation of free radical formation. These chemicals have been established to have detrimental effects on the vasculature, especially the stability of endothelial cells lining the vessels [Benowitz, 2003]. Some of the vascular effects that have been rigorously studied include the promotion of atherogenesis, development of aneurysms, and the increase of rupture of unstable plaques [Ambrose and Barua, 2004; Jonas *et al*, 1992]. Direct effects of smoking in the heart have also been documented (Reviewed in [Minicucci *et al*, 2009]). Although all the factors that contribute to the increased risk have not been fully elucidated, studies whereby smoking cessation has significantly decreased risk of cardiovascular events have definitively shown that smoking has significant consequences on cardiovascular health [Benowitz, 2003; Burns, 2003; Jonas *et al*, 1992].

D. CAVEAT

Life style changes have been shown to be effective in preventing the development and/or worsening of ischemic heart disease. These include regular cardio-intensive exercise, maintenance of healthy weight, and smoking cessation. There is an ongoing effort accompanied by significant spending aimed at increasing public awareness regarding the benefits of appropriate diet, exercise, and lifestyles in an attempt to improve quality of life, to decrease mortality and also decrease health care costs from heart disease [Lichtenstein *et al*, 2006; Tobacco Free Initiative]. However, the percentage of the North American population with a BMI ≥ 30 (obese) has not significantly declined, and cardiovascular disease remains the number one killer worldwide. Clearly, methods of prevention, and even post-injury interventions, are currently inadequate.

While there is no doubt that prevention is the first line of defense in dealing with ischemic heart disease, it is also evident that the improvement and expansion of available treatments and strategies are essential. Furthermore, understanding the mechanisms that govern cardioprotection from approaches that decrease risk (as described above) could contribute to promoting protection in populations whereby the lifestyle change is not feasible or realistic; for example, vigorous exercise for elderly or handicapped persons. Thus, continued research to further our understanding, and subsequently, the development (and promotion) of protective interventions that could contribute to improved cardiac outcomes, are urgently needed.

CHAPTER II

RODENT MODELS OF MYOCARDIAL INJURY

Several experimental models of myocardial injury *in vivo* have been developed for use in cardiovascular research. Amongst these, rat and mouse models have been and are being used extensively to simulate myocardial infarction-induced ischemic injury by: (1) surgically-induced coronary artery ligation, and (2) by high-dose isoproterenol administration

A. CORONARY LIGATION SURGERY

Coronary ligation surgery is designed to generate a wound similar to one resulting from myocardial infarction [Dixon *et al*, 1990; Pfeffer *et al*, 1984]. It is an invasive procedure involving sterile techniques in an open-heart surgery. During this surgery, the chest cavity is opened to expose the heart where a suture is tied around the left anterior descending (LAD) coronary artery. After successful ligation of the artery, the chest cavity is evacuated and sutured and the animal is allowed to recover. The tying of the artery significantly reduces or completely blocks flow of blood into the tissues supplied by that artery, resulting in ischemia and eventually, infarction (first reviewed in [WT Porter, 1893], and most recently in [Kolk *et al*, 2009]). There are some disadvantages in using this model including a requirement for advanced surgical skills, and expensive equipment such as respirators for small rodents, and the need for experienced technical assistance. Undoubtedly, the invasiveness of the procedure not only influences the response to the myocardial injury, but also to the chest wall injury. In addition, the

requirement of anaesthetics for the procedure introduces another variable that can influence cardiac repair response [TP Tan *et al*, 2003].

B. ISOPROTERENOL MODEL OF INJURY

To address the questions involving myocardial injury in mouse *in vivo*, the high dose isoproterenol method of injury was used. Isoproterenol (1-(3,4-dehydroxyphenyl)-2-isopropylaminoethanol hydrochloride) is a synthetic catecholamine that elicits its effects through the β -adrenergic receptors [Klein *et al*, 2001]. The isoproterenol model of injury has been widely used and characterized since the 1960's [Anderson *et al*, 2008; Beznak, 1962; Beznak and Hacker, 1964; Ferrans *et al*, 1964; Kahn *et al*, 1969; Lehr, 1972; Rona *et al*, 1959; Rona *et al*, 1963; Teerlink *et al*, 1994; Zbinden and Moe, 1969]. High dose isoproterenol, whether delivered by multiple infusions or one bolus, has been shown to result in myocardial damage/necrosis (lesions) very similar to that caused by a myocardial infarction [Ferrans *et al*, 1964; Teerlink *et al*, 1994], especially those caused by emotional and social stress [Sanchez *et al*, 2002]. Ease of delivery plus limited invasiveness and complications resulting from a thoracotomy, has led to the widespread use of the high-dose isoproterenol model of injury as an important tool in cardiovascular research.

Catecholamines are compounds bearing a catechol ring (six-sided carbon ring with two adjacent hydroxyl groups), which play a major role in the sympathetic nervous system [Vander, 1994]. They act as neurotransmitters or hormones, asserting their effect through adrenergic receptors and second messengers in many different cell types. Two

types of sympathetic adrenergic receptors have been classified: α -adrenergic receptors – generally involved in the control of arteriolar tone, and β receptors – which affect heart rate and inotropy (or force of muscle contraction) [Opie, 2004]. Both groups of receptors are subsequently broken down into further subtypes: α_1 , α_2 , β_1 (predominantly cardiac), β_2 (usually extracardiac), and β_3 (predominantly adipose). Adrenergic stimulation is cell type specific, therefore the systemic effect observed after one stimulus is the collective effect involving multiple system effects including those that negate each other. The effect of stimulation of the sympathetic nervous system is multi-factorial and is not yet fully understood. For example, naturally occurring catecholamines epinephrine and norepinephrine are both involved in the *fight-or-flight* response. Both can stimulate α as well as β receptors, but stimulation of epinephrine on α -adrenergic and β_2 receptors are greater than that of norepinephrine (a potent α -adrenergic agonist), whereas they have similar efficacies on the β_1 receptors [Brunton, 2006]. Thus, upon exercise, when epinephrine is released into the bloodstream an increase in cardiac output and peripheral blood flow ensues despite α -adrenergic stimulation causing constriction of peripheral blood vessels as the β -adrenergic stimulation constitutes a greater effect [Opie, 2004].

The effect of isoproterenol on contraction is mediated by the second messenger cyclic adenosine monophosphate (cAMP) and protein kinase A. Briefly, isoproterenol acts through β -adrenergic receptors that subsequently couple with stimulatory G protein to activate adenylyl cyclase. Activated adenylyl cyclase then converts adenosine triphosphate (ATP) to cAMP, mediating an increase in Ca^{2+} influx through protein kinase

A activation. Increased Ca^{2+} influx then increases inotropy and heart rate [Klabunde, 2005; Opie, 2004]; it can also promote cell death.

B.1. Proposed mechanisms for isoproterenol-induced myocardial injury

Isoproterenol is believed to cause myocardial damage through transient ischemic events. Beta-adrenergic stimulation in the myocardium increases heart rate and force of contraction, thereby increasing its oxygen requirement [Klabunde, 2005; Opie, 2004]. At the same time, isoproterenol stimulates vasculature vasodilation, resulting in decreased blood flow to the heart. The combined effect on the myocardium and the vasculature induces myocardial ischemic events that result in focal lesions similar in wound morphology to that resulting from the blockage of coronary vessels [Ferrans *et al*, 1964; Teerlink *et al*, 1994].

In addition to the effects on oxygen demand of the heart, evidence has also been reported whereby isoproterenol causes myocardial injury via cleavage of the protein dystrophin [Campos *et al*, 2008; Toyo-Oka *et al*, 2004]. Dystrophin is a membrane-associated protein found within muscle cells (including cardiac myocytes), responsible for linking intracellular actin to the external ‘anchor’ basement membrane protein laminin. Cell attachment to extracellular matrix contributes to cell and membrane stability [Deconinck and Dan, 2007; Hein *et al*, 2000; Kawada *et al*, 2005; Toyo-Oka *et al*, 2004]. Phenotype and other data gathered from studies of muscular dystrophy, the disease presenting upon mutation of the dystrophin gene, have revealed the importance of functional dystrophin in cell stability [Deconinck and Dan, 2007; Kawada *et al*, 2005;

McNally, 2007]. Cell death from dystrophin cleavage ultimately results from an increase in intracellular Ca^{2+} current, causing an imbalance in the ionic gradient leading to arrhythmic complications and apoptosis [Deconinck and Dan, 2007; Hein *et al*, 2000; Iwata *et al*, 2003; Kawada *et al*, 2005; McNally, 2007; Toyo-Oka *et al*, 2004; Yasuda *et al*, 2005]. This increase in Ca^{2+} influx results from sarcolemmal damage due to instability from insufficient basement membrane anchoring. Although normal cell repair occurs by vesicular exocytosis of membrane components, dystrophic cells undergo significantly increased damage from shear stress and cannot adequately sustain replacement of membrane components [Deconinck and Dan, 2007; Yasuda *et al*, 2005].

Whether this isoproterenol-induced increase in dystrophin cleavage is a direct or indirect effect is currently unknown. Rossi and colleagues have shown increased susceptibility of dystrophin to breakdown after isoproterenol administration, subsequently resulting in the breakdown of the other components of the dystrophin-to-laminin connection [Campos *et al*, 2008]. However, this susceptibility does not definitively implicate isoproterenol in direct dystrophin cleavage, and could be a downstream effect. Furthermore, another group has demonstrated that dystrophin cleavage occurs in both isoproterenol-induced and in coronary ligation-induced heart failure models, suggesting that this event is a downstream consequence of increased Ca^{2+} influx and subsequent calpain activation [Toyo-Oka *et al*, 2004].

CHAPTER III

MYOCARDIAL INJURY AND REPAIR

A. INTRODUCTION

The development of new strategies to promote better cardiac health requires improved understanding of the mechanisms that govern cardiac injury and its repair response. Here, the events surrounding myocardial injury and its subsequent repair are briefly described.

B. MYOCARDIAL INJURY

As discussed in the previous section, a primary reason for the development of heart failure results from myocardial injury stemming from irreversible damage to the heart due to the loss of the functional contractile units of the heart, the cardiac myocytes. The ability of adult heart to replace lost tissue by regeneration is limited and insufficient to replace large infarcts. Thus, cardiac repair essentially consists of a remodelling process that includes scar formation and which eventually leads to heart failure. The consequence of inadequate repair mechanisms in the heart contributes greatly to the prevalence of cardiac failure and mortality worldwide [Anversa *et al*, 1991; Beltrami *et al*, 2003; Beltrami *et al*, 2001; Kajstura *et al*, 2005; Lloyd-Jones *et al*, 2009; Quaini *et al*, 1994].

Cardiac myocytes contract ~70x in one minute; this constant activity requires a significant amount of ATP production to sustain cross-bridge cycling causing muscle contraction [Fuster *et al*, 2008; Opie, 2004; Sherwood, 1993]. Under normal physiological conditions, the cardiac myocyte produces energy primarily through fatty acid metabolism, which requires a constant supply of oxygen and fuel (fatty acids or glucose) molecules through the blood. When the blood and oxygen supply is compromised, the cardiac myocyte can initially compensate by decreasing contraction and switching to glucose metabolism via glycolysis to generate energy. Anaerobic metabolism cannot, however, produce sufficient energy metabolites to maintain work required of the heart. Therefore, severe or prolonged ischemia results in irreversible cardiac myocyte death [Opie, 2004; Ross, 1991].

As previously discussed, the majority of deaths from myocardial damage not resulting from genetic predispositions are due to atherosclerosis-related occlusions of a blood vessel feeding the myocardium. In addition to the physical occlusion of a blood vessel, non-obstructive myocardial infarctions can also occur; vasospasms and arrhythmias can result in the inadequate supply of blood to the myocardium. Evidence for increased catecholamine concentrations in the bloodstream of patients with large infarcts suggest that β -adrenergic stimulation contributes to the etiology of ischemic heart disease and myocardial infarction [Riles *et al*, 1993; Willerson and Buja, 1988]. It has been documented that cardiac patients can present symptoms of ischemia after intense physical or emotional exertion, supporting a role for catecholamines amongst possible triggers of an ischemic event. It is possible that intense adrenergic stimulus can trigger

plaque rupture leading to coronary thrombosis resulting in the physical blockage of a blood vessel, initiating an ischemic episode. In addition, catecholamine-induced arrhythmias or vasospasms have also been suspected to occur, similar to, if not a contributing cause, drug-induced infarctions such as those caused by cocaine abuse [Bacaner *et al*, 2004; Hollander and Hoffman, 1992].

To understand the molecular events that can lead to ischemic damage, an appreciation of the mechanisms required for its proper function is important. The exact changes (and timing) that occur at the tissue and molecular levels in the event of myocardial ischemia and infarction have not been fully elucidated, and likely vary depending on the circumstances. However, it is known that ischemia negatively affects the components of the cell structure, the cardiac myocyte ATP generation machinery, and its calcium handling system -- ultimately leading to the dysregulation of the fundamental balance of ionic gradients required for the proper function of the cardiac myocyte.

The major consequences of ischemia are (1) poor oxygen delivery, and (2) poor washout of metabolic by-products [Opie, 2004]. Poor oxygen delivery causes inadequate ATP production, which leads to multiple inadequacies in maintaining energy-dependent mechanisms including sodium pumps (resulting in increased Na^+ and depleted K^+ concentrations, inevitably leading to impaired electrochemical gradients), and Ca^{2+} pumps (leading to increased internal Ca^{2+} concentrations causing increased rate and force of contractions). Ion imbalance leads to arrhythmias and increased intracellular calcium causing the opening of the mitochondrial permeability transition (MPT) pore. Opening

of the MPT pore results in cytochrome C release, as well as ion influx into the mitochondria, further disturbing the delicate balance of electrochemical gradients between mitochondrial membranes. All these events contribute towards the development of cardiac myocyte cell death by apoptosis or necrosis [Opie, 2004].

C. CARDIAC RESPONSE TO INJURY

C.1. Inflammatory response and myocardial repair

Similar to classical wound healing observed in other tissues, the response to myocardial cell death and injury also involves the infiltration of immune cells and subsequent remodelling. The necessity of the inflammatory response in cardiac repair was discovered when patients undergoing anti-inflammatory treatment with methylprednisolone suffered from cardiac rupture [Roberts *et al*, 1976]. Further studies to support this finding showed that anti-inflammatory treatments result in a worse cardiac outcome resulting in increased infarct size and depressed cardiac function [Garcia *et al*, 2007; Hammerman *et al*, 1983; Mannisi *et al*, 1987]. However, evidence to contradict the unfavourable results has also been documented: treatment with steroids or other inhibitors of inflammation resulted in decreased infarct size and improved cardiac function [Kanzaki *et al*, 2001; Libby *et al*, 1973; X Liu *et al*, 2006; Spath *et al*, 1974; Valen *et al*, 2000]. Contradicting results of gross anti-inflammatory treatment suggest that certain inflammatory conditions likely can lead to beneficial events whereas some component/s of the inflammatory response can also exacerbate myocardial injury. As the inflammatory response may prove to be a possible target of therapy, furthering our

understanding of the conditions and the components involved in beneficial cardiac remodelling can lead to the development of strategies/procedures to promote improved patient recovery.

C.2. Infiltrating cells

Here, the main components of the myocardial infiltrate and their suspected contributions to myocardial repair are briefly described.

C.2.1. Neutrophils

Neutrophils are a type of granulocyte: phagocytic cells that look granulated under the microscope as they contain packets of lysosomes used for successful degradation of phagosomes. They are highly motile cells and are the first cell type to migrate into the injured tissue in response to (1) vasodilation and swelling of capillaries near injury, and (2) cytokines and chemokines released by injured/dying cells [Chandrasoma and Taylor]. The vasodilation and swelling assist the extravasation of neutrophils by increasing capillary intercellular permeability. Extravasation is also aided by slowing of blood flow by swelling, and physically, by expression of adhesion molecules on the vessel wall (e.g. selectins, and intercellular adhesion molecules or ICAMs) as well as by activation of neutrophils and their expression of integrins (which bind to ICAMs) [Frangogiannis, 2008; Frangogiannis *et al*, 2002; Hawkins *et al*, 1996; F Yang *et al*, 2002].

The role of neutrophils in cardiac injury is not well understood. They are the first type of cell that significantly increases in population after injury, and peaks 24 to 48 hours after induction of injury [Dewald *et al*, 2004; F Yang *et al*, 2002]. They are

responsible for the phagocytosis of dead cardiac cells and debris, and but may also contribute to cardiac remodeling following the initial phagocytic stage through secretion of various cytokines and factors. However, early studies using anti-neutrophil serum showed no significant effects on the formation of granulation tissue, suggesting that neutrophils may not have a significant contribution to the myocardial repair process beyond the initial phagocytosis of cell debris [Frangogiannis *et al*, 2002; Simpson and Ross, 1972]. Neutrophils are suspected to play a negative role in cardiac remodeling, as inhibition has resulted in decreased infarct size, and recently, a clinical study has observed a higher number of circulating neutrophils in patients correlating with larger infarct sizes [Husser *et al*, 2010; Litt *et al*, 1989; Romson *et al*, 1983]. However this observation may be an effect rather than a cause as studies using inter-cellular adhesion molecule 1 (ICAM-1) knockout mice have also shown less initial area of myocardial injury but no change in infarct or scar size weeks later, suggesting no significant detrimental effect on global myocardial repair [Metzler *et al*, 2001].

C.2.2. Monocytes and macrophages

Macrophages are phagocytic cells that play an important role in cardiac injury repair and remodelling. These are cells classified as monocytes while circulating until they migrate into a tissue where they differentiate into macrophages, which are larger and have increased cytosolic granules [Douglas and Ho]. Depletion of macrophages after myocardial injury results in incomplete debris clean up, as well as marked wall thinning and increased mortality [van Amerongen *et al*, 2007]. These cells migrate into the infarcted myocardium and their numbers peak between three and seven days post-

infarction in the mouse [Dewald *et al*, 2004; F Yang *et al*, 2002]. The role of the macrophages in cardiac repair appears to be multifactorial. In addition to their phagocytic properties, macrophages are also known to release growth factors and cytokines important in downstream remodelling stages including transforming growth factor (TGF)- β , FGF-2, platelet-derived growth factor (PDGF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) [Wernli *et al*, 2009]. Hence, in addition to a phagocytic role, macrophages appear to be important in contributing to the microenvironment that promotes the formation of granulation tissue, in the stimulation of proliferation of migrant cells, in the production and degradation of extracellular matrix, in differentiation of fibroblasts to myofibroblasts, in angiogenesis, and even in subsequent nerve growth following the cardiac insult [Drobic *et al*, 2007; Lijnen *et al*, 2003; Narine *et al*, 2004; Presta *et al*, 2009; Tsunoda *et al*, 2009; Valen *et al*, 2000; Wahl and Allen, 1988]. Clearly, macrophage infiltration following myocardial injury is a critical component to cardiac repair.

C.2.3. T Lymphocytes

The role of T-lymphocytes in myocardial injury has been given a lot less importance than the other mediators of the inflammatory response, neutrophils and macrophages. As such, publications on the role of T lymphocytes in healing after myocardial infarction are limited. This presumption that neutrophils and macrophages are more important players is surprising considering the well-established role of T lymphocytes in wound healing in other tissues such as the skin, where their interplay with fibroblasts contributes to improved healing [Barbul and Regan, 1990; Barbul *et al*, 1989;

Crowston *et al*, 1997; Efron *et al*, 1990; Tsunoda *et al*, 2009]. Myocardial response to injury progresses through the same basic stages of wound healing, and therefore a role for lymphocytes in repair and remodeling would be expected. A recent study using Rag 1 knockout mice (which lack mature T and B lymphocytes) showed decreased infarct sizes after ischemia/reperfusion injury compared with wild type controls, suggesting that lymphocytes (T and/or B) contribute to myocardial injury; however the number of infiltrating neutrophils were also significantly declined, raising the possibility that the event may also be neutrophil-mediated [Z Yang *et al*, 2005].

Studies revealing a detrimental effect of CD4-positive and not CD8-positive lymphocytes in myocardial damage, suggested that different lymphocytic subtypes might confer different effects on myocardial repair [Z Yang *et al*, 2006]. T lymphocyte interaction with fibroblasts has also been established in traditional wound healing, whereby growth factors and cytokines released by these cells work in a paracrine manner to stimulate production of the extracellular matrix. Evidence now emerging suggest that similar mechanisms to traditional wound healing may also be occurring in cardiac repair, which would suggest that infiltration of T cells into the injured myocardium is important for proper myocardial healing [Linfert *et al*, 2009]. T cell infiltration in the injured myocardium is significantly increased by the second day after induction of injury [F Yang *et al*, 2002]. This infiltration peaks at about seven days, reaching >8-fold increase from the initial infiltrating population 1 day after the cardiac event, then gradually declines. T cell presence at 28 days post-injury is still significantly higher than in sham control animals (baseline values) [F Yang *et al*, 2002].

C.2.4. Fibroblasts/Myofibroblasts

Fibroblasts are important cells in the healing process. These cells synthesize collagen and lay down the extracellular matrix (Reviewed in [Jugdutt, 2003]). In cardiac injury, fibroblasts migrate in response to local signals from the injured tissue as well as growth factors and cytokines secreted by immune cells at the injury site. There, they differentiate into smooth muscle actin-expressing cells called myofibroblasts, which are hypersecretory and responsible for excess collagen deposition leading to fibrosis [Faouzi *et al*, 1999; Ji Virag and Murry, 2003]. Myofibroblasts also secrete multiple growth factors and cytokines, further contributing to the microenvironment of the injured myocardium [KE Porter and Turner, 2009]. However, their exact spatial and temporal distribution in relation to the cardiac event has not been fully elucidated; conflicting reports as to the timing and persistence have been reported. One group has reported that myofibroblasts are significantly increased by three days post injury in the mouse, then subsequently declines [Dewald *et al*, 2004]. Another group using the same coronary ligation injury model and the same mouse strain (C57BL/6) has observed the increase in fibroblast/myofibroblast infiltration to occur 4 days after injury, which continues to increase for another 24 days [F Yang *et al*, 2002]. Although the timing of their infiltration is still up for debate, it has been established that myofibroblasts are ultimately responsible for the formation of cardiac scar tissue. Scar tissue formation is beneficial in most organs, and the process likely evolved from the organism's requirement to immediately seal any openings of the skin to protect against infiltration of invading microorganisms. However, excess scar tissue formation in the heart leads to a

diminished outcome. Although collagen-deposition and proper scar formation are critical in the prevention of cardiac rupture, its lack of elasticity and compliance eventually contributes to pathological remodelling and decreased cardiac function. Fibroblast differentiation into myofibroblasts is stimulated by TGF- β [Ishida *et al*, 2006; Meyer-Ter-Vehn *et al*, 2006; Meyer-ter-Vehn *et al*, 2006; Ramirez *et al*, 2006; Untergasser *et al*, 2005] and can be inhibited by increased FGF-2 availability as observed in other tissues [Cushing *et al*, 2008; Matthey *et al*, 1997]. There is no information as to how FGF-2 would affect myofibroblast formation in the heart.

C.2.5. Mast cells

Mast cells are leukocyte cells that are usually residents within a tissue. They are granulated cells involved in acute response to allergens and are also important players in wound healing (Reviewed in [Prussin and Metcalfe, 2006]). Mast cells have been shown to participate in the myocardial remodeling process by stimulation of collagen deposition as well as stimulation of angiogenesis [Frangogiannis *et al*, 1998; Somasundaram *et al*, 2005]. These cells reside within the tissue for long periods of time, and as such are hypothesized to play an important role in stimulating the initial response to injury in the myocardium [Frangogiannis, 2006]. It has been reported that ischemia/reperfusion injury can stimulate upregulation of stem cell factor [Frangogiannis *et al*, 1998], which acts as a chemotactic agent for mast cells that express the stem cell factor receptor c-kit (CD117). Thus, it is possible that upon injury to the myocardium, the mass efflux of cytokines and factors results in the recruitment of nearby mast cells to the area of injury, where they can contribute further to chemokine secretion. Changes on growth factor and chemokine

availability can define the injury microenvironment, which can then subsequently influence the overall remodeling process (Reviewed in [Balakumar *et al*, 2008]).

C.2.6. Stem and progenitor cells

Stem and progenitor cells are characterized as cells that are capable of both being able to renew themselves as well as create a daughter cell that is of a more differentiated lineage. Stem cells are less differentiated than progenitor cells and remain with a greater capacity to develop into a broader lineage. Progenitor cells are stem cells but which have already been dedicated to differentiate down a specific lineage (for example, hematopoietic). The main difference between the two is that a stem cell can theoretically undergo unlimited division (as permitted by its telomerase activity), whereas a progenitor is more limited. Nevertheless, multiple laboratories, including ours, have reported their presence in the injured myocardium. These cells are identified by their expression of the markers c-kit and Sca-1 [Oh *et al*, 2004; Orlic *et al*, 2001a; Orlic *et al*, 2001c]. The source of these cells is still under investigation, however, progenitor cells from the bone marrow have been reported [Orlic *et al*, 2001b; Orlic *et al*, 2001c] as well as cardiac stem cell niches within the myocardium [Urbanek *et al*, 2006]. Presumably, stem/progenitor cell migration into the injured myocardium results from chemotaxis resulting from the release of growth factors and cytokines released by the injured cardiac cells. They have also been shown to be able to *trans*-differentiate into the cells that make up the myocardium: cardiac myocytes, and endothelial cells and smooth muscle cells [Bearzi *et al*, 2009; Kajstura *et al*, 2005; Rota *et al*, 2007; Tillmanns *et al*, 2008; Urbanek *et al*, 2005]. Anversa and colleagues have shown that migration of these cells to the infarct

area and their subsequent *trans*-differentiation can occur without external manipulation. Additionally, there are several reports that manipulations to encourage the migration of these more premature cells into the infarct area with or without their *trans*-differentiation into the cells of the myocardium contribute to improved cardiac function [Beltrami *et al*, 2003; Kajstura *et al*, 2005; Orlic *et al*, 2001c]. These cells, therefore, present an exciting promise as possible candidate for stimulation of cardiac regeneration and ultimately, improved outcome to cardiac patients.

C.3. Stages of myocardial repair

The response to myocardial injury has been classified into three stages based on repair in other tissue types: (1) inflammatory, (2) proliferative, and (3) maturation (Figure 2; Reviewed in [Clark, 1985; Frangogiannis, 2006; Frangogiannis, 2008]).

The first stage, *inflammatory*, is the stage immediately following necrotic cell death. Dying cells release cytokines and factors that trigger chemotaxis/recruitment of leukocytes, and other cells, into the infarcted area. The spilled cytosolic contents of necrotic cells cause stimulation of the innate immune response [Frangogiannis, 2006]. At this stage, which lasts for approximately three days after the injury, a significant number of neutrophils and macrophages, as well as mast cells and lymphocytes migrate into the areas of injury. In mice specifically, leukocyte infiltration is significantly increased in the injured myocardium within 24 hours of injury [Dewald *et al*, 2004; F Yang *et al*, 2002]. The infiltrating immune cells in the *inflammatory* phase of the repair process are

primarily responsible for cleaning up dead cells and debris within the myocardium [Frangogiannis, 2006]. Similar to traditional wound healing responses, in the heart, the classical signals that govern the immune response have been shown to be involved: the activation of the complement system, Toll-like receptor responses which initiate the immune response and characterizes the cell subsets involved by the chemokine and cytokine cascades that are subsequently released, signal cascades triggered by reactive oxygen species, as well as proteinases that degrade the extracellular matrix [Frangogiannis, 2006; Frangogiannis, 2008; Heymans *et al*, 1999].

The *proliferation* stage follows within 72 hours of myocardial injury. This stage involves the migration of massive numbers of macrophages and fibroblasts, which lead to the formation of granulation tissue [Frangogiannis, 2006]. At 96 hours after injury, infiltration of macrophages peaks while fibroblast/myofibroblast infiltration occurs [F Yang *et al*, 2002]. These cells secrete cytokines and factors that stimulate deposition of collagen-rich extracellular matrix and scar tissue. These cells are also suspected to contribute to angiogenesis [Tsunoda *et al*, 2009]. At this stage, neutrophils begin to undergo apoptosis, a process which involves the secretion of TGF- β and subsequent phagocytosis by macrophages and is completed by the second week [Frangogiannis, 2006; F Yang *et al*, 2002]. Frangogiannis and colleagues report that these cells undergo apoptosis near the end of the *proliferation* stage, nearly 1 week after induction of injury [Frangogiannis, 2006], however, quantification of fibroblast infiltration by Carretero and colleagues show increased presence up to 28 days after injury [F Yang *et al*, 2002].

The *maturation* phase is marked by the completion of scar formation; this stage usually begins two weeks after induction of injury [Frangogiannis, 2006] and is usually complete by three weeks in the mouse [F Yang *et al*, 2002]. Evaluation of systolic function in mice at two weeks and at six months after injury did not reveal any significant changes [F Yang *et al*, 2002]. A slight (although non-significant) increasing trend was observed in left ventricular diastolic dimension between two weeks and two months post injury, therefore remodeling must still be occurring which can still affect cardiac function [F Yang *et al*, 2002]. From these observations, critical mechanisms of repair must occur within the first eight weeks after injury. It is now well accepted, however, that the scar tissue is neither dead nor acellular. It undergoes further remodelling and contributes to the continual remodelling of the remaining healthy myocardium.

An illustration depicting the model described by Frangogiannis (above) is shown in simple diagram form in Figure 2. Although the basic stages of the process of myocardial repair are generally accepted, the cellular players, their role, and their temporal and spatial distributions still need to be clarified. Many studies suggest that manipulation of certain cell types involved in the myocardial repair process may contribute to an improved cardiac outcome. Additionally, the mechanisms that govern transition from compensatory hypertrophy to decompensatory (pathologic) hypertrophy, which also involve the players of the myocardial repair process, have also not been fully elucidated. The transition to maladaptive cardiac hypertrophy leads to the development of heart failure. As such, the remodelling process and scar-formation (and subsequently

the development and progression of hypertrophy) are now being considered to be an attractive therapeutic target for the improvement of cardiac functional outcome.

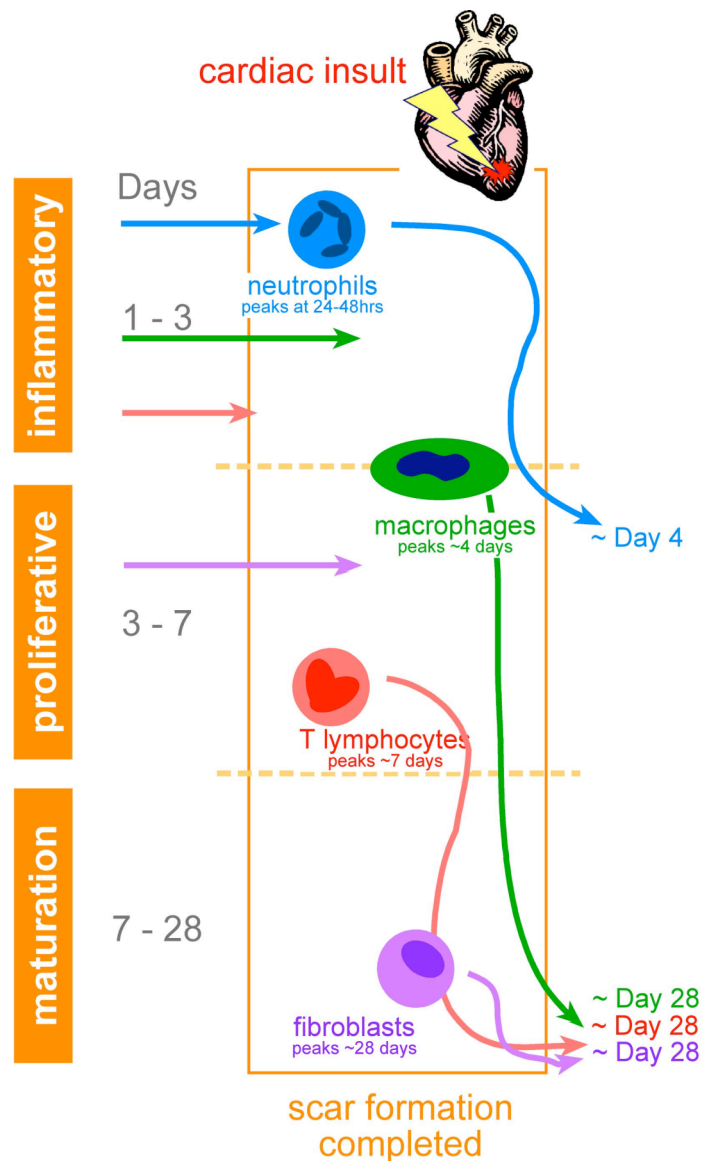


Figure 2. The stages of myocardial healing.

Immediately after myocardial injury occurs, repair begins, a process that involves the cells of the immune system. Within the first stage described by Frangogiannis, *inflammatory*, cells are recruited into the area of injury by massive cytokine release from dying and injured cardiac cells. Among the cells that migrate include neutrophils, macrophages, then followed by T lymphocytes. The ‘cocktail’ of cytokines released in the area of injury stimulate further recruitment of fibroblast and myofibroblast cells, as well as significant cell proliferation in the *proliferation* stage. The formation and establishment of the myocardial scar which involves significant decline in infiltrating cells and significant collagen deposition is completed in the *maturation* stage.

C.4. Cardiac regeneration

The concept that the heart is a terminally-differentiated organ originated from early observations that hearts suffering a myocardial infarction do not appear to regenerate to replace the lost muscle; regeneration was determined by measuring cardiomyocyte mitotic figures and thymidine incorporation (reviewed in [Anversa *et al*, 2006]). This idea was challenged by Piero Anversa as early as 1991 when he showed evidence for chromosomes in metaphase in adult rat cardiac myocytes, with the mitotic index increasing with age [Anversa *et al*, 1991]. Evidence for similar observations were later shown within the failing adult human heart [Beltrami *et al*, 2001; Quaini *et al*, 1994]. Despite evidence supporting the notion that the heart retains myocytes or myocyte progenitor populations capable of entering and traversing the cell cycle [Anversa *et al*, 1991; Engel *et al*, 2005], it is also true that these mechanisms are inadequate to repair lost tissue and as consequence ischemic heart disease and its complications still claim the most number of mortalities worldwide.

The potential to regenerate the heart after tissue loss is a very attractive concept. Evidence has shown that stem cells from the bone marrow or even resident cardiac stem cells harvested from the heart and expanded *ex vivo* can significantly improve cardiac function in rodents when injected at the border regions of the infarct [Beltrami *et al*, 2003; Orlic *et al*, 2001a; Orlic *et al*, 2001b; Orlic *et al*, 2001c]. Others have also shown similar observations [Bittira *et al*, 2003; Fransioli *et al*, 2008; Oh *et al*, 2004]. Results from basic science experiments have been promising, resulting in a push for clinical trials in an attempt to assess the efficacy of using bone marrow-derived cells to improve

myocardial repair in patients [Assmus *et al*, 2002; Britten *et al*, 2003; Dill *et al*, 2009]. Although results from the clinical trials have demonstrated the feasibility/safety of cell treatment, and its overall cardiac effects have been beneficial, there is significant room for therapeutic improvement. Thus, further studies to determine methods to optimally stimulate *trans*-differentiation of progenitor cells into functional cardiac myocytes are necessary, as well as massive clinical trials to ensure the efficacy and safety of such therapies.

D. CARDIOPROTECTION

Cells and tissue possess innate mechanisms that can confer resistance to stress and injury-induced cell death (cytoprotection). Understanding the mechanisms that govern innate cytoprotection and cardioprotection can indicate new “therapeutic” methods that can be used for the promotion of better cardiac health.

D.1. Ischemic preconditioning

First reported by Murry and colleagues, ischemic preconditioning is the phenomenon whereby brief episodes of ischemia followed by brief periods or reperfusion create a ‘cardioprotected state’ that is resistant to a subsequent more severe ischemic insult [Murry *et al*, 1986]. This observation was first described as early as the 1960’s by the same group [Jennings *et al*, 1960], and has since been observed to occur in a wide variety of species [Y Liu and Downey, 1992; Reimer *et al*, 1990; Schott *et al*, 1990;

Shizukuda *et al*, 1992]. Cardiac patients who suffered bouts of angina before onset of myocardial infarction had significantly improved recovery of contractile function (ejection fraction) compared to those who did not experience angina [Nakagawa *et al*, 1995; Noda *et al*, 1999; Papadopoulos *et al*, 2003]. Furthermore, patients undergoing surgery involving either multiple balloon-induced coronary occlusions during an angioplasty procedure, or multiple aortic clamping required for heart attachment to a heart-lung machine have also been observed to have improved outcome, supporting the increasing evidence that suggest that ischemic preconditioning also occurs in humans [Jenkins *et al*, 1995].

Preconditioning has been determined to confer cardioprotection in two distinct stages. The first “early” stage, happens essentially immediately after the stimulus although short-lived (about 4 hours). This early stage results from posttranslational protein modifications promoting improved cell resistance to injury. The second “late” stage of preconditioning occurs several hours after the initial event and can last several days. This second stage of protection results from changes in gene expression and subsequent *de novo* protein production. The exact mechanisms that govern preconditioning have not been fully elucidated, although it is known that it involves the activation of signal transduction pathways including (but not exclusively) Akt, Erk 1/2, and PKC. The resulting changes in cell proteins and gene expression inevitably promote changes in ionic channels on the mitochondrial membrane including the ATP-dependent potassium channels and the mitochondrial permeability transition pore that minimize the

massive ionic imbalance that occurs from ischemic events usually leading to cell death [Javadov *et al*, 2009].

Protection from acute ischemic events does not occur solely from events that happen prior to the massive ischemia. “Preconditioning” can also occur from multiple short bursts of ischemic events during, or after the infarction (“ischemic postconditioning”). Anaesthetics and other volatile substances can also be protective in a similar fashion (“pharmacological conditioning”). Additionally, ischemic events occurring in a remote organ (for example, kidneys or small intestine) has also been documented to confer cardioprotection similar to that observed in ischemic preconditioning (“remote ischemic preconditioning”).

Despite the phenomenon having been recognized since the 1990’s, and evidence that pre/postconditioning protection also occurs in human hearts, translation of this natural phenomenon to the bedside (due to the trigger and especially the small window of opportunity for delivery) has not been feasible.

D.2. Preconditioning stimulated by naturally-produced substances

The protective preconditioning effect has also been observed after triggers other than ischemia; for example, by stress or heat shock [Hale and Kloner, 1994; X Liu *et al*, 1992]. In addition, certain molecules have since been shown to trigger effects similar to ischemic preconditioning without the necessity of the brief periods of

ischemia/reperfusion. Tangible molecular triggers present more (and easier) means to exploit the naturally occurring protective mechanism. Among these non-ischemia triggers for preconditioning are naturally produced molecules including growth factors such as the growth factor FGF-2.

FGF-2 is produced by cardiac fibroblasts and cardiac myocytes, and is released into the extracellular space upon every contraction of the heart [Clarke *et al*, 1995; Clarke and Feedback, 1996; Kaye *et al*, 1996], making it an intriguing molecule given that it possesses preconditioning-like effects and is available at the target organ.

CHAPTER IV

FIBROBLAST GROWTH FACTOR-2

A. FGF-2 AND THE FGF FAMILY

FGF-2 belongs to the large FGF family of heparin-binding molecules, originally discovered as a substance in the bovine pituitary capable of stimulating proliferation of NIH 3T3 fibroblasts [Gospodarowicz, 1974; Gospodarowicz *et al*, 1978a; Gospodarowicz and Cheng, 1986; Gospodarowicz *et al*, 1984; Gospodarowicz *et al*, 1987; Gospodarowicz *et al*, 1985]. Purification of this mitogenic substance revealed the first two members of the FGF family, FGF-1 and FGF-2, which are distinct but highly related molecules. FGF-1 was originally called acidic FGF (or aFGF) because of its isoelectric point (pI) of ~5.6, while FGF-2, basic FGF (or bFGF) for its pI of 9.0 [Bohlen *et al*, 1984; Bohlen *et al*, 1985; Esch *et al*, 1985; Thomas *et al*, 1984]. Subsequent FGF family members to be discovered were designated numbers from 3 to 23 (recently reviewed in [Beenken and Mohammadi, 2009]). The FGF family members act through the membrane FGF receptors (FGFR1 to FGFR4), and they can all bind heparin (albeit with varying affinity), but that is where their similarities end (recently reviewed in [Beenken and Mohammadi, 2009]). These factors are multifunctional, and they can play important roles during mammalian development as well as normal tissue repair, or cancer; they affect cell proliferation, differentiation, migration, as well as survival [Arman *et al*, 1998; Beenken and Mohammadi, 2009; Dono *et al*, 1998; Gospodarowicz, 1976; Gospodarowicz *et al*, 1978b; Miller *et al*, 2000; Ortega *et al*, 1998; Quarto *et al*,

1989; Rossant *et al*, 1997; Sahni *et al*, 1999; Sekine *et al*, 1999; Sugi *et al*, 1993; Swain, 1995; Xu *et al*, 1998]. FGF-2 and FGF-1 have been implicated in acute as well as sustained cardioprotection [Buehler *et al*, 2002; Cuevas *et al*, 2000; Cuevas *et al*, 1999; Htun *et al*, 1998; Kardami *et al*, 1993; Padua *et al*, 1998; Padua *et al*, 1995; Palmen *et al*, 2004; Sheikh *et al*, 2001].

B. FGF-2 EXPRESSION AND FUNCTION

To determine processes in which FGF-2 is important, several strains of mice with depleted FGF-2 have been generated. Despite its involvement in several important biological processes, FGF-2 deficiency does not cause lethality. FGF-2 knockout mice have normal fecundity, and do not seem to have gross morphological differences compared to wild type mice [Dono *et al*, 1998; Miller *et al*, 2000; Ortega *et al*, 1998; Sheikh *et al*, 2001; Zhou *et al*, 1998]. However, multiple effects from the abolishment of FGF-2 have been demonstrated after different challenges or models of injury. Some of these include impaired bone densities due to effects on the parathyroid hormone stimulated effects on the osteoclasts and osteoblasts [Hurley *et al*, 2006; Okada *et al*, 2003; Sabbieti *et al*, 2009]; increased brain infarct sizes and impaired neuron proliferation after ischemic injury [Kiprianova *et al*, 2004; Yoshimura *et al*, 2001] although functional analysis showed improved sensory perception after nerve crushing injury [Jungnickel *et al*, 2010]; and delayed skin wound healing [Ortega *et al*, 1998].

In the heart, different effects of FGF-2 deletion have been observed in different mouse colonies. Cardiac dilatation was observed in one strain (C57BL/6 background) [Pellieux *et al*, 2001]; however, this was not observed in the colony used by the Schultz group, or in our laboratory (Black Swiss or CD-1 backgrounds) [Schultz *et al*, 1999; Sheikh *et al*, 2001]. Transgenic mouse models overexpressing FGF-2 have demonstrated the importance of FGF-2 in capillary growth: increased capillary density in hearts of transgenic FGF-2 mice was observed and is corroborated by evidence showing decreased postnatal capillary development in FGF-2 knockout mice [Amann *et al*, 2006; Sheikh *et al*, 2001].

Lack of lethality in FGF-2 knockout mice led to the hypothesis that FGF function may be compensated for by other FGF members when necessary. The majority of FGF molecules, including FGF-2 and FGF-1, signal through the same four extracellular FGFRs; FGF-1 has similar, high affinities, for all four receptors, suggesting a possibility for some redundancy between FGF-1 and FGF-2 [Beenken and Mohammadi, 2009]. However, in double knockout mice for both FGF-2 and FGF-1, phenotypic differences are limited to those previously observed in FGF-2 null animals [Miller *et al*, 2000], therefore questioning the potential for functional overlap between FGF-2 and FGF-1. Of course, this does not preclude the possibility of other FGF family members also having functional redundancies in conditions where FGF-2 or FGF-1 is not available.

C. FGF-2 STRUCTURE AND TISSUE FUNCTION

C.1. FGF-2 gene structure

The entire coding region of FGF-2 has been cloned and sequenced [Abraham *et al*, 1986a; Abraham *et al*, 1986b; Shimasaki *et al*, 1988]. Its sequence is highly conserved between multiple species including bovine, rabbit, and human, suggesting an evolutionarily important function. The FGF-2 gene has been mapped to band q26 – q27 on chromosome 4 in humans [Abraham *et al*, 1986a; Abraham *et al*, 1986b]. The gene contains three exons separated by two introns, and spans over 36 kilobases [Shibata *et al*, 1991]. Assessment of FGF-2 promoters revealed that the canonical TATA or CCAAT consensus sequences are absent [Shibata *et al*, 1991]. Transcription appears to be initiated by binding of transcription factors to several GC boxes (sequences with high GC content) within 300 base pairs upstream of the FGF-2 transcriptional start site [Shibata *et al*, 1991]. Other genes lacking TATA boxes have also been identified which rely on GC box motifs for transcription initiation [Araki *et al*, 1987; Ye *et al*, 1993]. In the FGF-2 gene, only one classical ATG (methionine) translation initiation site was located (at +492 of the human FGF-2 gene), however, the identification of several isoforms of FGF-2 (discussed in Chapter IV.C.2.) revealed several alternative translation initiation sites at CTG motifs (at +318, +355, and +369 of the human FGF-2 gene) [Shibata *et al*, 1991]. Translation start sites in the rat correspond to +250 (AUG), and +148 and +172 (CUG) [Pasumarthi *et al*, 1997]. Aligned human and rat FGF-2 promoter sequences were shown to have approximately 60% homology; with greater homology (~67%) at the proximal promoter region (-250/+1 in rat) compared to the more distal sequences (-500/-251) [Pasumarthi *et al*, 1997; Shibata *et al*, 1991], suggesting a possible greater importance in gene regulation at the proximal promoter region. Assessment of the proximal promoter

sequence revealed the presence of several GC rich regions within 100 bp of the transcription start site, including putative binding sites for transcription factors early growth factor-1 (Egr-1) overlapping with specificity protein 1 (Sp1) (Figure 7). The involvement of both zinc-finger Egr-1 and Sp1 transcription factors has been documented in FGF-2 promoter activation as well as transcription [Biesiada *et al*, 1996; Jin *et al*, 2000].

Egr-1 belongs to the immediate early response group of proteins, activated rapidly upon stress triggers, prior to any other proteins in the cell, and despite inhibition of protein synthesis [Sukhatme, 1990]. Activated transcription factor Egr-1 translocates into the nucleus where it can either stimulate or inhibit transcription of certain genes. In cardiac myocytes, a significant increase in complex between nuclear extract and a consensus Egr-1 DNA element was observed after stimulation using stress-inducers including phenylephrine, angiotensin II, and phorbol esters [Jin *et al*, 2000]. In the same study, it was shown that α -adrenergic-stimulated increase in FGF-2 promoter activity is mediated by Egr-1 using deletion analysis and transfection assays [Jin *et al*, 2000]. In addition, Egr-1 has also been shown to stimulate FGF-2 transcription in astrocytes [Biesiada *et al*, 1996].

Sp1 is the product of a housekeeping gene, consistently transcribed, and responsible for the upkeep of production of certain required proteins by the cell [NY Tan and Khachigian, 2009]. Its activation by phosphorylation has been shown to stimulate activation of transcription of several genes [Milanini-Mongiat *et al*, 2002; Opitz and

Rustgi, 2000; Pal *et al*, 1998]. Chromatin immunoprecipitation assay data suggest interaction of Sp1 with the FGF-2 promoter in neonatal rat cardiac myocytes *in situ* [Tang *et al*, 2009].

We have previously shown that FGF-2 can regulate its own synthesis at the transcriptional level in cardiac myocytes. FGF-2 gene regulation can also be mediated by both Egr-1 and Sp1 transcription factors, although their roles in autoregulation do not appear to be essential [Jimenez, 2003; Jimenez *et al*, 2004].

C.2. FGF-2 protein structure and subcellular distribution

FGF-2 protein exists in multiple isoforms, termed “low molecular weight” (18 kDa) and “high molecular weight” (>21 kDa) [Florkiewicz and Sommer, 1989]. These isoforms have overlapping, as well as completely distinct, biological functions (reviewed in [Kardami *et al*, 2007]). Multiple FGF-2 isoforms are generated from multiple alternative translation initiation sites (AUG, CUG) from the same mRNA [Florkiewicz and Sommer, 1989], as well as through proteolytic cleavage of high molecular weight isoforms [Doble *et al*, 1990; Klagsbrun *et al*, 1987]. All FGF-2 proteins share the core 155-amino acid sequence of the 18 kDa low molecular weight isoform. Crystalline analysis of the low molecular weight FGF-2 isoform revealed that it consists of a trigonal pyramidal structure made up of 12 antiparallel β sheets with helical structures at amino acid residues 13-30 and 131-136 [Eriksson *et al*, 1991; Moy *et al*, 1996; Zhu *et al*, 1991]. Helical structures have been shown to act as receptor-binding and heparin-binding

domains [Moy *et al*, 1996]. High molecular weight isoforms contain nuclear localization sequences to direct preferential subcellular localization to the nucleus [Pintucci *et al*, 1996]. However, classical nuclear localization sequence is not essential for targeting of FGF-2 into the nucleus as low molecular weight isoform within the nucleus has been documented [Choi *et al*, 2000; Claus *et al*, 2003; Sheng *et al*, 2004]. In the heart, cellular distribution of the different FGF-2 isoforms are the same; protein immunoblotting analyses of hearts from knockout mice of either low molecular weight or high molecular weight isoforms demonstrated the presence of the 18 kDa isoform in both the nucleus and the cytoplasm, whereas the high molecular weight isoform was only detected in the nucleus [Liao *et al*, 2009].

D. FGF-2 RELEASE

FGF-2 is devoid of the classical signal sequence required for exocytosis through the Golgi pathway [Szebenyi and Fallon, 1999]. However, FGF-2 is exported to the extracellular environment and signals through ligand binding to its cell surface receptor, which would clearly require a method of release. Passive FGF-2 release from the cytoplasm was proposed to occur through transient or permanent mechanical cell wounding [Clarke and Feedback, 1996; Kardami *et al*, 1991; McNeil *et al*, 1989], which is supported by evidence of increased FGF-2 release when contracting cells undergoing vigorous activity are stimulated further [Clarke *et al*, 1995; Clarke and Feedback, 1996; Kaye *et al*, 1996; Padua and Kardami, 1993]. FGF-2 release through the Na^+/K^+ ATPase has also been reported [Florkiewicz *et al*, 1998; Florkiewicz *et al*, 1995]. This group demonstrated that pharmacological inhibition of ion transport as well as treatment with

the Na⁺/K⁺ ATPase α subunit (for competition experiments) resulted in the significant decline of FGF-2 release. Additionally, a more 'active' (but ATP-independent) method of FGF-2 release through an ER-Golgi-independent exocytosis has also been reported [Schafer *et al*, 2004; Taverna *et al*, 2003]. This vesicle mediated release has been shown to occur through a single-direction translocation through the plasma membrane [Schafer *et al*, 2004]. Membrane vesicle shedding of FGF-2 has been shown in astrocytes, hepatoma cells (SK-Hep1), and fibroblasts (NIH 3T3) [Proia *et al*, 2008; Schiera *et al*, 2007; Taverna *et al*, 2003]. Thus, despite not having the classical signal sequence required for classical for ER-Golgi-dependent release, FGF-2 is both actively and passively released from mammalian cells.

E. FIBROBLAST GROWTH FACTOR RECEPTORS

FGF-2 and the majority of the FGF family of proteins bind with high affinity and signal through the cell surface FGF receptors (FGFR) (recently reviewed in [Beenken and Mohammadi, 2009]). Four types of FGFR have been isolated and studied (FGFR-1 to FGFR-4). A fifth receptor highly homologous to the other four has been identified, which is predominantly expressed in the pancreas, but no other study has looked further at its function [Kim *et al*, 2001; Sleeman *et al*, 2001]. FGFR-5 is devoid of an intracellular kinase domain, and binds neither FGF-2 nor FGF-1 [Kim *et al*, 2001; Sleeman *et al*, 2001].

The structures of the FGFRs are highly homologous, with 55% to 72% shared amino acid sequence [Johnson and Williams, 1993]. FGFR structure is shown in a simplified diagram in Figure 3. Briefly, these receptors consist of three main domains: a three-part extracellular immunoglobulin-like domain (ligand-binding), a transmembrane domain, and an intracellular tyrosine kinase domain (reviewed in [Beenken and Mohammadi, 2009; Klint and Claesson-Welsh, 1999]). The extracellular domain binds the FGF ligand with high affinity, and also contains a binding domain for heparin (which is proposed to increase binding affinities and potentially increase ligand-receptor stability). Also within the three-part immunoglobulin-like domain of the receptor molecule is a serine-rich region termed the “acid box” which may act to preferentially bind FGF isoforms to which it has higher affinity [Wang *et al*, 1995a; Wang *et al*, 1995b]. Splice variants of the FGFR also exist [Bernard *et al*, 1991; Chellaiah *et al*, 1994; Coutts and Gallagher, 1995; Shi *et al*, 1994; Wuechner *et al*, 1996]. The third immunoglobulin-like domain of the receptor, closest to the cell membrane, contains the region of variation in different isoforms of the same receptor. The biological significance of the FGFR splicing variants is not understood. However, splicing changes receptor affinities for the different FGF molecules, and receptor splicing appears to be tissue-specific [Beenken and Mohammadi, 2009; Yeh *et al*, 2003]. The specific affinities of the FGF family of proteins to the various isoforms of FGFR have been characterized. FGF-2 affinity is highest for FGFR-4, FGFR-3 (IIIc), and FGFR-1 (IIIc) while FGF-1 binds to all FGFR isoforms and splice variants with similar affinities [Ornitz *et al*, 1996]. In the heart, FGFR-1 is the major FGF-2 receptor [Kardami *et al*, 1995; L Liu *et al*, 1995; Pasumarthi *et al*, 1995].

For more information on FGFRs please refer to reviews on the subject [Eswarakumar *et al*, 2005; Mohammadi *et al*, 2005; Szebenyi and Fallon, 1999].

F. FGF-2 RELEASE AND SIGNALING IN THE HEART/CARDIAC MYOCYTE

Cardiac myocytes release FGF-2 on a beat-to-beat basis, presumably through transient mechanical wounding of the plasma membrane [Clarke *et al*, 1995; Kardami *et al*, 1991]. FGF-2 released from cardiac cells bind to the extracellular matrix as a consequence of its affinity to heparin sulfate proteoglycans (HSPG). There, it remains in “storage” until released or degraded [Folkman *et al*, 1988]. Presence of FGF-2 in the extracellular matrix surrounding cardiac cells is consistent with a paracrine or autocrine mode of action. Upon release from either the cell or its extracellular matrix stores, FGF-2 can act on a cell by binding cell surface FGFRs. FGF-2 can bind FGFR by itself, or in complex with HSPG [Detillieux *et al*, 2003]. HSPG binding to the second immunoglobulin-like domain of the FGFR has been shown to increase ligand-receptor stability. In fact, receptor signaling is proposed to occur by ligand binding to receptor, which may dimerize, leading to the subsequent formation of a complex consisting of two FGF-2:FGFR:HSPG, which is stabilized by the presence of the HSPG [Rapraeger *et al*, 1991] (Figure 3).

FGF-2 ligand binding to FGFR causes autophosphorylation and *trans*-phosphorylation (in case of dimers) of the intracellular C-terminal region tyrosine kinase

domain [Beenken and Mohammadi, 2009; Detillieux *et al*, 2003]. Phosphorylation of the tyrosine kinase domain can activate several different signal transduction pathways as summarized on Figure 3, which subsequently result in changes to gene expression. In the heart, FGF-2 has been shown to elicit its biological activities primarily through FGFR-1, which is expressed in both neonatal and adult cardiac myocytes [L Liu *et al*, 1995; Speir *et al*, 1992]. FGF-2 induces activation of several signal transduction pathways including those of PKC, MAPK (ERK 1/2, p38, JNK), IP3 and Akt in the heart [Beenken and Mohammadi, 2009; Kardami *et al*, 2007].

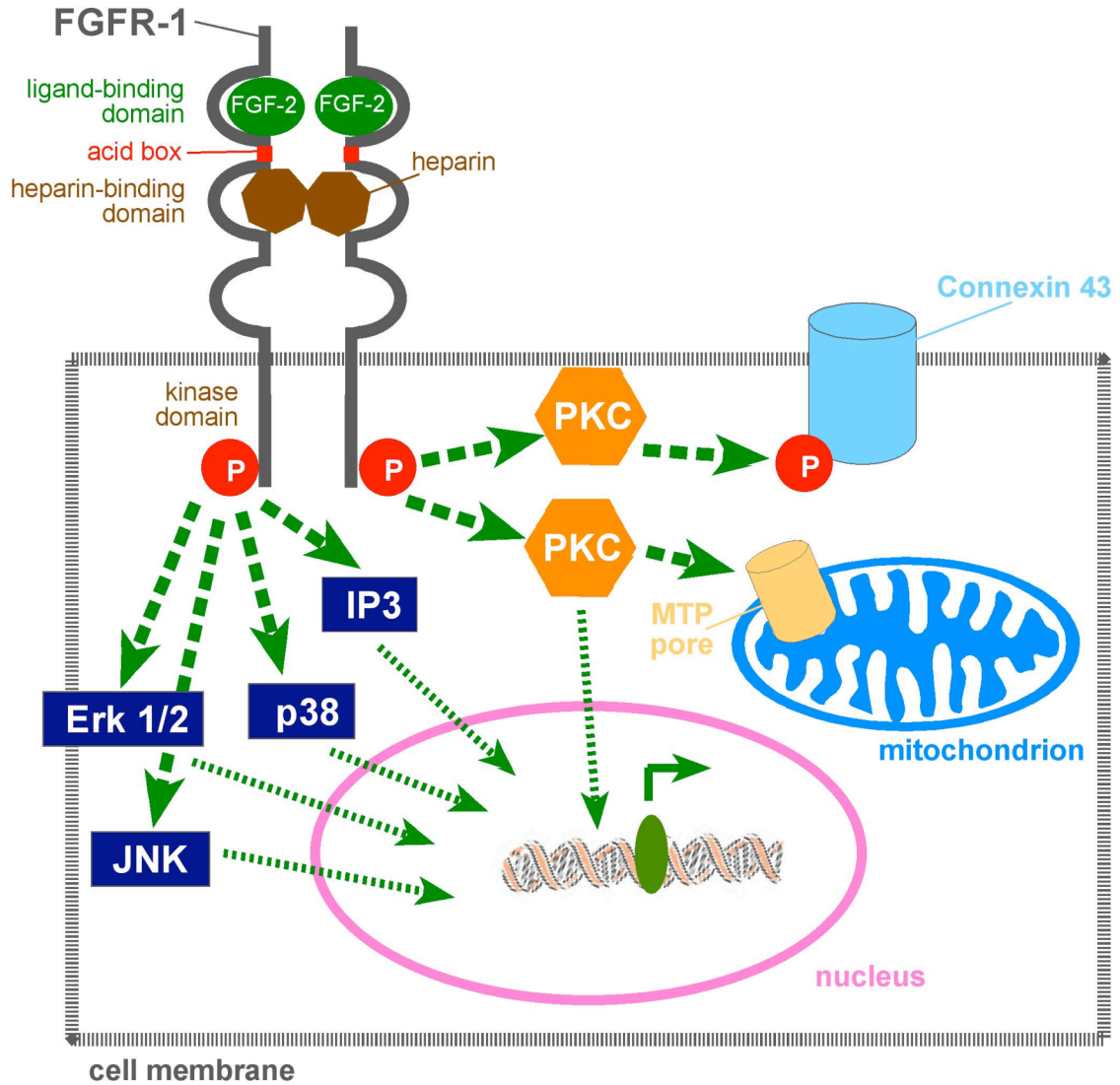


Figure 3. Downstream effects of FGF-2 binding to FGFR-1.

FGF-2/receptor-mediated events lead to tyrosine phosphorylation-mediated activation of downstream signaling effectors including Erk 1/2, p38 MAPK, JNK, IP3, and PKC. In turn, these effectors lead to changes in gene expression changes as well as effects on transmembrane channels like the mitochondrial permeability transition pore.

G. EFFECTS OF FGF-2 ON CELL PROLIFERATION

FGF-2 stimulates cell cycle entry and proliferation in many different cell types, including smooth muscle cells [Lindner and Reidy, 1991], endothelial cells [Schweigerer *et al*, 1987], neurons [Stemple *et al*, 1988], as well as neonatal cardiac myocytes *in vitro* [Kardami, 1990; Pasumarthi *et al*, 1994; Pasumarthi *et al*, 1996]. Induction of cell cycle progression by FGF-2 appears to be through interaction with FGFR1, and involves the PKC and MAPK pathways [Bansal *et al*, 2003; Engel *et al*, 2005; Frederick *et al*, 2007; Learish *et al*, 2000; Li *et al*, 2009; Skaletz-Rorowski *et al*, 2005; Skaletz-Rorowski *et al*, 1999].

Recently, considerable attention has been placed on the potential of progenitor-type cells to *trans*-differentiate into cardiac myocytes, and thereby replace myocytes lost after injury. Extensive studies are underway in an attempt to stimulate efficient proliferation and *trans*-differentiation of progenitor cells into functional cardiac myocytes with the view to improve cardiac function. FGF-2 has been implicated in cardiac stem cell proliferation and *trans*-differentiation [Rosenblatt-Velin *et al*, 2005] and has also been shown to be able to stimulate self-renewal of neural stem cells through MAPK pathways [Ma *et al*, 2009; Maric *et al*, 2007].

H. FGF-2 AND CARDIOPROTECTION

Acute delivery of FGF-2 can significantly improve cardiac myocyte cell viability and cardiac function upon injury, similar to effects conferred by ischemic preconditioning [Jiang *et al*, 2002; Jiang *et al*, 2004; Kardami *et al*, 1993; Nishida *et al*, 2003; Padua *et al*, 1995; Sheikh *et al*, 2001]. Immediate beneficial effects have been demonstrated by numerous studies showing significant preservation in cell numbers in FGF-2-treated versus non-treated cells *in vitro* after exposure to deleterious substances [Iwai-Kanai *et al*, 2002; Kardami *et al*, 1993]. In addition, several *ex vivo* studies have shown that hearts perfused with FGF-2 undergo significantly less damage (as assessed by infarct size and cardiac function) after isoproterenol-induced or ischemia/reperfusion-induced injury [Jiang *et al*, 2002; Jiang *et al*, 2004; Padua *et al*, 1998; Padua *et al*, 1995; Palmen *et al*, 2004; Sheikh *et al*, 2001]. *In vivo*, acute FGF-2 cardiac delivery also resulted in significant improvements in infarct size and cardiac function in several animal models after ischemia/reperfusion injury [Cuevas *et al*, 1997; Harada *et al*, 1994; Horrigan *et al*, 1996; Jiang *et al*, 2007; Jiang *et al*, 2002; Nishida *et al*, 2003].

FGF-2–induced cardioprotection appears to be multifactorial, requiring its FGFR1 receptor [Jiang *et al*, 2002; Liao *et al*, 2010]. Documented effects that contribute to improved cardiac function include: (1) increased cell survival (decreased apoptosis); (2) decreased inotropy (thus decreasing the increased energy requirement associated with the increase in force of contraction resulting from massive Ca^{2+} influx upon cardiac myocyte injury); (3) maintenance of ‘normal’ intercellular communication and rhythms (by

maintaining connexin43 phosphorylation status); (4) angiogenesis; and possibly (5) cardiac regeneration [Detillieux *et al*, 2003; Ishibashi *et al*, 1997; Jiang *et al*, 2004; Kardami *et al*, 2007; Kardami *et al*, 1993; Padua *et al*, 1998; Sheikh *et al*, 2001; Srisakuldee *et al*, 2006]. These effects have been shown to be mediated by signal transduction effectors including PKC, Erk 1/2 MAPK, JNK, and CK2 [House *et al*, 2003; House *et al*, 2005; Jiang *et al*, 2002; Jiang *et al*, 2004; Liao *et al*, 2007; Padua *et al*, 1998; Sheikh *et al*, 2001].

FGF-2-induced activation of PKC contributes to cardioprotection through changes in inotropy, and phosphorylation of connexin 43 [Ishibashi *et al*, 1997; Jiang *et al*, 2002; Padua *et al*, 1998]. Effects of PKC on inotropy remains to be clarified; FGF-2's negative inotropic effect was shown to be diminished with chelyrethrine treatment which may result from changes in myocyte oxygen consumption/efficiencies through translocation of PKC δ and PKC ϵ to the membrane [Ishibashi *et al*, 1997; Kang and Walker, 2005; Noguchi *et al*, 2001; Padua *et al*, 1998]. Effects of PKC activation on connexin 43 phosphorylation involve multiple paradigms that contribute to cardioprotection. For example, the prevention of mitochondrial permeability transition (MPT) pore opening leads to decreased susceptibility of cardiac myocytes to cell damage [Srisakuldee *et al*, 2009b]. In addition, FGF-2 administration contributes to the maintenance of intercellular communications and cardiac rhythm by maintaining the phosphorylation status of connexin 43 to pre-ischemic patterns [Srisakuldee *et al*, 2009a; Srisakuldee *et al*, 2006].

FGF-2–induced activation of the Erk1/2 MAPK pathway has long been established [Bailly *et al*, 2000; Bogoyevitch *et al*, 1994; Padua *et al*, 1998]. A link between Erk 1/2 activation and in cardioprotection is supported by decreased contractile function recovery after treatment with U-0126 (an inhibitor of Erk 1/2) in a low-flow model of ischemia reperfusion [House *et al*, 2005]. However, *in vivo* studies demonstrating that administration of a mutated version of FGF-2 (which does not activate the MAPK cascade) upon LAD ligation surgery remains cardioprotective suggests that Erk 1/2 activation is not essential for FGF-2–induced cardioprotection [Bailly *et al*, 2000; Jiang *et al*, 2004]. However, FGF-2-induced Erk 1/2 activation may be involved in the protection upon reperfusion, probably via an anti-apoptosis approach (may also involve PKC and the PI3kinase pathways) [Hausenloy *et al*, 2005; Kardami *et al*, 2007].

Other important proteins in FGF-2 signaling include casein kinase 2 (CK2) and Akt. FGF-2–induced mitogenesis has been shown to require the ligand-receptor complex translocation into the nucleus in association with translokin, and its subsequent activation of casein kinase 2 (CK2) [Bonnet *et al*, 1996; Bossard *et al*, 2003]. CK2 is not essential for acute cardioprotection by FGF-2, but has been shown to be important in long-term cardiac recovery from a permanent occlusion model of injury, presumably through its downstream mitogenic effects [Jiang *et al*, 2004]. Additionally, FGF-2–induced protection upon reperfusion has also been demonstrated to induce activation of PKC and Akt [Jiang *et al*, 2009].

FGF-2 effects post-injury may also involve effects on the remodelling process, including the stimulation of angiogenesis and cardiac regeneration. FGF-2-stimulated angiogenesis is well established and has been demonstrated in multiple animal models [Miyataka *et al*, 1998; Uchida *et al*, 1995; Unger *et al*, 1994; Watanabe *et al*, 1998; Yanagisawa-Miwa *et al*, 1992]. Improved function from exogenous FGF-2 treatment can be achieved using a non-angiogenic mutant FGF-2 [Jiang *et al*, 2004], however, FGF-2 can contribute to improved cardiac remodelling by stimulating angiogenesis. Consequently, assessment of feasibility of FGF-2 delivery for angiogenic purposes has been successfully executed with minimal complications in several clinical trials employing patients with chronic ischemic disease [Laham *et al*, 2000a; Laham *et al*, 1999; Lazarous *et al*, 2000; Lederman *et al*, 2002; Simons *et al*, 2002]. Clinical trials have resulted in primarily positive effects with reports of some improvement of angina, and tissue perfusion [Laham *et al*, 2000b; Laham *et al*, 1999; Lazarous *et al*, 2000; Lederman *et al*, 2002]. However, several trials have also resulted in non-significant results, in addition to some patients suffering from proteinuria and hypotension [Lederman *et al*, 2002; Simons *et al*, 2002; Unger *et al*, 2000]. The beneficial effects of FGF-2 treatment for the purposes of angiogenesis are certainly appealing. However, the varying (positive and null) results also indicate that further analysis, of patient status as well as of treatment modality, is necessary to optimize FGF-2-induced angiogenesis while limiting patient complications. Furthermore, well-designed large clinical trials will be important in trying to definitively determine whether FGF-2 delivery could significantly improve neovascularization at the clinic.

I. RESEARCH OBJECTIVES

As reviewed in the previous sections, an increase in FGF-2 provides acute beneficial effects by directly protecting cardiac cells from ischemic cell death; additionally, the ability of administered FGF-2 to promote cell migration, stimulate cell proliferation and new vessel formation provides long term benefits for tissue salvage after myocardial infarction.

While the effects of administered FGF-2 have been investigated in several studies, the effects of a chronic elevation in endogenous FGF-2 expression on recovery/remodeling after ischemic injury are not known. The effects of chronic FGF-2 elevation on cell populations infiltrating an ischemic lesion are also not known. The overall objective of this work is to investigate the relationship between increased FGF-2 expression in the heart and response to ischemic injury.

The main hypothesis is that chronic increases in FGF-2 expression are associated with decreased cardiac muscle loss and improved functional recovery in an *in vivo* model of ischemic injury (high dose isoproterenol administration).

Specific research objectives are:

1. To determine whether high levels of chronic FGF-2 overexpression in the heart has sustained beneficial effects for cardiac outcome after injury, using homozygous FGF-2 transgenic mice

2. To determine if modest levels of chronic FGF-2 overexpression in the heart are beneficial for cardiac outcome after injury, using heterozygous FGF-2 transgenic mice
3. To investigate the role of immunosuppression and T-cells in mediating the effects of FGF-2 on cardiac response to injury
4. To determine whether an acute model of swim exercise is adequate to confer cardioprotection from ischemic injury.

CHAPTER V

MATERIALS AND METHODS

A. ETHICS APPROVAL

All animals were housed and treated in accordance with the current *Guide for the Care and Use of Laboratory Animals*, published by the Canadian Council on Animal Care.

B. TRANSGENIC MOUSE MODELS

B.1. FGF-2 transgenic mouse line

A mouse model overexpressing FGF-2 was generated by pronuclear injection of a cDNA coding for the rat low molecular weight 18 kDa isoform of rat FGF-2 under the control of a Rous sarcoma virus promoter (RSVp.metFGF). These transgenic animals were generated using the CD-1 mouse strain. The mice are fertile and have no overt difference in phenotype from normal CD-1 mice. These transgenic mice were previously characterized and described [Sheikh *et al*, 2001].

C. ASSESSMENT OF TRANSGENIC ANIMALS

C.1. Genotyping by polymerase chain reaction (PCR)

Isolation of DNA from mouse-tail snips was originally conducted by a phenol

extraction method. DNA isolation using the DNeasy® Blood and Tissue Kit (Qiagen Inc, Mississauga, ON) was eventually adopted. To assess for transgenesis in our FGF-2 overexpressing mouse line, we developed a polymerase chain reaction (PCR) procedure to detect a DNA sequence within the transgenic mice where the RSV promoter conjoins with the rat FGF-2 gene. The sequences of the primers used are listed as FGF-2 forward and reverse primers for PCR in Table 1. A band of approximately 300 bp can be detected in a 1% agarose gel by electrophoresis for 45 minutes running at 100 volts.

C.2. Detection of RNA by Reverse Transcription (RT) and Quantitative PCR (qRT-PCR)

To detect FGF-2 expression levels in transgenic versus non-transgenic mouse hearts, semi-quantitative real time PCR (qRT-PCR) was performed. RNA was isolated using the RNeasy® fibrous tissue midi kit (Qiagen Inc., Mississauga, ON) from whole mouse hearts flash frozen in liquid nitrogen immediately after excision. Two µg of isolated RNA was used for reverse transcription using the QuantiTect Reverse Transcription kit (Qiagen Inc, Mississauga, ON). Semi-quantitative realtime PCR was conducted using an iCycler (BioRad) with total reaction volumes of 30 µL containing: 2.5 mM MgCl₂, 0.025% DMSO, 0.6 µL (1:1000) SYBR green (Sigma, catalogue #S9430), 0.3 µL (1:1000) fluorescein calibration dye (BioRad, Catalogue #170-8780), and 0.75 units platinum Taq DNA polymerase (Invitrogen). Primers used to detect FGF-2 and β-2-microglobulin (B2m), which is assumed to be constitutively active, are listed in Table 1. Gene expression was quantified by the comparative CT method as

described previously [Dharmaraj]. Calculated relative expression values were normalized to B2m values. Tests were run in triplicate on four independent samples.

C.3. Protein Immunoblotting

Western blotting was conducted as previously described with minor modifications [Srisakuldee *et al*, 2006]. Briefly, total protein was isolated from whole mouse hearts, and flash frozen in liquid nitrogen immediately after excision. Frozen mouse hearts were powdered in liquid nitrogen using a mortar and pestle and transferred to a 15 ml tube on ice. Further homogenization is accomplished using a glass and Teflon apparatus in a solution with approximately 10 volumes of TMN buffer (10 mM Tris-HCl pH=7.4, 100 mM NaCl, 300 mM sucrose, 2 mM MgCl₂, 1% thioglycol, 60 mM β -glycerophosphate, 10 mM NaF, 0.25% (v/v) NP40 containing the following inhibitors at a 1:100 dilution: Protease Inhibitor Cocktail Set II (EMD catalogue #524625), and Set IV (EMD catalogue #524628))/weight. Homogenized samples were transferred to 1.5 ml microfuge tubes where an equal volume of 2x SDS buffer (20% (v/v) glycerol, 100 mM Tris-HCl pH=6.8, 2% (w/v) SDS, 60 mM β -glycerophosphate, 5 mM EDTA, 5 mM EGTA, 2 mM NaOV, 1 mM NaF) with inhibitors was added. All samples were boiled for 5 minutes then sonicated at 40 Hz, then centrifuged at 21,000 x g for 15 minutes at 4°C to remove insoluble material.

All samples for SDS-PAGE were loaded and run on a SDS-PAGE gel (15%) as previously described, followed by transfer to a polyvinylidene fluoride (PVDF)

membrane [Srisakuldee *et al*, 2006]. Membranes were blocked for 1 hour in TBS-T (10 mM Tris-HCl pH 8.0 or 7.6, 150 mM NaCl, 0.5% v/v Tween-20) containing 10% skim milk powder. The blot was incubated with primary (FGF2) antibody in 1% milk TBS-T overnight at 4°C. The following day, blot was washed then incubated with secondary antibody (horseradish peroxidase-bound) for 1 hour at room temperature. After further washing, the membrane was incubated with ECL (Amersham Biosciences, Piscataway, NJ) and exposed to film.

D. *IN VIVO* MODELS OF INJURY

D.1. High-dose isoproterenol administration

D.1.1. Induction of injury

Transgenic mice and their non-transgenic counterparts (aged 8 to 14 weeks) were weighed on the day of drug delivery. Pre-treatment with the analgesic ketoprofen (Anafen®, MERIAL Canada, Baie d'Urfé, QC) at a dose of 5 mg/kg was given subcutaneously one hour prior to isoproterenol administration. (-)-Isoproterenol hydrochloride (Sigma, St. Louis, MO) was diluted in sterile saline to stock solutions varying between 30 and 50 mg/ml depending on the average weight of the group of animals so that final volume administered is between 100 and 180 µL. Isoproterenol was given intraperitoneally at a final dose of 160 mg/kg. Animals were monitored for at least one hour after drug administration, at which point symptoms have usually subsided. These symptoms can include bulging of eyes, piloerection, and excess salivation. Mortality rates due to isoproterenol delivery at the dosage given were close to 0%.

For experiments conducted in the R.O. Burrell animal facility within the St. Boniface General Hospital Research Centre, it is important that two separate incidences of increased mortality be documented. Both instances involved a large number of normal CD-1 male mice shipped from Charles River. The majority of the animals affected were males, and suffered from the inability to remove their urethral plugs leading to the inability to void their bladders. Administration of isoproterenol seemed to have exacerbated the situation. However, one mouse not used in the experiment (received neither saline nor isoproterenol treatment) housed in the same room, but was shipped in the same Charles River crate, also died of the same cause. Successful physical removal of urethral plugs by animal technicians allowed affected animals to live normally.

D.1.2. Immunosuppression with cyclosporine A or anti-CD3 antibodies

For immunosuppression, transgenic and non-transgenic mice (12 - 14 weeks old) were given daily gavage treatments of 75 mg/kg cyclosporine (Neoral; Novartis Pharmaceuticals Canada Inc, Dorval PQ), or 50 µg of anti-CD3ε antibodies intraperitoneally (BioLegend, San Diego, CA) for a period of four days before, and for 4 days following, isoproterenol administration. Isoproterenol injection was conducted as described above.

E. EXERCISE

Animals were weighed daily prior to swim. During the swim, mice were placed in a waterbath (50 x 30 x 20 cm) filled with fresh water kept at 32° to 37°C (10 mice maximum per waterbath). To eliminate potential effects of stress/fear from being placed in deep water, control “water” mice were also placed in an identical waterbath holding approximately 1 to 2 inches of water at 32° to 37°C so that mice were standing and walking in water but did not require swimming. This swimming protocol was based on previously published literature [Geisterfer-Lowrance *et al*, 1996; Kaplan *et al*, 1994], with slight modifications. For acute protocol, mice were placed in the waterbath and let swim once for a maximum of 30 minutes. For the chronic protocol, mice swam twice daily at increasing increments of 10 minutes daily, such that on day 1, mice swam for 10 minutes in the morning and 10 minutes in the afternoon, and the next day 20 minutes twice, and so on, until a maximal swim time of 90 minutes twice daily, was reached on the 9th day of exercise. The chronic protocol was for a total of 28 days/5 days per week. Mice were monitored closely throughout the duration of the swim. After swimming, mice were placed under a heat lamp on fluffed clean dry towels to help shorten time for drying fur and to help keep their body temperature from dropping. Once animals were dry, they were returned to their designated cages. Animals were given one bolus of high dose isoproterenol as previously described 48 hours after swim. Cardiac function was assessed by echocardiography 24 hours, 2 weeks, and 4 weeks after isoproterenol administration.

F. ASSESSMENT OF CARDIAC FUNCTION

F.1. Echocardiography

Echocardiographic analysis (via M-mode echocardiography and tissue Doppler imaging) was performed in collaboration with Dr. Davinder Jassal in the Institute of Cardiovascular Sciences at the St. Boniface Hospital Research Centre (Winnipeg, Canada). Briefly, M-mode echocardiography is used for the assessment of heart measurements including left ventricular internal systolic dimension and left ventricular internal diastolic dimensions. These values are used to determine cardiac function, specifically, values for ejection fraction and fractional shortening. Tissue Doppler imaging on the other hand, is a less load-dependent measure of contractility. Measures of left ventricular endocardial velocity and strain rate are determined using this technique, demonstrating the cardiac myocytes' capacity to contract.

Transthoracic echocardiography was conducted using a 13-MHz probe (Vivid 7, GE Medical Systems, Milwaukee, WI) in unanaesthetized mice at baseline, and 24 hours, 2 weeks and 4 weeks post-injection. Hearts were imaged in the 2D parasternal short axis view and three different frames of a M-mode echocardiogram were recorded. LV end-diastolic diameter (LVEDD) and LV fractional shortening (FS) were measured. The LV ejection fraction (EF) was calculated using the prolate ellipsoid geometric model and parasternal long axis views for measurement of LV end-systolic and diastolic volumes [Rodrigues *et al*, 2004].

Tissue Doppler Imaging (TDI) was acquired on a parasternal short axis view at the level of the papillary muscles, at a rate of 483 frames per second [Neilan *et al*, 2006]. For peak systolic endocardial velocity (V_{ENDO}), a region of interest (0.2 x 0.2 mm) in the posterior wall was analyzed. Radial strain rate (SR) was measured over a distance of 0.6 mm (Echopac PC, GE Medical, Milwaukee, WI). The temporal smoothing filters were turned off for all measurements. The values obtained in five consecutive cardiac cycles were averaged.

G. ASSESSMENT OF MYOCARDIAL INJURY *IN VIVO* AND EFFECTS OF TREATMENT

G.1. Heart weight – to – body weight ratio

Mice were weighed immediately prior to euthanization by cervical dislocation, at which point hearts were rapidly excised and atria were removed. Cardiac ventricles were blotted on paper towels to remove excess blood, then weighed. The ratio of heart weight and body weight are calculated and plotted on a graph.

G.2. Morphology

G.2.1. Preparation of paraffin-embedded slides

Hearts stained for morphological assessment were removed directly after mouse euthanasia and fixed in PBS-buffered formalin. Formalin-fixed hearts were embedded in paraffin and cut into 7 μm cross-sections, and prepared for haematoxylin and eosin

staining (see below). Tissue sections were deparaffinized in xylene for 5 minutes 3 times (using fresh xylene), and gradually rehydrated in decreasing concentrations of ethanol in water (95% ethanol in water for 2 minutes twice, 90% ethanol for 10 minutes, 80% ethanol for 10 minutes, 70% ethanol for 10 minutes). Slides were then placed in PBS.

G.2.2. Haematoxylin & eosin staining

Deparaffinized sections were immersed in haematoxylin (Harris Hematoxylin, Protocol®, Fisher Scientific Company LLC, Kalamazoo, MI) for 5 minutes using a staining dish, and subsequently washed with tap water (2 to 5 minutes) to remove excess dye. After dipping several times in distilled water, slides were immersed in acid alcohol for 3 to 10 dips then washed in tap water for 5 minutes. Slides were again dipped in distilled water and subsequently submerged in ammonia water for 2 minutes, then again rinsed in tap water for 5 minutes. Counterstaining with eosin was accomplished by placing the slides in another staining dish containing eosin (Accustain® Eosin Y Aqueous Solution, Sigma-Aldrich Inc., St. Louis, MO) for 15 seconds to 2 minutes, and followed by a tap water rinse until the water run off was colourless. After staining, the tissue sections were put through a dehydration process involving gradual introduction to increasing concentrations of ethanol in water (10 dips in 50%, 10 dips in 70%, 2x- 5 minutes in 80%, 2x- 5 minutes in 90%, 5 minutes in 100% ethanol). To complete the dehydration process, slides were submerged in xylene for 4 minutes prior to mounting of coverslip using Permount (Fisher Scientific, Fair Lawn NJ).

G.2.3. Morphometric analysis

To assess the effects of chronic upregulation of FGF-2 on myocardial injury *in vivo*, deparaffinated sections were stained with haematoxylin and eosin to enable detection of myocardial organization or disarray, and cellular infiltration. Haematoxylin and eosin-stained heart sections from isoproterenol-treated transgenic and non-transgenic mice, cyclosporine-treated or vehicle-treated, were evaluated for severity of “damage” by quantitation of areas with vacuolar formation, cellular infiltration, edema, and fibrosis. Hearts were sectioned into 13 cross section ‘levels’ from apex to base, whereby each level is separated by approximately 300 μm . Each cross section level was assessed for such areas of “damage” at 5 distinct regions (each 0.3 mm^2) using the “lasso” and “fill” features in Adobe Photoshop, or the Nikon NIS-Elements Basic Research v.2.10 software, then subsequently quantified using either ImageTool v.2.0. or Nikon NIS-Elements Basic Research software v.2.10. The number and volume of areas of “damage” per level was determined using ImageTool v.2.0.

G.2.4. Assessment of “lesion” volume and number

Ventricular tissue was fixed in formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned from apex to base, whereby every 400 μm a series of 4- μm sections were obtained. Sections were stained with haematoxylin and eosin to assess tissue morphology.

G.3. Identification of infiltrating cells

G.3.1. Bright-field microscopy

To assess for the type of immune cells present within the myocardial infiltrate, two pathologists (Drs. Carmen Morales and Yijun Fan) were asked independently to assess haematoxylin and eosin stained tissue sections for the presence of immune cells including neutrophils, macrophages, and T cells using slides stained for H&E, *e.g.*, Figure 12. They both independently noted the significant presence of a large T lymphocyte population within the myocardium by 24 hours post-isoproterenol administration. Immune cells were photographed using brightfield optics.

G.3.2. Immunofluorescence and Nomarski microscopy

To specifically identify T cells within the areas of infiltration, antibodies were used against the CD3 ϵ cell surface marker specifically expressed by T cells. Transgenic FGF-2 mice were given high dose isoproterenol as previously described and euthanized 24 hours after drug administration. Hearts were extracted, embedded in O.C.T. Compound (Tissue-Tek, Sakura Finetek U.S.A., Torrance, CA), flash frozen in an ethanol/dry ice slurry, and tissue blocks were subsequently cryosectioned into 7 μ m thick sections on Superfrost®/Plus slides (Fisherbrand, Fisher Scientific, U.S.A.). Tissue sections were fixed in cold acetone for 10 minutes then stained with haematoxylin for 5 seconds to stain the nuclei. Sections were then blocked with 2% normal goat serum-1% bovine serum albumin-phosphate buffered saline (BSA-PBS) for 30 minutes at room temperature, and incubated for two hours at room temperature with anti-CD3 antibodies (1:25; DAKO Diagnostics, Mississauga, ON) diluted in 1% BSA-PBS. The secondary

antibody Cy3 (donkey anti-rabbit; 1:50; Jackson ImmunoResearch Laboratories; West Grove, PA) was applied for one hour at room temperature. Control slides were incubated with identical solutions with the exception of the primary antibody was replaced with buffer. Coverslips were mounted using CrystalMount mounting medium. Slides were examined by confocal microscopy and Nomarski microscopy.

H. STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad InStat® software. One-way ANOVA was followed by the appropriate post-test for multiple comparisons, and $p < 0.05$ was considered statistically significant.

Table 1. Oligonucleotide sequences used

PCR	FGF-2 forward primer	GTGCCTAGCTCGATACAATAAACGCC
PCR	FGF-2 reverse primer	GTCCATCTTCCTTCATAG
qRT-PCR	FGF-2 forward primer	CCAGTTGGTATGTGGCACTG
qRT-PCR	FGF-2 reverse primer	TCAGCTCTTAGCAGACATTGGA
qRT-PCR	B2m forward primer	GCTATCCAGAAAACCCCTCAAA
qRT-PCR	B2m reverse primer	GCGGGTGGAACTGTGTTACG

PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; FGF, fibroblast growth factor; B2m, β -2-microglobulin.

CHAPTER VI

RESULTS

A. EFFECTS OF INCREASED ENDOGENOUS FGF-2 ON MYOCARDIAL INJURY *IN VIVO*

In this chapter, the effect of increased FGF-2 in the heart on the extent of myocardial damage after injury *in vivo* is assessed. A previously described transgenic mouse line characterized by overexpression of FGF-2 in the heart, and a high-dose of the β -adrenergic agonist isoproterenol to induce myocardial injury, are used.

A.1. FGF-2 transgenic mice have increased FGF-2 RNA and protein in their hearts compared with non-transgenic counterparts

To assess the role of increased FGF-2 in the heart upon myocardial injury *in vivo*, a transgenic mouse line overexpressing rat FGF-2 under the control of the Rous sarcoma virus (RSV) promoter was used (RSVp.metFGF-2) [Sheikh *et al*, 2001] (Figure 4). Homozygous FGF-2 transgenic mice have been characterized previously, and shown to have significantly increased FGF-2 RNA compared to their non-transgenic counterparts. Assessment of protein levels in the heart revealed a 22-fold increase in FGF-2 in transgenic versus non-transgenic mice (Figure 4). These transgenic animals are of

normal phenotype and fecundity, with the exception of a small but statistically significant increase in the capillary density in their hearts [Sheikh *et al*, 2001].

It is important to note that the overexpression of FGF-2 was achieved by placing the rat FGF-2 gene under the control of the constitutively expressed RSV promoter [Gorman *et al*, 1982]. Consequently, expression of the transgene in the FGF-2 transgenic mice was also detected in skeletal muscle, but not in lung, brain, kidney, spleen, or liver [Sheikh *et al*, 2001].

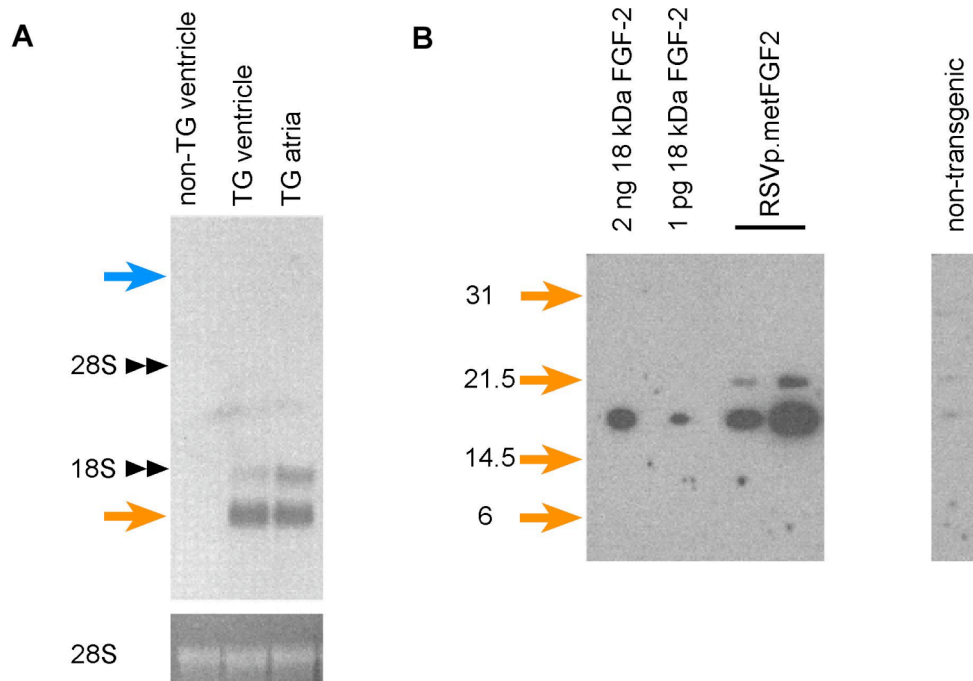


Figure 4. Increased FGF-2 mRNA and protein levels in the homozygous FGF-2 transgenic mice.

(A) FGF-2 RNA as detected by RNA (northern) blotting. The FGF-2 transgene (1.3 kb, orange arrow), and endogenous FGF-2 transcript (6.1 kb, blue arrow) are shown. Double arrows indicate 28S and 18S RNA mobility. The 28S RNA band is shown for loading comparison. (B) FGF-2 protein as detected by protein (western) immunoblotting. Purified 18 kDa FGF-2 was used as a positive control. Orange arrowheads show mobilities of molecular weight markers (kDa) as indicated. Low molecular weight 18 kDa and high molecular weight 21 kDa isoforms are identified using specific monoclonal antibodies against FGF-2. Data provided by Dr. F. Sheikh; modified from [Sheikh *et al*, 2001].

A.2. *In vivo* model of cardiac injury: high dose isoproterenol treatment induces ischemic damage in the mouse myocardium

To assess the role of increased endogenous FGF-2 in the heart upon injury *in vivo*, the high-dose isoproterenol method of myocardial injury was utilized. The β -adrenergic agonist isoproterenol stimulates an increase in heart rate and in cardiac myocyte force of contraction, resulting in an increase in cardiac tissue oxygen demand [Opie, 2004; Rona *et al*, 1959]. In addition, β -agonist stimulation results in the dilatation of the vasculature, which, in combination with the increase in force of contraction, results in an overall-decreased blood, flow to the heart [Beznak, 1962; Beznak and Hacker, 1964; Opie, 2004; Rona *et al*, 1959]. Together, the simultaneous increase in oxygen demand and decrease in blood flow result in the development of ischemic regions within the cardiac myocardium, leading to focal regions of myocardial injury.

To determine whether this mode of injury is effective in the CD-1 mouse strain, the background strain for the FGF-2 transgenic (RSVp.metFGF) animals, mice were injected with isoproterenol dissolved in saline by the intraperitoneal (i.p.) route. At doses of 80 mg/kg isoproterenol or greater, morphological assessment of myocardial tissue sections after drug administration revealed areas of injury and cellular infiltration that are similar to those resulting from the occlusion of coronary arteries (Figure 5). Haematoxylin and eosin-stained sections revealed characteristic features of myocardial lesions including an increased cellular infiltration, as evidenced by numerous cells with large nuclei and relatively small cytoplasmic domains. Other characteristics observed

(and previously characterized) from isoproterenol-induced myocardial injury include evidence of vacuolization and disarray of the cardiac myocytes [Beznak, 1962; Beznak and Hacker, 1964; Meij *et al*, 2002; Opie, 2004; Rona *et al*, 1959; Teerlink *et al*, 1994].

The areas of myocardial damage increase in size relative to the dose of isoproterenol administered [Teerlink *et al*, 1994]. Mortality rates also increase proportionally with isoproterenol dose. A dose of 160 mg/kg was ultimately selected for experimental use based on its ability to provide the greatest measurable effects on the heart, for example, as reflected in measurements of areas of injury, with mortality rates remaining at near 0%.

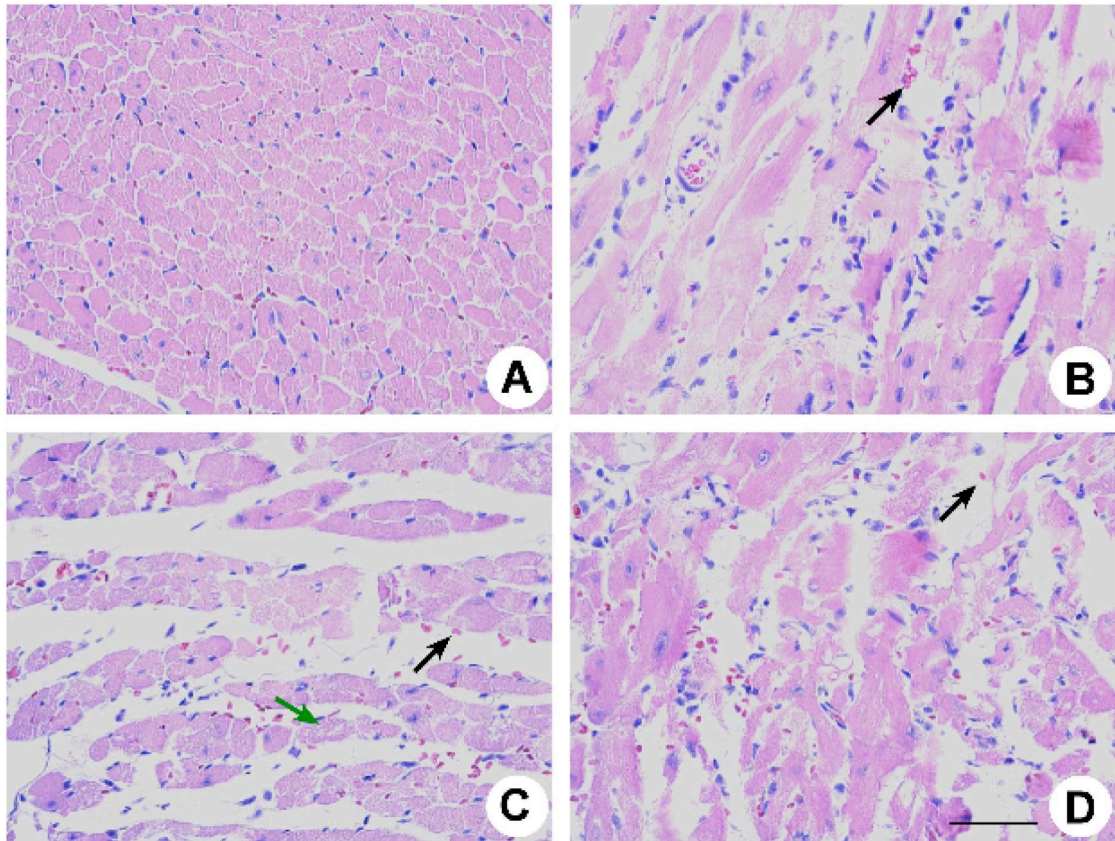


Figure 5. The characteristics of isoproterenol-induced myocardial injury.

Isoproterenol-induced myocardial injury induces apparent damage as reflected by increased edema, vacuolation (green arrow) and myocyte disarray, as are shown here (B, C, D) in comparison to the remaining healthy myocardium unaffected by the treatment (A). Hearts from mice given one bolus of high-dose isoproterenol were paraffin-embedded, sectioned, and stained for haematoxylin (blue/purple – nuclei) and eosin (pink – cytoplasm). Red blood cells appear as darker pink dots (arrows). Bar is equivalent to 50 μ m.

A.3. Increased heart weight-to-body weight ratio was observed in FGF-2 transgenic mice 28 days after isoproterenol administration

To assess the effects of isoproterenol on heart weight-to-body weight ratios in FGF-2 transgenic and non-transgenic CD-1 mice, weights were measured at 1, 7, and 28 days post isoproterenol administration and compared with corresponding values from saline-injected animals. Isoproterenol was given in either 80 or 160 mg/kg doses. A significant 54% increase in heart weight-to-body weight ratio was observed only in the FGF-2 transgenic mice at four weeks after administration of 160 mg/kg isoproterenol (Figure 6). No significant change from controls was observed in other groups. These results suggest that the high dose isoproterenol administration promoted a hypertrophic effect only in hearts with increased FGF-2 expression.

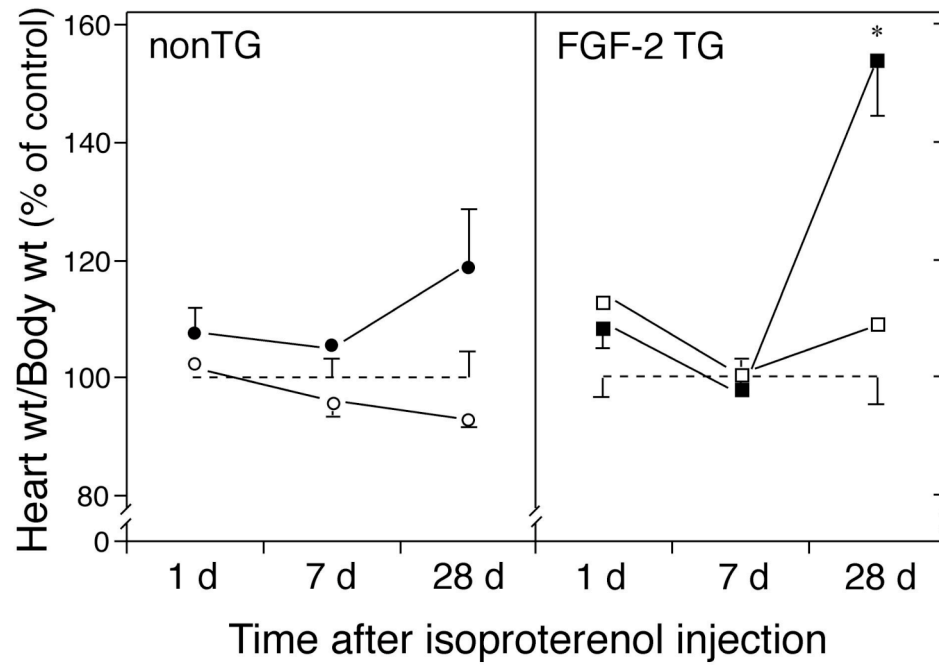


Figure 6. Increased heart weight-to-body weight ratio was observed in FGF-2 transgenic mice 28 days after high dose isoproterenol

FGF-2 transgenic and non-transgenic mice were given saline (dashed lines), 80 mg/kg isoproterenol (open symbols), or 160 mg/kg isoproterenol (closed symbols), and euthanized 1, 7, or 28 days later. Heart weight-to-body weight ratios are expressed as a percentage of the mean value of the matching saline-treated group. * $p < 0.01$ vs. saline-treated FGF-2 transgenic group. Data reproduced with permission from [Meij *et al*, 2002].

A.4. Increased myocardial infiltration and disarray were observed in FGF-2 transgenic mice after isoproterenol-induced myocardial injury

Due to previous evidence of FGF-2–induced cardioprotection upon acute exogenous administration, it was hypothesized that a chronic increase in endogenous FGF-2 would be cardioprotective in an *in vivo* model of injury. To address this hypothesis, homozygous FGF-2 transgenic mice were subjected to the high dose isoproterenol model of injury. FGF-2 transgenic and non-transgenic mice were given one bolus of 160 mg/kg isoproterenol i.p., then euthanized at 6, 12, and 24 hours and 2, 4, and 6 weeks post-injury. Mouse hearts were immediately fixed in formalin and subsequently embedded in paraffin. Tissue blocks were sectioned at 400 μ m intervals from apex to aorta then assessed as previously described [Ferrans *et al*, 1964; Meij *et al*, 2002; Rona *et al*, 1959; Rona *et al*, 1963]. Observation of haematoxylin and eosin-stained heart sections using brightfield microscopy revealed no clear myocardial disturbance (looked identical to saline-treated control hearts) at 6 hours post-injury in neither the transgenic nor non-transgenic group (unpublished results). This is in agreement with observations from Ferrans and colleagues using adult rats whereby infarct-like “lesions” were not detected until 12 hours after isoproterenol administration despite enzymatic and mitochondrial changes earlier on [Ferrans *et al*, 1964]. However, morphological assessment of heart tissue from both transgenic and non-transgenic groups from time points 12 hours and later revealed a clear increase in cellular infiltration and disarray in myocardial tissue of mice that received high-dose isoproterenol (Figures 7 and 8). At every time point assessed starting at 12 hours post-isoproterenol, cardiomyocyte disarray

was always larger in transgenic compared to non-transgenic mice at the same time point, and remained larger until 6 weeks post-injury, the last time point assessed (Figures 7 and 8). Although the cellular infiltration in some transgenic animals was more exaggerated than others within the same group, as a whole, the transgenic group had larger “lesion”-like/cellular infiltration areas. Therefore, instead of the hypothesized decreased injury based on the demonstrated cardioprotective properties of FGF-2, our data suggested the opposite, namely increased injury inferred by the increased cellular infiltration associated with a chronic increase in endogenous FGF-2 levels. The rapidity of the response (within 12 hours) raised the possibility that this was potentially an exacerbated inflammatory response.

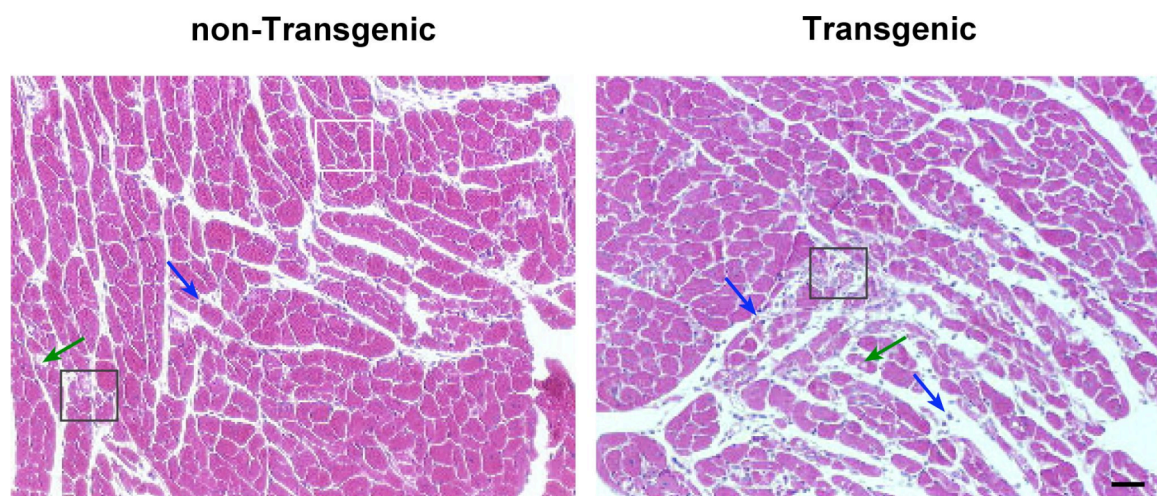


Figure 7. Transgenic mouse hearts have increased “lesion”-like/cellular infiltration regions in comparison with non-transgenic animals after isoproterenol-induced myocardial injury.

Transgenic and non-transgenic mice were given a 160 mg/kg isoproterenol bolus and then euthanized 48 hours later. Hearts were sectioned and stained for haematoxylin and eosin. “Lesions” were defined as areas with increased edema, vacuolization of cells (green arrows), myocyte disarray (grey boxes; compare with uninjured regions within the white box on left), and cellular infiltration (blue arrows). Bar is equivalent to 50 μ m.

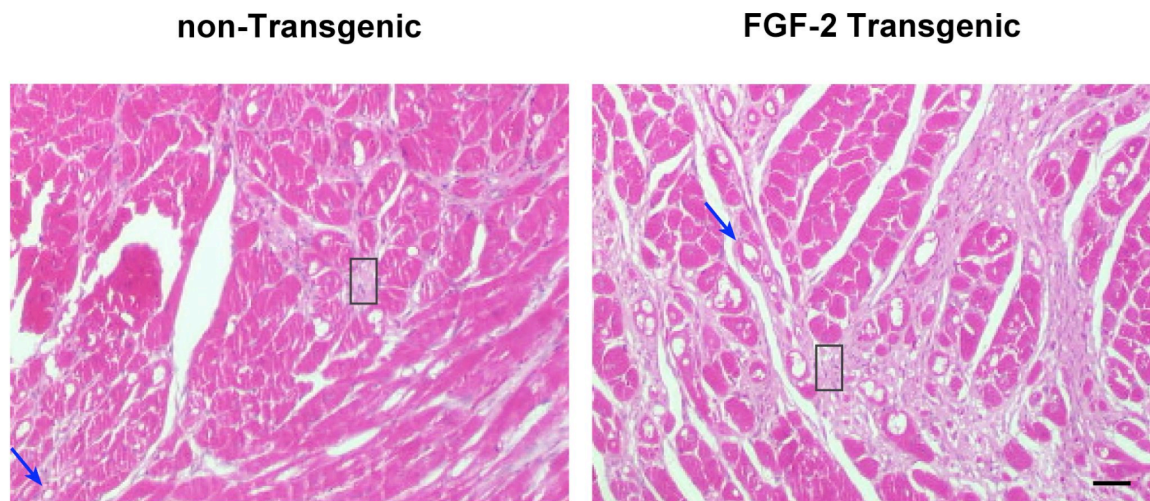


Figure 8. Transgenic mouse hearts had increased regions of scarring 6 weeks after isoproterenol-induced myocardial injury.

Transgenic and non-transgenic mice were given a single bolus of isoproterenol (160 mg/kg) and euthanized 6 weeks later. Hearts were sectioned and stained for haematoxylin and eosin. Scars are defined as areas lacking cardiomyocytes and presenting with increased extracellular matrix (grey boxes). Structures that resemble blood vessels are also visible (blue arrows). Bar is equivalent to 50 μ m.

A.5. Quantitation of areas of cellular infiltration show significantly increased affected myocardium in FGF-2 transgenic mice compared with non-transgenic mice after myocardial injury *in vivo*

To quantitatively determine areas of disarray in the injured hearts, formalin-fixed haematoxylin and eosin-stained tissue sections from FGF-2 transgenic and non-transgenic mice were assessed using Adobe Photoshop and ImageTool software. The tissue sections assessed were taken from 10 to 13 ‘levels’ of each heart cut from apex to base at 400 μm intervals (Figure 9). Features of areas considered part of the “injury” included interstitial areas with increased cellular infiltration, edema, vacuolization, and myocyte disarray. Areas of apparent damage were assessed as a percentage of the total field of view assessed per ‘level’ of the heart (Figure 10). Quantitation revealed a significant 6-fold increase in areas of apparent injury in FGF-2 transgenic mice when compared to non-transgenic mice after isoproterenol-induced myocardial injury ($p < 0.05$, $n=4$).

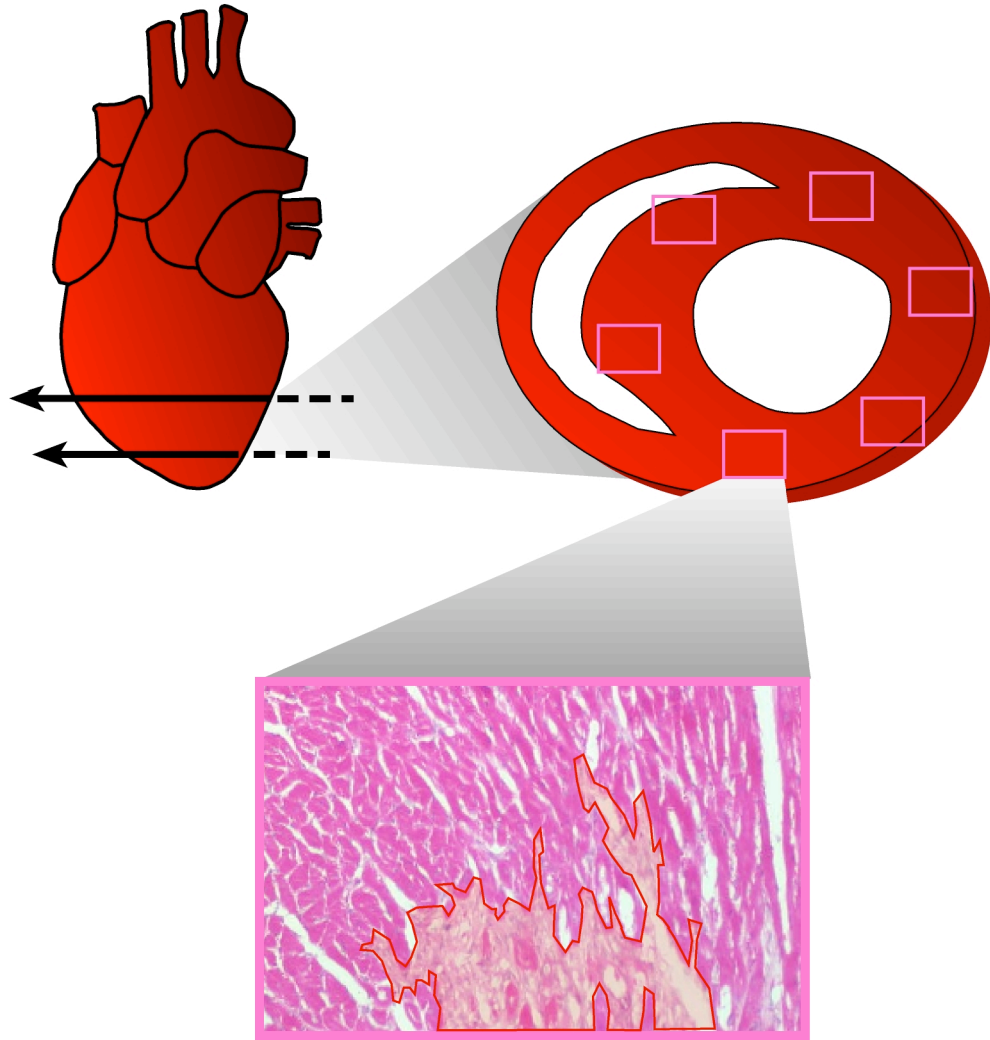


Figure 9. Quantitation of “lesion”-like/cellular infiltration regions in the isoproterenol-injured myocardium.

Tissue sections were stained for haematoxylin and eosin. Areas with the characteristics of damage are defined in Chapter V.G.2.3 and V.G.2.4 using Adobe Photoshop and the area determined using ImageTool software.

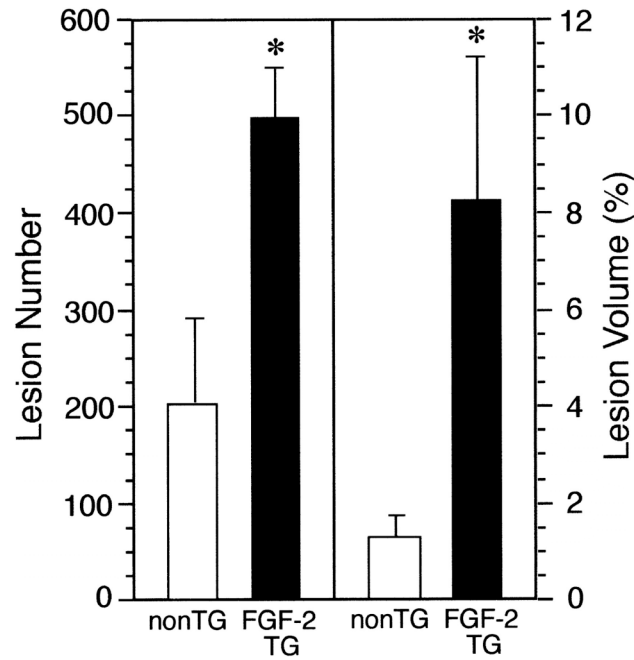


Figure 10. Quantitation revealed more, and significantly larger, “lesion”-like/cellular infiltration areas in transgenic mouse hearts compared with non-transgenic mice after isoproterenol-induced myocardial injury.

Mice were injected with 160-mg/kg isoproterenol and euthanized one day later. Paraffin-embedded heart tissue sections were stained with haematoxylin and eosin and assessed as shown in Figure 9 and described in Chapter V.G.2.3-4. Five areas per ventricular section and five sections per heart were assessed per animal. Lesion-like volume (mean) is expressed as a percentage of the total cardiac ventricular volume assessed ($5.25 \times 10^7 \mu\text{m}^3$). * $p < 0.05$ when compared to non-transgenic controls. Error bars indicate standard deviation. Original data by Sarah K. Jimenez and reproduced with permission from [Meij *et al*, 2002].

A.6. Pathological assessment of the myocardium after isoproterenol-induced myocardial injury revealed a significant increase in infiltrating cells in FGF-2 transgenic mouse hearts

The increase in myocardial disarray appeared to coincide with increased numbers of infiltrating cells. To examine for any changes in cell populations between FGF-2 transgenic and non-transgenic mice, cells were assessed in six areas of the myocardium in each animal for numbers of cardiac myocytes (healthy and damaged), as well as infiltrating cells. A significant increase in damage (as defined by edema, vacuolation, and myocyte disarray) was noted in transgenic hearts, whereas the total numbers of cardiac myocytes counted in each group in the assessment were comparable (Figure 11). Additionally, an increase in infiltrating cells in hearts of FGF-2 transgenic animals was also observed.

Increased cellular infiltration suggested an exacerbated inflammatory response. To assess for cells potentially involved in this response, “lesion”-like/cellular infiltration areas were examined closely using brightfield microscopy. With the aid of two trained pathologists, Dr. Carmen Morales (Medical Director of Hematology, Health Sciences Centre, and Professor, Department of Pathology, University of Manitoba, Winnipeg, Manitoba) and Dr. Yijun Fan (Research Associate, Department of Medical Microbiology, University of Manitoba) cells within the cellular infiltrate of haematoxylin and eosin-stained heart sections were identified. Specifically, cells that play a role in the inflammatory response, including neutrophils, macrophages, and T lymphocytes, were

thus identified within the myocardial infiltrate (Figure 12). The identification of these cells used the following criteria: neutrophils, ~10 μm diameter cells containing pink granules, and whose nuclei contain 3 or 4 lobes; macrophages, granular cells of about 10 to 30 μm in diameter, with an irregular shape and kidney-shaped nuclei; and lymphocytes, ~10 μm -diameter cells with round nuclei and little cytoplasm [Gartner, 2007]. It was also apparent that a relatively large number of T cells were present within the myocardium 24 hours after initiation of injury.

These observations concerning T cells were validated by cell staining using specific markers. FGF-2 transgenic mice were injected with isoproterenol and euthanized 24 hours later. Hearts were excised, embedded (O.C.T. compound), and subsequently cut into 7 μm sections. T lymphocytes were identified within the cellular infiltrate in transgenic mouse hearts after myocardial injury. Due to the tendency of mouse cardiac tissue to display autofluorescence (especially within the blue to green spectrum), identification of T cells by immunofluorescence techniques required optimization of a protocol combining confocal and Nomarski microscopy. Fixed heart sections were stained with haematoxylin to enable identification of cell nuclei, then rinsed well to prevent the dye from masking the sought-after epitopes. Haematoxylin-stained slides were subsequently treated with antibodies against CD3 (a protein expressed on the cell surface of T cells) and secondary antibodies conjugated to fluorescent tags, allowing T cell identification (Figure 13). To ensure specificity of CD3 tags, samples of mouse spleen were used as positive controls, and tissue sections not treated with primary anti-CD3 antibodies were used as negative controls. CD3-positive cells were identified

within the cellular infiltrate of isoproterenol-treated mouse myocardium. Thus, areas of myocardial injury in the transgenic FGF-2 animals contained a population of infiltrating T lymphocytes.

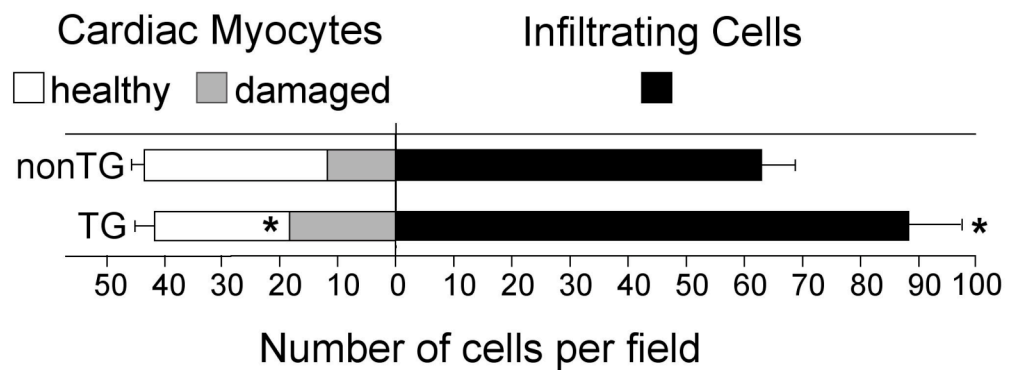


Figure 11. Increased infiltrating cells correlates with number of damaged cardiac myocytes in FGF-2 transgenic mice

FGF-2 transgenic and non-transgenic mice were administered 160 mg/kg isoproterenol and euthanized 24 hours later. Six sections per heart were analyzed for healthy and damaged cardiac myocytes, as well as infiltrating cells within the field of view. Stacked bars show comparable numbers of myocytes in areas assessed. * $p < 0.05$ versus non-transgenic mice. Reproduced with permission from [Meij *et al*, 2002].

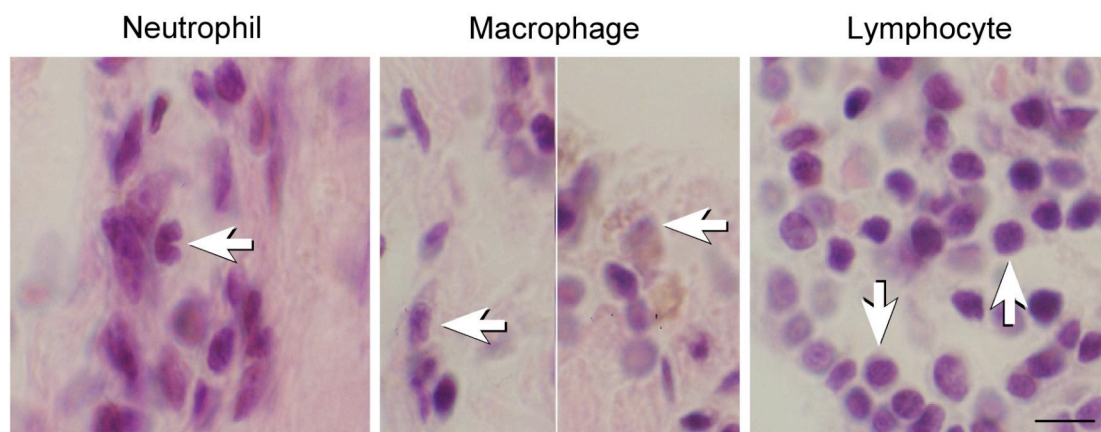


Figure 12. Initial assessment of cellular infiltrate of hearts from mice given high-dose isoproterenol revealed the presence of immune cells.

Haematoxylin and eosin-stained heart tissue sections were examined under brightfield optics. Neutrophils, macrophages, and T lymphocytes were identified within the cellular infiltrate of mouse hearts after isoproterenol administration, as indicated by white arrows. Figure provided by Sarah K. Jimenez and modified/reprinted with permission from [Meij *et al*, 2002].

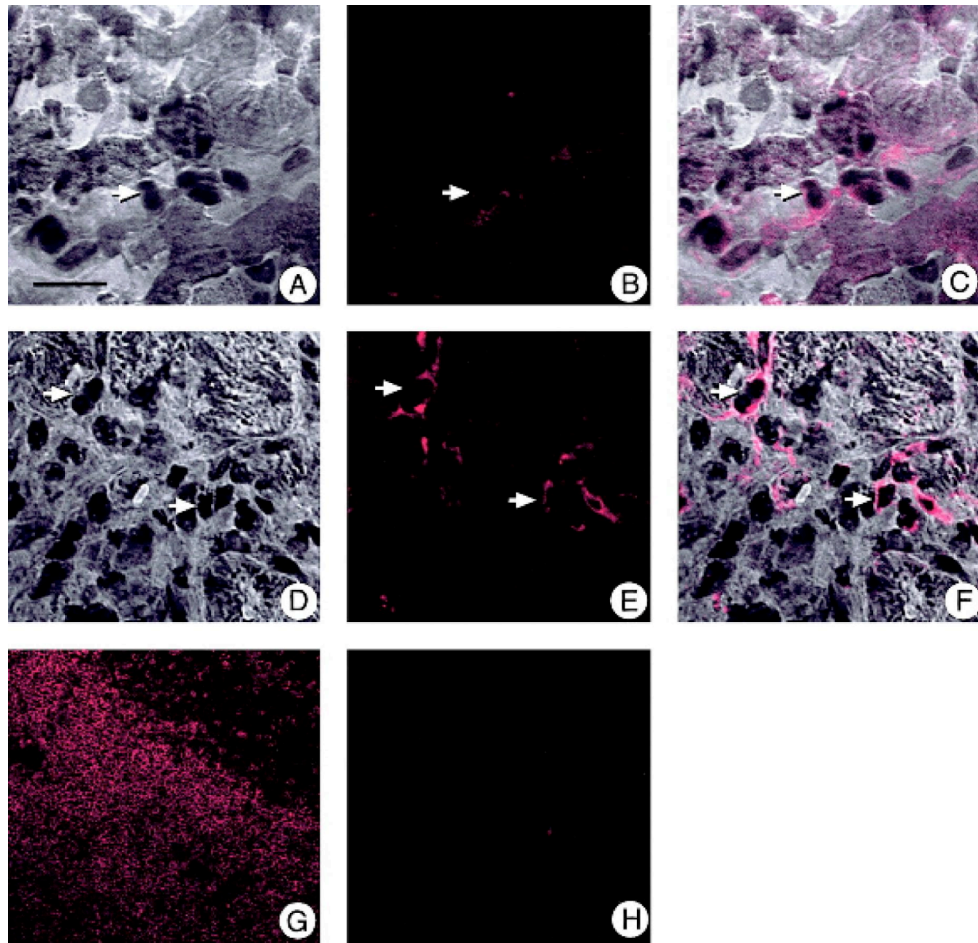


Figure 13. Assessment of the cellular infiltrate of hearts from mice given high-dose isoproterenol revealed CD3-positive cells.

FGF-2 transgenic mice were injected with 160 mg/kg IsP and subsequently euthanized 1 or 4 days later. Hearts were embedded in OCT compound then cryosectioned to 7 μ m thick, and subsequently fixed using acetone. Ventricular sections from 1-day (A-C) and 4-day (D-F) animals were stained with haematoxylin to identify nuclei (A and D) and CD3 antibodies to identify T cells (B and E). Arrows in images separately showing nuclei and CD3 staining identify CD3-positive cells. A spleen section stained for CD3 (G) and a ventricular section from a FGF-2 transgenic mouse without primary antibody incubation (H) are shown as positive and negative controls, respectively. Bar is equivalent to 20 μ m (A-F) and 80 μ m (G and H). Original data from Sarah K. Jimenez. The image is reprinted with permission from [Meij *et al*, 2002].

A.7. CD4-positive cells co-stain for FGFR-1 within the areas of infiltration after isoproterenol-induced myocardial injury

To examine whether the cells within the myocardial infiltrate of animals subjected to high dose isoproterenol could be directly influenced by FGF-2, they were probed for FGFR-1 expression. Mice were given high-dose isoproterenol, euthanized 24 hours later, and hearts excised and prepared for immunohistochemistry. Heart tissues were sectioned (7 μ m), acetone-fixed, and then probed with antibodies against the T cell marker CD4 (a protein expressed on the cell surface of a subset of T lymphocytes) as well as FGFR-1. Nuclei were visualized by dipping slides in bisbenzimide (Hoechst 33258). All slides were mounted and examined by epifluorescence microscopy. Cells co-staining for both CD4 and FGFR-1 were identified in the myocardial infiltrate (Figure 14). These results indicate the presence of FGFR-1-expressing T cells within the areas of infiltration, and suggest a possible direct effect of FGF-2 on the T cells that migrate into the heart as a result of isoproterenol-induced myocardial injury.

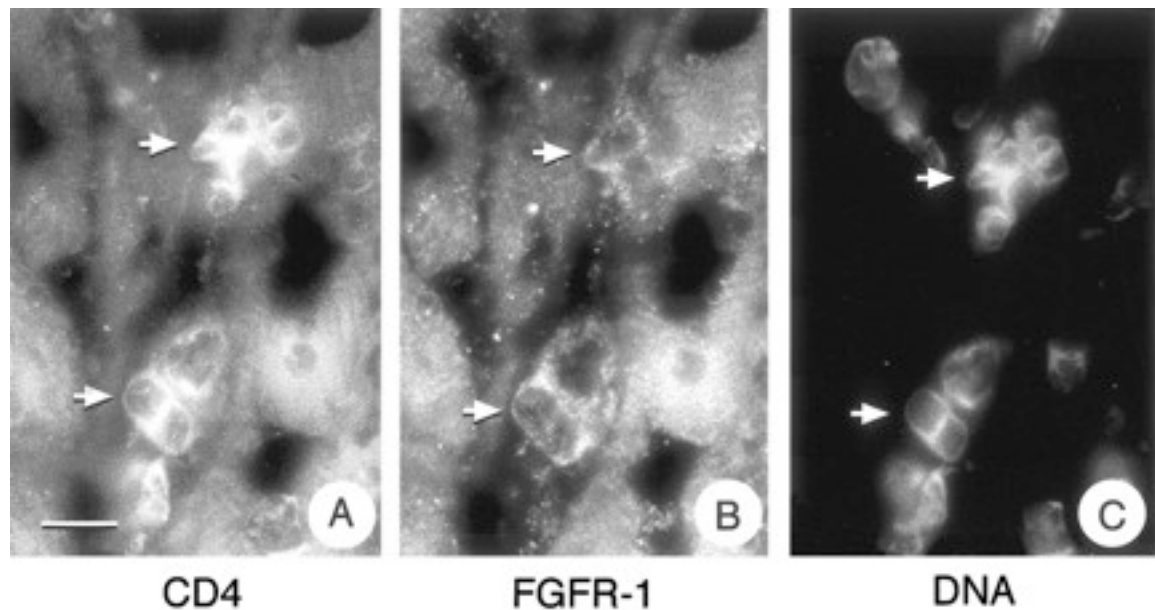


Figure 14. Cells co-expressing the T cell marker CD4 and the FGF-2 receptor FGFR-1 are identified within the myocardial infiltrate

FGF-2 transgenic mice were given a high dose bolus of 160 mg/kg isoproterenol then euthanized the following day. Hearts were excised, sectioned in OCT blocks, and subsequently fixed in acetone. Cryosections of 7 μ m thickness were probed with antibodies against CD4 and Flg (FGFR-1). Nuclei were visualized using Hoechst staining. Cells co-expressing CD4 and FGFR-1 are indicated by arrows. Bar is equivalent to 10 μ m. Original data supplied by Dr. Hanneke Meij, and image modified from [Meij *et al*, 2002].

A.8. Inhibition of T cells by immunosuppression using cyclosporine A or anti-CD3 ϵ antibodies decreases cellular infiltrate and myocardial disarray in FGF-2 transgenic and non-transgenic mouse hearts after myocardial injury

The finding of increased T cell numbers within the FGF-2 transgenic heart infiltrate suggested that T lymphocytes are potential candidates for affecting cardiac outcome, and was therefore investigated. The effect of immunosuppression (specifically targeting T-cell activation) on “lesion”-like/cellular infiltration area size was assessed. T cell activation was inhibited using either cyclosporine A or antibodies against CD3 ϵ . Antibodies against CD3 ϵ were also used in addition to cyclosporine to determine whether a more specific inhibition of T cells would have similar results to a broad immunosuppressive treatment. CD3 ϵ is a molecule expressed on the T lymphocyte cell surface and is required for proper development of mature lymphocytes [Dave, 2009]. Therefore, inhibition of this molecule would specifically prevent the activation of T cells. Mice were given either (1) 75 mg/kg cyclosporine A by gavage, or (2) 50 μ g anti-CD3 antibodies i.p. or (3) vehicle for controls, for 4 days before and 4 days after administration of 160 mg/kg isoproterenol. The dose and timing of immunosuppressive treatments were chosen to be those that have been shown to be effective in mice *in vivo* [Batiuk *et al*, 1996; Hirsch *et al*, 1990]. Formalin-fixed, deparaffinized heart sections from transgenic and non-transgenic groups were stained with haematoxylin and eosin and assessed for cellular infiltration. Observations using bright-field microscopy revealed a decline in lesion areas and numbers, as shown in representative images in Figure 15. Cellular infiltration after immunosuppression and isoproterenol-induced injury is

comparable between FGF-2 transgenic and non-transgenic mice, and between cyclosporine A- and anti-CD3 ϵ antibody-treated mouse hearts.

Haematoxylin and eosin-stained tissue sections were assessed for areas of “lesion”-like/ cellular infiltration using Adobe Photoshop and ImageTool software as described in Chapter V. Immunosuppression was associated with a significant decrease in “lesion”-like areas and volumes in FGF-2 transgenic mice compared to non-immunosuppressed transgenic mice after isoproterenol-treatment ($*p<0.05$, $n=3-4$). Apparent “lesion”/infiltration size after immunosuppression with either cyclosporine or CD3 ϵ antibodies were comparable within groups and lower (although not significantly different) than the isoproterenol only-treated non-transgenic group (Figure 16).

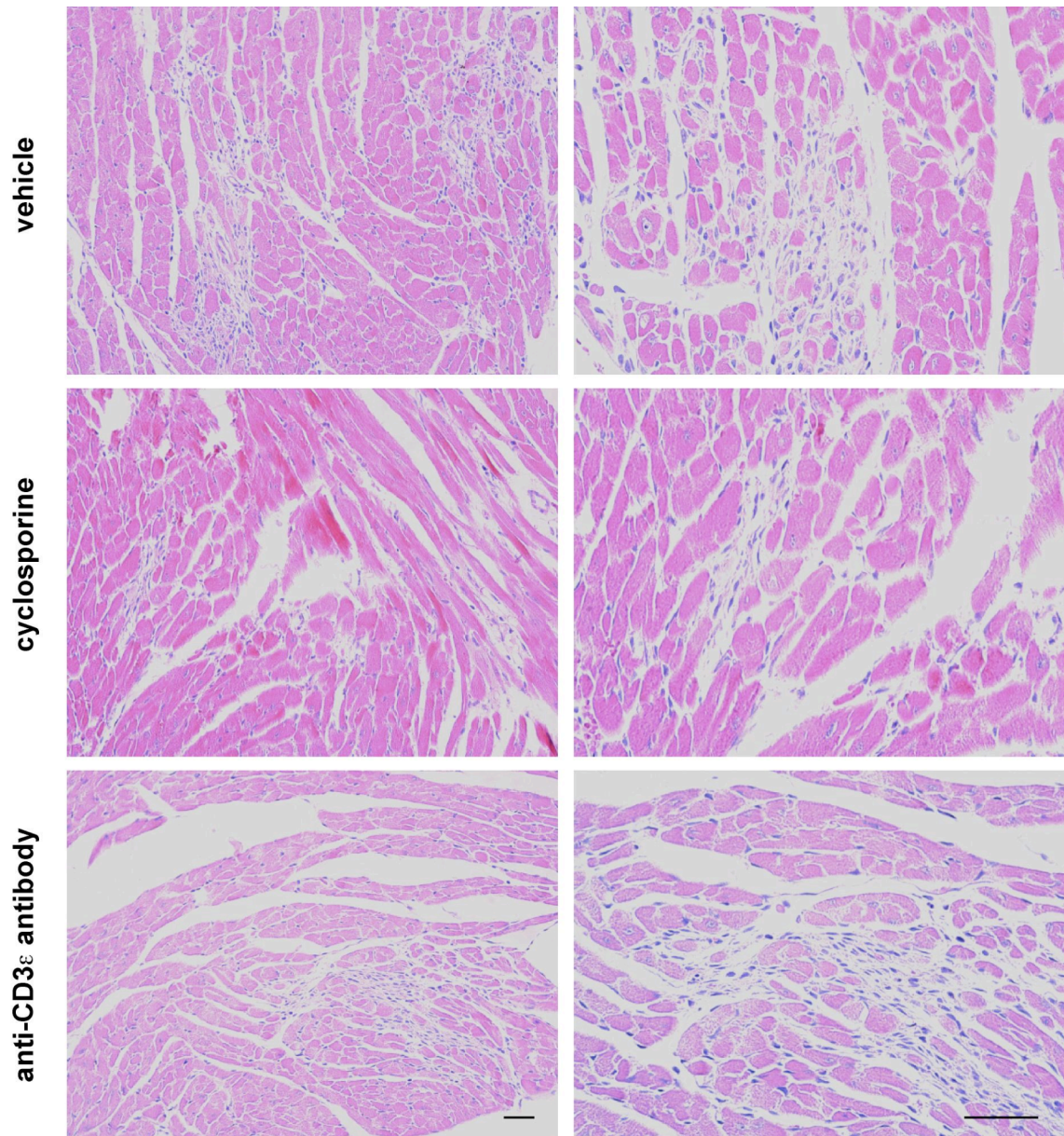


Figure 15. Haematoxylin and eosin-stained sections of hearts treated with immunosuppressive agents show decreased cellular infiltration.

Transgenic FGF-2 mice were given one of: (1) cyclosporine A, (2) antibody against CD3 ϵ , or (3) vehicle, for 4 days prior and 4 days after high-dose isoproterenol administration. Heart sections (7 μ m) were stained with haematoxylin and eosin to enable morphological assessment of cellular infiltration. Bar is equivalent to 50 μ m. These images show the quality of the lesions and are not representative of quantity. Quantification of lesion volume is shown in Figure 16.

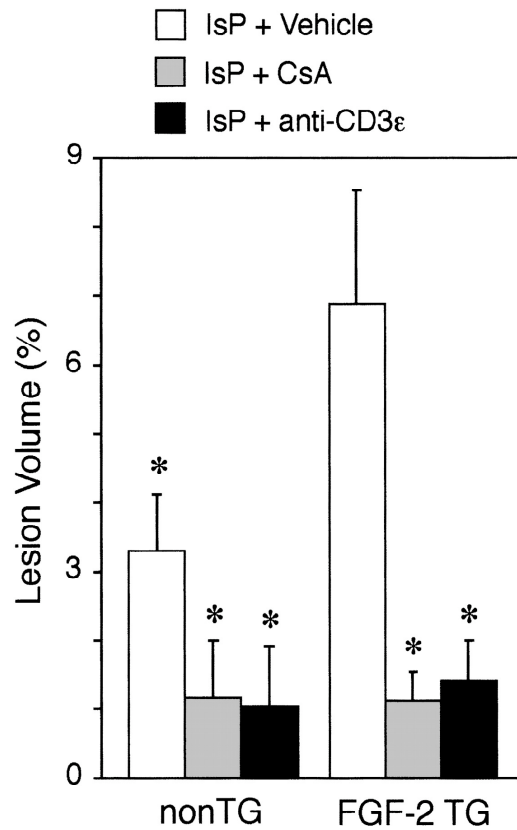


Figure 16. Effect of immunosuppression on the degree of cellular infiltration and “lesion”-like areas.

FGF-2 transgenic and non-transgenic mice were treated daily with immunosuppressants cyclosporine A or anti-CD3ε antibodies, or vehicle for 4 days before and 4 days after 160 mg/kg isoproterenol administration. Heart tissue sections were stained with haematoxylin and eosin and assessed as described in Chapter V.G.2.3-4. Five areas per ventricular section and five sections per heart were assessed per animal. “Lesion” volume (mean) is expressed as a percentage of the total cardiac ventricular volume assessed ($5.25 \times 10^7 \mu\text{m}^3$). Error bars indicate standard deviation. * $P < 0.05$. Original data from Sarah K. Jimenez and reproduced with permission from [Meij *et al*, 2002].

Chapter findings:

In this chapter, the question of whether increased endogenous FGF-2 would affect cardiac injury and repair upon β -agonist-induced myocardial injury was investigated.

Observations made from this study include:

1. Isoproterenol induces myocardial injury in CD-1 mice, as measured by morphological analysis of heart tissue sections.
2. Myocardial “lesions,” as determined by myocardial disarray and cellular infiltration, increased in area in an isoproterenol dose-dependent manner.
3. Increased endogenous FGF-2 resulted in increased heart weight to body weight ratio 28 days after administration of 160 mg/kg isoproterenol, suggesting hypertrophy.
4. Isoproterenol treatment resulted in a significant increase in damaged cardiac myocytes as well as infiltrating cells in FGF-2 transgenic mice compared with their non-transgenic counterparts.
5. After isoproterenol-induced injury, a significant 6-fold increase in areas of cellular infiltration (appeared “lesion”-like) was observed in FGF-2 transgenic when compared with non-transgenic mice.
6. Assessment of the myocardial infiltrate revealed the presence of inflammatory cells including neutrophils, macrophages, and T lymphocytes.
7. T cells expressing the FGF-2 receptor FGFR-1 were identified within the myocardial infiltrate of FGF-2 transgenic mice, suggesting a possible role for T cells in the phenomenon observed.
8. Inhibition of T cell activation by cyclosporine A or by antibodies against CD3 ϵ resulted in the significant decline in “lesion”-like/cellular infiltration area and volume in both FGF-2 transgenic and non-transgenic hearts after isoproterenol-induced injury. Following immunosuppression, the areas of cellular infiltration in FGF-2 transgenic mice were comparable in size to those of non-transgenic animals receiving isoproterenol alone.

CHAPTER VII

RESULTS

B. CARDIAC FUNCTION IN FGF-2 TRANSGENIC MICE AFTER MYOCARDIAL INJURY *IN VIVO*

B.1. Assessment of left ventricular function in isoproterenol-induced myocardial injury in transgenic mice overexpressing FGF-2 by tissue Doppler imaging

In the previous chapter it was shown that a chronic increase in FGF-2 expression in transgenic mice is associated with a significant increase in infiltrating cells after isoproterenol-induced myocardial injury and a, presumably related, increase in myocardial disarray, which would appear to contrast with the acute and sustained beneficial effects of administered FGF-2 [Detillieux *et al*, 2003; House *et al*, 2005; Jiang *et al*, 2009; Kardami *et al*, 2007; Padua *et al*, 1998; Sheikh *et al*, 2001; Srisakuldee *et al*, 2009a]. In this chapter, the aim was to gain a better insight into this apparent conflicting data. Firstly, instead of using FGF-2 homozygous transgenic mice, which express high levels of FGF-2, heterozygous FGF-2 mice were pursued to provide a more modest increase in endogenous FGF-2. Secondly, cardiac outcome was examined instead in terms of cardiac function through Doppler Imaging.

B.2. Heterozygous FGF-2 transgenic mice have increased FGF-2 RNA and protein in their hearts compared with non-transgenic counterparts

Like their homozygous counterparts, heterozygous (RSVp.metFGF2) FGF-2 transgenic mice are of normal phenotype and fecundity. To assess FGF-2 expression in heterozygous FGF-2 transgenic mice, RNA levels from hearts of both FGF-2 transgenic and non-transgenic CD-1 mice were compared. Total RNA was extracted from whole hearts and used for reverse transcription-PCR; products were subsequently used for quantitative realtime PCR analyses. FGF-2 RNA levels were determined using the CT method and normalized using values for β -2-microglobulin (B2m) [Mahoney *et al*, 2004], which was assumed to be expressed constitutively. A significant ~16-fold increase in FGF-2 mRNA was observed in heterozygous transgenic mouse hearts compared to non-transgenic controls ($p < 0.001$, $n = 4$) (Figure 17).

Total protein from whole hearts was also extracted from heterozygous transgenic and non-transgenic animals. Tissue homogenization was performed using a mortar and pestle with liquid nitrogen, and samples were kept constantly on ice to prevent proteolysis. Total protein was separated by SDS-PAGE and transferred to PVDF membrane that was probed using antibodies specific against FGF-2, and then re-probed for GAPDH to normalize for loading. FGF-2 protein levels in heterozygous FGF-2 transgenic mouse hearts was ~2.2-fold higher compared with non-transgenic control hearts ($p < 0.01$, $n = 3$) (Figure 18).

Thus, the 2.2-fold increase in FGF-2 levels in heterozygous FGF-2 transgenic mouse hearts is ~10x less than the 22-fold stimulation of FGF-2 levels in homozygotes, but remain significantly higher compared with non-transgenic controls.

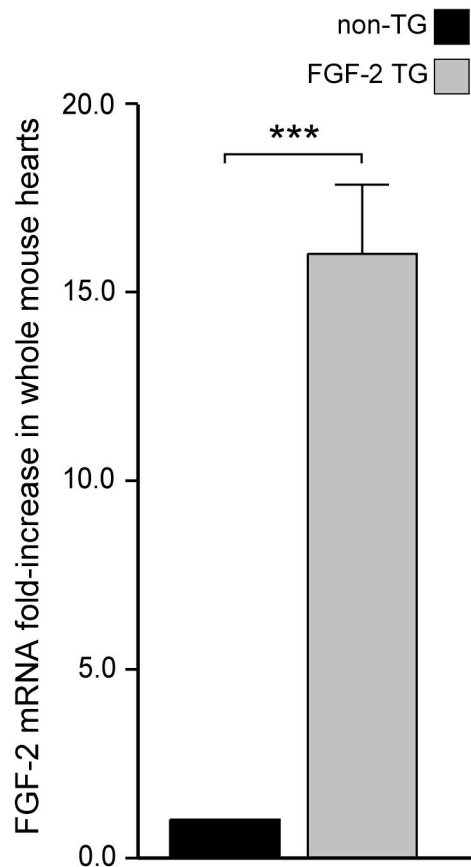


Figure 17. Heterozygous FGF-2 transgenic mice have increased cardiac FGF-2 RNA levels compared to non-transgenic mice.

Total RNA was extracted from heterozygous transgenic (TG) and non-transgenic mouse hearts. RNA expression levels were assessed by quantitative realtime PCR analysis. Using the $\Delta\Delta\text{Ct}$ method of calculation, FGF-2 RNA in heterozygous FGF-2 TG mouse hearts was found to be ~16-fold greater than non-transgenic animals (values arbitrarily set to 1). FGF-2 expression was normalized using beta-2-microglobulin. *** $p < 0.001$ compared to non-transgenic controls. Error bars indicate standard error from the mean.

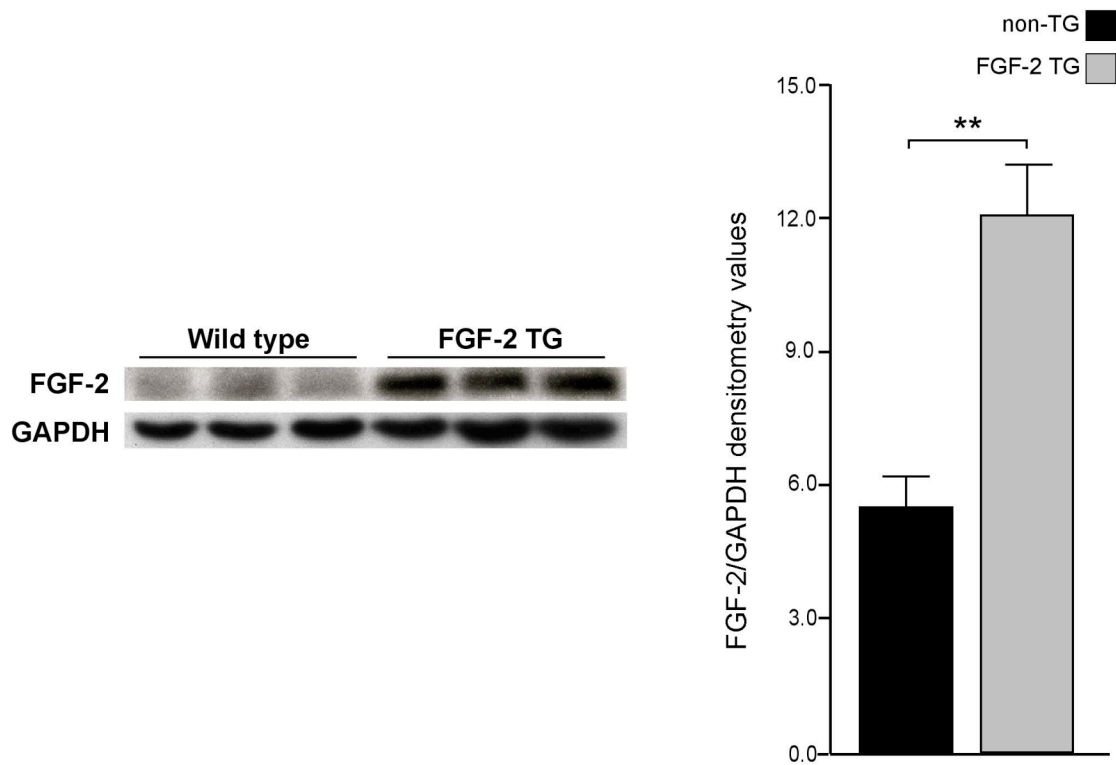


Figure 18. FGF-2 protein levels are significantly higher in the heterozygote FGF-2 transgenic mouse heart when compared to wild type CD-1 mice.

Total protein was extracted from hearts of three transgenic (TG) and three non-transgenic (non-TG) wild type CD-1 mice and subsequently assessed in a Western blot using antibodies specific for FGF-2. Heterozygous FGF-2 transgenic mice were shown to have a ~2-fold increase in FGF-2 protein in their hearts compared with wild type CD-1 mice. ** $P < 0.01$.

B.3. High-dose isoproterenol treatment induces infarct-like histological changes in both FGF-2 transgenic and non-transgenic mice

Previously, it was shown that isoproterenol administration results in myocardial injury as determined by assessment of cardiac morphology in CD-1 mice; this injury is significantly increased in homozygous FGF-2 transgenic animals (Chapter VI). Assessment of myocardial injury after high dose isoproterenol administration was also conducted using heterozygous mice. Heterozygous mice as well as age- and sex-matched non-transgenic control mice were given one bolus of isoproterenol i.p. at a dosage of 160 mg/kg. Sections of mouse heart tissue at 24 hours were stained for haematoxylin and eosin and assessed morphologically for areas of myocardial disarray and cellular infiltration. Heterozygous transgenic mouse hearts displayed increased cellular infiltration compared with non-transgenic controls (Figure 19). Thus, increased endogenous FGF-2 in the heart (even as low as a 2.2-fold protein increase) appears to increase cellular infiltration following *in vivo* isoproterenol-induced myocardial injury.

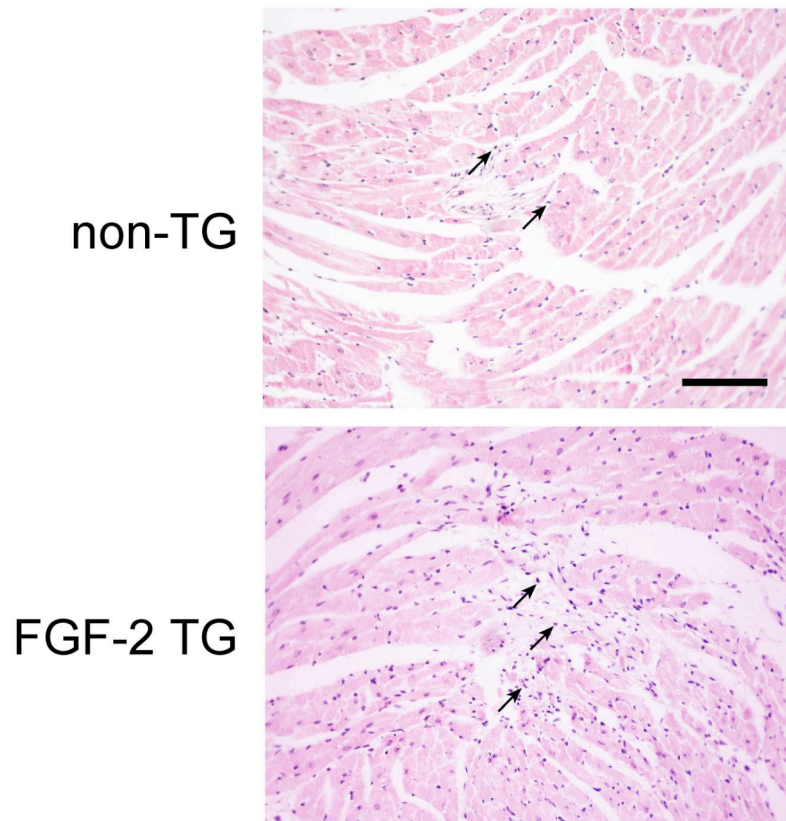


Figure 19. Heterozygous FGF-2 transgenic mice also have greater cellular infiltration than their non-transgenic counterparts.

Heterozygous FGF-2 transgenic (TG) and non-transgenic (non-TG) mice were given one bolus of high-dose isoproterenol (160 mg/kg) and euthanized 48 hours later. Heart tissue were sectioned and stained for haematoxylin and eosin. Areas of infiltration are observed as increased blue-stained nuclei (arrows). Bar is equivalent to 80 μ m.

B.4. Tissue Doppler imaging predicts improved outcome in FGF-2 transgenic mice after isoproterenol-induced myocardial injury

To assess cardiac function, two methods of echocardiography were utilized: standard M-mode, and tissue Doppler imaging. These procedures were conducted in collaboration with cardiologist Dr. Davinder Jassal (St. Boniface Hospital Research Centre, Winnipeg, Manitoba).

Functional parameters were measured by M-mode echocardiography in mice at 24 hours, 2 weeks and 4 weeks after administration of high dose isoproterenol. Baseline values for left ventricular end diastolic diameter, fractional shortening, and ejection fraction all fall within the normal expected values range [Jassal *et al*, 2009]. Under the conditions used, one bolus of 160 mg/kg isoproterenol did not cause significant changes at the organ level as measured by M-mode echocardiography (Table 2, Figures 20 and 21). Although a trend towards a decrease in fractional shortening was observed (from 55 ± 4 to 52 ± 3), the change was not significant (Figure 21).

In contrast to M-mode echocardiography results, significant changes were observed in mice after assessment of tissue Doppler imaging parameters left ventricular endocardial velocity and strain rate (a derivative of left ventricular endocardial velocity). Tissue Doppler imaging parameters were measured prior to isoproterenol administration (baseline), as well as 24 hours, 2 weeks, and 4 weeks post injury in both FGF-2 transgenic and non-transgenic mice. Quantitative results are shown in Figure 24 and representative tracings are shown in Figures 23. Baseline values for both left ventricular

endocardial velocity and strain rate were within the normal expected range for mice. A significant decrease in each parameter was observed in both groups by 24 hours post-isoproterenol injection (Figure 24). Importantly, transgenic mice had improved function when compared to non-transgenic values. For left ventricular endocardial velocity, non-transgenic animals averaged ~48% of baseline by 24 hours, declining to ~36% of baseline by 2 weeks with no further change to 4 weeks post-injection. Transgenic animals, by contrast, averaged ~76% of baseline at 24 hours, with no further decline by 4 weeks post-injection. Strain rate measurements showed a similar pattern, with non-TG animals declining to ~53% of baseline by 24 hours and no further significant decline, and TG animals declining to ~75% of baseline by 24 hours with no further decline.

Table 2. M-mode echocardiography values of FGF-2 transgenic and non-transgenic mice before and after high-dose isoproterenol administration

	timepoint	<i>n</i>	LVEDD	FS	EF
FGF-2 non-transgenics	baseline	10	3.0±0.2	55±4	81±3
	24 h	9	3.1±0.2	55±5	80±3
	2 w	7	3.1±0.2	53±2	80±2
	4 w	7	3.2±0.3	52±3	81±2
FGF-2 transgenics	baseline	11	3.2±0.2	55±5	80±4
	24 h	9	3.1±0.1	55±5	79±3
	2 w	7	3.1±0.2	53±2	80±2
	4 w	7	3.1±0.3	53±3	81±1

LVEDD, Left ventricular end diastolic diameter; FS, fractional shortening; EF, ejection fraction

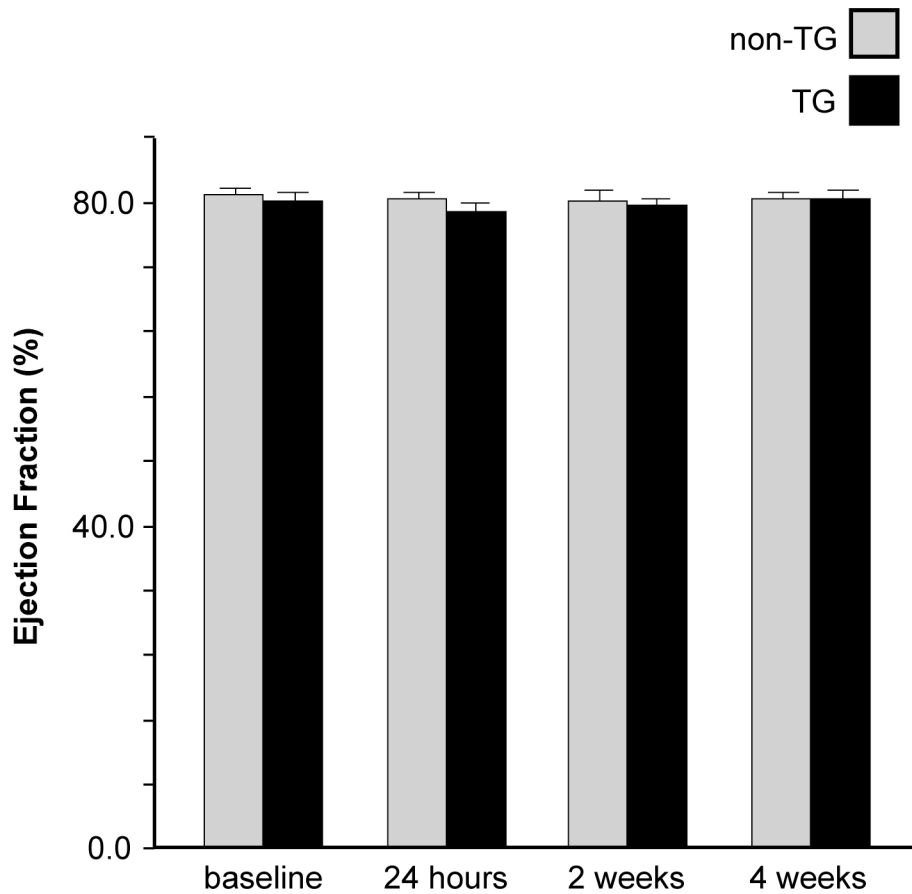


Figure 20. No difference in ejection fraction was observed after isoproterenol-administration as measured by M-mode echocardiography.

Heterozygous FGF-2 transgenic (TG) and non-transgenic (non-TG) mice were given one bolus of high-dose isoproterenol (160 mg/kg). Cardiac function was assessed by measuring ejection fraction by echocardiography at baseline, at 24 hours, 2 weeks, and at 4 weeks post-injury. (n=7-11).

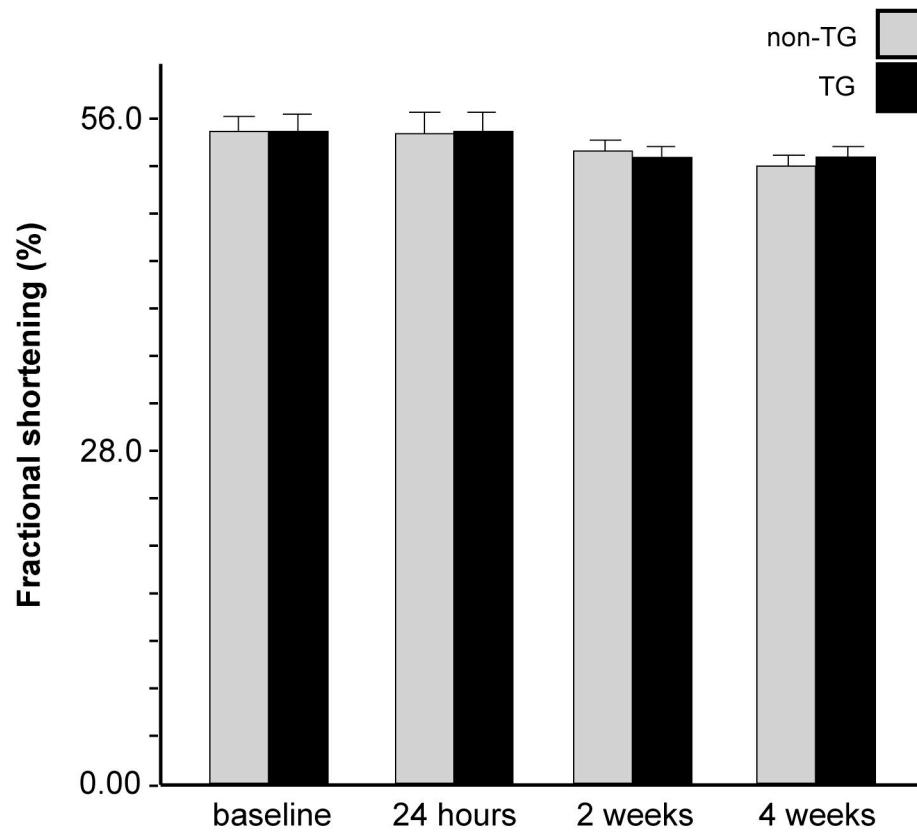


Figure 21. No difference in fractional shortening was observed after isoproterenol-administration as measured by M mode echocardiography.

Heterozygous FGF-2 transgenic (TG) and non-transgenic (non-TG) mice were given one bolus of high-dose isoproterenol (160 mg/kg). Cardiac function was assessed by measuring fractional shortening by echocardiography at baseline, as well as 24 hours, 2 weeks, and 4 weeks post-injury. (n=7-11).

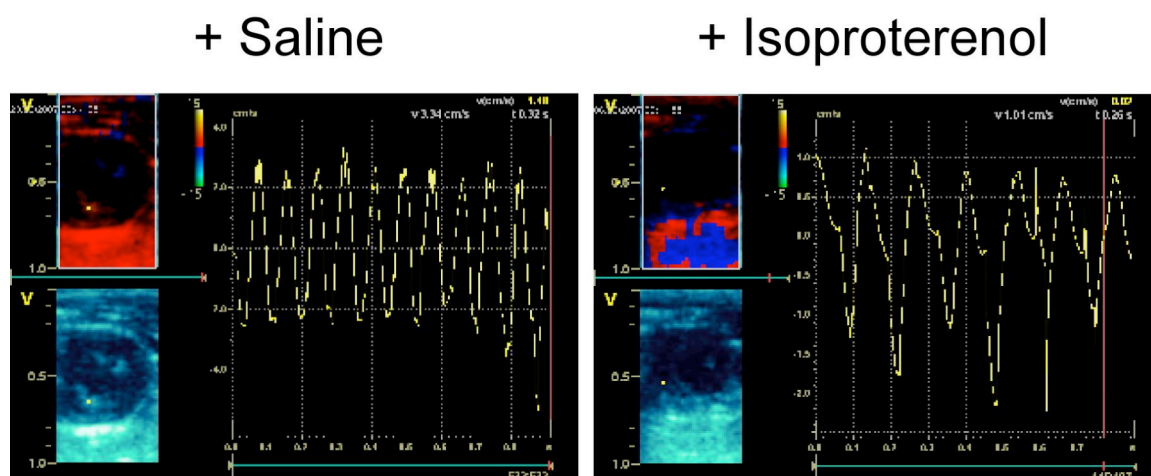


Figure 22. A significant decline in cardiac function as assessed by of tissue Doppler imaging was observed 24 hours after high-dose isoproterenol administration.

Representative images of left ventricular endocardial velocity tracings are presented above. Normal CD-1 mice were given 160 mg/kg isoproterenol or vehicle, then assessed 24 hours later. The left panel shows a mouse treated with vehicle, whose left ventricular endocardial velocity is reading at 3.34 cm/s. In contrast, in the right panel, a mouse treated with isoproterenol shows a decline in cardiac function, reading at 1.01 cm/s.

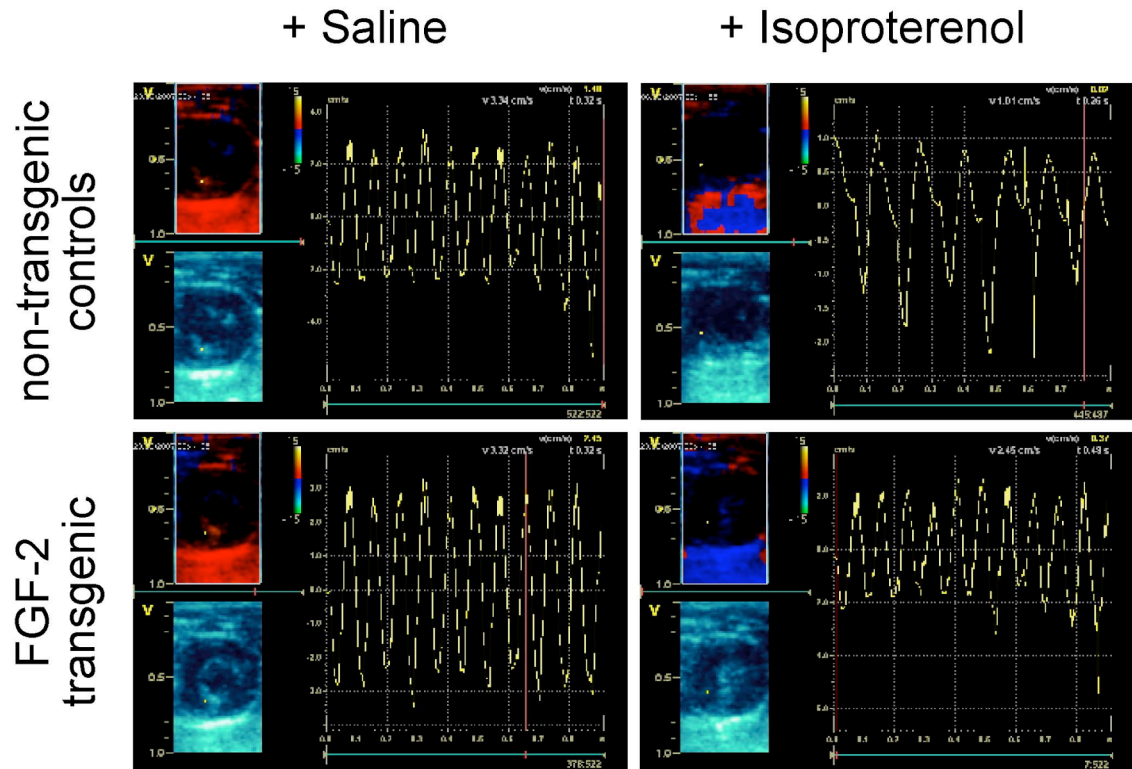


Figure 23. Representative tissue Doppler tracings of FGF-2 transgenic and non-transgenic mouse hearts.

Measurement of left ventricular endocardial velocity was taken from FGF-2 transgenic (TG) mice (bottom two panels) and non-transgenic (non-TG) controls (top two panels), given vehicle (left panels) or high-dose 160 mg/kg isoproterenol (right panels). Non-injured, saline-treated myocardium show comparable tissue Doppler parameter values between TG mouse hearts and controls (3.32 cm/s and 3.34 cm/s, respectively). After isoproterenol administration, endocardial velocities of both TG and non-TG mouse hearts (2.45 cm/s and 1.01 cm/s, respectively) decline compared to baseline and/or vehicle-treated hearts.

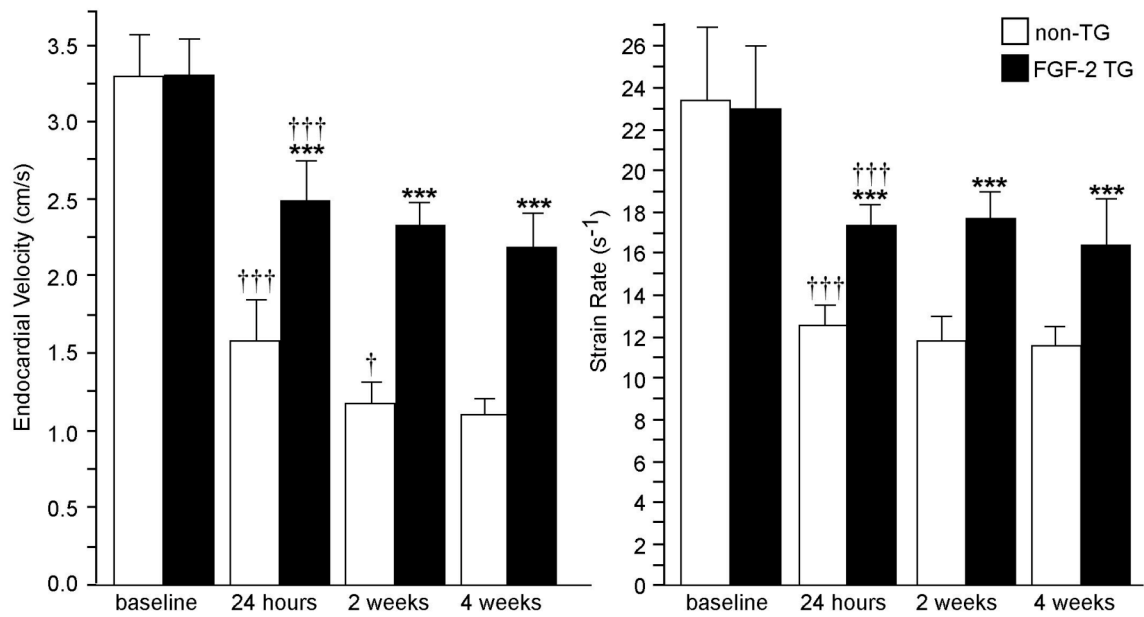


Figure 24. Mice overexpressing FGF-2 show improved recovery of myocyte function after isoproterenol-induced myocardial injury.

Transgenic (TG) and non-transgenic (non-TG) mice were given high dose 160 mg/kg isoproterenol. Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate are presented. Statistical analyses are presented as * for transgenic versus non-transgenic mouse values at the same time point; † for transgenic or non-transgenic mouse values compared with the previous time point. Error bars indicate standard deviation. *** $p < 0.001$; † and ††† $p < 0.05$ and 0.001 , respectively.

B.5. Pre-treatment of mice with cyclosporine A reduces functional recovery in FGF-2 transgenic mice after isoproterenol-induced myocardial injury

Previously, we demonstrated that the increased cellular infiltration in FGF-2 transgenic mouse hearts after isoproterenol-induced myocardial injury was significantly reduced to levels comparable to non-transgenic mice after treatment with cyclosporine A (Figure 16). We have now examined the effect of cyclosporine A on the recovery of cardiac function in FGF-2 transgenic, as well as non-transgenic, mice after injection with isoproterenol. Tissue Doppler measurements in the presence of cyclosporine A treatment are shown in Figure 25. At 24 hours post-isoproterenol treatment, endocardial velocity values for non-transgenic mice averaged ~48% of baseline by 24 hours after isoproterenol injection, declining to ~38% of baseline at 2 weeks, with no further significant change at 4 weeks post-treatment. FGF-2 transgenic mice treated with cyclosporine A showed a statistically significant improvement in endocardial velocity values, compared to non-transgenic mice, at all time points. However, strain rate values were not significantly different in the FGF-2 transgenic mice compared to non-transgenic mice, at 2 and 4 weeks post-injury.

Figure 26 shows the effect of cyclosporine A treatment on tissue Doppler measurements from FGF-2 transgenic mice injected with isoproterenol. Values from transgenic FGF-2 mice treated with or without cyclosporine A are shown side-by-side for comparison: at 24 hours post-isoproterenol, neither left ventricular endocardial velocity nor strain rate were significantly affected by cyclosporine A treatment. This changed at 2

and 4 weeks post-isoproterenol whereby cyclosporine A treatment caused significant decreases in endocardial velocity, and strain rate measurements. These results suggest that the cardioprotection observed in FGF-2 transgenic mice is partially dependent on a cyclosporine-sensitive component that becomes active at relatively later stages of cardiac repair/remodelling.

The effect of cyclosporine A on functional parameters was also assessed for the non-transgenic mice, and results are shown in Figure 27. There were no differences observed between cyclosporine A-treated and untreated groups at any time point assessed.

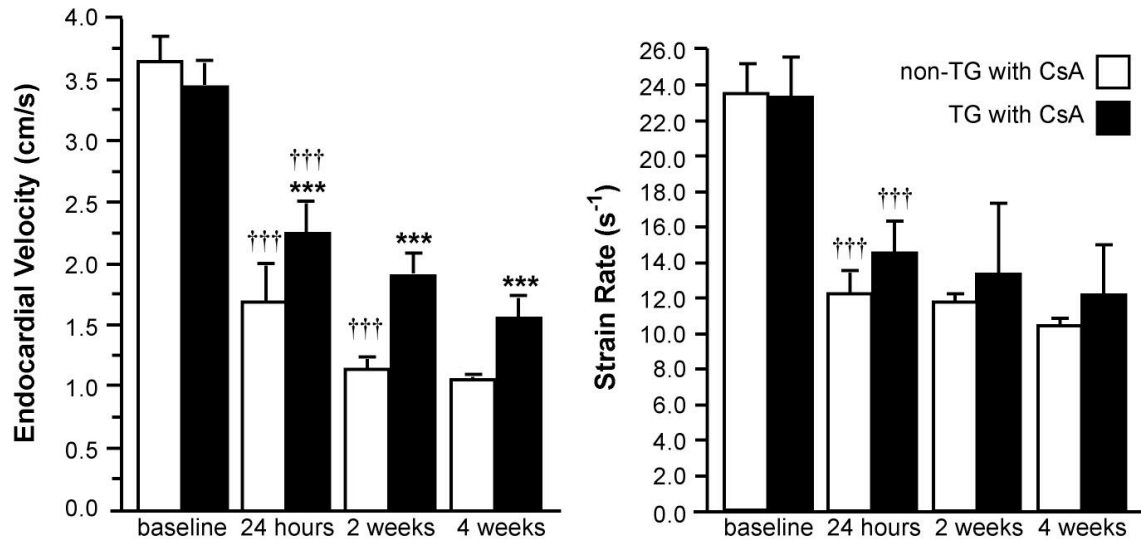


Figure 25. Mice overexpressing FGF-2 continue to display improved recovery of endocardial velocity after isoproterenol-induced myocardial injury in the presence of cyclosporine A (immunosuppressive) treatment

Heterozygous FGF-2 transgenic (TG) and non-transgenic (non-TG) mice were treated with cyclosporine A (CsA) for 4 days before and 4 days after receiving one high-dose bolus of isoproterenol (160 mg/kg). Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate are presented. Statistical analyses are presented as * for values at the same time point, and † for values compared with the previous time point. *** and ††† $P < 0.001$. Error bars indicate standard deviation.

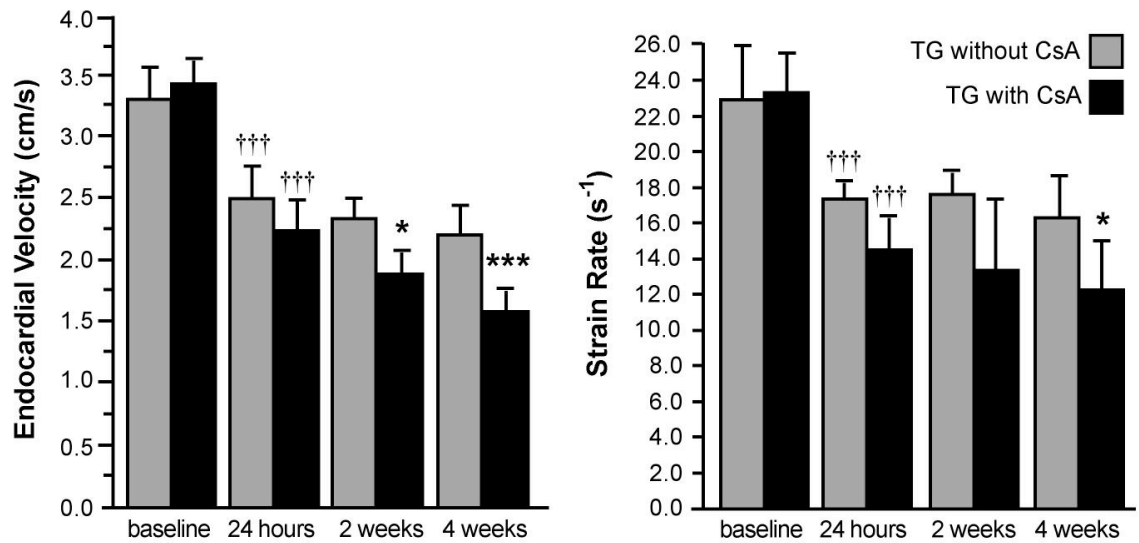


Figure 26. Cyclosporine A decreases cardiac function in transgenic mice overexpressing FGF-2 and subjected to isoproterenol-induced myocardial injury.

Heterozygous FGF-2 transgenic (TG) mice were treated with cyclosporine A (CsA) for 4 days before and 4 days after receiving one high-dose bolus of isoproterenol (160 mg/kg). Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate in comparison with transgenic mice that did not receive CsA are presented. Statistical analyses are presented as * for values at the same time point, and † for values compared with the previous time point. *, *** $p < 0.05$ and 0.001, respectively. ††† $P < 0.001$. Error bars indicate standard deviation.

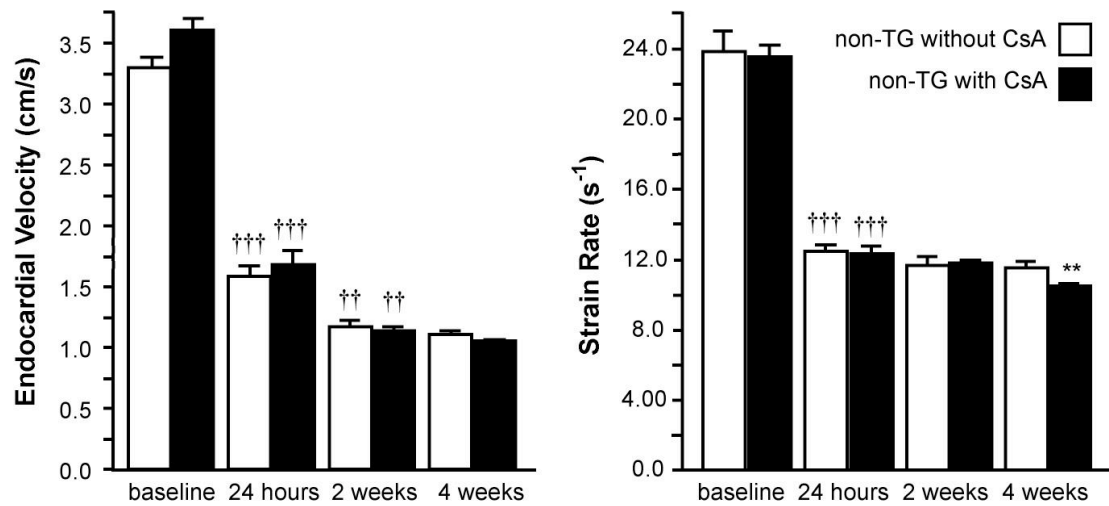


Figure 27. Cyclosporine A does not affect tissue Doppler indices after isoproterenol-induced myocardial injury in non-transgenic mice

Non-transgenic (non-TG) mice were treated with cyclosporine A (CsA) for 4 days before and 4 days after receiving one high-dose bolus of isoproterenol (160 mg/kg). Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate in comparison with non-transgenic mice that did not receive CsA are presented. Statistical analyses are presented as * for values at the same time point, and † for values compared with the previous time point. ** $P < 0.01$; †† and ††† $P < 0.01$ and 0.001, respectively. Error bars indicate standard error from the mean.

B.6. Treatment with anti-CD3 antibodies has no effect on cardiac function of FGF-2 transgenic mice after isoproterenol-induced myocardial injury

To assess whether the decrease in cardiac tissue Doppler measurements observed in FGF-2 transgenic mice after cyclosporine A treatment was a T cell-mediated effect, the study was repeated using antibodies against CD3 ϵ in place of cyclosporine A as a more specific inhibitor of T cells (as introduced in Chapter V). CD3 ϵ , found on the cell surface of T cells, is required for cell activation. Antibodies against CD3 ϵ were administered intraperitoneally for 4 days before and 4 days after isoproterenol administration as previously discussed. Cardiac function was assessed at baseline before anti-CD3 ϵ antibody delivery, and at 24 hours, 2 weeks, and 4 weeks post-injury (Figure 28).

In agreement with previous results, baseline values were within the normal expected range, and isoproterenol administration resulted in a significant decline in left ventricular endocardial velocity and strain rate values in all mice at all the post-injury time points assessed. Similarly, cardiac functional parameters of FGF-2 transgenic mice were significantly improved, compared to those of their corresponding non-transgenic counterparts. The anti-CD3 ϵ treatment did not elicit any significant decline in cardiac functional parameters in either the FGF-2 transgenic or the non-transgenic groups (Figures 29 and 30). A decreasing trend was observed in the cardiac parameters of FGF-2 transgenic mice that received antibodies compared to vehicle, but this observation is not statistically significant (n=3-6). These results indicate that treatment with anti-CD3 ϵ antibodies to inhibit T cell activation does not have same effect observed in mice

treated with cyclosporine A. These issues will be discussed in Chapter XII.A.2., considering cyclosporine A and CD3 ϵ as effective immunosuppressants and neutralizing agents of all or some T cells in the heart.

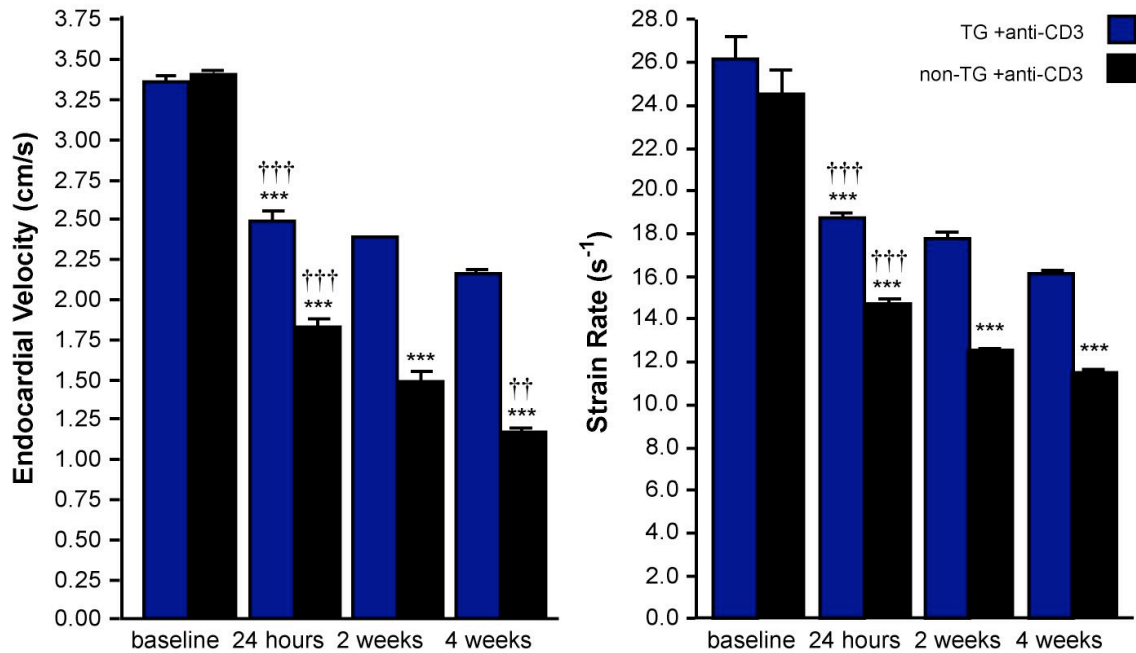


Figure 28. Mice overexpressing FGF-2 continue to display improved recovery of endocardial velocity after isoproterenol-induced myocardial injury in the presence of immunosuppressive treatment using antibodies against CD3 ϵ

Heterozygous FGF-2 transgenic (TG) and non-transgenic (non-TG) mice were treated with antibodies against CD3 ϵ (anti-CD3) for 4 days before and 4 days after receiving one high-dose bolus of isoproterenol (160 mg/kg). Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate are presented. Statistical analyses are presented as * for values at the same time point; † for values compared with the previous time point. ***, ††† $P < 0.001$. Error bars indicate standard error from the mean.

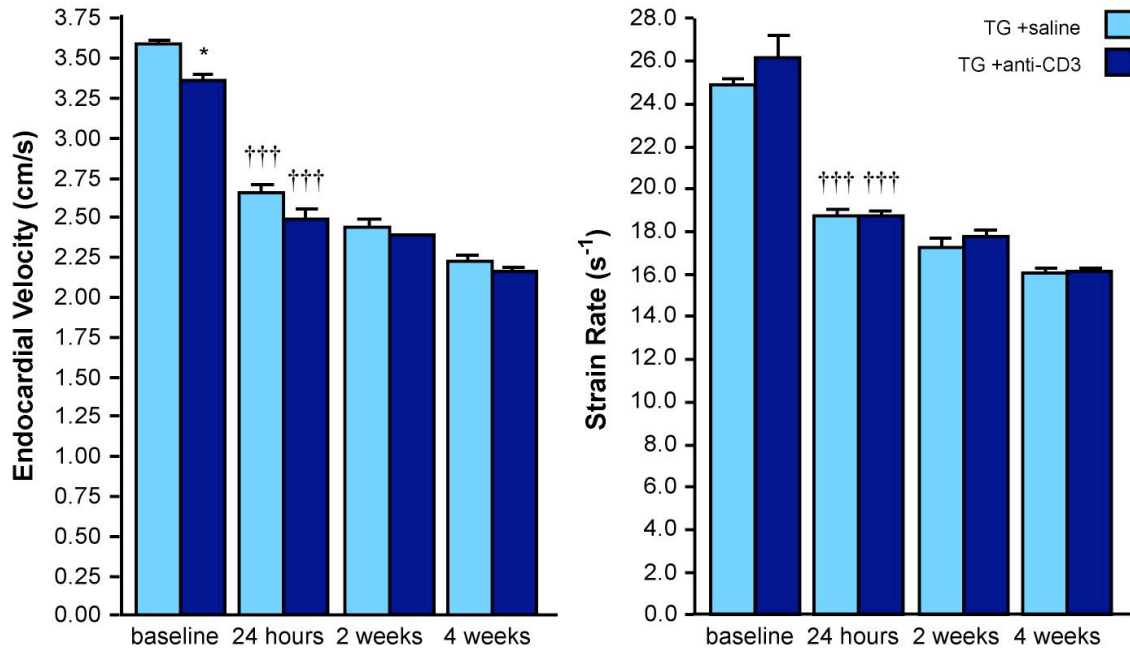


Figure 29. Anti-CD3 ϵ antibody treatment has no significant effect on cardiac function in transgenic mice overexpressing FGF-2 and subjected to isoproterenol-induced myocardial injury.

Heterozygous FGF-2 transgenic (TG) mice were treated with antibodies against CD3 ϵ (anti-CD3) for 4 days before and 4 days after receiving one high-dose bolus of isoproterenol (160 mg/kg). Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate in comparison with transgenic mice that did not receive anti-CD3 ϵ antibodies are presented. Statistical analyses are presented as * for values at the same time point; † for values compared with the previous time point. * P <0.05, and ††† P <0.001. Error bars indicate standard error from the mean.

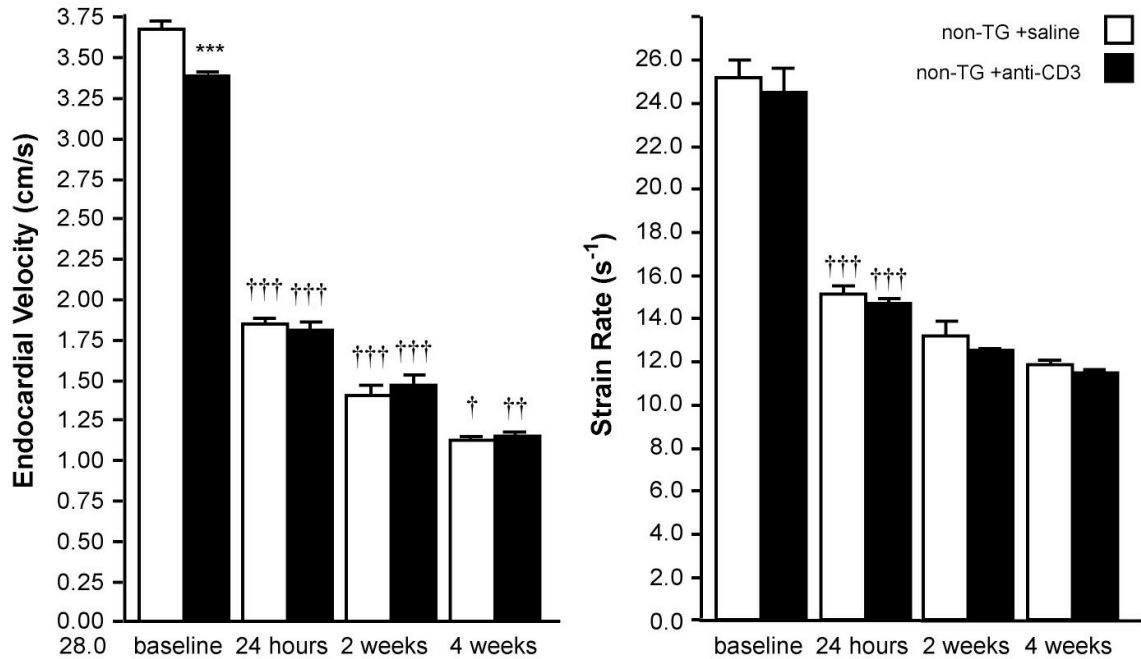


Figure 30. Anti-CD3 ϵ antibody treatment A does not affect tissue Doppler indices after isoproterenol-induced myocardial injury in non-transgenic mice

Non-transgenic (non-TG) mice were treated with antibodies against CD3 ϵ (anti-CD3) for 4 days before and 4 days after receiving one high-dose bolus of isoproterenol (160 mg/kg). Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity and strain rate in comparison with non-transgenic mice that did not receive anti-CD3 are presented. Statistical analyses are presented as † for +anti-CD3 or – anti-CD3 compared with the previous time point. † and ††† $p < 0.05$ and 0.001 , respectively. Error bars indicate standard error from the mean.

Chapter findings:

Previous experiments have shown improved cardiac viability in transgenic mice overexpressing FGF-2 in their hearts after an *ex vivo* model of ischemia-reperfusion injury. However, in the studies described in Chapter VI, increased endogenous FGF-2 levels was associated with a significant increase in inflammatory cell infiltration in response to isoproterenol treatment, which would be indicative of cardiac damage. In this chapter, an attempt was made to reconcile these observations made about FGF-2 and a cardioprotective role with *ex vivo* and *in vivo* models, by assessing cardiac function and its relationship to cellular infiltration. Observations made from this study include:

1. FGF-2 levels are increased about 2-fold in heterozygous FGF-2 transgenic mouse hearts.
2. Isoproterenol induces myocardial injury and increased cellular infiltration and myocyte disarray in heterozygous FGF-2 transgenic mice.
3. Myocardial lesions, as determined by myocardial disarray and cellular infiltration, are decreased in both heterozygous FGF-2 transgenic and non-transgenic mice after cyclosporine A treatment.
4. Isoproterenol-induced myocardial injury resulted in a significant decline in cardiac function in both heterozygous FGF-2 transgenic and non-transgenic mice as assessed by tissue Doppler imaging.
5. Despite increased cellular infiltration, significant functional recovery was observed in FGF-2 transgenic mouse hearts after isoproterenol-induced injury compared with non-transgenic animals. This finding is in agreement with previous results showing a beneficial effect of FGF-2 on the heart upon injury.
6. Treatment with cyclosporine A resulted in a significant decline in functional parameters in heterozygous FGF-2 transgenic mouse hearts at 2 and 4 weeks after isoproterenol injection.
7. Treatment with antibodies against CD3 ϵ resulted in no significant changes in left ventricular tissue Doppler indices compared with non-treated animals, regardless of FGF-2 overexpression.

CHAPTER VIII

RESULTS

C. EFFECTS OF PRE-INJURY ACUTE EXERCISE ON CARDIAC FUNCTION AFTER INJURY

In this chapter, we examined the effect of acute swimming exercise on functional recovery in hearts after isoproterenol-induced myocardial injury and addressed the question of whether acute exercise is sufficient to offer protection to the heart.

C.1. Acute swimming significantly improves tissue Doppler imaging parameters after isoproterenol-induced myocardial injury

Wild type CD-1 mice were subjected to an acute swimming protocol (1 bout, 30 minute duration; described in Chapter V.E; Figure 31) to assess the effect of one bout of exercise on cardioprotection from myocardial injury. Two sets of controls were included: (i) In the “water” group, mice were treated in an identical manner as the “swim” group, except the depth of the water in the bath was limited so that it allowed the mice to touch the bottom of the tank and keep their head above water and kept them from “swimming”; and (ii) in the “cage” group, mice were not subjected to swim or exposed to water. There were four mice per group. Twenty-four hours later, both swim and control mouse groups were given a high-dose bolus of isoproterenol (160 mg/kg) to cause myocardial injury (see Chapter V). Cardiac function was assessed by tissue Doppler imaging at 24 hours, 2 weeks, and 4 weeks post injury (Figures 32 and 33). As expected, left ventricular

endocardial velocity significantly declined in all groups at 24 hours after isoproterenol-induced injury and further declined at 2 weeks but not declining further at 4 weeks after injury. Left ventricular endocardial velocity parameters were not significantly different between cage (1.76 ± 0.08) and water (1.73 ± 0.09) groups at 24 hours post-injury, but were significantly lower than the swim group (2.12 ± 0.06). The values further declined by 2 weeks, where the cage group (1.36 ± 0.03) showed a trend towards declining compared to the water group (1.49 ± 0.03) although the difference did not reach significance. The water group control values remained significantly lower than those of the swim group (1.8 ± 0.03), indicating that swimming induced cardioprotection. Similar findings were obtained at 4 weeks post-injury. Thus, one bout of swimming can stimulate cardioprotection as measured by tissue Doppler imaging parameters after isoproterenol-induced myocardial injury.

Evidence of cardioprotection from one bout of swimming was observed using normal CD-1 mice. These results, although preliminary ($n=4$ per group), show significant improvement in tissue Doppler indices in mice that were put through a stressful and vigorous swim procedure compared with either water or cage control groups (Figures 32 and 33). Interestingly, tissue Doppler indices for the water group showed a trend towards some improvement compared with the cage group at the later time points of 2 and 4 weeks when compared individually though differences did not reach significance. One can speculate that even a minor increase in stress from being in water and outside their normal “cage” environment might elicit some benefits.



Figure 31. Image of mice swimming in a water bath.

Mice were individually identified using toe tattoos as well as coloured marks using a non-toxic permanent marker.

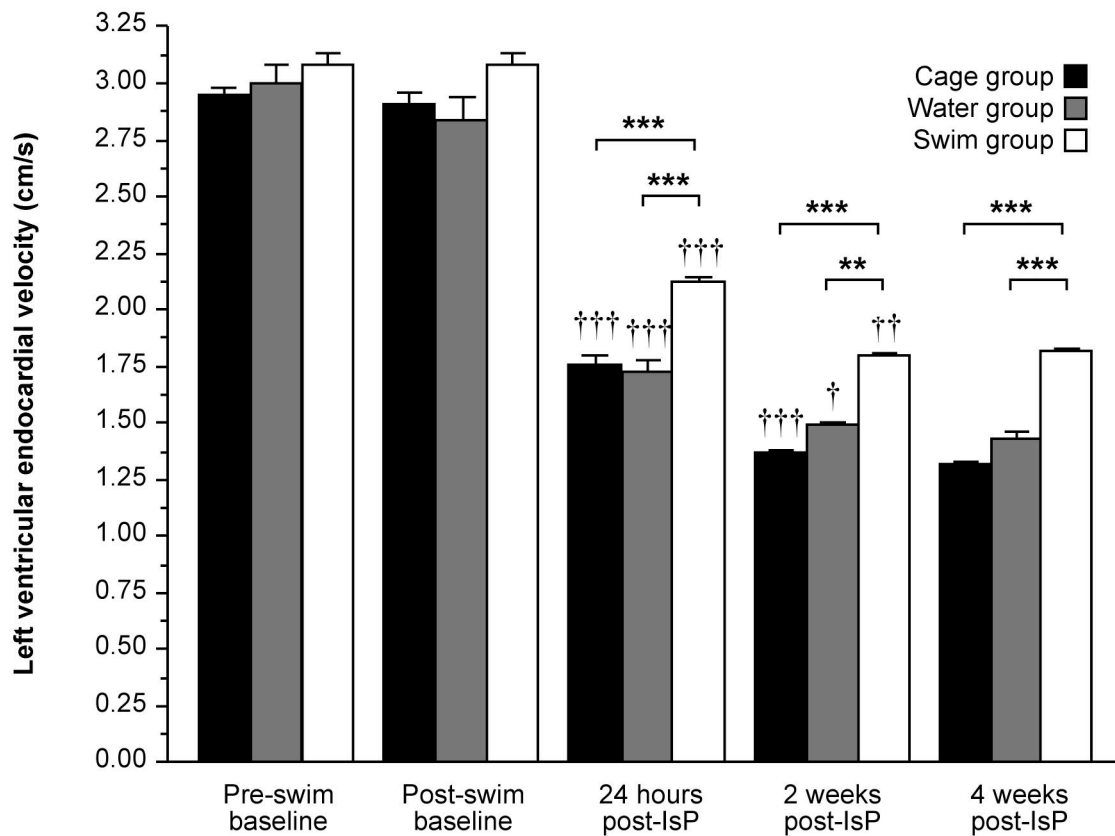


Figure 32. Left ventricular endocardial velocity values predict significant improvement in cardiac function in the exercise group up to 4 weeks post-injury.

Wild type CD-1 mice put through an acute exercise protocol consisting of one bout of 30 minute swim. Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for left ventricular endocardial velocity for swim in comparison with water and cage groups are presented. Statistical analyses are presented as * for significant changes between groups at the same time point; † for the same group compared with its value at the previous time point. **, *** $p < 0.01$ and 0.001 , respectively. †, and ††† $p < 0.05$, and 0.001 respectively. Error bars indicate standard error from the mean. $n=4$ each.

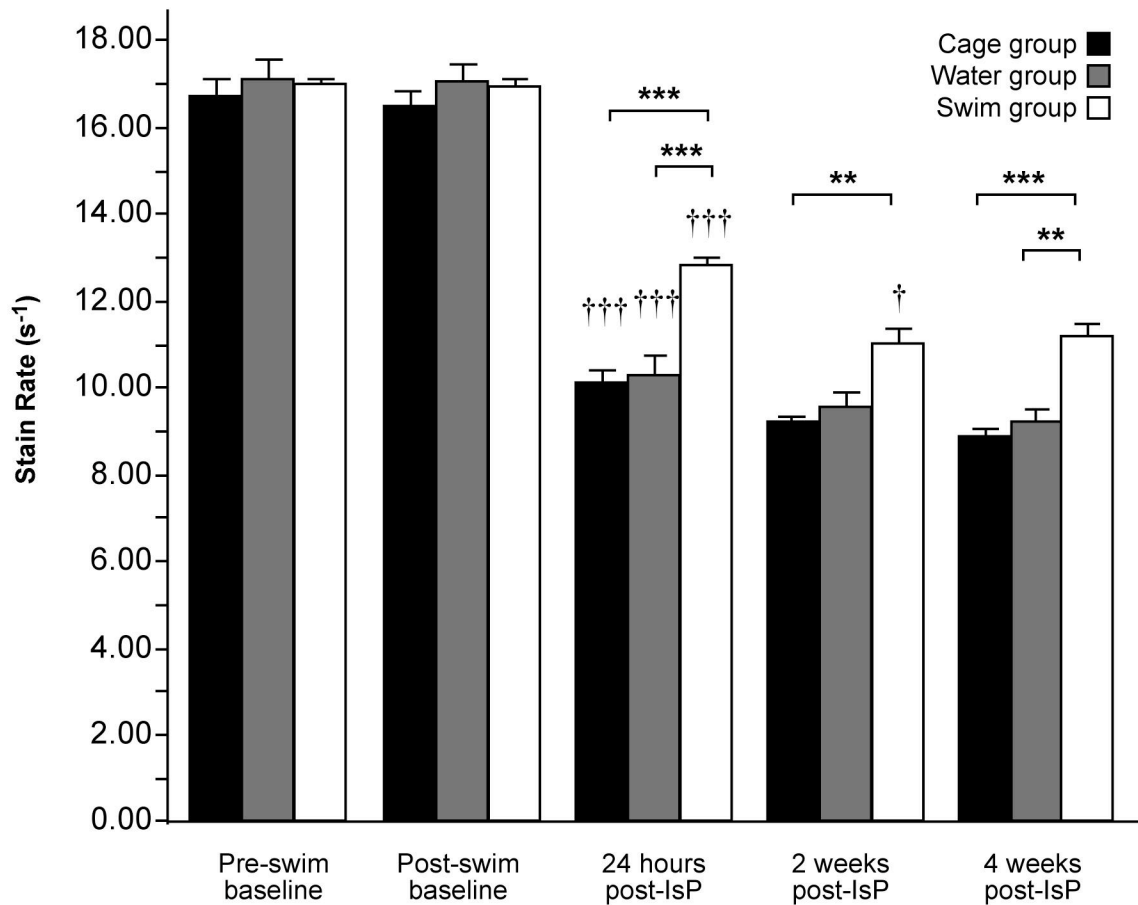


Figure 33. Strain rate predict significant improvement in cardiac function in the exercise group up to 4 weeks post-injury.

Wild type CD-1 mice put through an acute exercise protocol consisting of one bout of 30 minute swim. Cardiac function was assessed using tissue Doppler imaging at baseline prior to injury, as well as 24 hours, 2 weeks, and 4 weeks after injury. Values for strain rate for swim in comparison with water and cage groups are presented. Statistical analyses are presented as * for significant changes between groups at the same time point; † for the same group compared with its value at the previous time point. **, *** $p < 0.01$ and 0.001 , respectively. †, ††, ††† $p < 0.05$, and 0.01 respectively. Error bars indicate standard error from the mean. $n=4$ each.

C.2. Chronic swimming significantly increases heart weight:body weight ratios in non-transgenic CD-1 mice

Calculation of heart weight-to-body weight ratios after chronic swimming at 24 hours after the last swim was carried out to assess for development of physiological hypertrophy. Mice from the swim group were compared to the water group control mice in shallow water. Heart weight:body weight ratios were 1.14-fold higher in mice within the swim group compared to both the water group ($p<0.001$, $n=9=10$), and 1.15-fold higher than the cage group ($p<0.01$, $n=4-9$; Figure 34). As chronic exercise results in the physiological enlargement of hearts, detection of hypertrophy in the chronic swimming group validated the swim protocol as exercise for the mice.

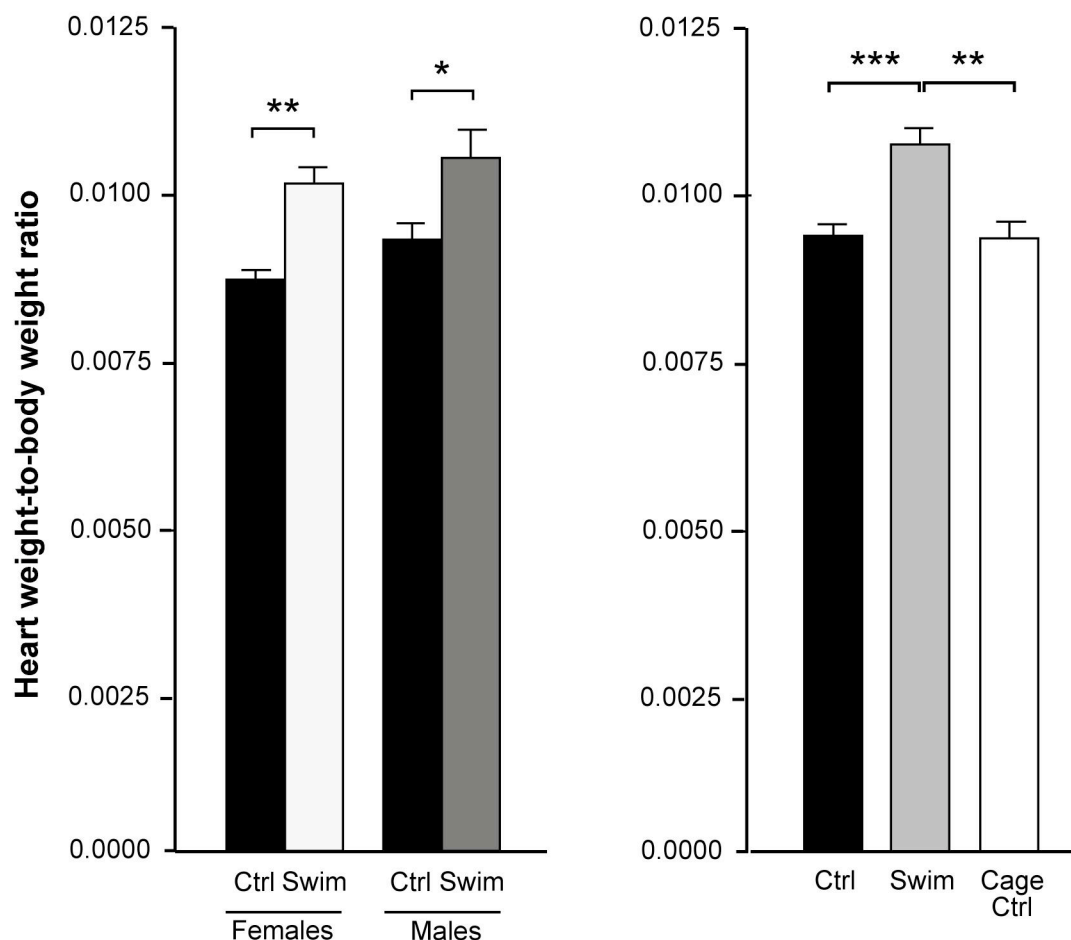


Figure 34. Chronic swimming significantly increased heart weight-to-body weight ratios of mice.

Mice were put through a swim protocol in a waterbath of up to 1.5 hours in duration per session, with 2 sessions per day (Swim), as described in Chapter V. Two groups of control animals were used: (1) water controls (Ctrl), which consisted of mice placed in an identical waterbath, but whose water levels remained about 1-2 inches deep so that the mice are in water but do not require swimming to stay above water; and (2) cage controls (Cage Ctrl), which consisted of mice that stayed within their cages throughout the duration of the experiment. The chronic swim protocol consisted of one month of swimming (5 days per week). Immediately prior to euthanasia, all mice were weighed. Immediately after euthanasia, hearts were removed, blotted to remove excess blood, and weighed. The ratios of heart weight-to-body weight are presented. *, **, and *** $p < 0.05$, 0.01, and 0.001, respectively. Error bars indicate standard error from the mean.

Chapter findings:

In this chapter an attempt was made to address the effect of acute exercise on cardiac outcome following injury. Observations made from this study include:

1. One acute bout of swimming 24 hours prior to isoproterenol-induced myocardial injury resulted in a significant improvement in cardiac outcome compared with control animals as assessed by tissue Doppler imaging. This improvement was evident at least until 4 weeks post-injury.
2. Chronic swimming significantly increased heart weight to body weight ratios compared to control animals, suggesting that the swimming protocol used involves cardiac work similar to that of physiological exercise.

CHAPTER IX

DISCUSSION

A. DISCUSSION OF OBSERVATIONS PRESENTED IN CHAPTER VI: EFFECTS OF INCREASED ENDOGENOUS FGF-2 ON MYOCARDIAL INJURY *IN VIVO*

A.1. Summary of findings

In this study, the effects of FGF-2 overexpression in the mouse heart upon myocardial injury *in vivo* were investigated. Isoproterenol-induced myocardial damage resulted in a significant increase in area and volume of myocardial cellular infiltration and disarray in transgenic mice overexpressing FGF-2 in the heart compared to their non-transgenic counterparts. A significant increase in heart weight-to-body weight ratios was also observed in FGF-2 transgenic mice 28 days after isoproterenol administration; hypertrophy was not observed in non-transgenic injured mice or in non-injured FGF-2 transgenic mice. The increase in cellular infiltration and disarray were attributed to an amplified number of T lymphocytes, as immunosuppressive treatments with either cyclosporine A or antibodies against CD3 ϵ resulted in significant reduction of cellular disarray and infiltration. These results suggest that an increase in FGF-2 availability in the heart can stimulate cardiac hypertrophy, and exacerbate myocardial injury through an increase in the inflammatory response following the injury; this phenomenon appears to be mediated by T lymphocytes.

A.2. Cellular infiltration after isoproterenol-induced myocardial injury

Assessment of cardiac tissue after myocardial injury reveals classical wound healing-associated changes including dead and dying myocytes, edema, and cellular infiltration, followed by a decline in infiltrate and the subsequent formation of a scar tissue [Frangogiannis, 2008; Frangogiannis *et al*, 2002; F Yang *et al*, 2002]. This description agrees with the observed morphological properties of hearts from mice given isoproterenol in the studies presented here (Figure 5).

Cellular infiltration in the myocardium is generally considered to be part of an immune and inflammatory response to wounding. Previous characterization of the immune response in mice revealed increased infiltration involving neutrophils and macrophages [Frangogiannis, 2008; Ji Virag and Murry, 2003]. The cellular components of the immune system have long been known to play important roles in myocardial injury, repair, and remodelling, similar to classical wound healing in other tissues, but the contributions of individual players are still under heavy investigation. It has been shown that the migration of neutrophils, macrophages, T cells, and other immune cells into the areas of myocardium can occur as a result of cardiac injury. Their migration into the myocardium is thought to exacerbate injury, as many studies whereby immune mediators were suppressed resulted in smaller infarct sizes [Ikeda *et al*, 2001; Kanzaki *et al*, 2001; X Liu *et al*, 2006]. Thus, based on these observations, it would appear that an increase in immune cell infiltration post-injury leads to, and indicates, a more detrimental event. However, clinical studies using steroids to limit inflammation after injury have shown no significant benefits to myocardial repair, therefore questioning the convention that

immune cell infiltration mediates a diminished cardiac outcome, or at least that it does so in all cases (reviewed in [LeGal and Morrissey, 1990; Linfert *et al*, 2009]).

A.3. Increased cellular infiltration with increased FGF-2 availability

Considering previous observations of FGF-2-induced cardioprotection, an increase in myocardial disarray and cellular infiltration with increased FGF-2 availability in the FGF-2 transgenic mouse was not anticipated. It was originally hypothesized that an increase in endogenous FGF-2 would induce sustained beneficial effects, including decreased cardiac cell injury and death when observed histopathologically. Migration of inflammatory cells into the areas of myocardial injury is well documented, and occurs as a result of free radical generation and complement activation [Frangogiannis *et al*, 2002]. In the case of the increase in cell infiltration in the FGF-2 transgenic mice observed in this study, the question remains whether the increased inflammatory response in the heart was indeed a direct measure of increased myocardial damage or whether the increased number is a direct effect of FGF-2 on the inflammatory cells.

The data do not support a direct role for FGF-2 in the increased “lesion”-formation observed after isoproterenol-induced myocardial injury, but rather to an indirect effect by increasing cellular infiltration. When immunosuppressive agents (cyclosporine A and anti-CD3 ϵ antibodies) were given in addition to isoproterenol, cellular infiltration and myocyte disarray were decreased and levels were comparable between FGF-2 transgenic and non-transgenic mice (Figure 16). This suggests that

despite increased endogenous FGF-2, the increase in cellular infiltration observed can be reduced upon immunosuppressive treatments. This observation is consistent with previous publications suggesting that cellular infiltration contributes to greater damage after injury [LeGal and Morrissey, 1990].

A.4. T lymphocytes and the myocardial infiltrate

Closer assessment of the myocardial infiltrate after isoproterenol-induced injury in mice revealed an increased presence of T cells (Figures 12 and 13). Reports that β -adrenergic-stimulation is associated with increased T cell survival, accumulation, and mobilization [Anane *et al*, 2009; Loza *et al*, 2007; Mills *et al*, 1997] is consistent with the increase in T cell numbers detected in the myocardium upon injury. Thus, it appears that isoproterenol-induced myocardial injury stimulates the infiltration of a greater number of T cells into the myocardium, in addition to the “usual” responders to myocardial injury, specifically, macrophages and neutrophils [Frangogiannis, 2006; Frangogiannis, 2008; Frangogiannis *et al*, 2002].

Furthermore, it appears that an increase in T cell infiltration might also result from an increase in endogenous FGF-2 levels, as well as an increase in T cell activation in response to β -adrenergic stimulation. FGF-2 release is increased upon contraction and from damaged cardiac cells [Clarke *et al*, 1995; Clarke and Feedback, 1996; Kaye *et al*, 1996]. Therefore, FGF-2 availability in the FGF-2 transgenic mice may increase due to constitutively higher levels in the heart, as well as increased “release” upon contraction

and injury (transient sarcolemmal disruption), and from the eventual necrosis of cells in the ischemic regions of the heart. T cells were shown to express the predominant FGF-2 receptor in the heart, FGFR-1 [Meij *et al*, 2002]. This finding is supported by the results of a study done in Jurkat T cells showing expression of FGFR-1 [Zhao *et al*, 1995]. To my knowledge, expression of other FGF receptors by T lymphocytes has not been reported. However, the presence of FGFR-1-positive T lymphocytes within the myocardial infiltrate of the FGF-2 transgenic mice suggests that increased FGF-2 availability can stimulate and/or influence T cell signaling/behaviour. In Jurkat T cells, it has been shown that co-stimulation of anti-CD3 antibodies with FGF-1 resulted in a significant increase in IL-2 release [Zhao *et al*, 1995], confirming that FGF-FGFR interaction on the T lymphocyte can significantly affect its cytokine production.

CD3-positive cells were identified within the myocardial infiltrate (Figure 13), and CD4-positive cells co-expressing FGFR-1 were also observed (Figure 14). The observed higher numbers of T cells could be attributed to an FGF-2-stimulated (1) increase in basal T cell levels (potentially by acting as a chemotactic and/or survival agent, or both, or (2) activation and/or proliferation of T cells in response to injury. FGF-2 is not essential for T lymphocyte development, but some effect cannot be discounted as multiple isoforms of the FGFR family, including FGFR-1, are highly expressed in the thymus, and may therefore contribute to T cell maturation [Hughes, 1997; Zhou *et al*, 1998]. However, basal T cell numbers were not assessed in non-transgenic versus FGF-2 transgenic mice; it is therefore not known whether the increase in T cells in the myocardial infiltrate stems from an increase in basal levels from

gestation. FGF-2 is a chemotactic agent, and has been reported to stimulate significant T lymphocyte infiltration into mammary tumour stroma in response to exogenous addition [Tsunoda *et al*, 2009]. In our study, the T cell population was increased after injury in FGF-2 transgenic mice compared to their non-transgenic counterparts (Figures 11 and 12), suggesting that it is likely due to a direct effect of the increased availability of “endogenous” FGF-2 on the T cells. Whether or not this increase in cell number involves an FGF-2-induced mitotic mechanism or an increase in T lymphocyte activation in response to FGF-2 remains to be determined.

A.5. T lymphocytes and the recruitment of the myocardial infiltrate

In the studies presented here, inhibition of T cells using cyclosporine A or antibodies against CD3 ϵ resulted in the significant decline of cellular infiltrate to levels comparable between FGF-2 transgenic and non-transgenic groups (Figures 15 and 16). Infiltrate levels after immunosuppression and injury decreased to levels lower than observed in injured non-transgenic hearts without immunosuppression. Examples of CD3, CD4, and CD8 positive cells were detected in an assessment of T cells within the myocardial infiltrate; it is clear, however, that the T cell population does not compose the majority of the cells in the infiltrate [Meij *et al*, 2002]. These results show that inhibition of T cells significantly decreases myocardial cellular infiltration (in addition to T cells), indicating that they play a significant role in the presence of the increased cellular infiltration observed in both FGF-2 transgenic and non-transgenic mouse hearts after isoproterenol-induced myocardial injury.

A.6. Immunosuppression and the myocardial infiltrate

An attempt was made to determine the role of T cells in the cardiac infiltration by immunosuppression using either cyclosporine A or antibodies against CD3 ϵ . Quantitative analysis of cardiac cross-sectional tissue showed a significant decline in cellular infiltration in both groups of animals that received immunosuppressive treatments. Immunosuppression, using either treatment modality, resulted in (comparable) minimal cellular infiltration after isoproterenol-induced myocardial injury (Figure 16). The difference between the suppressive effects of cyclosporine A versus CD3 ϵ antibodies on infiltrate count was not significant. However, a trend was observed by visual assessment that slightly more infiltrate is present within the areas of injury/disarray in the anti-CD3 antibody-treated group compared to the cyclosporine A-treated group (Figure 15). The actual number of cells within the infiltrate was not quantified, however; thus this apparent difference, if true, might be explained by the ability of the drug cyclosporine A to affect multiple different pathways. Although used commonly as an immunosuppressive agent to inhibit T cells in the clinic, cyclosporine A does not specifically inhibit T cells alone. Its mechanism of action is to inhibit the translocation of the transcription factor nuclear factor of activated T cells (NFAT), an event which proceeds from the direct inhibition by cyclosporine A of the phosphatase calcineurin [Matsuda and Koyasu, 2000]. Both calcineurin and NFAT have been documented to have effects on many different cell types including cells involved in the wound inflammatory response as well as cardiac myocytes. Assuming that antibodies against CD3 ϵ target T cells specifically, it would be expected that less cells would

infiltrate the damaged myocardium in animals that have received cyclosporine A. However, the possibility that this might also translate into a functional difference in the heart cannot be determined by morphometric analysis alone; rather a measure of cardiac function is required. Perhaps more importantly, given the apparent contradiction between evidence in support of the cardioprotective effects of FGF-2 versus increased infiltrate as a reflection of increased cardiac damage in the FGF-2 transgenic mouse, could only be resolved through an assessment of cardiac function, as pursued in Chapter IV through tissue Doppler imaging.

A.7. FGF-2 and cardiac hypertrophy

FGF-2 has been shown previously to stimulate hypertrophic growth of the heart [Detillieux *et al*, 2003; Jiang *et al*, 2007; Kardami *et al*, 2004]. A significant 54% increase in heart weight-to-body weight ratio was observed in FGF-2 transgenic mice 28 days after one high-dose bolus of isoproterenol (Figure 6). This increase in heart size was not observed in non-transgenic animals or in the transgenic mice at the earlier time points assessed. This result suggests that the increase in endogenous FGF-2 may play a role in the development of cardiac hypertrophy. Furthermore, the FGF-2-induced increase in cardiac mass occurred in the context of myocardial injury; no significant difference in heart weight-to-body weight ratio was detected between uninjured FGF-2 transgenic and control mice. In addition, heart weight-to-body weight ratios in FGF-2 transgenic mouse hearts were not significantly different from non-transgenic animals of the same age and sex [Sheikh *et al*, 2001]. Accordingly, no significant difference in ANF

RNA levels (a gene classically upregulated upon cardiac hypertrophy) in heart tissue was detected between groups without cardiac challenge [Sheikh *et al*, 2001].

This increase in cardiac:body weight ratio following injury may be attributed to a pro-hypertrophic property of FGF-2, particularly its high molecular weight isoform (hi-FGF-2). Previously published data show that administration of hi-FGF-2 in adult rats subsequent to LAD ligation surgery resulted in a significant increase in heart size compared to rats that received vehicle alone, or those that received the 18 kDa low molecular weight FGF-2 (lo-FGF-2); studies with myocytes in culture corroborated the *in vivo* results, by showing that hi- but not lo- FGF-2 stimulated hypertrophy [Jiang *et al*, 2007]. FGF-2 transgenic mice were generated using constructs that predominantly express lo-FGF-2. However, analysis of FGF-2 protein expression in transgenic mouse hearts showed an increase not only in lo-FGF-2 but also in hi-FGF-2 as well when compared with non-transgenic controls [Sheikh *et al*, 2001]. This might be related to the ability of FGF-2 to stimulate its own gene expression in cardiac myocytes [Jimenez, 2003; Jimenez *et al*, 2004]. As a result, even exogenous addition of lo-FGF-2 to cardiac myocyte cultures could result in increased production of hi-FGF-2 contributing to a pro-hypertrophic response. Thus, while an effect of lo-FGF-2 in our system cannot be ruled out, the cardiac hypertrophy observed could be attributed to the increase in the availability of hi-FGF-2. Nevertheless, this increase in heart size is associated with both the isoproterenol-induced injury obtained, and the augmented levels of FGF-2.

Cardiac hypertrophy is a compensatory response to changes in cardiac workload. An increase in heart size can be considered beneficial as an adaptive response to increased workload due to physiological reasons such as exercise. However, cardiac hypertrophy can also be maladaptive when the increase in cardiac size is the result of a continuous increase in cardiac stress and workload, for example, in hypertension and following myocardial tissue loss and injury. In the mice overexpressing FGF-2, the increase in cellular infiltration and myocyte disarray are typically considered to be indicators of myocardial damage, and would therefore suggest that the increased heart:body weight ratio observed 28 days after isoproterenol administration is evidence of a hypertrophic maladaptive response leading to loss of function and subsequent failure. However, whether the increase in heart size in mice overexpressing FGF-2 is linked to adaptive or maladaptive response cannot be definitively determined by morphometric analysis of injury alone and, again for this, functional analysis of the hearts is necessary.

A.8. Cellular infiltration and late-stage remodeling of the myocardium

As shown in Figure 5, the morphological appearance of cardiac tissue from FGF-2 transgenic mice after isoproterenol-induced myocardial injury, and specifically the increase in T cell infiltration, would suggest a worsening of function and thus worse prognosis. However, most publications on the inflammatory cells that migrate post myocardial injury focus on macrophages and neutrophils, likely because T cells are not the predominant infiltrating cell observed within the first 48 hours after myocardial infarction or ischemia/reperfusion injury [Fishbein *et al*, 1978; Hawkins *et al*, 1996].

Thus, whether T cell presence post myocardial injury and repair contributes in a beneficial or detrimental manner has not been elucidated.

Images of cardiac tissue stained for haematoxylin and eosin from FGF-2 transgenic mice euthanized 6 weeks post isoproterenol-induced injury show larger areas of scarring than non-transgenic mice, consistent with the increased myocardial disarray and cellular infiltration 24 hours after injury (Figure 8). However, haematoxylin and eosin images of FGF-2 transgenic hearts also show structures with morphological features of blood vessels. It is well established that FGF-2 is a potent angiogenic agent (Reviewed in [Detillieux *et al*, 2003]). In addition, based on a study of a model of breast cancer, Tsunoda and colleagues suggested that angiogenesis can be mediated by T lymphocytes in an environment with increased FGF-2 availability [Tsunoda *et al*, 2009]. Thus, it is probable that the increase in endogenous FGF-2 levels in the transgenic mouse heart, as well as the increase in T lymphocyte presence, stimulated an induction of angiogenesis in the myocardium after isoproterenol-induced injury.

The data presented in Chapter VI describe the morphology of the myocardium after isoproterenol induced injury, but did not include any measure of cardiac function. This is important as the morphology seen in FGF-2 transgenic mice after isoproterenol induced injury can be interpreted as both negative and positive in terms of anticipated outcome. While the cardiac morphology and exacerbated inflammatory response observed within 1 week of injury suggests more damage, by 6 weeks structures resembling blood vessels are apparent suggesting a well perfused and potentially more

viable myocardium (Figures 7 and 8). It was therefore possible that an improvement in cardiac function may still ensue in FGF-2 transgenic mice after isoproterenol-induced cardiac injury. Furthermore, the increases in FGF-2 availability and cellular infiltrate may also contribute to blood vessel formation as suggested by other studies of angiogenesis [Jiang *et al*, 2004; Presta *et al*, 2009; Tsunoda *et al*, 2009].

CHAPTER X

DISCUSSION

B. DISCUSSION OF OBSERVATIONS PRESENTED IN CHAPTER VII: CARDIAC FUNCTION IN FGF-2 TRANSGENIC MICE AFTER MYOCARDIAL INJURY *IN VIVO*

B.1. Summary of findings

In the current study, a heterozygous transgenic model of FGF-2 overexpression was used to examine the effect of increased endogenous FGF-2 on isoproterenol-induced injury in the heart. Assessment was conducted on both tissue morphology as well as long term functional performance *in vivo*, by standard M-mode echocardiography and tissue Doppler imaging. As also observed in the homozygous FGF-2 transgenic mice (discussed in the previous section), increased cellular infiltration, often associated with myocyte disarray and cell vacuolation, and interpreted as ‘damage’, was more pronounced at 24 hours as well as 2 and 4 weeks post-treatment in tissue sections of heterozygous FGF-2 transgenic compared to non-transgenic mouse hearts. *In vivo* tissue Doppler imaging, however, revealed that recovery of left ventricular function, as indicated by left ventricular endocardial velocity and strain rate, was significantly improved in the presence of increased FGF-2, in a sustained fashion, up to 4 weeks post-isoproterenol treatment. Improvement in tissue Doppler imaging indices suggests increased cardiac myocyte survival and predicts improved cardiac outcome in FGF-2 transgenic mice and, thus, a positive and not negative correlation with cellular

infiltration. Furthermore, when cellular infiltration was inhibited through pretreatment with cyclosporine A, there was a corresponding partial but significant decrease in the beneficial effect of FGF-2 overexpression as assessed by tissue Doppler imaging. This implies that a cyclosporine A-sensitive component contributes, but may not be solely responsible for, to the sustained beneficial effect of FGF-2 in transgenic hearts. An attempt at the inhibition of T cell activation by treatment using antibodies against CD3 ϵ did not result in any decrease in the beneficial effects of FGF-2 overexpression, which, if true, would suggest no effect of T cells on FGF-2–induced cardioprotection. However, the efficacy of the CD3 ϵ antibody treatment was not assessed, and thus, cannot be considered veritable results.

B.2. Cyclosporine A-sensitive component of FGF-2-induced beneficial effects

Cyclosporine use in the clinic is predominantly for the inhibition of T cell activation in organ transplantation to prevent rejection [Kahan, 2009]. As shown in the studies with homozygous FGF-2 mice (Figure 15), treatment with cyclosporine A or antibodies against CD3 ϵ results in the virtual abolishment of cellular infiltration after isoproterenol-induced myocardial injury [Meij *et al*, 2002]. In the current study, cyclosporine A treatment decreased the beneficial functional effect of FGF-2 overexpression (Figure 27).

Cyclosporine A is effective in preventing the activation of T lymphocytes by way of inhibition of calcineurin and NFAT [Matsuda and Koyasu, 2000; McCaffrey *et al*,

1993; Meij *et al*, 2002]. The mechanism of action of cyclosporine A is through the inhibition of calcineurin, which, normally promotes the translocation of the transcription factor NFAT into the nucleus to induce transcription of a set of genes. However, the calcineurin/NFAT pathway is not specific only to T cells; as such it can affect cells other than T lymphocytes including macrophages, neutrophils, mast cells, basophils, and stem cells [Arras *et al*, 1998; Benson and Ziegler, 1989; Cirillo *et al*, 1990; Draberova, 1990; Kiani *et al*, 2004; Spisani *et al*, 2001; Wenzel-Seifert *et al*, 1991]. In addition, cyclosporine A is also able to affect multiple cell types through NFAT-independent mechanisms [Chan *et al*, 2007]. Cardiac myocytes can also be affected by cyclosporine A [Altschuld *et al*, 1992; Nazareth *et al*, 1991]. The cyclosporine A target calcineurin is a major calcium-activated phosphatase, which is linked to the development of abnormal cardiac growth [Wilkins *et al*, 2004]. In addition, calcineurin is involved in the dephosphorylation of several cardiac proteins, such as, for example, the gap junction phosphoprotein connexin-43 [Chu *et al*, 2002]. It also is an important mediator of the transcription of genes involved in cardiac hypertrophy [Bourajjaj *et al*, 2008; Wilkins *et al*, 2004]. Thus a role for other cyclosporine A-sensitive cell types within (or outside) the infiltrate, in FGF-2-mediated cardioprotection, cannot be ruled out. Furthermore, it is possible that a direct effect of cyclosporine A on cardiomyocytes of the FGF-2 overexpressing mice contributed to a decrease in tissue Doppler imaging performance, independently of or in addition to its effects on T cell activation/cellular infiltration.

It is noteworthy that cyclosporine A by itself has been documented to have cardioprotective benefits including a mechanism by which it inhibits the opening of the

mitochondrial permeability transition pore [Altschuld *et al*, 1992; Halestrap *et al*, 1997; Massoudy *et al*, 1997; Nazareth *et al*, 1991; Novgorodov *et al*, 1992]. However, its mechanism of action for cardioprotection remains under contention as many studies show the inability of cyclosporine to improve infarct sizes, or to overcome cardiovascular risk, for example, in obesity [Huhn *et al*, 2009; Lie *et al*, 2010]. In our hands, cyclosporine had no significant effect on cardioprotection in non-transgenic mice (Figure 27) showing it has no detrimental cardiac effect; rather, its effects are on a function in FGF-2 transgenic animals (Figure 26). The decline in cardiac parameters with cyclosporine A treatment was observed at later time points (2 and 4 weeks post-injury), despite treatment ending 4 days after induction of injury, suggesting that cyclosporine A treatment does not have any immediate detrimental effects on FGF-2–induced functions. A decline in function observed weeks after injury suggests that it is likely an effect on angiogenesis or late-stage remodelling. Calcineurin has been shown to contribute to FGF-2–induced angiogenesis [X Liu *et al*, 2008]. In addition, cyclosporine has also been shown to downregulate angiogenic proteins including VEGF, and has been shown to inhibit FGF-2–induced angiogenesis by inhibiting endothelial cell proliferation [Shah *et al*, 2008; Sharpe *et al*, 1989]. Therefore, it is likely that the decline in function after injury observed in cyclosporine A-treated FGF-2 transgenic mice is, at least in part, due to cyclosporine effects on angiogenesis, possibly affecting endothelial cell proliferation and migration.

B.3. T lymphocytes in myocardial injury and repair

A direct role for T lymphocytes in recruiting inflammatory cells to a wound area is well-documented (reviewed in [Barbul and Regan, 1990]). In addition to physically stimulating lysis of cells that have been tagged for death, T lymphocytes also release cytokines that act as chemotactic and proliferative agents. Additionally to cell recruitment and proliferation, cytokine release also contributes to the microenvironment of an area of injury as it determines which cells are recruited as well as their response. For example, the cytokines interleukin (IL)-1, IL-6 and tumour necrosis factor can usually stimulate a pro-inflammatory immune response, whereas IL-4 and IL-10 would stimulate an anti-inflammatory cascade of events [Diwan *et al*, 2003; Opal and DePalo, 2000]. Thus, increased T cell presence in the hearts of FGF-2 overexpressing mice would be expected to influence the balance of cytokines in the area of injury and create a distinct microenvironment. T lymphocyte-related changes in cytokines, therefore, might also play a role in the improved functional characteristics observed in the FGF-2 transgenic mice, a role not previously attributed to T lymphocytes. Overall, it is likely that increased FGF-2 resulted in an “alternate” microenvironment for myocardial repair, influenced by a change in cytokine production. Further studies are needed to address this question.

B.4. Cellular infiltrate and myocardial repair

In addition to T lymphocytes, cyclosporine A has been shown to affect macrophages, and neutrophils, amongst many other cell types [Martin and Muir, 1990; Wahl and Allen, 1988]. FGF-2 has been shown to be able to stimulate progenitor stem

cell proliferation, survival and mobilization [Baddoo *et al*, 2003; Ma *et al*, 2009; Maric *et al*, 2007; Rosenblatt-Velin *et al*, 2005; Schmidt *et al*, 2006]. It is therefore logical to expect that these types of cells may also be components of the cellular infiltrate observed in the FGF-2 overexpressing mice. Progenitor/stem cells can exert beneficial effects through a paracrine contribution [Balsam *et al*, 2004; Murry *et al*, 2004] and may also contribute by transdifferentiating into cell types that contribute to a regenerative response (reviewed in [Gnecchi *et al*, 2008]). Progenitor cells *trans*-differentiating into functional cardiac myocytes leading to improved contractility has been documented previously in other animal models, and might also contribute to the FGF-2-induced improvement of outcome [Beltrami *et al*, 2003; Kajstura *et al*, 2005].

B.5. Cyclosporine A-insensitive component of the beneficial effect of FGF-2 on recovery as assessed by tissue Doppler imaging.

FGF-2 exerts major cytoprotective/cardioprotective effects, and it does so in both acute (bolus administration) and chronic scenarios, as previous studies in our and others' laboratories using FGF-2 overexpressing transgenic mice have shown [Detillieux *et al*, 2003; House *et al*, 2003; Nishida *et al*, 2003; Padua *et al*, 1998; Padua *et al*, 1995; Sheikh *et al*, 2001]. FGF-2 would be expected to continue to exert its cytoprotective effects within the viable myocardium during the events following isoproterenol-induced injury. We have shown that even after the cyclosporine A-induced decrease, functional recovery in the FGF-2 transgenic mice remained higher than that of non-transgenic mice. Thus, it is reasonable to suggest that cytoprotective effects on the viable myocardium are

responsible for the cyclosporine A-insensitive component of the improvement detected by tissue Doppler imaging.

B.6. The isoproterenol model of injury and its functional assessment

Isoproterenol, under the conditions employed, did not induce enough damage to the heart to be detected by M-mode echocardiography (Figures 20 and 21). Systolic function (as measured by ejection fraction and fractional shortening) was not significantly different between the isoproterenol-treated transgenic and non-transgenic groups. Although chronic isoproterenol treatment normally results in hypertrophy and subsequent changes in M-mode echocardiography indices, some studies employing acute treatment with isoproterenol resulted in detrimental diastolic function, but not in systolic function unless challenged by infusion with polyvinylpyrrolidone [Anderson *et al*, 2008; Beznak, 1962]. Furthermore, other studies using short-term isoproterenol infusion (within one week) demonstrated significant heart mass increase (hypertrophy) but ejection fraction and fractional shortening parameters remained unchanged between control and treated animal groups [Anderson *et al*, 2008; TP Tan *et al*, 2003]. It is therefore not unusual for a short-term acute isoproterenol treatment, as used here, to be unable to stimulate significant changes in M-mode echocardiography indices. It is likely that the damage induced by high dose isoproterenol treatment did not affect enough myocytes to sufficiently affect function at the organ level. Nevertheless, functional changes were observed by tissue Doppler imaging, the more sensitive technique that has been shown to be able to confidently predict cardiac functional outcome.

Based on a review of the literature, this also appears to be the first study to employ the more sensitive ultrasound method of tissue Doppler imaging to measure cardiac function after myocardial injury resulting from a high-dose β -adrenergic agonist administration. The tissue Doppler imaging indices of left ventricular function, including maximal systolic endocardial velocity and myocardial strain rate, are purportedly less load-dependent compared to fractional shortening and ejection fraction, and can be used to quantify regional and global systolic and diastolic function with greater accuracy [Urheim *et al*, 2000]. Tissue Doppler imaging allows for quantitative assessments of cardiac parameters at specific regions of the heart in real time, even before any irregularities can be measured in the whole organ by standard M-mode echocardiography. In a canine model of Duchenne's muscular cardiomyopathy, strain rate was affected before any changes to fractional shortening were observed [Chetboul *et al*, 2004]. In addition, tissue Doppler imaging but not conventional echocardiography was shown to be sensitive enough to distinguish between physiological (exercise induced) and pathological (pressure overload) left ventricular hypertrophy in rats [Derumeaux *et al*, 2002]. Furthermore, the sensitivity of tissue Doppler imaging has been used in clinical settings for early detection of cardiomyopathy and prediction of disease progression and functional outcome [Nagueh *et al*, 2001; Nagueh *et al*, 2003]. Here, we have demonstrated the increased sensitivity of tissue Doppler imaging in mice compared with standard gray scale echocardiography, since the damage caused by isoproterenol injection was evidenced by significant changes in both left ventricular endocardial velocity and strain rate (Figure 26), but not ejection fraction or fractional shortening (Table 2). Thus,

these results not only demonstrate that isoproterenol has an immediate effect on cardiac function, but also that increased myocardial FGF-2 contributes to an injury-resistant state.

CHAPTER XI

DISCUSSION

C. DISCUSSION OF OBSERVATIONS PRESENTED IN CHAPTER VIII: EFFECT OF PRE-INJURY ACUTE EXERCISE ON CARDIAC FUNCTION AFTER INJURY

C.1. Acute exercise-induced cardioprotection

Exercise has long been established to be beneficial: it improves resistance to disease and promotes the prevention and/or improved management of chronic diseases such as diabetes and osteoporosis, as well as decreasing the risk for cardiovascular disease [Dominguez *et al*, 2010; Hackam *et al*, 2010; Ignarro *et al*, 2007; Williams *et al*, 2007]. However, the question whether the cardiovascular benefits from exercise occurs through effects on cardiac cell biology and physiology, or rather simply a direct effect of healthy weight management subsequent to chronic exercise, remains to be determined. Additionally, the question of “How much exercise is *sufficient* to impart cardiovascular benefits?” also remains to be answered.

In Chapter VIII, a single bout of exercise was shown to be adequate to impart beneficial effects on the heart, as measured by tissue Doppler imaging. Significant improvements in left ventricular endocardial velocity and strain rate parameters were observed with exercise after isoproterenol-induced myocardial injury (Figure 31). These results suggest that an acute effect of exercise alone, without the contribution of weight management or long-term changes on metabolism, can contribute to improved cardiac

outcome. These results are supported by a recent study whereby significant changes in circulating angiogenic cells (assumed to be associated with improved prognosis) were observed in human cardiac patients after a single bout of exercise [Van Craenenbroeck *et al*, 2010]. However, the improvement in function in the swim group in the studies in this thesis was observed soon after injury, suggesting a preconditioning-like effect rather than an improvement in function due to the promotion of angiogenesis. An effect of angiogenesis is strongly suspected to be a contributing factor resulting from chronic exercise; however, in this study, the exercise was of an acute nature (30 minutes), and improvement in cardiac function at the late timepoint (4 weeks) was not observed beyond that at 24 hours and 2 weeks.

It is more likely that the cardioprotective effect observed here was similar to a remote ischemic preconditioning effect. Previously, it has been shown that an ischemic event in a remote organ prior to myocardial injury can stimulate improved functional recovery [Kharbanda *et al*, 2009]. Remote preconditioning has been attributed to activation of molecules including Erk1/2 and PKC ϵ [Shimizu *et al*, 2009]. The physical nature of exercise, in this case, swimming, result in brief intermittent “ischemic” events (by increased oxygen demand of the leg muscles, an event similar to that leading to the generation of lactic acid in exercising muscle in humans), which could contribute to the improved cardiac function as observed here by tissue Doppler imaging.

C.2. The swimming protocol

The question arose as to whether the exercise as used in this study was sufficient to cause physiological changes in the heart. Several laboratories have demonstrated the ability of different swimming protocols to create exercise-induced physiological hypertrophy in mice [Kaplan *et al*, 1994]. In humans, exercise (including swimming) is known to increase the demand on the heart, and as a result, increase heart rate. However, “arm cuffs” to quantitatively assess mouse heart rates were not available for this project. Thus, evidence of cardiac hypertrophy was used indirectly to determine whether the swimming protocol used here generated enough work (requiring an increased cardiac output) for the experimental mice. Significant increases in heart weight-to-body weight ratio were determined after a chronic one month swim protocol using wild type CD-1 mice when compared to control mice (Figure 34). It was thus reasonable to conclude that swimming is a valid dynamic “exercise” that involves increased demand on the heart.

CHAPTER XII

DISCUSSION

A. FGF-2-INDUCED CARDIOPROTECTION: WHAT COULD BE HAPPENING?

A.1. FGF-2-induced cardioprotection

Studies using FGF-2 transgenic mice and immunosuppression implicates chronic FGF-2 increase in cardioprotection against isoproterenol-induced myocardial injury. A two-stage process was observed where protection could be important. The first, “early stage” of cardioprotection occurs within the first 24 hours of injury. A benefit of increased FGF-2 availability is apparent and likely implicates the well-documented cytoprotective effects of FGF-2. This would require FGF-2 ligand binding with its receptor FGFR-1, but not necessarily FGF-imparted angiogenic properties [Jiang *et al*, 2004]. Protection by FGF-2 has been reported to occur via activation of protein kinase C and other downstream proteins associated with this signaling pathway [Jiang *et al*, 2002; Jiang *et al*, 2009; Padua *et al*, 1998; Srisakuldee *et al*, 2009a; Srisakuldee *et al*, 2006]. A contribution of FGF-2-induced protection through the phosphorylation of connexin 43 (Cx43) is also expected likely leading to the inhibition of the opening of the mitochondrial permeability pore [Srisakuldee *et al*, 2009a; Srisakuldee *et al*, 2006]. As such, this component of the protective mechanism is likely an effect of lo-FGF-2 rather than hi-FGF-2 [Srisakuldee *et al*, 2010]. In addition, a component of this FGF-2-induced first stage protection must be partially sensitive to cyclosporine A. Although the raw data

are not significantly different, a subtle decline in left ventricular endocardial velocity values was observed in FGF-2 transgenic mice (from ~76% of baseline) compared with FGF-2 transgenic mice treated with cyclosporine A (~65% of baseline). This decline in function 24 hours after injury, if real, is likely not a direct effect of cyclosporine A alone, but rather a combinatory effect with FGF-2, as it is observed in FGF-2 transgenic animals given cyclosporine A, but not in non-transgenic mice that also received cyclosporine A.

Limited damage within the early stages of injury is expected to contribute to a better outcome. This is observed in all groups assessed in the studies reported here. However, it is also clear that a continual decline in tissue Doppler imaging parameters occurs, as is also observed in all groups. A second, later stage of the remodeling process also seemingly transpires. This “late stage” event occurs sometime between 24 hours and 2 weeks after induction of injury. This second stage is evident as a decline in tissue Doppler imaging indices after injury in all groups assessed with the exception of the FGF-2 transgenic group. This suggests that a protective mechanism against this second stage of injury is in place in FGF-2 transgenic animals. Cyclosporine A treatment partially abolishes this protection; a significant decrease in tissue Doppler imaging indices was observed from ~65% of baseline at 24 hours after injury to ~55% by 2 weeks, and to ~45% by 4 weeks in FGF-2 transgenic animals. This is the greatest percent decline from the initial 24 hour timepoint observed between all groups in the studies reported here. This suggests that a significant detrimental effect of cyclosporine A acts on FGF-2-induced effects at the later stage of the remodeling process after isoproterenol-induced injury.

Thus, FGF-2's cardioprotective benefits include protection at an early stage, immediately after injury, as well as at a later stage of the cardiac repair response (sometime between 24 hours and 4 weeks). This protection is partially dependent on a cyclosporine-sensitive event, but this sensitivity plays a greater role in the late stage protection. This is the first report showing a cyclosporine-sensitive component to FGF-2-induced cardioprotection at multiple stages of the remodeling process.

A.2. Increased endogenous FGF-2-induced cardioprotection: conclusions and new hypotheses

Assessment of the data reveals some hints as to the mechanisms that may be occurring in chronic increased FGF-2-induced cardioprotection. The early-stage cardioprotection imparted in a preconditioning manner was previously discussed (previous section), and was not unexpected. Here, the potential contribution of FGF-2 cardioprotection in late-stage remodeling is discussed.

Although many different cells are likely involved in the FGF-2-induced cardioprotection at the late stages of remodeling, the data generated after cyclosporine A treatment suggested that T lymphocytes may play an important role. This was also supported by the observation that decreased cellular infiltrate corresponds to a decline in tissue Doppler imaging parameters; and cellular infiltration was observed to be significantly decreased upon treatment with cyclosporine A. However, an attempt at

inhibiting T cells using antibodies against CD3 ϵ did not result in any significant changes in tissue Doppler indices in either FGF-2 transgenic or non-transgenic groups after isoproterenol-induced myocardial injury. This anti-CD3 ϵ study, however, did not include assessment of antibody activity (a positive control), and thus cannot be used to draw proper conclusions. However, assuming the immunosuppression using the antibodies was sufficient to inhibit T cells, and then it is likely that the protection is related to a cyclosporine A-sensitive event, rather than a T cell-mediated event.

Cyclosporine A is commonly used as an immunosuppressant because of its efficient inhibition of T cell activation [Batiuk *et al*, 1996; Matsuda and Koyasu, 2000]. However, it is also known to have other effects as its mechanism of action is through the inhibition of the transcription factor NFAT (which is not tissue/cell specific) [Benson and Ziegler, 1989; Chan *et al*, 2007; Halestrap *et al*, 1997; Matsuda and Koyasu, 2000; Spisani *et al*, 2001]. Based on the gavage method of administration of cyclosporine A, systemic action is enabled and, hence, multi-factorial effects are possible, and likely. In the heart alone, cyclosporine A has been shown to inhibit calcineurin-mediated hypertrophic growth [Wilkins *et al*, 2004]. Here, treatment with cyclosporine A resulted in a significant decline in cardiac function, but only in FGF-2 transgenic animals. This raises the possibility that at least part of the FGF-2-mediated cardioprotection at the later stage is through a hypertrophic remodeling process involving calcineurin. This was not observed in non-transgenic mice, suggesting that an increase in FGF-2 levels above normal physiological concentrations may be required to stimulate this second late-stage cardioprotection.

An FGF-2 effect on protective mechanisms other than compensatory hypertrophy may also be occurring. Cyclosporine A can influence scar formation as well as new collateral vessel formation through effects on fibroblasts, macrophages, and endothelial cells. This is substantiated by a study by Murry and colleagues which demonstrated a significant increase in proliferating myofibroblast cells and an earlier peak to proliferating endothelial cells in FGF-2 transgenic animals [JA Virag *et al*, 2007]. Cyclosporine A has been shown to inhibit angiogenesis, by downregulation of VEGF receptor [Shah *et al*, 2008]. FGF-2-induced proliferation of endothelial cells has also been shown to be inhibited by cyclosporine A [Sharpe *et al*, 1989]. In addition, studies using other FGF-2 transgenic mouse lines as well as studies in cancer research in combination with our data hint at a cooperative mechanism between multiple cell types to stimulate remodeling and growth of collateral vessels [Tsunoda *et al*, 2009; JA Virag *et al*, 2007]. Furthermore, studies of myocardial injury where FGF-2 was administered to the myocardium also showed increased c-kit-positive cells [Kardami *et al*, 2007], suggesting a possible role for FGF-2 in the chemotaxis of progenitor cells (or other c-kit-positive cells) that may contribute to improved cardiac function. Considering that cyclosporine A treatment was completed by 4 days post-injury, and the decline in function observed was not until 2 and 4 weeks, it is likely that cyclosporine effects were on the microenvironment (composition or events) at an earlier stage of the remodelling process. This further suggests that the effects must be of a multi-factorial nature. Thus, FGF-2 could have effects on multiple cell types that migrate into the area of injury and are involved in cardiac wound healing. Further study is required to delineate exactly

which cells contribute, and how, to the FGF-2–induced cardioprotection observed in this study.

Therefore, FGF-2 is cardioprotective when increased in the heart, and contributes to at least two stages of myocardial remodeling: one in an immediate preconditioning-like manner evident within 24 hours of injury, and in a second, later-stage role that is cyclosporine-sensitive.

CHAPTER XIII

FUTURE DIRECTIONS

A. RATIONALE

The finding that a chronic increase in FGF-2 levels in the heart confers cardioprotection (observed by significant improvement in cardiac function) by 24 hours after injury as well as 2 and 4 weeks later, provides an attractive option to exploit for improved cardiac outcome for the general population. However, before such a possibility can be considered, a better understanding of the mechanisms governing the cardioprotection is necessary.

B. CARDIOPROTECTION FROM INCREASED ENDOGENOUS FGF-2 LEVELS

The cardioprotection observed in FGF-2 transgenic mice as assessed by tissue Doppler imaging was partially mediated by a cyclosporine-sensitive event, and coincided with a significant increase in cellular infiltrate. To be able to understand the mechanisms that govern FGF-2-induced cardioprotection, these cells/inflammatory events need to be identified and characterized.

Treatment with cyclosporine A was originally intended to knock down T cell activity to determine their role in FGF-2-induced cardioprotection. Due to the broad

effects of cyclosporine A, additional, non T cell-dependent effects cannot be excluded. A similar experiment, but using a more specific inhibitor of T cells (antibodies against CD3 ϵ), suggested that T lymphocytes were not critical in the cardioprotection observed. However, this experiment was preliminary, and imperfect: although visual assessment of cellular infiltration suggested that an immunosuppressive effect was achieved, its efficacy was not quantified. Thus, partial immunosuppression could have occurred, or that certain subsets of cells were more sensitive to the treatment. To verify a T cell-mediated effect on FGF-2-induced cardioprotection, a more comprehensive experiment repeating the immunosuppression experiments using antibodies against CD3 ϵ would be necessary. The effect of immunosuppression would also have to be verified. Testing can be achieved by quantification of T cells and activated T cells circulating in the bloodstream (plasma samples from the animals on the last day of antibody treatment) of antibody-treated animals compared to their baseline levels prior to immunosuppression and those of control animals. Assessment of cytokines released could also verify antibody activity. Verification of immunosuppression can present a better picture of the role of T cells in FGF-2-induced cardioprotection.

However, due to the likelihood of “incomplete” immunosuppressive therapies, a more definitive method of assessment for the role of T lymphocytes in FGF-2-induced cardioprotection, would be to utilize progeny from a cross between the FGF-2 transgenic mouse (RSVp.metFGF2) and a Rag null mouse, and use their offspring to assess cardioprotective effects after injury as described in this thesis. Rag null mice are characterized by an inability to produce mature T cells or B cells and, thus, their

phenotype can be described as akin to that of a “non-leaky” severe combined immune deficient mouse [Chen *et al*, 1994]. If such studies showed no change in cardiac function after isoproterenol-induced injury, then it is likely that T lymphocytes do not have a significant role in FGF-2–induced cardioprotection.

An assessment of the basal levels of circulating cells (that were identified within the myocardial infiltrate in Chapter VI) should also be done and compared between FGF-2 transgenic mice and non-transgenic mice. Such an experiment could be easily conducted by flow cytometry, or using hematology analyzer technology, to assess whether the initial blood populations of the animals used in the study are comparable. It is possible that basal cell numbers/populations could be altered by the availability of increased FGF-2 from gestation and, thus, could also affect wound repair, for example, in the heart.

To assess the contributions of FGF-2 on angiogenesis at the later stages of cardiac injury as previously discussed, blood vessel densities need to be assessed using specific stains for markers of blood vessels (for example, von Willebrand factor to identify endothelial cells and highlight the capillaries, and α -smooth muscle actin to identify larger vessels). Quantitation of blood vessels and comparing them to the density at baseline would provide better insight into whether angiogenesis is a contributing factor to FGF-2–induced cardioprotection in our model, and whether the declined cardiac function with cyclosporine A treatment also coincides with decreased blood vessel densities. Endothelial cells begin migrating and proliferating into a wound area several days after

injury. Quantitation of these cells in transgenic FGF-2 versus non-transgenic mouse hearts after isoproterenol and cyclosporine A treatment could also give insights as to the effects of cyclosporine on FGF-2-induced angiogenesis. Another option would be to generate mice whose FGF-2 gene has an alanine substituted for the serine at amino acid position 117, which allows FGF-2-induced mitogenesis, but whose mutation diminishes its ability to activate CK2 and subsequent cell proliferation [Bailly *et al*, 2000; Jiang *et al*, 2004]. This construct has previously been shown to be unable to stimulate FGF-2-induced angiogenesis. These mice could be used in identical experiments to those used in this thesis to determine whether angiogenesis (which requires CK2 activation) is responsible for the improved cardiac function observed in the later (2 and 4 week) time points.

The contributions of different cell types could also be assessed by doing similar immunosuppression studies conducted here, but using specific inhibitors of possible target cells (for example, semapimod to inhibit macrophage activation [Kherani *et al*, 2004], or PR-39 to inhibit neutrophils [Hoffmeyer *et al*, 2000]). Results from such studies would at least suggest whether any of the other components of the myocardial infiltrate contribute to FGF-2-induced cardioprotection.

Contributions of compensatory hypertrophy (since previously reported to be inhibited by cyclosporine A) could also be assessed. Firstly, a heart weight-to-body weight or heart weight-to-tibia length ratio would need to be conducted to show effects on hypertrophy, if any. Direct measure of cardiac myocyte size would confirm

hypertrophic growth. Experiments using pharmacologically inhibitory molecules against any of the important pathways in the development of hypertrophy [Bernardo *et al*, 2010] and subsequent assessment of cardiac function after injury could also suggest whether the protection observed here could, at least partially, be attributed to an effect on the hypertrophic process.

To determine the molecular mechanisms involved in cardioprotection observed with increased endogenous FGF-2, studies assessing the levels of known mediators of FGF-2 effects could be done. It has been shown previously that FGF-2 mediates its effects through its cell surface receptor FGFR1 and intracellularly via PKC as well as connexin 43 [Jiang *et al*, 2002]. New evidence has now emerged from our laboratory that the ischemic preconditioning-like FGF-2-induced cardioprotection (at least in an *ex vivo* preparation) occurs through the inhibition of the opening of the mitochondrial permeability transition pore [Srisakuldee *et al*, 2010]. Whether this mechanism contributes to the results observed in the FGF-2 transgenic mice in the studies described here remains to be determined.

C. EXERCISE-INDUCED CARDIOPROTECTION

The fact that exercise can induce cardioprotection has previously been established [Ignarro *et al*, 2007; Williams *et al*, 2007]. The observations of exercise-induced cardioprotection presented in this thesis showed some similarity to the effects seen with overexpression of FGF-2 in the heart. Thus, the possibility exists that an increase in

FGF-2 bioavailability in the heart contributes to exercise-induced cardioprotection. The data presented here to support the exercise-induced protection is preliminary, and therefore, repeating the experiment would be necessary.

The question whether exercise can stimulate an increase in FGF-2 in the heart remains unanswered. Preliminary studies (n=3) using a swimming regimen on mice showed an increasing trend for FGF-2 RNA in their hearts, but the difference compared to shallow water controls is not significant. This could certainly be due to the small number of animals tested, however, duration/extent of (1) exercise, and (2) timing of RNA and protein isolation also need to be taken into account. In addition, consideration of the exercise routine could also make a difference; a leisurely activity would not have the same effects of a stressful forced one. For example, swimming is not a leisurely activity for mice, but they voluntarily run on treadmills if given the opportunity. Exercise has been shown to increase serum FGF-2 levels in men after a 6-month exercise regime [Seida *et al*, 2003]. If exercise can be demonstrated to acutely increase FGF-2 levels in the heart, the next question to be addressed would be whether an exercise regime could be optimized to enable an increase in endogenous FGF-2 levels in the heart in a more chronic manner.

Confirmation of FGF-2 participation in exercise-induced cardioprotection could be addressed by conducting the same experiments here using FGF-2 null mice. Abolishment of protection would definitively implicate the importance of FGF-2 in exercise-induced cardioprotection. However, if protection is still observed, it is

important to note that functional redundancies among the other FGF molecules (for example, FGF-1) may exist, and that negative results may not necessarily indicate a non-involvement of FGF-2 in exercise-induced cardioprotection.

D. FINAL THOUGHTS / RELEVANCE

Lessons learned from the studies conducted in this thesis could contribute to possible therapeutic strategies translatable to the clinic. The greatest challenge for delivery of cardioprotection via ischemic preconditioning has been timing, due to the inability to predict the occurrence of a cardiac insult, and the window of opportunity remains small. A potential to exploit ischemic preconditioning-like protection (whether by FGF-2 or not) is perhaps by simulating the protein profiles found in the cardiac myocyte during its “protected” state. Such states could only be achieved after being able to identify the protein complexes necessary for cardioprotection. The possibility of exercise promoting a protein state similar to the cardioprotected cardiac myocyte also remains to be identified.

In addition to mimicking the protein states in the myocyte upon ischemic pre/postconditioning, it is also possible that players external to the cardiac myocyte could mediate the promotion of a “protected” state. In this thesis, a cyclosporine-sensitive component has been identified to be contributing to cardioprotection. Whether this component is internal to the myocyte, or external (perhaps a component of the cellular infiltrate) remains to be identified, as discussed previously. Clearly, identification of

either molecules or cells that can contribute to an improved cardiac functional outcome after myocardial injury could be exploited at the clinic.

Such discoveries, as well as identification of the mechanisms mediating the protective benefits of exercise, could be beneficial for a large patient population. Additionally, patients who cannot perform vigorous exercise (for example, elderly or physically handicapped persons) could also potentially benefit from protection artificially, by delivery of the essential molecular events from exercise-induced protection downstream from physical exertion.

Perhaps most importantly, the cyclosporine-sensitive event observed in this thesis was shown to contribute beneficially at the later stages of remodeling (2 and 4 weeks after injury). Identification of the molecular events involved in this process could be exploited for improved cardiac remodelling after injury. This therapeutic strategy would benefit a greater number of people because: (1) of the increased window of opportunity for therapeutic delivery, and (2) this would also include not only patients at risk for MI, but also those that could benefit from improved cardiac remodelling (for example, those presenting with heart failure additionally to those who have already suffered from an MI).

CHAPTER XIV

REFERENCES

- JA Abraham, A Mergia, JL Whang, A Tumolo, J Friedman, KA Hjerrild, D Gospodarowicz and JC Fiddes. (1986a) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**: 545-8.
- JA Abraham, JL Whang, A Tumolo, A Mergia, J Friedman, D Gospodarowicz and JC Fiddes. (1986b) Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *Embo J* **5**: 2523-8.
- VJ Adlam, JC Harrison, CM Porteous, AM James, RA Smith, MP Murphy and IA Sammut. (2005) Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. *Faseb J* **19**: 1088-95.
- RA Altschuld, CM Hohl, LC Castillo, AA Garleb, RC Starling and GP Brierley. (1992) Cyclosporin inhibits mitochondrial calcium efflux in isolated adult rat ventricular cardiomyocytes. *Am J Physiol* **262**: H1699-704.
- K Amann, J Faulhaber, V Campean, V Balajew, R Dono, G Mall and H Ehmke. (2006) Impaired myocardial capillarogenesis and increased adaptive capillary growth in FGF2-deficient mice. *Lab Invest* **86**: 45-53.
- JA Ambrose and RS Barua. (2004) The pathophysiology of cigarette smoking and cardiovascular disease: an update. *J Am Coll Cardiol* **43**: 1731-7.
- LH Anane, KM Edwards, VE Burns, MT Drayson, NE Riddell, JJ van Zanten, GR Wallace, PJ Mills and JA Bosch. (2009) Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. *Brain Behav Immun*
- M Anderson, D Moore and D Larson. (2008) Comparison of isoproterenol and dobutamine in the induction of cardiac hypertrophy and fibrosis. *Perfusion* **23**: 231-5.
- P Anversa, D Fitzpatrick, S Argani and JM Capasso. (1991) Myocyte mitotic division in the aging mammalian rat heart. *Circ Res* **69**: 1159-64.
- P Anversa, A Leri and J Kajstura. (2006) Cardiac regeneration. *J Am Coll Cardiol* **47**: 1769-76.
- E Araki, F Shimada, H Uzawa, M Mori and Y Ebina. (1987) Characterization of the promoter region of the human insulin receptor gene. Evidence for promoter activity. *J Biol Chem* **262**: 16186-91.

E Arman, R Haffner-Krausz, Y Chen, JK Heath and P Lonai. (1998) Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. *Proc Natl Acad Sci U S A* **95**: 5082-7.

M Arras, R Strasser, M Mohri, R Doll, P Eckert, W Schaper and J Schaper. (1998) Tumor necrosis factor-alpha is expressed by monocytes/macrophages following cardiac microembolization and is antagonized by cyclosporine. *Basic Res Cardiol* **93**: 97-107.

SM Artham, CJ Lavie, RV Milani, DA Patel, A Verma and HO Ventura. (2009) Clinical impact of left ventricular hypertrophy and implications for regression. *Prog Cardiovasc Dis* **52**: 153-67.

E Ashley and J Niebauer. (2004) Cardiology explained. Retrieved from www.accessmedicine.com.

B Assmus, V Schachinger, C Teupe, M Britten, R Lehmann, N Dobert, F Grunwald, A Aicher, C Urbich, H Martin, D Hoelzer, S Dimmeler and AM Zeiher. (2002) Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* **106**: 3009-17.

F Atsma, ML Bartelink, DE Grobbee and YT van der Schouw. (2006) Postmenopausal status and early menopause as independent risk factors for cardiovascular disease: a meta-analysis. *Menopause* **13**: 265-79.

M Bacaner, J Brietenbucher and J LaBree. (2004) Prevention of ventricular fibrillation, acute myocardial infarction (myocardial necrosis), heart failure, and mortality by bretylium: is ischemic heart disease primarily adrenergic cardiovascular disease? *Am J Ther* **11**: 366-411.

M Baddoo, K Hill, R Wilkinson, D Gaupp, C Hughes, GC Kopen and DG Phinney. (2003) Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem* **89**: 1235-49.

K Bailly, F Soulet, D Leroy, F Amalric and G Bouche. (2000) Uncoupling of cell proliferation and differentiation activities of basic fibroblast growth factor. *Faseb J* **14**: 333-44.

P Balakumar, AP Singh, SS Ganti, P Krishan, S Ramasamy and M Singh. (2008) Resident cardiac mast cells: are they the major culprit in the pathogenesis of cardiac hypertrophy? *Basic Clin Pharmacol Toxicol* **102**: 5-9.

LB Balsam, AJ Wagers, JL Christensen, T Kofidis, IL Weissman and RC Robbins. (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* **428**: 668-73.

R Bansal, S Magge and S Winkler. (2003) Specific inhibitor of FGF receptor signaling: FGF-2-mediated effects on proliferation, differentiation, and MAPK activation are inhibited by PD173074 in oligodendrocyte-lineage cells. *J Neurosci Res* **74**: 486-93.

A Barbul and MC Regan. (1990) The regulatory role of T lymphocytes in wound healing. *J Trauma* **30**: S97-100.

A Barbul, T Shawe, SM Rotter, JE Efron, HL Wasserkrug and SB Badawy. (1989) Wound healing in nude mice: a study on the regulatory role of lymphocytes in fibroplasia. *Surgery* **105**: 764-9.

TD Batiuk, J Urmson, D Vincent, RW Yatscoff and PF Halloran. (1996) Quantitating immunosuppression. Estimating the 50% inhibitory concentration for in vivo cyclosporine in mice. *Transplantation* **61**: 1618-24.

C Bearzi, A Leri, F Lo Monaco, M Rota, A Gonzalez, T Hosoda, M Pepe, K Qanud, C Ojaimi, S Bardelli, D D'Amario, DA D'Alessandro, RE Michler, S Dimmeler, AM Zeiher, K Urbanek, TH Hintze, J Kajstura and P Anversa. (2009) Identification of a coronary vascular progenitor cell in the human heart. *Proc Natl Acad Sci U S A* **106**: 15885-90.

A Beenken and M Mohammadi. (2009) The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov* **8**: 235-53.

AP Beltrami, L Barlucchi, D Torella, M Baker, F Limana, S Chimenti, H Kasahara, M Rota, E Musso, K Urbanek, A Leri, J Kajstura, B Nadal-Ginard and P Anversa. (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**: 763-76.

AP Beltrami, K Urbanek, J Kajstura, SM Yan, N Finato, R Bussani, B Nadal-Ginard, F Silvestri, A Leri, CA Beltrami and P Anversa. (2001) Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* **344**: 1750-7.

NL Benowitz. (2003) Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment. *Prog Cardiovasc Dis* **46**: 91-111.

A Benson and HK Ziegler. (1989) Macrophages as targets for inhibition by cyclosporine. *Transplantation* **47**: 696-703.

O Bernard, M Li and HH Reid. (1991) Expression of two different forms of fibroblast growth factor receptor 1 in different mouse tissues and cell lines. *Proc Natl Acad Sci U S A* **88**: 7625-9.

BC Bernardo, KL Weeks, L Pretorius and JR McMullen. (2010) Molecular distinction between physiological and pathological cardiac hypertrophy: Experimental findings and therapeutic strategies. *Pharmacol Ther*

M Beznak. (1962) Hemodynamics during the acute phase of myocardial damage caused by isoproterenol. *Can J Biochem Physiol* **40**: 25-30.

M Beznak and P Hacker. (1964) Hemodynamics During the Chronic Stage of Myocardial Damage Caused by Isoproterenol. *Can J Physiol Pharmacol* **42**: 269-74.

E Biesiada, M Razandi and ER Levin. (1996) Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. *J Biol Chem* **271**: 18576-81.

B Bittira, D Shum-Tim, A Al-Khaldi and RC Chiu. (2003) Mobilization and homing of bone marrow stromal cells in myocardial infarction. *Eur J Cardiothorac Surg* **24**: 393-8.

MA Bogoyevitch, PE Glennon, MB Andersson, A Clerk, A Lazou, CJ Marshall, PJ Parker and PH Sugden. (1994) Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J Biol Chem* **269**: 1110-9.

P Bohlen, A Baird, F Esch, N Ling and D Gospodarowicz. (1984) Isolation and partial molecular characterization of pituitary fibroblast growth factor. *Proc Natl Acad Sci U S A* **81**: 5364-8.

P Bohlen, F Esch, A Baird and D Gospodarowicz. (1985) Acidic fibroblast growth factor (FGF) from bovine brain: amino-terminal sequence and comparison with basic FGF. *Embo J* **4**: 1951-6.

H Bonnet, O Filhol, I Truchet, P Brethenou, C Cochet, F Amalric and G Bouche. (1996) Fibroblast growth factor-2 binds to the regulatory beta subunit of CK2 and directly stimulates CK2 activity toward nucleolin. *J Biol Chem* **271**: 24781-7.

C Bossard, H Laurell, L Van den Berghe, S Meunier, C Zanibellato and H Prats. (2003) Translokin is an intracellular mediator of FGF-2 trafficking. *Nat Cell Biol* **5**: 433-9.

M Bourajjaj, AS Armand, PA da Costa Martins, B Weijts, R van der Nagel, S Heeneman, XH Wehrens and LJ De Windt. (2008) NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. *J Biol Chem* **283**: 22295-303.

E Braunwald. (2008). Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine. Philadelphia: Saunders Elsevier.

EC Breen, EC Johnson, H Wagner, HM Tseng, LA Sung and PD Wagner. (1996) Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J Appl Physiol* **81**: 355-61.

MB Britten, ND Abolmaali, B Assmus, R Lehmann, J Honold, J Schmitt, TJ Vogl, H Martin, V Schachinger, S Dimmeler and AM Zeiher. (2003) Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. *Circulation* **108**: 2212-8.

LL Brunton. (2006) Goodman and Gilman's The Pharmacological Basis of Therapeutics. Retrieved from www.accessmedicine.com.

A Buehler, A Martire, C Strohm, S Wolfram, B Fernandez, M Palmen, XH Wehrens, PA Doevendans, WM Franz, W Schaper and R Zimmermann. (2002) Angiogenesis-independent cardioprotection in FGF-1 transgenic mice. *Cardiovasc Res* **55**: 768-77.

DM Burns. (2003) Epidemiology of smoking-induced cardiovascular disease. *Prog Cardiovasc Dis* **46**: 11-29.

EC Campos, MM Romano, CM Prado and MA Rossi. (2008) Isoproterenol induces primary loss of dystrophin in rat hearts: correlation with myocardial injury. *Int J Exp Pathol* **89**: 367-81.

S Capewell, ES Ford, JB Croft, JA Critchley, KJ Greenlund and DR Labarthe. (2010) Cardiovascular risk factor trends and potential for reducing coronary heart disease mortality in the United States of America. *Bull World Health Organ* **88**: 120-30.

MW Chan, PD Arora and CA McCulloch. (2007) Cyclosporin inhibition of collagen remodeling is mediated by gelsolin. *Am J Physiol Cell Physiol* **293**: C1049-58.

P Chandrasoma and CR Taylor. Chapter 3. The Acute Inflammatory Response. Retrieved from <http://www.accessmedicine.com/content.aspx?aID=183347>.

AT Chellaiah, DG McEwen, S Werner, J Xu and DM Ornitz. (1994) Fibroblast growth factor receptor (FGFR) 3. Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. *J Biol Chem* **269**: 11620-7.

J Chen, Y Shinkai, F Young and FW Alt. (1994) Probing immune functions in RAG-deficient mice. *Curr Opin Immunol* **6**: 313-9.

V Chetboul, C Escriviou, D Tessier, V Richard, JL Pouchelon, H Thibault, F Lallemand, C Thuillez, S Blot and G Derumeaux. (2004) Tissue Doppler imaging detects early asymptomatic myocardial abnormalities in a dog model of Duchenne's cardiomyopathy. *Eur Heart J* **25**: 1934-9.

J Choi, MK Ko and EP Kay. (2000) Subcellular localization of the expressed 18 kDa FGF-2 isoform in corneal endothelial cells. *Mol Vis* **6**: 222-31.

G Chu, AN Carr, KB Young, JW Lester, A Yatani, A Sanbe, MC Colbert, SM Schwartz, KF Frank, PD Lampe, J Robbins, JD Molkentin and EG Kranias. (2002) Enhanced myocyte contractility and Ca²⁺ handling in a calcineurin transgenic model of heart failure. *Cardiovasc Res* **54**: 105-16.

R Cirillo, M Triggiani, L Siri, A Ciccarelli, GR Pettit, M Condorelli and G Marone. (1990) Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interacting with cyclophilin. *J Immunol* **144**: 3891-7.

RA Clark. (1985) Cutaneous tissue repair: basic biologic considerations. I. *J Am Acad Dermatol* **13**: 701-25.

MS Clarke, RW Caldwell, H Chiao, K Miyake and PL McNeil. (1995) Contraction-induced cell wounding and release of fibroblast growth factor in heart. *Circ Res* **76**: 927-34.

MS Clarke and DL Feedback. (1996) Mechanical load induces sarcoplasmic wounding and FGF release in differentiated human skeletal muscle cultures. *Faseb J* **10**: 502-9.

P Claus, F Doring, S Gringel, F Muller-Ostermeyer, J Fuhrlott, T Kraft and C Grothe. (2003) Differential intranuclear localization of fibroblast growth factor-2 isoforms and specific interaction with the survival of motoneuron protein. *J Biol Chem* **278**: 479-85.

JC Coutts and JT Gallagher. (1995) Receptors for fibroblast growth factors. *Immunol Cell Biol* **73**: 584-9.

JG Crowston, M Salmon, PT Khaw and AN Akbar. (1997) T-lymphocyte-fibroblast interactions. *Biochem Soc Trans* **25**: 529-31.

P Cuevas, F Carceller, RM Lozano, A Crespo, M Zazo and G Gimenez-Gallego. (1997) Protection of rat myocardium by mitogenic and non-mitogenic fibroblast growth factor during post-ischemic reperfusion. *Growth Factors* **15**: 29-40.

P Cuevas, F Carceller, V Martinez-Coso, E Asin-Cardiel and G Gimenez-Gallego. (2000) Fibroblast growth factor cardioprotection against ischemia-reperfusion injury may involve K⁺ ATP channels. *Eur J Med Res* **5**: 145-9.

P Cuevas, F Carceller, V Martinez-Coso, B Cuevas, A Fernandez-Ayerdi, D Reimers, E Asin-Cardiel and G Gimenez-Gallego. (1999) Cardioprotection from ischemia by fibroblast growth factor: role of inducible nitric oxide synthase. *Eur J Med Res* **4**: 517-24.

MC Cushing, PD Mariner, JT Liao, EA Sims and KS Anseth. (2008) Fibroblast growth factor represses Smad-mediated myofibroblast activation in aortic valvular interstitial cells. *Faseb J* **22**: 1769-77.

VP Dave. (2009) Hierarchical role of CD3 chains in thymocyte development. *Immunol Rev* **232**: 22-33.

G de Simone, RB Devereux, SR Daniels and RA Meyer. (1995) Gender differences in left ventricular growth. *Hypertension* **26**: 979-83.

N Deconinck and B Dan. (2007) Pathophysiology of duchenne muscular dystrophy: current hypotheses. *Pediatr Neurol* **36**: 1-7.

HA Demirel, SK Powers, MA Zergeroglu, RA Shanely, K Hamilton, J Coombes and H Naito. (2001) Short-term exercise improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *J Appl Physiol* **91**: 2205-12.

G Derumeaux, P Mulder, V Richard, A Chagraoui, C Nafeh, F Bauer, JP Henry and C Thuillez. (2002) Tissue Doppler imaging differentiates physiological from pathological pressure-overload left ventricular hypertrophy in rats. *Circulation* **105**: 1602-8.

KA Detillieux, F Sheikh, E Kardami and PA Cattini. (2003) Biological activities of fibroblast growth factor-2 in the adult myocardium. *Cardiovasc Res* **57**: 8-19.

O Dewald, G Ren, GD Duerr, M Zoerlein, C Klemm, C Gersch, S Tincey, LH Michael, ML Entman and NG Frangogiannis. (2004) Of mice and dogs: species-specific differences in the inflammatory response following myocardial infarction. *Am J Pathol* **164**: 665-77.

S Dharmaraj. RT-PCR: The Basics. Retrieved from <http://www.ambion.com/techlib/basics/rtpcr/index.html>.

T Dill, V Schachinger, A Rolf, S Mollmann, H Thiele, H Tillmanns, B Assmus, S Dimmeler, AM Zeiher and C Hamm. (2009) Intracoronary administration of bone marrow-derived progenitor cells improves left ventricular function in patients at risk for adverse remodeling after acute ST-segment elevation myocardial infarction: results of the Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction study (REPAIR-AMI) cardiac magnetic resonance imaging substudy. *Am Heart J* **157**: 541-7.

A Diwan, T Tran, A Misra and DL Mann. (2003) Inflammatory mediators and the failing heart: a translational approach. *Curr Mol Med* **3**: 161-82.

IM Dixon, SL Lee and NS Dhalla. (1990) Nitrendipine binding in congestive heart failure due to myocardial infarction. *Circ Res* **66**: 782-8.

BW Doble, RR Fandrich, L Liu, RR Padua and E Kardami. (1990) Calcium protects pituitary basic fibroblast growth factors from limited proteolysis by co-purifying proteases. *Biochem Biophys Res Commun* **173**: 1116-22.

LJ Dominguez, R Scalisi and M Barbagallo. (2010) Therapeutic options in osteoporosis. *Acta Biomed* **81 Suppl 1**: 55-65.

R Dono, G Texido, R Dussel, H Ehmke and R Zeller. (1998) Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *Embo J* **17**: 4213-25.

SD Douglas and W Ho. (2007) Morphology of Monocytes and Macrophages. Retrieved from www.accessmedicine.com/content.aspx?aID=2145926.

L Draberova. (1990) Cyclosporin A inhibits rat mast cell activation. *Eur J Immunol* **20**: 1469-73.

V Drobic, RH Cunnington, KM Bedosky, JE Raizman, VV Elimban, SG Rattan and IM Dixon. (2007) Differential and combined effects of cardiotrophin-1 and TGF-beta1 on cardiac myofibroblast proliferation and contraction. *Am J Physiol Heart Circ Physiol* **293**: H1053-64.

JE Efron, HL Frankel, SA Lazarou, HL Wasserkrug and A Barbul. (1990) Wound healing and T-lymphocytes. *J Surg Res* **48**: 460-3.

FB Engel, M Schebesta, MT Duong, G Lu, S Ren, JB Madwed, H Jiang, Y Wang and MT Keating. (2005) p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev* **19**: 1175-87.

AE Eriksson, LS Cousens, LH Weaver and BW Matthews. (1991) Three-dimensional structure of human basic fibroblast growth factor. *Proc Natl Acad Sci U S A* **88**: 3441-5.

F Esch, A Baird, N Ling, N Ueno, F Hill, L Denoroy, R Klepper, D Gospodarowicz, P Bohlen and R Guillemin. (1985) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. *Proc Natl Acad Sci U S A* **82**: 6507-11.

VP Eswarakumar, I Lax and J Schlessinger. (2005) Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* **16**: 139-49.

S Faouzi, B Le Bail, V Neaud, L Boussarie, J Saric, P Bioulac-Sage, C Balabaud and J Rosenbaum. (1999) Myofibroblasts are responsible for collagen synthesis in the stroma of human hepatocellular carcinoma: an in vivo and in vitro study. *J Hepatol* **30**: 275-84.

VJ Ferrans, RG Hibbs, WC Black and DG Weilbaecher. (1964) Isoproterenol-Induced Myocardial Necrosis. a Histochemical and Electron Microscopic Study. *Am Heart J* **68**: 71-90.

MC Fishbein, D Maclean and PR Maroko. (1978) The histopathologic evolution of myocardial infarction. *Chest* **73**: 843-9.

RZ Florkiewicz, J Anchin and A Baird. (1998) The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na⁺,K⁺-ATPase. *J Biol Chem* **273**: 544-51.

RZ Florkiewicz, RA Majack, RD Buechler and E Florkiewicz. (1995) Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. *J Cell Physiol* **162**: 388-99.

RZ Florkiewicz and A Sommer. (1989) Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc Natl Acad Sci U S A* **86**: 3978-81.

J Folkman, M Klagsbrun, J Sasse, M Wadzinski, D Ingber and I Vlodavsky. (1988) A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. *Am J Pathol* **130**: 393-400.

NG Frangogiannis. (2006) The mechanistic basis of infarct healing. *Antioxid Redox Signal* **8**: 1907-39.

NG Frangogiannis. (2008) The immune system and cardiac repair. *Pharmacol Res* **58**: 88-111.

NG Frangogiannis, JL Perrard, LH Mendoza, AR Burns, ML Lindsey, CM Ballantyne, LH Michael, CW Smith and ML Entman. (1998) Stem cell factor induction is associated with mast cell accumulation after canine myocardial ischemia and reperfusion. *Circulation* **98**: 687-98.

NG Frangogiannis, CW Smith and ML Entman. (2002) The inflammatory response in myocardial infarction. *Cardiovasc Res* **53**: 31-47.

J Fransioli, B Bailey, NA Gude, CT Cottage, JA Muraski, G Emmanuel, W Wu, R Alvarez, M Rubio, S Ottolenghi, E Schaefer and MA Sussman. (2008) Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. *Stem Cells* **26**: 1315-24.

TJ Frederick, J Min, SC Altieri, NE Mitchell and TL Wood. (2007) Synergistic induction of cyclin D1 in oligodendrocyte progenitor cells by IGF-I and FGF-2 requires differential stimulation of multiple signaling pathways. *Glia* **55**: 1011-22.

V Fuster, RA O'Rourke, RA Walsh and P Poole-Wilson. (2008). Hurst's THE HEART. China: The McGraw-Hill Companies, Inc.

RA Garcia, KV Go and FJ Villarreal. (2007) Effects of timed administration of doxycycline or methylprednisolone on post-myocardial infarction inflammation and left ventricular remodeling in the rat heart. *Mol Cell Biochem* **300**: 159-69.

LPaH Gartner, J. L. (2007). Color Textbook of Histology. Philadelphia, PA: Elsevier.

AA Geisterfer-Lowrance, M Christe, DA Conner, JS Ingwall, FJ Schoen, CE Seidman and JG Seidman. (1996) A mouse model of familial hypertrophic cardiomyopathy. *Science* **272**: 731-4.

A Gluba, M Banach, DP Mikhailidis and J Rysz. (2009) Genetic determinants of cardiovascular disease: the renin-angiotensin-aldosterone system, paraoxonases, endothelin-1, nitric oxide synthase and adrenergic receptors. *In Vivo* **23**: 797-812.

M Gnechhi, Z Zhang, A Ni and VJ Dzau. (2008) Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* **103**: 1204-19.

CM Gorman, GT Merlino, MC Willingham, I Pastan and BH Howard. (1982) The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc Natl Acad Sci U S A* **79**: 6777-81.

D Gospodarowicz. (1974) Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature* **249**: 123-7.

D Gospodarowicz. (1976) Humoral control of cell proliferation: the role of fibroblast growth factor in regeneration, angiogenesis, wound healing, and neoplastic growth. *Prog Clin Biol Res* **9**: 1-19.

D Gospodarowicz, H Bialecki and G Greenburg. (1978a) Purification of the fibroblast growth factor activity from bovine brain. *J Biol Chem* **253**: 3736-43.

D Gospodarowicz and J Cheng. (1986) Heparin protects basic and acidic FGF from inactivation. *J Cell Physiol* **128**: 475-84.

D Gospodarowicz, J Cheng, GM Lui, A Baird and P Bohlent. (1984) Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc Natl Acad Sci U S A* **81**: 6963-7.

D Gospodarowicz, N Ferrara, L Schweigerer and G Neufeld. (1987) Structural characterization and biological functions of fibroblast growth factor. *Endocr Rev* **8**: 95-114.

D Gospodarowicz, S Massoglia, J Cheng, GM Lui and P Bohlen. (1985) Isolation of pituitary fibroblast growth factor by fast protein liquid chromatography (FPLC): partial chemical and biological characterization. *J Cell Physiol* **122**: 323-32.

D Gospodarowicz, AL Mescher and CR Birdwell. (1978b) Control of cellular proliferation by the fibroblast and epidermal growth factors. *Natl Cancer Inst Monogr* **109**: 30.

DG Hackam, NA Khan, BR Hemmelgarn, SW Rabkin, RM Touyz, NR Campbell, R Padwal, TS Campbell, MP Lindsay, MD Hill, RR Quinn, JL Mahon, RJ Herman, EL Schiffrin, M Ruzicka, P Larochelle, RD Feldman, M Lebel, L Poirier, JM Arnold, GW Moe, JG Howlett, L Trudeau, SL Bacon, RJ Petrella, A Milot, JA Stone, D Drouin, JM Boulanger, M Sharma, P Hamet, G Fodor, GK Dresser, SG Carruthers, G Pylypchuk, ED Burgess, KD Burns, M Vallee, GV Prasad, RE Gilbert, LA Leiter, C Jones, RI Ogilvie, V Woo, PA McFarlane, RA Hegele and SW Tobe. (2010) The 2010 Canadian Hypertension Education Program recommendations for the management of hypertension: part 2 - therapy. *Can J Cardiol* **26**: 249-58.

SL Hale and RA Kloner. (1994) Protection of myocardium by transient, preischemic administration of phenylephrine in the rabbit. *Coron Artery Dis* **5**: 605-10.

AP Halestrap, CP Connern, EJ Griffiths and PM Kerr. (1997) Cyclosporin A binding to mitochondrial cyclophilin inhibits the permeability transition pore and protects hearts from ischaemia/reperfusion injury. *Mol Cell Biochem* **174**: 167-72.

H Hammerman, RA Kloner, S Hale, FJ Schoen and E Braunwald. (1983) Dose-dependent effects of short-term methylprednisolone on myocardial infarct extent, scar formation, and ventricular function. *Circulation* **68**: 446-52.

K Harada, W Grossman, M Friedman, ER Edelman, PV Prasad, CS Keighley, WJ Manning, FW Sellke and M Simons. (1994) Basic fibroblast growth factor improves myocardial function in chronically ischemic porcine hearts. *J Clin Invest* **94**: 623-30.

DJ Hausenloy, A Tsang, MM Mocanu and DM Yellon. (2005) Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. *Am J Physiol Heart Circ Physiol* **288**: H971-6.

HK Hawkins, ML Entman, JY Zhu, KA Youker, K Berens, M Dore and CW Smith. (1996) Acute inflammatory reaction after myocardial ischemic injury and reperfusion. Development and use of a neutrophil-specific antibody. *Am J Pathol* **148**: 1957-69.

S Hein, S Kostin, A Heling, Y Maeno and J Schaper. (2000) The role of the cytoskeleton in heart failure. *Cardiovasc Res* **45**: 273-8.

S Heymans, A Lutun, D Nuyens, G Theilmeier, E Creemers, L Moons, GD Dyspersin, JP Cleutjens, M Shipley, A Angellilo, M Levi, O Nube, A Baker, E Keshet, F Lupu, JM Herbert, JF Smits, SD Shapiro, M Baes, M Borgers, D Collen, MJ Daemen and P Carmeliet. (1999) Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med* **5**: 1135-42.

R Hirsch, JA Bluestone, L DeNenno and RE Gress. (1990) Anti-CD3 F(ab')₂ fragments are immunosuppressive in vivo without evoking either the strong humoral response or morbidity associated with whole mAb. *Transplantation* **49**: 1117-23.

MR Hoffmeyer, R Scalia, CR Ross, SP Jones and DJ Lefer. (2000) PR-39, a potent neutrophil inhibitor, attenuates myocardial ischemia-reperfusion injury in mice. *Am J Physiol Heart Circ Physiol* **279**: H2824-8.

JE Hollander and RS Hoffman. (1992) Cocaine-induced myocardial infarction: an analysis and review of the literature. *J Emerg Med* **10**: 169-77.

MC Horrigan, AI MacIsaac, FA Nicolini, DG Vince, P Lee, SG Ellis and EJ Topol. (1996) Reduction in myocardial infarct size by basic fibroblast growth factor after temporary coronary occlusion in a canine model. *Circulation* **94**: 1927-33.

SL House, C Bolte, M Zhou, T Doetschman, R Klevitsky, G Newman and J Schultz Jel. (2003) Cardiac-specific overexpression of fibroblast growth factor-2 protects against myocardial dysfunction and infarction in a murine model of low-flow ischemia. *Circulation* **108**: 3140-8.

SL House, K Branch, G Newman, T Doetschman and J Schultz Jel. (2005) Cardioprotection induced by cardiac-specific overexpression of fibroblast growth factor-2 is mediated by the MAPK cascade. *Am J Physiol Heart Circ Physiol* **289**: H2167-75.

P Htun, WD Ito, IE Hofer, J Schaper and W Schaper. (1998) Intramyocardial infusion of FGF-1 mimics ischemic preconditioning in pig myocardium. *J Mol Cell Cardiol* **30**: 867-77.

SE Hughes. (1997) Differential expression of the fibroblast growth factor receptor (FGFR) multigene family in normal human adult tissues. *J Histochem Cytochem* **45**: 1005-19.

R Huhn, A Heinen, MW Hollmann, W Schlack, B Preckel and NC Weber. (2009) Cyclosporine A administered during reperfusion fails to restore cardioprotection in prediabetic Zucker obese rats in vivo. *Nutr Metab Cardiovasc Dis*

MM Hurley, Y Okada, L Xiao, Y Tanaka, M Ito, N Okimoto, T Nakamura, CJ Rosen, T Doetschman and JD Coffin. (2006) Impaired bone anabolic response to parathyroid hormone in Fgf2^{-/-} and Fgf2^{+/-} mice. *Biochem Biophys Res Commun* **341**: 989-94.

O Husser, V Bodi, J Sanchis, J Nunez, L Mainar, FJ Chorro, MP Lopez-Lereu, JV Monmeneu, F Chaustre, MJ Forteza, I Trapero, F Dasi, I Benet, GA Riegger and A Llacer. (2010) White Blood Cell Subtypes after STEMI: Temporal Evolution, Association with Cardiovascular Magnetic Resonance-Derived Infarct Size and Impact on Outcome. *Inflammation*

- J Huzen, RA de Boer, DJ van Veldhuisen, WH van Gilst and P van der Harst. (2010) The emerging role of telomere biology in cardiovascular disease. *Front Biosci* **15**: 35-45.
- LJ Ignarro, ML Balestrieri and C Napoli. (2007) Nutrition, physical activity, and cardiovascular disease: an update. *Cardiovasc Res* **73**: 326-40.
- Y Ikeda, LH Young, R Scalia, CR Ross and AM Lefer. (2001) PR-39, a proline/arginine-rich antimicrobial peptide, exerts cardioprotective effects in myocardial ischemia-reperfusion. *Cardiovasc Res* **49**: 69-77.
- W Insull, Jr. (2009) The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. *Am J Med* **122**: S3-S14.
- Y Ishibashi, Y Urabe, H Tsutsui, S Kinugawa, M Sugimachi, M Takahashi, S Yamamoto, H Tagawa, K Sunagawa and A Takeshita. (1997) Negative inotropic effect of basic fibroblast growth factor on adult rat cardiac myocyte. *Circulation* **96**: 2501-4.
- W Ishida, Y Mori, G Lakos, L Sun, F Shan, S Bowes, S Josiah, WC Lee, J Singh, LE Ling and J Varga. (2006) Intracellular TGF-beta receptor blockade abrogates Smad-dependent fibroblast activation in vitro and in vivo. *J Invest Dermatol* **126**: 1733-44.
- E Iwai-Kanai, K Hasegawa, M Fujita, M Araki, T Yanazume, S Adachi and S Sasayama. (2002) Basic fibroblast growth factor protects cardiac myocytes from iNOS-mediated apoptosis. *J Cell Physiol* **190**: 54-62.
- Y Iwata, Y Katanosaka, Y Arai, K Komamura, K Miyatake and M Shigekawa. (2003) A novel mechanism of myocyte degeneration involving the Ca²⁺-permeable growth factor-regulated channel. *J Cell Biol* **161**: 957-67.
- DS Jassal, SY Han, C Hans, A Sharma, T Fang, R Ahmadie, M Lytwyn, JR Walker, RS Bhalla, A Czarnecki, T Moussa and PK Singal. (2009) Utility of tissue Doppler and strain rate imaging in the early detection of trastuzumab and anthracycline mediated cardiomyopathy. *J Am Soc Echocardiogr* **22**: 418-24.
- S Javadov, M Karmazyn and N Escobales. (2009) Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. *J Pharmacol Exp Ther* **330**: 670-8.
- DP Jenkins, SE Steare and DM Yellon. (1995) Preconditioning the human myocardium: recent advances and aspirations for the development of a new means of cardioprotection in clinical practice. *Cardiovasc Drugs Ther* **9**: 739-47.
- RB Jennings, HM Sommers, GA Smyth, HA Flack and H Linn. (1960) Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch Pathol* **70**: 68-78.

ZS Jiang, M Jeyaraman, GB Wen, RR Fandrich, IM Dixon, PA Cattini and E Kardami. (2007) High- but not low-molecular weight FGF-2 causes cardiac hypertrophy in vivo; possible involvement of cardiotrophin-1. *J Mol Cell Cardiol* **42**: 222-33.

ZS Jiang, RR Padua, H Ju, BW Doble, Y Jin, J Hao, PA Cattini, IM Dixon and E Kardami. (2002) Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C. *Am J Physiol Heart Circ Physiol* **282**: H1071-80.

ZS Jiang, W Srisakuldee, F Soulet, G Bouche and E Kardami. (2004) Non-angiogenic FGF-2 protects the ischemic heart from injury, in the presence or absence of reperfusion. *Cardiovasc Res* **62**: 154-66.

ZS Jiang, GB Wen, ZH Tang, W Srisakuldee, RR Fandrich and E Kardami. (2009) High molecular weight FGF-2 promotes postconditioning-like cardioprotection linked to activation of the protein kinase C isoforms Akt and p70 S6 kinase. *Can J Physiol Pharmacol* **87**: 798-804.

SK Jimenez. (2003) Autoregulation of FGF-2 in cardiac myocytes: Effect of FGF-2 and beta-adrenergic stimulation on FGF-2 promoter activity in transfected neonatal rat cardiac myocyte cultures and transgenic mice. Winnipeg: University of Manitoba.

SK Jimenez, F Sheikh, Y Jin, KA Detillieux, J Dhaliwal, E Kardami and PA Cattini. (2004) Transcriptional regulation of FGF-2 gene expression in cardiac myocytes. *Cardiovasc Res* **62**: 548-57.

Y Jin, F Sheikh, KA Detillieux and PA Cattini. (2000) Role for early growth response-1 protein in alpha(1)-adrenergic stimulation of fibroblast growth factor-2 promoter activity in cardiac myocytes. *Mol Pharmacol* **57**: 984-90.

DE Johnson and LT Williams. (1993) Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* **60**: 1-41.

MA Jonas, JA Oates, JK Ockene and CH Hennekens. (1992) Statement on smoking and cardiovascular disease for health care professionals. American Heart Association. *Circulation* **86**: 1664-9.

BI Jugdutt. (2003) Remodeling of the myocardium and potential targets in the collagen degradation and synthesis pathways. *Curr Drug Targets Cardiovasc Haematol Disord* **3**: 1-30.

J Jungnickel, K Haastert, M Grzybek, N Thau, E Lipokatic-Takacs, A Ratzka, A Nolle, P Claus and C Grothe. (2010) Mice lacking basic fibroblast growth factor showed faster sensory recovery. *Exp Neurol* **223**: 166-72.

BD Kahan. (2009) Forty years of publication of transplantation proceedings--the second decade: the cyclosporine revolution. *Transplant Proc* **41**: 1423-37.

DS Kahn, G Rona and CI Chappel. (1969) Isoproterenol-induced cardiac necrosis. *Ann N Y Acad Sci* **156**: 285-93.

J Kajstura, M Rota, B Whang, S Cascapera, T Hosoda, C Bearzi, D Nurzynska, H Kasahara, E Zias, M Bonafe, B Nadal-Ginard, D Torella, A Nascimbene, F Quaini, K Urbanek, A Leri and P Anversa. (2005) Bone marrow cells differentiate in cardiac cell lineages after infarction independently of cell fusion. *Circ Res* **96**: 127-37.

M Kang and JW Walker. (2005) Protein kinase C delta and epsilon mediate positive inotropy in adult ventricular myocytes. *J Mol Cell Cardiol* **38**: 753-64.

Y Kanzaki, F Terasaki, M Okabe, T Hayashi, H Toko, H Shimomura, S Fujioka, Y Kitaura, K Kawamura, Y Horii, T Isomura and H Suma. (2001) Myocardial inflammatory cell infiltrates in cases of dilated cardiomyopathy as a determinant of outcome following partial left ventriculectomy. *Jpn Circ J* **65**: 797-802.

ML Kaplan, Y Cheslow, K Vikstrom, A Malhotra, DL Geenen, A Nakouzi, LA Leinwand and PM Buttrick. (1994) Cardiac adaptations to chronic exercise in mice. *Am J Physiol* **267**: H1167-73.

E Kardami. (1990) Stimulation and inhibition of cardiac myocyte proliferation in vitro. *Mol Cell Biochem* **92**: 129-35.

E Kardami, K Detillieux, X Ma, Z Jiang, JJ Santiago, SK Jimenez and PA Cattini. (2007) Fibroblast growth factor-2 and cardioprotection. *Heart Fail Rev* **12**: 267-77.

E Kardami, ZS Jiang, SK Jimenez, CJ Hirst, F Sheikh, P Zahradka and PA Cattini. (2004) Fibroblast growth factor 2 isoforms and cardiac hypertrophy. *Cardiovasc Res* **63**: 458-66.

E Kardami, L Liu and BW Doble. (1991) Basic fibroblast growth factor in cultured cardiac myocytes. *Ann N Y Acad Sci* **638**: 244-55.

E Kardami, L Liu, SK Pasumarthi, BW Doble and PA Cattini. (1995) Regulation of basic fibroblast growth factor (bFGF) and FGF receptors in the heart. *Ann N Y Acad Sci* **752**: 353-69.

E Kardami, KBS Pasumarthi, L Liu, BW Doble, SE Davey and PA Cattini. (1993) Basic fibroblast growth factor in cardiac myocytes: expression and effects. In: *Growth factors and the cardiovascular system*. P Cummins (Ed.). Norwell: Kluwer Academic Publishers.

T Kawada, F Masui, A Tezuka, T Ebisawa, H Kumagai, M Nakazawa and T Toyo-Oka. (2005) A novel scheme of dystrophin disruption for the progression of advanced heart failure. *Biochim Biophys Acta* **1751**: 73-81.

D Kaye, D Pimental, S Prasad, T Maki, HJ Berger, PL McNeil, TW Smith and RA Kelly. (1996) Role of transiently altered sarcolemmal membrane permeability and basic fibroblast growth factor release in the hypertrophic response of adult rat ventricular myocytes to increased mechanical activity in vitro. *J Clin Invest* **97**: 281-91.

LM Khachigian. (2006) Early growth response-1 in cardiovascular pathobiology. *Circ Res* **98**: 186-91.

RK Kharbanda, TT Nielsen and AN Redington. (2009) Translation of remote ischaemic preconditioning into clinical practice. *Lancet* **374**: 1557-65.

AR Kherani, GW Moss, H Zhou, A Gu, G Zhang, AR Schulman, JM Fal, R Sorabella, T Plasse, L Rui, S Homma, D Burkhoff, MC Oz and J Wang. (2004) Macrophage inhibitor, semapimod, reduces tumor necrosis factor-alpha in myocardium in a rat model of ischemic heart failure. *J Cardiovasc Pharmacol* **44**: 665-71.

A Kiani, I Habermann, M Haase, S Feldmann, S Boxberger, MA Sanchez-Fernandez, C Thiede, M Bornhauser and G Ehninger. (2004) Expression and regulation of NFAT (nuclear factors of activated T cells) in human CD34+ cells: down-regulation upon myeloid differentiation. *J Leukoc Biol* **76**: 1057-65.

I Kim, S Moon, K Yu, U Kim and GY Koh. (2001) A novel fibroblast growth factor receptor-5 preferentially expressed in the pancreas(1). *Biochim Biophys Acta* **1518**: 152-6.

I Kiprianova, K Schindowski, O von Bohlen und Halbach, S Krause, R Dono, M Schwaninger and K Unsicker. (2004) Enlarged infarct volume and loss of BDNF mRNA induction following brain ischemia in mice lacking FGF-2. *Exp Neurol* **189**: 252-60.

RE Klabunde. (2005). Cardiovascular Physiology Concepts. Lippincott Williams & Wilkins.

M Klagsbrun, S Smith, R Sullivan, Y Shing, S Davidson, JA Smith and J Sasse. (1987) Multiple forms of basic fibroblast growth factor: amino-terminal cleavages by tumor cell- and brain cell-derived acid proteinases. *Proc Natl Acad Sci U S A* **84**: 1839-43.

AL Klatsky. (2009) Alcohol and cardiovascular diseases. *Expert Rev Cardiovasc Ther* **7**: 499-506.

TE Klein, JT Chang, MK Cho, KL Easton, R Ferguson, M Hewett, Z Lin, Y Liu, S Liu, DE Oliver, DL Rubin, F Shafa, JM Stuart and RB Altman. (2001) Integrating genotype and phenotype information: an overview of the PharmGKB project. Pharmacogenetics Research Network and Knowledge Base. *Pharmacogenomics J* **1**: 167-70.

P Klint and L Claesson-Welsh. (1999) Signal transduction by fibroblast growth factor receptors. *Front Biosci* **4**: D165-77.

RA Kloner. (2006) Natural and unnatural triggers of myocardial infarction. *Prog Cardiovasc Dis* **48**: 285-300.

MV Kolk, D Meyberg, T Deuse, KR Tang-Quan, RC Robbins, H Reichenspurner and S Schrepfer. (2009) LAD-ligation: a murine model of myocardial infarction. *J Vis Exp*

RJ Laham, NA Chronos, M Pike, ME Leimbach, JE Udelson, JD Pearlman, RI Pettigrew, MJ Whitehouse, C Yoshizawa and M Simons. (2000a) Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study. *J Am Coll Cardiol* **36**: 2132-9.

RJ Laham, M Rezaee, M Post, D Novicki, FW Sellke, JD Pearlman, M Simons and D Hung. (2000b) Intrapericardial delivery of fibroblast growth factor-2 induces neovascularization in a porcine model of chronic myocardial ischemia. *J Pharmacol Exp Ther* **292**: 795-802.

RJ Laham, FW Sellke, ER Edelman, JD Pearlman, JA Ware, DL Brown, JP Gold and M Simons. (1999) Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. *Circulation* **100**: 1865-71.

DF Lazarous, EF Unger, SE Epstein, A Stine, JL Arevalo, EY Chew and AA Quyyumi. (2000) Basic fibroblast growth factor in patients with intermittent claudication: results of a phase I trial. *J Am Coll Cardiol* **36**: 1239-44.

RD Learish, MD Bruss and M Haak-Frendscho. (2000) Inhibition of mitogen-activated protein kinase blocks proliferation of neural progenitor cells. *Brain Res Dev Brain Res* **122**: 97-109.

RJ Lederman, FO Mendelsohn, RD Anderson, JF Saucedo, AN Tenaglia, JB Hermiller, WB Hillegass, K Rocha-Singh, TE Moon, MJ Whitehouse and BH Annex. (2002) Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. *Lancet* **359**: 2053-8.

YM LeGal and LL Morrissey. (1990) Methylprednisolone interventions in myocardial infarction: a controversial subject. *Can J Cardiol* **6**: 405-10.

D Lehr. (1972) Healing of myocardial necrosis caused by sympathomimetic amines. *Recent Adv Stud Cardiac Struct Metab* **1**: 526-50.

M Lenz, T Richter and I Muhlhauser. (2009) The morbidity and mortality associated with overweight and obesity in adulthood: a systematic review. *Dtsch Arztebl Int* **106**: 641-8.

F Li, C Fan, T Cheng, C Jiang and B Zeng. (2009) Efficient inhibition of fibroblast proliferation and collagen expression by ERK2 siRNAs. *Biochem Biophys Res Commun* **382**: 259-63.

S Liao, J Bodmer, D Pietras, M Azhar, T Doetschman and J Schultz Jel. (2009) Biological functions of the low and high molecular weight protein isoforms of fibroblast growth factor-2 in cardiovascular development and disease. *Dev Dyn* **238**: 249-64.

S Liao, JR Bodmer, M Azhar, G Newman, JD Coffin, T Doetschman and J Schultz Jel. (2010) The influence of FGF2 high molecular weight (HMW) isoforms in the development of cardiac ischemia-reperfusion injury. *J Mol Cell Cardiol* **48**: 1245-54.

S Liao, D Porter, A Scott, G Newman, T Doetschman and J Schultz Jel. (2007) The cardioprotective effect of the low molecular weight isoform of fibroblast growth factor-2: the role of JNK signaling. *J Mol Cell Cardiol* **42**: 106-20.

P Libby, PR Maroko, CM Bloor, BE Sobel and E Braunwald. (1973) Reduction of experimental myocardial infarct size by corticosteroid administration. *J Clin Invest* **52**: 599-607.

AH Lichtenstein, LJ Appel, M Brands, M Carnethon, S Daniels, HA Franch, B Franklin, P Kris-Etherton, WS Harris, B Howard, N Karanja, M Lefevre, L Rudel, F Sacks, L Van Horn, M Winston and J Wylie-Rosett. (2006) Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. *Circulation* **114**: 82-96.

RH Lie, N Stoettrup, E Sloth, JM Hasenkam, R Kroyer and TT Nielsen. (2010) Post-conditioning with cyclosporine A fails to reduce the infarct size in an in vivo porcine model. *Acta Anaesthesiol Scand* **54**: 804-13.

P Lijnen, V Petrov, K Rumilla and R Fagard. (2003) Transforming growth factor-beta 1 promotes contraction of collagen gel by cardiac fibroblasts through their differentiation into myofibroblasts. *Methods Find Exp Clin Pharmacol* **25**: 79-86.

V Lindner and MA Reidy. (1991) Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc Natl Acad Sci U S A* **88**: 3739-43.

D Linfert, T Chowdhry and H Rabb. (2009) Lymphocytes and ischemia-reperfusion injury. *Transplant Rev (Orlando)* **23**: 1-10.

MR Litt, RW Jeremy, HF Weisman, JA Winkelstein and LC Becker. (1989) Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. *Circulation* **80**: 1816-27.

L Liu, KB Pasumarthi, RR Padua, H Massaeli, RR Fandrich, GN Pierce, PA Cattini and E Kardami. (1995) Adult cardiomyocytes express functional high-affinity receptors for basic fibroblast growth factor. *Am J Physiol* **268**: H1927-38.

X Liu, RM Engelman, Moraru, II, JA Rousou, JE Flack, 3rd, DW Deaton, N Maulik and DK Das. (1992) Heat shock. A new approach for myocardial preservation in cardiac surgery. *Circulation* **86**: II358-63.

X Liu, X Wu, L Cai and S Sun. (2008) Calreticulin downregulation is associated with FGF-2-induced angiogenesis through calcineurin pathway in ischemic myocardium. *Shock* **29**: 140-8.

X Liu, W Xie, P Liu, M Duan, Z Jia, W Li and J Xu. (2006) Mechanism of the cardioprotection of rhEPO pretreatment on suppressing the inflammatory response in ischemia-reperfusion. *Life Sci* **78**: 2255-64.

Y Liu and JM Downey. (1992) Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol* **263**: H1107-12.

D Lloyd-Jones, R Adams, M Carnethon, G De Simone, TB Ferguson, K Flegal, E Ford, K Furie, A Go, K Greenlund, N Haase, S Hailpern, M Ho, V Howard, B Kissela, S Kittner, D Lackland, L Lisabeth, A Marelli, M McDermott, J Meigs, D Mozaffarian, G Nichol, C O'Donnell, V Roger, W Rosamond, R Sacco, P Sorlie, R Stafford, J Steinberger, T Thom, S Wasserthiel-Smoller, N Wong, J Wylie-Rosett and Y Hong. (2009) Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **119**: e21-181.

MJ Loza, SP Peters, S Foster, IU Khan and RB Penn. (2007) beta-Agonist enhances type 2 T-cell survival and accumulation. *J Allergy Clin Immunol* **119**: 235-44.

DK Ma, K Ponnusamy, MR Song, GL Ming and H Song. (2009) Molecular genetic analysis of FGFR1 signalling reveals distinct roles of MAPK and PLCgamma1 activation for self-renewal of adult neural stem cells. *Mol Brain* **2**: 16.

DJ Mahoney, K Carey, MH Fu, R Snow, D Cameron-Smith, G Parise and MA Tarnopolsky. (2004) Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* **18**: 226-31.

JA Mannisi, HF Weisman, DE Bush, P Dudeck and B Healy. (1987) Steroid administration after myocardial infarction promotes early infarct expansion. A study in the rat. *J Clin Invest* **79**: 1431-9.

D Maric, A Fiorio Pla, YH Chang and JL Barker. (2007) Self-renewing and differentiating properties of cortical neural stem cells are selectively regulated by basic

fibroblast growth factor (FGF) signaling via specific FGF receptors. *J Neurosci* **27**: 1836-52.

M Marini, E Falcieri, V Margonato, D Trere, R Lapalombella, S di Tullio, C Marchionni, S Burattini, M Samaja, F Esposito and A Veicsteinas. (2008) Partial persistence of exercise-induced myocardial angiogenesis following 4-week detraining in the rat. *Histochem Cell Biol* **129**: 479-87.

CW Martin and IF Muir. (1990) The role of lymphocytes in wound healing. *Br J Plast Surg* **43**: 655-62.

P Massoudy, S Zahler, C Kupatt, E Reder, BF Becker and E Gerlach. (1997) Cardioprotection by cyclosporine A in experimental ischemia and reperfusion--evidence for a nitric oxide-dependent mechanism mediated by endothelin. *J Mol Cell Cardiol* **29**: 535-44.

P Mathieu, I Lemieux and JP Despres. (2010) Obesity, inflammation, and cardiovascular risk. *Clin Pharmacol Ther* **87**: 407-16.

S Matsuda and S Koyasu. (2000) Mechanisms of action of cyclosporine. *Immunopharmacology* **47**: 119-25.

DL Matthey, PT Dawes, NB Nixon and H Slater. (1997) Transforming growth factor beta 1 and interleukin 4 induced alpha smooth muscle actin expression and myofibroblast-like differentiation in human synovial fibroblasts in vitro: modulation by basic fibroblast growth factor. *Ann Rheum Dis* **56**: 426-31.

PG McCaffrey, C Luo, TK Kerppola, J Jain, TM Badalian, AM Ho, E Burgeon, WS Lane, JN Lambert, T Curran and et al. (1993) Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**: 750-4.

EM McNally. (2007) New approaches in the therapy of cardiomyopathy in muscular dystrophy. *Annu Rev Med* **58**: 75-88.

PL McNeil, L Muthukrishnan, E Warder and PA D'Amore. (1989) Growth factors are released by mechanically wounded endothelial cells. *J Cell Biol* **109**: 811-22.

A Mehta. (2010) Management of cardiovascular risk associated with insulin resistance, diabetes, and the metabolic syndrome. *Postgrad Med* **122**: 61-70.

JT Meij, F Sheikh, SK Jimenez, PW Nickerson, E Kardami and PA Cattini. (2002) Exacerbation of myocardial injury in transgenic mice overexpressing FGF-2 is T cell dependent. *Am J Physiol Heart Circ Physiol* **282**: H547-55.

B Metzler, J Mair, A Lercher, C Schaber, F Hintringer, O Pachinger and Q Xu. (2001) Mouse model of myocardial remodelling after ischemia: role of intercellular adhesion molecule-1. *Cardiovasc Res* **49**: 399-407.

T Meyer-Ter-Vehn, S Gebhardt, W Sebald, M Buttmann, F Grehn, G Schlunck and P Knaus. (2006) p38 inhibitors prevent TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci* **47**: 1500-9.

T Meyer-ter-Vehn, S Sieprath, B Katzenberger, S Gebhardt, F Grehn and G Schlunck. (2006) Contractility as a prerequisite for TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci* **47**: 4895-904.

J Milanini-Mongiat, J Pouyssegur and G Pages. (2002) Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription. *J Biol Chem* **277**: 20631-9.

DL Miller, S Ortega, O Bashayan, R Basch and C Basilico. (2000) Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol Cell Biol* **20**: 2260-8.

PJ Mills, RS Karnik and E Dillon. (1997) L-selectin expression affects T-cell circulation following isoproterenol infusion in humans. *Brain Behav Immun* **11**: 333-42.

MF Minicucci, PS Azevedo, SA Paiva and LA Zornoff. (2009) Cardiovascular remodeling induced by passive smoking. *Inflamm Allergy Drug Targets* **8**: 334-9.

M Miyataka, K Ishikawa and R Katori. (1998) Basic fibroblast growth factor increased regional myocardial blood flow and limited infarct size of acutely infarcted myocardium in dogs. *Angiology* **49**: 381-90.

M Mohammadi, SK Olsen and OA Ibrahimi. (2005) Structural basis for fibroblast growth factor receptor activation. *Cytokine Growth Factor Rev* **16**: 107-37.

FJ Moy, AP Seddon, P Bohlen and R Powers. (1996) High-resolution solution structure of basic fibroblast growth factor determined by multidimensional heteronuclear magnetic resonance spectroscopy. *Biochemistry* **35**: 13552-61.

CE Murry, RB Jennings and KA Reimer. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**: 1124-36.

CE Murry, MH Soonpaa, H Reinecke, H Nakajima, HO Nakajima, M Rubart, KB Pasumarthi, JI Virag, SH Bartelmez, V Poppa, G Bradford, JD Dowell, DA Williams and LJ Field. (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* **428**: 664-8.

SF Nagueh, LL Bachinski, D Meyer, R Hill, WA Zoghbi, JW Tam, MA Quinones, R Roberts and AJ Marian. (2001) Tissue Doppler imaging consistently detects myocardial abnormalities in patients with hypertrophic cardiomyopathy and provides a novel means for an early diagnosis before and independently of hypertrophy. *Circulation* **104**: 128-30.

SF Nagueh, J McFalls, D Meyer, R Hill, WA Zoghbi, JW Tam, MA Quinones, R Roberts and AJ Marian. (2003) Tissue Doppler imaging predicts the development of hypertrophic cardiomyopathy in subjects with subclinical disease. *Circulation* **108**: 395-8.

Y Nakagawa, H Ito, M Kitakaze, H Kusuoka, M Hori, T Kuzuya, Y Higashino, K Fujii and T Minamino. (1995) Effect of angina pectoris on myocardial protection in patients with reperfused anterior wall myocardial infarction: retrospective clinical evidence of "preconditioning". *J Am Coll Cardiol* **25**: 1076-83.

K Narine, O DeWever, K Cathenis, M Mareel, Y Van Belleghem and G Van Nooten. (2004) Transforming growth factor-beta-induced transition of fibroblasts: a model for myofibroblast procurement in tissue valve engineering. *J Heart Valve Dis* **13**: 281-9; discussion 289.

W Nazareth, N Yafei and M Crompton. (1991) Inhibition of anoxia-induced injury in heart myocytes by cyclosporin A. *J Mol Cell Cardiol* **23**: 1351-4.

TG Neilan, DS Jassal, TM Perez-Sanz, MJ Raher, AD Pradhan, ES Buys, F Ichinose, DB Bayne, EF Halpern, AE Weyman, G Derumeaux, KD Bloch, MH Picard and M Scherrer-Crosbie. (2006) Tissue Doppler imaging predicts left ventricular dysfunction and mortality in a murine model of cardiac injury. *Eur Heart J* **27**: 1868-75.

S Nishida, H Nagamine, Y Tanaka and G Watanabe. (2003) Protective effect of basic fibroblast growth factor against myocyte death and arrhythmias in acute myocardial infarction in rats. *Circ J* **67**: 334-9.

T Noda, S Minatoguchi, K Fujii, M Hori, T Ito, K Kanmatsuse, M Matsuzaki, T Miura, H Nonogi, M Tada, M Tanaka and H Fujiwara. (1999) Evidence for the delayed effect in human ischemic preconditioning: prospective multicenter study for preconditioning in acute myocardial infarction. *J Am Coll Cardiol* **34**: 1966-74.

T Noguchi, Z Chen, SP Bell, L Nyland and MM LeWinter. (2001) Activation of PKC decreases myocardial O₂ consumption and increases contractile efficiency in rats. *Am J Physiol Heart Circ Physiol* **281**: H2191-7.

SA Novgorodov, TI Gudiz, YM Milgrom and GP Brierley. (1992) The permeability transition in heart mitochondria is regulated synergistically by ADP and cyclosporin A. *J Biol Chem* **267**: 16274-82.

H Oh, X Chi, SB Bradfute, Y Mishina, J Pocius, LH Michael, RR Behringer, RJ Schwartz, ML Entman and MD Schneider. (2004) Cardiac muscle plasticity in adult and embryo by heart-derived progenitor cells. *Ann N Y Acad Sci* **1015**: 182-9.

Y Okada, A Montero, X Zhang, T Sobue, J Lorenzo, T Doetschman, JD Coffin and MM Hurley. (2003) Impaired osteoclast formation in bone marrow cultures of Fgf2 null mice in response to parathyroid hormone. *J Biol Chem* **278**: 21258-66.

IM Olfert, EC Breen, O Mathieu-Costello and PD Wagner. (2001) Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. *J Appl Physiol* **91**: 1176-84.

SM Opal and VA DePalo. (2000) Anti-inflammatory cytokines. *Chest* **117**: 1162-72.

LH Opie. (2004). Heart physiology: from cell to circulation. Philadelphia: Lippincott Williams & Wilkins.

OG Opitz and AK Rustgi. (2000) Interaction between Sp1 and cell cycle regulatory proteins is important in transactivation of a differentiation-related gene. *Cancer Res* **60**: 2825-30.

D Orlic, J Kajstura, S Chimenti, DM Bodine, A Leri and P Anversa. (2001a) Transplanted adult bone marrow cells repair myocardial infarcts in mice. *Ann N Y Acad Sci* **938**: 221-9; discussion 229-30.

D Orlic, J Kajstura, S Chimenti, I Jakoniuk, SM Anderson, B Li, J Pickel, R McKay, B Nadal-Ginard, DM Bodine, A Leri and P Anversa. (2001b) Bone marrow cells regenerate infarcted myocardium. *Nature* **410**: 701-5.

D Orlic, J Kajstura, S Chimenti, F Limana, I Jakoniuk, F Quaini, B Nadal-Ginard, DM Bodine, A Leri and P Anversa. (2001c) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A* **98**: 10344-9.

DM Ornitz, J Xu, JS Colvin, DG McEwen, CA MacArthur, F Coulier, G Gao and M Goldfarb. (1996) Receptor specificity of the fibroblast growth factor family. *J Biol Chem* **271**: 15292-7.

S Ortega, M Ittmann, SH Tsang, M Ehrlich and C Basilico. (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci U S A* **95**: 5672-7.

RR Padua and E Kardami. (1993) Increased basic fibroblast growth factor (bFGF) accumulation and distinct patterns of localization in isoproterenol-induced cardiomyocyte injury. *Growth Factors* **8**: 291-306.

RR Padua, PL Merle, BW Doble, CH Yu, P Zahradka, GN Pierce, V Panagia and E Kardami. (1998) FGF-2-induced negative inotropism and cardioprotection are inhibited by chelerythrine: involvement of sarcolemmal calcium-independent protein kinase C. *J Mol Cell Cardiol* **30**: 2695-709.

RR Padua, R Sethi, NS Dhalla and E Kardami. (1995) Basic fibroblast growth factor is cardioprotective in ischemia-reperfusion injury. *Mol Cell Biochem* **143**: 129-35.

S Pal, KP Claffey, HT Cohen and D Mukhopadhyay. (1998) Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. *J Biol Chem* **273**: 26277-80.

M Palmen, MJ Daemen, LJ De Windt, J Willems, WR Dassen, S Heeneman, R Zimmermann, M Van Bilsen and PA Doevendans. (2004) Fibroblast growth factor-1 improves cardiac functional recovery and enhances cell survival after ischemia and reperfusion: a fibroblast growth factor receptor, protein kinase C, and tyrosine kinase-dependent mechanism. *J Am Coll Cardiol* **44**: 1113-23.

CE Papadopoulos, HI Karvounis, IT Gourasas, GE Parharidis and GE Louridas. (2003) Evidence of ischemic preconditioning in patients experiencing first non-ST-segment elevation myocardial infarction (NSTEMI). *Int J Cardiol* **92**: 209-17.

KB Pasumarthi, BW Doble, E Kardami and PA Cattini. (1994) Over-expression of CUG- or AUG-initiated forms of basic fibroblast growth factor in cardiac myocytes results in similar effects on mitosis and protein synthesis but distinct nuclear morphologies. *J Mol Cell Cardiol* **26**: 1045-60.

KB Pasumarthi, Y Jin, ME Bock, A Lytras, E Kardami and PA Cattini. (1995) Characterization of fibroblast growth factor receptor 1 RNA expression in the embryonic mouse heart. *Ann N Y Acad Sci* **752**: 406-16.

KB Pasumarthi, Y Jin and PA Cattini. (1997) Cloning of the rat fibroblast growth factor-2 promoter region and its response to mitogenic stimuli in glioma C6 cells. *J Neurochem* **68**: 898-908.

KB Pasumarthi, E Kardami and PA Cattini. (1996) High and low molecular weight fibroblast growth factor-2 increase proliferation of neonatal rat cardiac myocytes but have differential effects on binucleation and nuclear morphology. Evidence for both paracrine and intracrine actions of fibroblast growth factor-2. *Circ Res* **78**: 126-36.

C Pellieux, A Foletti, G Peduto, JF Aubert, J Nussberger, F Beermann, HR Brunner and T Pedrazzini. (2001) Dilated cardiomyopathy and impaired cardiac hypertrophic response to angiotensin II in mice lacking FGF-2. *J Clin Invest* **108**: 1843-51.

SM Penckofer, D Hackbarth and DW Schwartz. (2003) Estrogen plus progestin therapy: the cardiovascular risks exceed the benefits. *J Cardiovasc Nurs* **18**: 347-55.

JM Pfeffer, MA Pfeffer, PJ Fletcher and E Braunwald. (1984) Ventricular performance in rats with myocardial infarction and failure. *Am J Med* **76**: 99-103.

G Pintucci, N Quarto and DB Rifkin. (1996) Methylation of high molecular weight fibroblast growth factor-2 determines post-translational increases in molecular weight and affects its intracellular distribution. *Mol Biol Cell* **7**: 1249-58.

KE Porter and NA Turner. (2009) Cardiac fibroblasts: at the heart of myocardial remodeling. *Pharmacol Ther* **123**: 255-78.

WT Porter. (1893) On the Results of Ligation of the Coronary Arteries. *J Physiol* **15**: 121-248 1.

SK Powers, JC Quindry and AN Kavazis. (2008) Exercise-induced cardioprotection against myocardial ischemia-reperfusion injury. *Free Radic Biol Med* **44**: 193-201.

DS Prasad, Z Kabir, AK Dash and BC Das. (2009) Smoking and cardiovascular health: a review of the epidemiology, pathogenesis, prevention and control of tobacco. *Indian J Med Sci* **63**: 520-33.

M Presta, G Andres, D Leali, P Dell'era and R Ronca. (2009) Inflammatory cells and chemokines sustain FGF2-induced angiogenesis. *Eur Cytokine Netw* **20**: 39-50.

P Proia, G Schiera, M Mineo, AM Ingrassia, G Santoro, G Savettieri and I Di Liegro. (2008) Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor. *Int J Mol Med* **21**: 63-7.

C Prussin and DD Metcalfe. (2006) 5. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* **117**: S450-6.

F Quaini, E Cigola, C Lagrasta, G Saccani, E Quaini, C Rossi, G Olivetti and P Anversa. (1994) End-stage cardiac failure in humans is coupled with the induction of proliferating cell nuclear antigen and nuclear mitotic division in ventricular myocytes. *Circ Res* **75**: 1050-63.

N Quarto, D Talarico, A Sommer, R Florkiewicz, C Basilico and DB Rifkin. (1989) Transformation by basic fibroblast growth factor requires high levels of expression: comparison with transformation by hst/K-fgf. *Oncogene Res* **5**: 101-10.

AM Ramirez, Z Shen, JD Ritzenthaler and J Roman. (2006) Myofibroblast transdifferentiation in obliterative bronchiolitis: tgf-beta signaling through smad3-dependent and -independent pathways. *Am J Transplant* **6**: 2080-8.

AC Rapraeger, A Krufka and BB Olwin. (1991) Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**: 1705-8.

KA Reimer, CE Murry and RB Jennings. (1990) Cardiac adaptation to ischemia. Ischemic preconditioning increases myocardial tolerance to subsequent ischemic episodes. *Circulation* **82**: 2266-8.

TS Riles, FS Fisher, S Schaefer, PF Pasternack and FG Baumann. (1993) Plasma catecholamine concentrations during abdominal aortic aneurysm surgery: the link to perioperative myocardial ischemia. *Ann Vasc Surg* **7**: 213-9.

R Roberts, V DeMello and BE Sobel. (1976) Deleterious effects of methylprednisolone in patients with myocardial infarction. *Circulation* **53**: 1204-6.

AC Rodrigues, R Hataishi, F Ichinose, KD Bloch, G Derumeaux, MH Picard and M Scherrer-Crosbie. (2004) Relationship of systolic dysfunction to area at risk and infarction size after ischemia-reperfusion in mice. *J Am Soc Echocardiogr* **17**: 948-53.

JL Romson, BG Hook, SL Kunkel, GD Abrams, MA Schork and BR Lucchesi. (1983) Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* **67**: 1016-23.

G Rona, CI Chappel, T Balazs and R Gaudry. (1959) An infarct-like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat. *AMA Arch Pathol* **67**: 443-55.

G Rona, DS Kahn and CI Chappel. (1963) Studies on Infarct-Like Myocardial Necrosis Produced by Isoproterenol: a Review. *Rev Can Biol* **22**: 241-55.

N Rosenblatt-Velin, MG Lepore, C Cartoni, F Beermann and T Pedrazzini. (2005) FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. *J Clin Invest* **115**: 1724-33.

J Ross, Jr. (1991) Myocardial perfusion-contraction matching. Implications for coronary heart disease and hibernation. *Circulation* **83**: 1076-83.

J Rossant, B Ciruna and J Partanen. (1997) FGF signaling in mouse gastrulation and anteroposterior patterning. *Cold Spring Harb Symp Quant Biol* **62**: 127-33.

M Rota, J Kajstura, T Hosoda, C Bearzi, S Vitale, G Esposito, G Iaffaldano, ME Padin-Iruegas, A Gonzalez, R Rizzi, N Small, J Muraski, R Alvarez, X Chen, K Urbanek, R Bolli, SR Houser, A Leri, MA Sussman and P Anversa. (2007) Bone marrow cells adopt the cardiomyogenic fate in vivo. *Proc Natl Acad Sci U S A* **104**: 17783-8.

MG Sabbieti, D Agas, L Xiao, L Marchetti, JD Coffin, T Doetschman and MM Hurley. (2009) Endogenous FGF-2 is critically important in PTH anabolic effects on bone. *J Cell Physiol* **219**: 143-51.

M Sahni, DC Ambrosetti, A Mansukhani, R Gertner, D Levy and C Basilico. (1999) FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev* **13**: 1361-6.

O Sanchez, A Arnau, M Pareja, E Poch, I Ramirez and M Soley. (2002) Acute stress-induced tissue injury in mice: differences between emotional and social stress. *Cell Stress Chaperones* **7**: 36-46.

T Schafer, H Zentgraf, C Zehe, B Brugger, J Bernhagen and W Nickel. (2004) Unconventional secretion of fibroblast growth factor 2 is mediated by direct translocation across the plasma membrane of mammalian cells. *J Biol Chem* **279**: 6244-51.

JF Scherrer, T Chrusciel, A Zeringue, LD Garfield, PJ Hauptman, PJ Lustman, KE Freedland, RM Carney, KK Bucholz, R Owen and WR True. (2010) Anxiety disorders increase risk for incident myocardial infarction in depressed and nondepressed Veterans Administration patients. *Am Heart J* **159**: 772-9.

G Schiera, P Proia, C Alberti, M Mineo, G Savettieri and I Di Liegro. (2007) Neurons produce FGF2 and VEGF and secrete them at least in part by shedding extracellular vesicles. *J Cell Mol Med* **11**: 1384-94.

A Schmidt, D Ladage, T Schinkothe, U Klausmann, C Ulrichs, FJ Klinz, K Brixius, S Arnhold, B Desai, U Mehlhorn, RH Schwinger, P Staib, K Addicks and W Bloch. (2006) Basic fibroblast growth factor controls migration in human mesenchymal stem cells. *Stem Cells* **24**: 1750-8.

RJ Schott, S Rohmann, ER Braun and W Schaper. (1990) Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* **66**: 1133-42.

JE Schultz, SA Witt, ML Nieman, PJ Reiser, SJ Engle, M Zhou, SA Pawlowski, JN Lorenz, TR Kimball and T Doetschman. (1999) Fibroblast growth factor-2 mediates pressure-induced hypertrophic response. *J Clin Invest* **104**: 709-19.

L Schweigerer, G Neufeld, J Friedman, JA Abraham, JC Fiddes and D Gospodarowicz. (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature* **325**: 257-9.

A Seida, J Wada, M Kunitomi, Y Tsuchiyama, N Miyatake, M Fujii, S Kira, K Takahashi, K Shikata and H Makino. (2003) Serum bFGF levels are reduced in Japanese overweight men and restored by a 6-month exercise education. *Int J Obes Relat Metab Disord* **27**: 1325-31.

K Sekine, H Ohuchi, M Fujiwara, M Yamasaki, T Yoshizawa, T Sato, N Yagishita, D Matsui, Y Koga, N Itoh and S Kato. (1999) Fgf10 is essential for limb and lung formation. *Nat Genet* **21**: 138-41.

G Shah, FA Middleton, KL Gentile, S Tripathi, D Bruch, KG Maier and DS Kittur. (2008) Cyclosporine inhibition of angiogenesis involves the transcription factor HESR1. *J Surg Res* **149**: 171-6.

RJ Sharpe, KA Arndt, SI Bauer and TE Maione. (1989) Cyclosporine inhibits basic fibroblast growth factor-driven proliferation of human endothelial cells and keratinocytes. *Arch Dermatol* **125**: 1359-62.

F Sheikh, DP Sontag, RR Fandrich, E Kardami and PA Cattini. (2001) Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts. *Am J Physiol Heart Circ Physiol* **280**: H1039-50.

Z Sheng, JA Lewis and WJ Chirico. (2004) Nuclear and nucleolar localization of 18-kDa fibroblast growth factor-2 is controlled by C-terminal signals. *J Biol Chem* **279**: 40153-60.

L Sherwood. (1993). Human physiology: from cells to systems. St. Paul: West Publishing Company.

DL Shi, C Launay, V Fromentoux, JJ Feige and JC Boucaut. (1994) Expression of fibroblast growth factor receptor-2 splice variants is developmentally and tissue-specifically regulated in the amphibian embryo. *Dev Biol* **164**: 173-82.

F Shibata, A Baird and RZ Florkiewicz. (1991) Functional characterization of the human basic fibroblast growth factor gene promoter. *Growth Factors* **4**: 277-87.

YH Shim. (2010) Cardioprotection and ageing. *Korean J Anesthesiol* **58**: 223-30.

S Shimasaki, N Emoto, A Koba, M Mercado, F Shibata, K Cooksey, A Baird and N Ling. (1988) Complementary DNA cloning and sequencing of rat ovarian basic fibroblast growth factor and tissue distribution study of its mRNA. *Biochem Biophys Res Commun* **157**: 256-63.

M Shimizu, M Tropak, RJ Diaz, F Suto, H Surendra, E Kuzmin, J Li, G Gross, GJ Wilson, J Callahan and AN Redington. (2009) Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: evidence suggesting cross-species protection. *Clin Sci (Lond)* **117**: 191-200.

Y Shizukuda, RT Mallet, SC Lee and HF Downey. (1992) Hypoxic preconditioning of ischaemic canine myocardium. *Cardiovasc Res* **26**: 534-42.

M Simons, BH Annex, RJ Laham, N Kleiman, T Henry, H Dauerman, JE Udelson, EV Gervino, M Pike, MJ Whitehouse, T Moon and NA Chronos. (2002) Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. *Circulation* **105**: 788-93.

DM Simpson and R Ross. (1972) The neutrophilic leukocyte in wound repair a study with antineutrophil serum. *J Clin Invest* **51**: 2009-23.

A Skaletz-Rorowski, H Eschert, J Leng, B Stallmeyer, JR Sindermann, E Pulawski and G Breithardt. (2005) PKC delta-induced activation of MAPK pathway is required for bFGF-stimulated proliferation of coronary smooth muscle cells. *Cardiovasc Res* **67**: 142-50.

A Skaletz-Rorowski, J Waltenberger, JG Muller, E Pawlus, K Pinkernell and G Breithardt. (1999) Protein kinase C mediates basic fibroblast growth factor-induced proliferation through mitogen-activated protein kinase in coronary smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19**: 1608-14.

M Sleeman, J Fraser, M McDonald, S Yuan, D White, P Grandison, K Kumble, JD Watson and JG Murison. (2001) Identification of a new fibroblast growth factor receptor, FGFR5. *Gene* **271**: 171-82.

P Somasundaram, G Ren, H Nagar, D Kraemer, L Mendoza, LH Michael, GH Caughey, ML Entman and NG Frangogiannis. (2005) Mast cell tryptase may modulate endothelial cell phenotype in healing myocardial infarcts. *J Pathol* **205**: 102-11.

JA Spath, Jr., DL Lane and AM Lefer. (1974) Protective action of methylprednisolone on the myocardium during experimental myocardial ischemia in the cat. *Circ Res* **35**: 44-51.

E Speir, V Tanner, AM Gonzalez, J Farris, A Baird and W Casscells. (1992) Acidic and basic fibroblast growth factors in adult rat heart myocytes. Localization, regulation in culture, and effects on DNA synthesis. *Circ Res* **71**: 251-9.

S Spisani, E Fabbri, M Muccinelli, A Cariani, L Barbin, F Trotta and L Dovigo. (2001) Inhibition of neutrophil responses by cyclosporin A. An insight into molecular mechanisms. *Rheumatology (Oxford)* **40**: 794-800.

W Srisakuldee, MM Jeyaraman, BE Nickel, S Tanguy, ZS Jiang and E Kardami. (2009a) Phosphorylation of connexin-43 at serine 262 promotes a cardiac injury-resistant state. *Cardiovasc Res* **83**: 672-81.

W Srisakuldee, BE Nickel, RR Fandrich, ZS Jiang and E Kardami. (2006) Administration of FGF-2 to the heart stimulates connexin-43 phosphorylation at protein kinase C target sites. *Cell Commun Adhes* **13**: 13-9.

W Srisakuldee, BE Nickel, X Ma and E Kardami. (2010) High (but not low) molecular weight FGF2 causes the release of cytochrome C and 30 kDa connexin-43 fragment from isolated cardiac mitochondria. Palm Springs: American Heart Association Basic Cardiovascular Sciences 2010 Scientific Sessions.

W Srisakuldee, BE Nickel, F Zhang, KB Pasumarthi and E Kardami. (2009b) Fibroblast growth factor-2 stimulates mitochondrial connexin-43 phosphorylation at protein kinase C target sites and prevents its degradation by mitochondrial calpain. Winnipeg: University of Manitoba.

Statistics Canada. Table 102-0563 - Leading causes of death, total population, by sex, Canada, provinces and territories, annual. (2005) Retrieved from http://cansim2.statcan.gc.ca/cgi-win/cnsmcgi.exe?Lang=E&CNSM-Fi=CII/CII_1-eng.htm.

DL Stemple, NK Mahanthappa and DJ Anderson. (1988) Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: a sequence of events in sympathetic development. *Neuron* **1**: 517-25.

S Su, R Lampert, F Lee, JD Bremner, H Snieder, L Jones, NV Murrah, J Goldberg and V Vaccarino. (2010) Common genes contribute to depressive symptoms and heart rate variability: the Twins Heart Study. *Twin Res Hum Genet* **13**: 1-9.

Y Sugi, J Sasse and J Lough. (1993) Inhibition of precardiac mesoderm cell proliferation by antisense oligodeoxynucleotide complementary to fibroblast growth factor-2 (FGF-2). *Dev Biol* **157**: 28-37.

VP Sukhatme. (1990) Early transcriptional events in cell growth: the Egr family. *J Am Soc Nephrol* **1**: 859-66.

JL Swain. (1995) Fibroblast growth factor signalling and myogenic development. *Trans Am Clin Climatol Assoc* **106**: 109-12; discussion 112-3.

G Szebenyi and JF Fallon. (1999) Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* **185**: 45-106.

H Takano, H Hasegawa, T Nagai and I Komuro. (2003) Implication of cardiac remodeling in heart failure: mechanisms and therapeutic strategies. *Intern Med* **42**: 465-9.

NY Tan and LM Khachigian. (2009) Sp1 phosphorylation and its regulation of gene transcription. *Mol Cell Biol* **29**: 2483-8.

TP Tan, XM Gao, M Krawczynszyn, X Feng, H Kiriazis, AM Dart and XJ Du. (2003) Assessment of cardiac function by echocardiography in conscious and anesthetized mice: importance of the autonomic nervous system and disease state. *J Cardiovasc Pharmacol* **42**: 182-90.

YY Tan, GC Gast and YT van der Schouw. (2010) Gender differences in risk factors for coronary heart disease. *Maturitas* **65**: 149-60.

W Tang, Q Pan, F Sun, J Ma, S Tang, K Le, Y Wan, Q Chen and P Liu. (2009) Involvement of Sp1 binding sequences in basal transcription of the rat fibroblast growth factor-2 gene in neonatal cardiomyocytes. *Life Sci* **84**: 421-7.

S Taverna, G Gherzi, A Ginestra, S Rigogliuso, S Pecorella, G Alaimo, F Saladino, V Dolo, P Dell'Era, A Pavan, G Pizzolanti, P Mignatti, M Presta and ML Vittorelli. (2003) Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells. *J Biol Chem* **278**: 51911-9.

JR Teerlink, JM Pfeffer and MA Pfeffer. (1994) Progressive ventricular remodeling in response to diffuse isoproterenol-induced myocardial necrosis in rats. *Circ Res* **75**: 105-13.

KA Thomas, M Rios-Candelore and S Fitzpatrick. (1984) Purification and characterization of acidic fibroblast growth factor from bovine brain. *Proc Natl Acad Sci U S A* **81**: 357-61.

J Tillmanns, M Rota, T Hosoda, Y Misao, G Esposito, A Gonzalez, S Vitale, C Parolin, S Yasuzawa-Amano, J Muraski, A De Angelis, N Lecapitaine, RW Siggins, M Loredi, C Bearzi, R Bolli, K Urbanek, A Leri, J Kajstura and P Anversa. (2008) Formation of large coronary arteries by cardiac progenitor cells. *Proc Natl Acad Sci U S A* **105**: 1668-73.

Tobacco Free Initiative. Retrieved from <http://www.who.int/tobacco/en/>.

T Toyo-Oka, T Kawada, J Nakata, H Xie, M Urabe, F Masui, T Ebisawa, A Tezuka, K Iwasawa, T Nakajima, Y Uehara, H Kumagai, S Kostin, J Schaper, M Nakazawa and K Ozawa. (2004) Translocation and cleavage of myocardial dystrophin as a common pathway to advanced heart failure: a scheme for the progression of cardiac dysfunction. *Proc Natl Acad Sci U S A* **101**: 7381-5.

S Tsunoda, H Sakurai, Y Saito, Y Ueno, K Koizumi and I Saiki. (2009) Massive T-lymphocyte infiltration into the host stroma is essential for fibroblast growth factor-2-promoted growth and metastasis of mammary tumors via neovascular stability. *Am J Pathol* **174**: 671-83.

Y Uchida, A Yanagisawa-Miwa, F Nakamura, K Yamada, T Tomaru, K Kimura and T Morita. (1995) Angiogenic therapy of acute myocardial infarction by intrapericardial injection of basic fibroblast growth factor and heparin sulfate: an experimental study. *Am Heart J* **130**: 1182-8.

EF Unger, S Banai, M Shou, DF Lazarous, MT Jaklitsch, M Scheinowitz, R Correa, C Klingbeil and SE Epstein. (1994) Basic fibroblast growth factor enhances myocardial collateral flow in a canine model. *Am J Physiol* **266**: H1588-95.

EF Unger, L Goncalves, SE Epstein, EY Chew, CB Trapnell, RO Cannon, 3rd and AA Quyyumi. (2000) Effects of a single intracoronary injection of basic fibroblast growth factor in stable angina pectoris. *Am J Cardiol* **85**: 1414-9.

G Untergasser, R Gander, C Lilg, G Lepperdinger, E Plas and P Berger. (2005) Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* **126**: 59-69.

K Urbanek, D Cesselli, M Rota, A Nascimbene, A De Angelis, T Hosoda, C Bearzi, A Boni, R Bolli, J Kajstura, P Anversa and A Leri. (2006) Stem cell niches in the adult mouse heart. *Proc Natl Acad Sci U S A* **103**: 9226-31.

K Urbanek, M Rota, S Cascapera, C Bearzi, A Nascimbene, A De Angelis, T Hosoda, S Chimenti, M Baker, F Limana, D Nurzynska, D Torella, F Rotatori, R Rastaldo, E Musso, F Quaini, A Leri, J Kajstura and P Anversa. (2005) Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. *Circ Res* **97**: 663-73.

S Urheim, T Edvardsen, H Torp, B Angelsen and OA Smiseth. (2000) Myocardial strain by Doppler echocardiography. Validation of a new method to quantify regional myocardial function. *Circulation* **102**: 1158-64.

G Valen, T Kawakami, P Tahepold, A Dumitrescu, C Lowbeer and J Vaage. (2000) Glucocorticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72. *Am J Physiol Heart Circ Physiol* **279**: H836-43.

MJ van Amerongen, MC Harmsen, N van Rooijen, AH Petersen and MJ van Luyn. (2007) Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am J Pathol* **170**: 818-29.

EM Van Craenenbroeck, VY Hoymans, PJ Beckers, NM Possemiers, K Wuyts, BP Paelinck, CJ Vrints and VM Conraads. (2010) Exercise training improves function of circulating angiogenic cells in patients with chronic heart failure. *Basic Res Cardiol*

AJS Vander, James H.; Luciano, Dorothy S. (1994). Human physiology: the mechanisms of body function. New York: McGraw-Hill, Inc.

JA Virag, ML Rolle, J Reece, S Hardouin, EO Feigl and CE Murry. (2007) Fibroblast growth factor-2 regulates myocardial infarct repair: effects on cell proliferation, scar contraction, and ventricular function. *Am J Pathol* **171**: 1431-40.

JI Virag and CE Murry. (2003) Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. *Am J Pathol* **163**: 2433-40.

SM Wahl and JB Allen. (1988) T lymphocyte-dependent mechanisms of fibrosis. *Prog Clin Biol Res* **266**: 147-60.

F Wang, M Kan, J Xu, G Yan and WL McKeegan. (1995a) Ligand-specific structural domains in the fibroblast growth factor receptor. *J Biol Chem* **270**: 10222-30.

F Wang, M Kan, G Yan, J Xu and WL McKeegan. (1995b) Alternately spliced NH₂-terminal immunoglobulin-like Loop I in the ectodomain of the fibroblast growth factor (FGF) receptor 1 lowers affinity for both heparin and FGF-1. *J Biol Chem* **270**: 10231-5.

E Watanabe, DM Smith, J Sun, FW Smart, JB Delcarpio, TB Roberts, CH Van Meter, Jr. and WC Claycomb. (1998) Effect of basic fibroblast growth factor on angiogenesis in the infarcted porcine heart. *Basic Res Cardiol* **93**: 30-7.

KA Webster, DJ Discher and NH Bishopric. (1993) Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxic cardiac myocytes. *J Biol Chem* **268**: 16852-8.

K Wenzel-Seifert, L Grunbaum and R Seifert. (1991) Differential inhibition of human neutrophil activation by cyclosporins A, D, and H. Cyclosporin H is a potent and effective inhibitor of formyl peptide-induced superoxide formation. *J Immunol* **147**: 1940-6.

G Wernli, W Hasan, A Bhattacharjee, N van Rooijen and PG Smith. (2009) Macrophage depletion suppresses sympathetic hyperinnervation following myocardial infarction. *Basic Res Cardiol* **104**: 681-93.

C Wilhelmsson, A Vedin and L Wilhelmsen. (1981) Cost-benefit aspects of post-myocardial infarction intervention. *Acta Med Scand Suppl* **651**: 317-21.

BJ Wilkins, YS Dai, OF Bueno, SA Parsons, J Xu, DM Plank, F Jones, TR Kimball and JD Molkentin. (2004) Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res* **94**: 110-8.

JT Willerson and LM Buja. (1988) Beta-adrenergic mechanisms during severe myocardial ischemia and evolving infarction. *Postgrad Med Spec No*: 27-32.

MA Williams, WL Haskell, PA Ades, EA Amsterdam, V Bittner, BA Franklin, M Gulanick, ST Laing and KJ Stewart. (2007) Resistance exercise in individuals with and without cardiovascular disease: 2007 update: a scientific statement from the American Heart Association Council on Clinical Cardiology and Council on Nutrition, Physical Activity, and Metabolism. *Circulation* **116**: 572-84.

World Health Organization. The top 10 causes of death. (2008) Retrieved from <http://www.who.int/mediacentre/factsheets/fs310/en/index.html>.

C Wuechener, AC Nordqvist, A Winterpacht, B Zabel and M Schalling. (1996) Developmental expression of splicing variants of fibroblast growth factor receptor 3 (FGFR3) in mouse. *Int J Dev Biol* **40**: 1185-8.

X Xu, M Weinstein, C Li, M Naski, RI Cohen, DM Ornitz, P Leder and C Deng. (1998) Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* **125**: 753-65.

A Yanagisawa-Miwa, Y Uchida, F Nakamura, T Tomaru, H Kido, T Kamijo, T Sugimoto, K Kaji, M Utsuyama, C Kurashima and et al. (1992) Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science* **257**: 1401-3.

F Yang, YH Liu, XP Yang, J Xu, A Kapke and OA Carretero. (2002) Myocardial infarction and cardiac remodelling in mice. *Exp Physiol* **87**: 547-55.

Z Yang, YJ Day, MC Toufektsian, SI Ramos, M Marshall, XQ Wang, BA French and J Linden. (2005) Infarct-sparing effect of A2A-adenosine receptor activation is due primarily to its action on lymphocytes. *Circulation* **111**: 2190-7.

Z Yang, YJ Day, MC Toufektsian, Y Xu, SI Ramos, MA Marshall, BA French and J Linden. (2006) Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on CD4+ T lymphocytes. *Circulation* **114**: 2056-64.

S Yasuda, D Townsend, DE Michele, EG Favre, SM Day and JM Metzger. (2005) Dystrophic heart failure blocked by membrane sealant poloxamer. *Nature* **436**: 1025-9.

K Ye, CA Dinarello and BD Clark. (1993) Identification of the promoter region of human interleukin 1 type I receptor gene: multiple initiation sites, high G+C content, and constitutive expression. *Proc Natl Acad Sci U S A* **90**: 2295-9.

BK Yeh, M Igarashi, AV Eliseenkova, AN Plotnikov, I Sher, D Ron, SA Aaronson and M Mohammadi. (2003) Structural basis by which alternative splicing confers specificity in fibroblast growth factor receptors. *Proc Natl Acad Sci U S A* **100**: 2266-71.

S Yoshimura, Y Takagi, J Harada, T Teramoto, SS Thomas, C Waeber, JC Bakowska, XO Breakefield and MA Moskowitz. (2001) FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. *Proc Natl Acad Sci U S A* **98**: 5874-9.

G Zbinden and RA Moe. (1969) Pharmacological studies on heart muscle lesions induced by isoproterenol. *Ann N Y Acad Sci* **156**: 294-308.

XM Zhao, VM Byrd, WL McKeehan, MB Reich, GG Miller and JW Thomas. (1995) Costimulation of human CD4+ T cells by fibroblast growth factor-1 (acidic fibroblast growth factor). *J Immunol* **155**: 3904-11.

M Zhou, RL Sutliff, RJ Paul, JN Lorenz, JB Hoying, CC Haudenschild, M Yin, JD Coffin, L Kong, EG Kranias, W Luo, GP Boivin, JJ Duffy, SA Pawlowski and T Doetschman. (1998) Fibroblast growth factor 2 control of vascular tone. *Nat Med* **4**: 201-7.

X Zhu, H Komiya, A Chirino, S Faham, GM Fox, T Arakawa, BT Hsu and DC Rees. (1991) Three-dimensional structures of acidic and basic fibroblast growth factors. *Science* **251**: 90-3.

Y Zou, H Takano, H Akazawa, T Nagai, M Mizukami and I Komuro. (2002) Molecular and cellular mechanisms of mechanical stress-induced cardiac hypertrophy. *Endocr J* **49**: 1-13.