AUTOREGULATION OF FGF-2 IN CARDIAC MYOCYTES:

EFFECT OF FGF-2 AND β-ADRENERGIC STIMULATION ON FGF-2 PROMOTER ACTIVITY IN TRANSFECTED NEONATAL RAT CARDIAC MYOCYTE CULTURES AND TRANSGENIC MICE

BY

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A thesis submitted to the Faculty of Graduate Studies

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ABSTRACT

Cardiac disease is the leading cause of death in North America. Although there are plenty of therapies available for survivors, there is still an increasing need for the prevention (or reduction of severity) of heart attacks (myocardial infarction). In this context, there are naturally-occurring proteins that have been shown to exhibit cardioprotective properties, such as Fibroblast Growth Factor-2 (FGF-2), which is currently in clinical trials for its angiogenic function, and could be exploited further for its other beneficial properties.

FGF-2 is naturally produced in the heart and evidence shows that it is released on a beat-to-beat basis. Clearly, continuous FGF-2 release would require a method for its replacement, and the hypothesis proposed is that FGF-2 can regulate its own synthesis at the transcriptional level. A combination of *in vitro* and *in vivo* experiments were used to study the effect of FGF-2 on its own synthesis. Neonatal rat cardiac myocytes were transiently transfected with the firefly luciferase gene under the control of approximately 1000 base pairs (bp; nucleotides -1058/+54) of the rat FGF-2 proximal promoter region (-1058FGFp.*luc*) then treated with FGF-2. Conditions, including the dose of FGF-2 to be used and the period of treatment, were established. A significant ~2.5-fold increase in FGF-2 promoter activity was observed when compared to vehicle-treated myocytes under basal conditions.

A transgenic (TG) mouse expressing the -1058FGFp.*luc* was used in an attempt to assess the regulation of FGF-2 promoter activity *in vivo*. FGF-2 has been reported to be released from cardiac myocytes on contraction. The β-adrenergic agonist, isoproterenol (IsP), is known to increase heart rate and inotropy, and was used to indirectly stimulate an increase in heart rate in TG mice, and, indirectly, FGF-2 release. IsP treatment significantly increased FGF-2 promoter activity in adult TG mice. However, *in vitro*, the administration of isoproterenol did not induce an increase in FGF-2 promoter activity in transfected cardiac myocytes. This may reflect, among many things, the diminished extracellular FGF-2 stores *in vitro* when compared to whole hearts, and differences in developmental stage.

The 1000 bp FGF-2 promoter was truncated to approximately 150 bp (nucleotides -110/+42), and retested for response to direct administration of FGF-2 *in vitro* in an attempt to elucidate sequences that may be responsible for FGF-2 autoregulation. Treatment of cardiac myocytes transfected with luciferase under the control of nucleotides -110/+42 (-110FGFp.*luc*) showed a significant increase in promoter activity comparable to that of -1058FGFp.*luc* after FGF-2 treatment. This suggests that the ~150 bp region contains sufficient sequence information for FGF-2 responsiveness. The sequences within the proximal FGF-2 promoter region are GC-rich and contain multiple sites with the potential for binding transcription factors, including overlapping sites for early growth response-1 (Egr-1) and stimulatory protein-1 (Sp1). The ability of Egr-1 or Sp1

to interact with sequences in the region corresponding to nucleotides -7/+42 of the FGF-2 promoter region was suggested by DNA binding studies. Specifically, the electrophoretic mobility shift assay was used to assess the ability of sites within -7/+42 to compete for heart protein binding to radiolabeled consensus Egr-1 or Sp1 DNA elements. Also, overexpression of Egr-1 or Sp1 in cardiac myocytes transfected with -1058FGFp.*luc* showed a significant increase in FGF-2 promoter activity, but mutation of these sequences did not abolish the effects (fold-effect) that resulted from direct FGF-2 treatment. However, mutation of the Egr-1 and Sp1 binding sequences within nucleotides -110/+42 significantly decreased basal luciferase activity, which suggests that these sequences can play a role in FGF-2 gene regulation.

Thus, the results suggest that FGF-2 has the capacity to regulate its own synthesis at the transcriptional level in cardiac myocytes. Also, β-adrenergic stimulation results in an increase in FGF-2 promoter activity *in vivo* although the mechanism, direct or indirect, is unknown. Transfection studies *in vitro*, however, are consistent with an indirect mechanism and the possibility of FGF-2 autoregulation. The transcription factors Egr-1 and Sp1 can regulate FGF-2 gene regulation in cardiac myocytes, although, their role in autoregulation does not appear to be essential.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
μg	microgram
μl	microliter
ACE	angiotensin converting enzyme
aFGF	acidic fibroblast growth factor; also FGF-1
ANF	atrial natriuretic factor
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor; also FGF-2
bp	base pairs
CABG	coronary artery bypass graft
cAMP	cyclic AMP (adenosine monophosphate)
CMF	calcium- and magnesium-free
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNAzymes	DNA-based enzymes
ECM	extracellular matrix
eg.	for example
Egr-1	early growth response protein-1
EMSA	Electromobility Shift Assay
FBS	fetal bovine serum

FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
g	gram
g	acceleration due to gravity
g_i	G protein - inhibitory
g_o	G protein - "other"
g_s	G protein – stimulatory
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
HDL	high density lipoprotein
HEPES	N-{2-Hydroxyethyl}piperazine-N'-{2-ethanesulfonic acid}
HMW	high molecular weight
HSPG	heparin sulfate proteoglycans
Ig	immunoglobulin
i.p.	intraperitoneal
IRES	internal ribosome entry site
IsP	isoproterenol
kg	kilogram
l	liter
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LMW	low molecular weight

LVAD	left ventricular assist device
m	mutant
MAPK	mitogen-activated protein kinase
mg	milligram
ml	milliliter
mM	millimolar
MI	myocardial infarction
ng	nanogram
nm	nanometer
PBS	phosphate-buffered saline
pI	isoelectric point
PKC	protein kinase C
PLC	phospholipase C
PTCA	percutaneous transluminal coronary angioplasty
rlu	relative light units
RNA	ribonucleic acid
Sp1	stimulatory protein-1
SEM	standard error from the mean
tPA	tissue plasminogen activator
TG	transgenic
TRANSFAC	The Transcription Factor Database
TK	thymidine kinase
U	units

UV	ultraviolet
WT	wild type

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Chapter I

INTRODUCTION

A. HEART DISEASE

A.1. Overview

Cardiac disease is the leading cause of death in Canada [Heart and Stroke Foundation of Canada[a]]. Although multiple strategies now exist to reduce the chances of cardiac disease and as a consequence, decrease the number of fatalities due to cardiac failure, it still remains the leading cause of death, and is predicted to become the number one killer worldwide [Hennekens, 1999].

The heart's main function of pumping blood through the body requires the close coordination of myocyte contractility. The heart is composed of many cell types including working cardiomyocytes which comprise most of the heart's mass and contributing to contractile function, Purkinje fibers, fibroblasts, and cells of the circulatory system such as smooth muscle and endothelial cells.

Constant contractions require high amounts of energy, and thus a continuous supply of oxygen is crucial for proper heart function and myocyte integrity. The high energy requirement of cardiac myocytes is evidenced by their high numbers of mitochondria (approximately 30% and 20% of myocyte volume in the ventricle in the adult rat and man, respectively) [Opie, 2001]. If the blood flow to the heart ceases at any point (myocardial ischemia), be it due to the

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[a] Heart and Stroke – Just the facts 2002/2003 edition. Retrieved November 20, 2003, from http://www.heartandstroke.ca

blockage of vasculature, injury, spasm, or disease, the cardiac cells are able to briefly revert to anaerobic metabolism. However, anaerobic glycolysis produces much lower amounts of adenosine triphosphate (ATP) production, and is not enough to sustain normal heart function. In addition, anaerobic metabolism also has a lactic acid end product, which can accumulate and result in acidosis. After a certain period of time under lower energy and acidosis, the heart cells, specifically the cardiac myocytes, begin to undergo cell death (myocardial infarction). The cell death, through necrosis or apoptosis (programmed cell death that requires energy), extends to all heart tissue where blood perfusion has ceased. Injury to the cardiac cells broadens even further to surrounding undamaged cells, as cell lysis in the injured area affects neighboring cells by their released proteases and other factors that degrade cell material [Henderson, 1996].

If blood flow is quickly restored, tissue areas that have not undergone necrosis may be salvaged. However, there is also the phenomenon of "reperfusion injury," whereby the initial re-flow of blood causes further injury in the affected tissue through sudden massive ionic concentration gradient changes [Reviewed in Allen and Xiao, 2003]. After ion concentration gradients are returned to normal, the repair response ensues. From the cellular standpoint, the damaged cells within the infarct region release cytokines and other factors that recruit immune cells to begin the repair response. Phagocytes digest dead, dying, and injured cells, allowing space into which heart cells later migrate. The immune cells – and presumably other cells in the infarct border – release factors

and other cytokines that recruit fibroblasts and myofibroblasts into the region of the infarct [Reviewed in Ren *et al*, 2003]. These cells are highly efficient at producing and laying down collagen into their extracellular matrix, thus causing the formation of a less elastic and fibrous scar tissue. This scar tissue, although eventually becoming vascularized, cannot replace the elasticity or the force generated by contractile cardiac myocytes that used to occupy the area and, as a result, the heart workload increases. The heart tries to compensate using multiple different strategies, including hypertrophy, as will be discussed in the following section [Reviewed in Dhalla *et al*, 1996].

A.2. Pathologies that may result from Myocardial Infarction

Myocardial infarction (MI) affects all of the cell types that reside in the heart, resulting in tissue loss and contractile dysfunction, and eventually hypertrophy of the myocytes and the heart [Vinten-Johansen *et al*, 1999].

Tissue loss (via necrosis and apoptosis). Cell death, as previously mentioned, leaves an empty niche into which other cells may migrate. The change in the cell population now inhabiting the area once occupied by cardiac myocytes, results in different tissue properties including changes in elasticity and conduction potential.

Contractility dysfunction or stunning. Acute MI may result in "myocardial stunning" where myocardial contractile function is diminished due to an ischemic episode [Reviewed in Bolli, 1990; Heyndrickx, 2003]. Although this phenomenon

is reversible, the duration of ischemia and the number of its occurrence play a role in the degree of injury [Heyndrickx, 2003].

Arrhythmias. Cardiac myocytes that used to occupy the infarct region are connected end-to-end by gap junctions. These channels allow the cardiac cells to communicate and coordinate electrical impulses to synchronize their contraction. Certain gap junctions found in the heart's ventricles are highly expressed in cardiac myocytes [Gourdie, 1995; Gros and Jongsma, 1996]. It has been observed that upon ischemia, the expression of Connexin 43, a gap junction protein, is decreased, alongside changes in its phosphorylation [Reviewed in Kanno and Saffitz, 2001]. Changes in gap junction properties due can result in many different conduction-associated pathologies often observed in MI patients, such as arrhythmias, the disregulation of cardiac rhythms due to irregular cardiac myocyte contractions (bradycardia, tachychardia).

Hypertrophy. The loss of cardiac myocytes and their replacement with more fibrous tissue presents further challenges to the injured heart. This loss increases the workload presented to the remaining viable myocardium, which compensates by increasing in cell size (hypertrophy) but not in cell number (hyperplasia) [Anversa *et al*, 1986].

Vasculature defects. MI-induced cell death also includes the cells that make up the vasculature that perfuse the heart tissue with blood. Although the increase in concentration of cytokines and growth factors released within the injured area may trigger neovascularization, not all blood vessels formed result in functional connections to major bloodstreams. The cells of the vasculature are

capable of regeneration. Angiogenesis eventually occurs within the scar tissue that replaced the damaged myocardium, but may include irregularities such as aneurysms (the ballooning of blood vessels).

Other complications that may result from a MI include, but are not limited to, recurrent angina (chest pain), recurrent ischemia/infarction, pericarditis (disease of the pericardium, the sac that envelopes the heart), and embolisms (obstruction of a blood vessel) [Antman and Braunwald, 2001].

Unfortunately, beyond a certain amount of compensation by the remaining healthy myocardium (revascularization of scar tissue, hypertrophy), the heart may still undergo failure.

A.3. Therapies available post-MI

There are now many therapies in place to try to prevent patient death from heart failure. These range from physical vascular interventions (such as the implantation of stents or balloon angioplasties to increase the diameter of arteries with plaques, and coronary artery bypass grafts (CABG)), pharmacological vascular interventions (such as diuretics and vasodilators), electrical devices (such as pacemakers that stimulate and synchronize the heart's electrical impulses), physical assist devices (such as left ventricular assist devices, LVADs, which assist the movement of blood from the left ventricle to the aorta), to actually replacing the heart through transplantation [Katz, 2000].

An effective "treatment" would be to prevent the injury from occurring in the first place. After treating vascular obstruction, another priority is to increase

the heart's resistance to injury. We now know that it is possible to make cardiac myocytes less susceptible to the consequences of ischemia, a process called cardioprotection. This includes any method that may limit the myocardium's susceptibility to damage, ranging from exercise, pharmacological interventions, to molecular interventions such as the preservation of mitochondrial function [Monteiro *et al*, 2003], and cardiac myocyte membrane integrity [Detillieux *et al*, 1999]. This thesis focuses on fibroblast growth factor-2 (FGF-2), a molecule that has been shown to have cardioprotective properties [Reviewed in Detillieux *et al*, 2003].

B. HEART DISEASE: AT THE LEVEL OF THE CARDIAC MYOCYTE

The cardiac myocyte makes up approximately 30% of the heart cells, but over 75% of its volume [Opie, 2001]. It is the cell containing the contractile mechanism responsible for the contraction of the heart, and thus the pumping of the blood through the body. Unlike the other cell types in the heart, the cardiac myocyte is considered to be terminally differentiated, or non-regenerative [Soonpaa *et al*, 1995]. The view, however, that the heart does not have the capacity to regenerate after injury is currently under much debate [Laflamme *et al*, 2002; Glaser *et al*, 2002; Beltrami *et al*, 2003]. Many studies now suggest that there may be circulating or local cardiac stem cells that allow the regeneration of cardiac myocytes in infarcted regions [Orlic *et al*, 2001; Yeh *et al*, 2003]. A recent study has also shown the presence of a population of stem cells that reside within the heart tissue [Beltrami *et al*, 2003]. Whether enough cardiac

myocytes can re-enter the cell cycle to repopulate infarcted areas, this is still open to debate; however there is currently very limited data supporting this hypothesis [Pasumarthi and Field, 2002; Nadal-Ginard *et al*, 2003]. It is therefore essential to minimize the extent of cardiac damage which in turn necessitates a solid understanding of cardioprotective mechanisms.

C. POSSIBLE PREVENTATIVE MEASURES

C.1. The common practice: fighting atherosclerosis

There are many different strategies currently being studied and tested that are believed to help prevent or decrease the chances of cardiac disease, and most focus on preventing coronary heart disease. The leading cause of cardiac failure and mortality is coronary heart disease [Heart and Stroke Foundation of Ontario[b]]. This can lead to myocardial ischemia and infarction, or a stroke (ischemia and infarction in the brain). The most common reason for ischemia is the blockage of the main coronary arteries due to atherosclerosis (the formation of plaque in blood vessels). Briefly, atherosclerosis results from the build up of fatty cells, containing low density lipoprotein (LDL), on the smooth muscle cell layer within the blood vessel wall which can form calcified plaques upon oxidization of the LDL. LDL is a package of fat containing a high concentration of cholesterol. These plaques build up and decrease the diameter of the blood vessel. If an embolism occurs or any further immune response to the

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[b] Heart and Stroke - Just the facts 2002/2003 edition. Retrieved November 20, 2003, from http://www.heartandstroke.ca

atherosclerotic plaque, blood flow ceases, and results in ischemia [Sherwood, 1993].

Coronary heart disease preventative measures include vitamin E supplementation [Manson *et al*, 2003] and other anti-oxidative molecules, and increasing blood levels of high density lipoprotein (HDL, a circulating lipoprotein containing a high concentration of protein and a low concentration of cholesterol) and decreasing LDL concentrations to help prevent atherosclerosis [Sherwood, 1993], intake of angiotensin converting enzyme (ACE; an enzyme that decreases the uptake of fluids into the kidneys) inhibitors [Baker, 2002; Sherwood, 1993], or blood platelet aggregation inhibitors such as aspirin [Shannon and Harrigan, 2001] to decrease high blood pressure; and immediate delivery of enzymes such as tissue plasminogen activator (tPA), Streptokinase, and Urokinase that break down blood clots immediately upon a patient's arrival into the emergency room suffering from a MI [Antman and Braunwald, 2001].

C.2. Adjunct treatment: Cardioprotection

In addition to preventative approaches, treatments known to increase cardiac resistance to ischemic injury are the subject of intense interest and investigation. For example, resveratrol in red wine is reported to be cardioprotective [Sato *et al*, 2002; Shigematsu *et al*, 2003]. In regards to cardioprotection, one practice now becoming more common is the oral intake of suspected cardioprotective agents such as aspirin and resveratrol, which is found in red wine. Research laboratories try to understand mechanisms of suspected

cardio-protective agents. Currently undergoing clinical trials are the exogenous administration of naturally-occurring proteins, such as Cardiotrophin-1 [Ghosh *et al*, 2000], and FGF-2, which have shown evidence for cardioprotection [Padua *et al*, 1995; Jiang *et al*, 2002].

D. FIBROBLAST GROWTH FACTORS

D.1. The FGF family

The Fibroblast Growth Factor (FGF) family is a group of 22 structurally similar, naturally-occurring proteins [Wilkie *et al*, 2002]. FGF was first isolated from the bovine pituitary in the 1970s, and was initially noted for its capacity to promote growth and proliferation (incorporation of tritiated thymidine) in fibroblast cells (NIH 3T3) [Gospodarowicz *et al*, 1975]. Members of this family have since been shown to promote growth and proliferation in numerous cell types, and have also been shown to play an important role in mammalian development [Sugi *et al*, 1993; Swain, 1994; Leconte *et al*, 1998]. The first two members of the FGF family that were isolated were termed “acidic” FGF (aFGF) and “basic” FGF (bFGF), based on their isoelectric point (pI) values [Maciag *et al*, 1984, Esch *et al*, 1985]. As more members of the family were discovered, aFGF and bFGF were renamed FGF-1 and FGF-2, thus designating subsequent FGF members with numbers as they were discovered [Baird and Klagsbrun, 1991].

The FGF family share several distinct properties: (1) they are all structurally related, (2) they are all able to bind heparin molecules, and (3) they all bind (and possibly act through) the cell surface Fibroblast Growth Factor

Receptor (FGFR) family [Reviewed in Powers *et al*, 2000; Klint and Claesson-Welsh, 1999].

D.2. The FGF receptors

FGF receptors can be broadly divided into two types, high affinity receptors, and low affinity receptors.

High affinity FGF receptors. The FGFR family consists of four well-characterized transmembrane receptors (FGFR-1 through -4) of the tyrosine kinase family [Hughes and Hall, 1993; Reviewed in Bikfalvi *et al*, 1997; Klint and Claesson-Welsh, 1999; Powers *et al*, 2000]. A fifth member, FGFR-5, has recently been identified, however, this has not yet been fully characterized beyond knowing that it is devoid of an intracellular kinase domain [Sleeman *et al*, 2001; Kim *et al*, 2001]. The FGF receptors are mainly found on the cell surface, but also in association with the nucleus [Stachowiak *et al*, 1996]. There are also repots of secreted FGFR isoforms [Duan *et al*, 1992; Givol and Yayon, 1992].

The first four members of the FGFR family share between 55% and 72% amino acid sequence homology [Johnson and Williams, 1993], and their structures consist of an extracellular ligand binding immunoglobulin-like (Ig-like) domain, an acidic region, a heparin-binding domain [Kan *et al*, 1993], a transmembrane domain, and an intracellular tyrosine kinase domain [McKeehan and Kan, 1994; Reviewed in Powers *et al*, 2000]. The variation in structure lies mainly in the extracellular Ig-like domains, which have been designated Ig domain I, II, and III, where Ig domain III is closest to the plasma membrane.

These Ig-like domains bind the FGF ligand, and mediate the stimulation of signal transduction cascades within the cell through the phosphorylation of their tyrosine kinase domain [Reviewed in Powers *et al*, 2000]. The most conserved region of the FGFR proteins belong to these kinase domains and are found intracellularly (up to 92% amino acid homology) [Reviewed in Powers *et al*, 2000].

Low affinity FGF receptors. Heparan sulfate proteoglycans (HSPG) also act as low-affinity receptors for the FGF family, and are important mediators in the interactions between FGFs and FGFRs [Pellegrini, 2001].

This heparin-binding property has been proposed to play quite an important role in FGF function. FGF in complex with heparin, alone or in multiple tandems, has been shown to be protected from thermal denaturation [Gospodarowicz and Cheng, 1986] and from proteolysis [Damon *et al*, 1989]. Besides adding to FGF stability, binding to heparan sulfate proteoglycans on the cell surface and heparin and other related molecules in the extracellular matrix also serves another important function relating to FGF biological activity: maintaining a local reservoir [Vlodavsky *et al*, 1991]. Once released by the cell, the sequestering of FGF-2 by heparin molecules prevents its transport to distant cells, allowing for its biological activity to be contained near the cell that produced it, thus providing the potential for autocrine or paracrine activities [Reviewed in Ornitz, 2000]. Autocrine defines the ability of a molecule produced by a certain cell to be released and act back on the same cell that produced it. Paracrine describes the action of a molecule from one cell on a nearby neighboring cell.

Proteases that degrade heparin molecules may also act as a mechanism of FGF release from extracellular reserves. Injury may also act in the same manner and release FGF stores [Reviewed in Powers *et al*, 2000]. The sequestering of FGF molecules to the extracellular matrix also acts to control FGF concentration gradients, which plays an important role in development [Alanko *et al*, 1994; Kengaku and Okamoto, 1995].

E. FIBROBLAST GROWTH FACTOR-2

The subject of this thesis is fibroblast growth factor-2 (FGF-2), a member of the FGF family [Reviewed in Bikfalvi *et al*, 1997]. It has been shown to promote growth and proliferation cells of neuroectoderm and mesoderm origin [Basilico and Moscatelli, 1992]. FGF-2 is ubiquitously expressed, but does not bind to all four FGFRs with the same affinity [Baird, 1994]. Among the high affinity FGF receptors, FGF-2 binds preferentially to FGFR-1 and FGFR-3, and with lower affinity to FGFR-4, and even less to FGFR-2 [Ornitz *et al*, 1996].

E.1. FGF-2 structure

The FGF-2 protein exists in several different isoforms: 18, 21, 23, 24, and 34 kDa proteins [Florkiewicz and Sommer, 1989; Arnaud *et al*, 1999]. The crystalline structure of the 18 kDa form of the FGF-2 protein has been determined to be in a trigonal pyramidal form made up of 12 antiparallel β-sheets. Two amino acid sequences within the structure have also been

discovered which are important in receptor binding – both to high- and low-affinity receptors [Baird *et al*, 1988].

The term "low molecular weight FGF-2" (LMW) is now the designated term for 18 kDa FGF-2, and "high molecular weight FGF-2" (HMW) for other isoforms [Pasumarthi *et al*, 1996; Reviewed in Bikfalvi *et al*, 1997]. In the heart, HMW FGF-2 predominates in the neonatal stage while LMW FGF-2 predominates in the adult [Liu *et al*, 1993]. Overexpression of HMW in neonatal rat cardiac myocyte cultures has also been linked with nuclear binucleation and DNA compaction [Pasumarthi *et al*, 1996; Hirst *et al*, 2003]. Overexpression of HMW FGF-2 in cardiac myocytes has also been implicated in apoptotic cell death as determined by DNA laddering [Hirst *et al*, 2003]. HMW FGF-2 has, in its amino-terminal sequence, a nuclear localization-like sequence, and the protein itself has been shown to be localized in the nucleus [Bugler *et al*, 1991]. The LMW FGF-2 lacks a conventional signal peptide for secretion and is thus considered to be an intracellular molecule, where it can be found in the nucleus and cytosol. It is also found at the ECM, although its method of release is not understood.

E.2. Proposed methods of FGF-2 release

Several laboratories have reported many different methods of FGF-2 release. These include release upon cell lysis or wounding upon injury or death (expulsion along with other cytosolic proteins) [Muthukrishnan *et al*, 1991; Mignatti *et al*, 1992; Clarke *et al*, 1993], exocytosis through the Na+/K+ ATPase (inhibition of ion transport also inhibits FGF-2 export from the cell) [Florkiewicz *et*

al, 1998; Wakisaka *et al*, 2002], complement-mediated injury [Floege *et al*, 1992], and release through transient disruptions of the plasma membrane upon cell movement [Sterpetti *et al*, 1994; Clarke *et al*, 1995; Kaye *et al*, 1996]. A recent study has also shown FGF-2 release through vesicle shedding from human hepatoma cells, a phenomenon that has not been studied as of yet in the cardiac myocyte [Taverna *et al*, 2003].

E.3. Effect of FGF-2 on cells

FGF-2 is known to have multiple biological roles in many different cell types. The different effects presumably reflect the ability of FGF-2 to activate several through different transduction pathways, ultimately affecting nuclear processes [Reviewed in Bikfalvi *et al*, 1997].

Directly, FGF-2 can be imported into the nucleus through the nuclear localization-like sequences found on the HMW isoforms. Although this pathway has been shown to cause transformation of NIH 3T3 cell with tumorigenic properties in high concentrations [Quarto *et al*, 1991], most laboratories have focused on the effects of FGF-2 through its extracellular receptors.

Indirectly, FGF-2 causes its effect by binding to its extracellular receptors, which causes signal cascades that ultimately result in an effect on the chromatin in the nucleus [Goldfarb, 2001]. Extracellular FGF signaling is mediated by both high- (FGFR family) and low-affinity (heparin and heparin sulfate proteoglycans) receptors. As FGF molecules are released from the cell, they are sequestered by the extracellular matrix. FGF-2 can also bind to its low-affinity heparan sulfate

proteoglycan receptors associated with the cell surface, which facilitate binding to the high-affinity FGF receptors [Yayon *et al*, 1991]. It is believed that facilitation consists of dimerization of the ligand-receptor complex, which can then act to autophosphorylate certain tyrosine residues in the receptor intracellular kinase domain, starting a signal cascade of events. Tyrosine phosphorylation can then act to recruit further signal cascade molecules required for FGF-2 signaling within the nucleus [Reviewed in Klint and Claesson-Welsh, 1999].

In addition to the well-documented effects of FGF-2/FGF receptor during development (including the heart) [Sugi *et al*, 1993; Swain, 1994; Mima *et al*, 1995], the two most important properties of FGF-2 in the heart fall under the categories of "angiogenesis" and "cardioprotection" [Reviewed in Detillieux *et al*, 2003].

E.4. FGF-2 and angiogenesis

FGF-2 is a potent angiogenic molecule [Lyons *et al*, 1991; Reviewed in Slavin, 1995]. It stimulates the migration and proliferation of smooth muscle and endothelial cells, allowing the formation of new blood vessels. Studies in canine, rabbit, and porcine models have shown that administration of FGF-2 prior to ischemia results in increased blood flow through collateral vessels after treatment [Rajanayagam *et al*, 2000; Lazarous *et al*, 1996; Landau *et al*, 1995].

Clinical trials. The potency to promote angiogenesis has been tested in clinical trials whereby FGF-2 is administered (intracoronary) to patients suffering from ischemic heart disease. FGF-2 treatment was well-tolerated, except for a

few cases of hypotension [Unger *et al*, 2000; Laham *et al*, 2000]. Patients showed capillary formation [Schumacher *et al*, 1998], an improved exercise tolerance [Laham *et al*, 2000], and decreased recurrence of angina [Ruel *et al*, 2002]. Patients who have undergone coronary artery bypass and were given treatments of FGF-2 have improved left ventricular ejection fractions three years after treatment when compared to those who received placebo treatments [Pecher and Schumacher, 2000]. Further trials are ongoing [Reviewed in Detillieux *et al*, 2003].

E.5. FGF-2 and cardioprotection

Early studies have shown that FGF-2 treated cells in culture are less susceptible to the damage caused by hypoxia [Noxakie *et al*, 1993; Maiese *et al*, 1993; Akaneya *et al*, 1993; Kardami *et al*, 1993]. These observations led to the theory that FGF-2 may also be a cardioprotective agent. This idea was supported by the report that FGF-2 treated rat cardiac myocytes in culture were less susceptible to injury after treatment with hydrogen peroxide [Kardami *et al*, 1993]. Studies that focus on FGF-2 for its direct cardioprotective properties (rather than angiogenic properties) have shown the decreased release of markers of injury from the heart after ischemia/reperfusion injury after FGF-2 treatment [Padua *et al*, 1995; Padua *et al*, 1998; Jiang *et al*, 2002]. Examination of hearts after FGF-2 treatment and ischemia/reperfusion injury in rat models have shown decreased infarct sizes, TUNEL-positive cardiac myocytes, and ventricular tachycardia, whereas left ventricular function (measured developed

pressure) was improved [Jiang *et al*, 2002; Nishida *et al*, 2003]. Transgenic mice overexpressing FGF-2 in the heart were generated and shown to have decreased injury after ischemia/reperfusion *ex vivo* [Sheikh *et al*, 2001; House *et al*, 2003].

FGF-2 Cardioprotection: mechanism. FGF-2 signaling through its cell surface receptors stimulates the signal transduction pathways involving mitogen-activated protein kinase (MAPK) and protein kinase c (PKC). Both pathways have been proposed to play a role in cardioprotection [Iwai-Kanai *et al*, 2002; Padua *et al*, 1995; Padua *et al*, 1998]. Administration of FGF-2 in rats before or after ischemia from coronary artery ligation resulted in decreased damage and improved left ventricular function when compared to sham animals [Jiang *et al*, 2002]. This cardioprotection was abrogated when a mutant FGF-2 (lower binding affinity to FGFR-1) was administered prior to surgery, implicating a role for FGFR-1 in FGF-2 cardioprotection [Jiang *et al*, 2002]. FGF-2 was also cardioprotective when administered during reperfusion of the ischemic heart [Jiang *et al*, 2002; Cuevas *et al*, 2001]. The protective effects of FGF-2 are mediated by PKC, most likely the PKCε subtype [Padua *et al*, 1998; Jiang *et al*, 2002].

Although we have some understanding on the mechanisms of FGF-2 cardioprotection, in order to maximally exploit these properties, it is important to understand how the endogenous gene is regulated.

E.6. FGF synthesis/regulation

The FGF-2 gene promoter region is devoid of the classical TATA or CAAT boxes [Shibata *et al*, 1991, Pasumarthi *et al*, 1997]. However, a region of the FGF-2 human and rat promoter has been shown to be GC-rich, characteristic of sequences known to regulate other TATA-less promoters such as that of interleukin-1 [Ye *et al*, 1993]. A 152 bp proximal region of the FGF-2 promoter (nucleotides –110/+42) containing these GC-rich sequences, and specifically adjacent to a major transcription initiation site (nucleotides –7/+42), was shown to be sufficient for the detection of FGF-2 promoter activity (through α-adrenergic stimulation) using a reporter gene construct [Jin *et al*, 2000].

Transcriptional regulation. Studies of FGF-2-induced effects on human glia cells have shown evidence for the autoregulation of FGF-2 [Wang *et al*, 1997]. Autoregulation is the ability of a gene product to affect its own synthesis. There has also been evidence for the involvement of the transcription factor, Egr-1 in FGF-2 synthesis [Biesiada *et al*, 1996]. Recent studies using RNA-cleaving phosphodiester-linked DNA-based enzymes (DNAzymes) that were engineered to specifically cleave Egr-1 mRNA have shown the inhibition of angiogenesis by FGF-2 in subcutaneous Matrigel plugs in mice [Fahmy *et al*, 2003], suggesting involvement of Egr-1 in FGF-2 activity.

Translational regulation. Despite the presence of multiple FGF-2 isoforms, there is only one FGF-2 gene, found on chromosome 4 in humans, and chromosome 3 in mice [Fukushima *et al*, 1990]. The presence of multiple isoforms is due to the alternate translation at multiple start sites on the same

mRNA. HMW FGF-2 is the product of translation from a CUG start site(s) upstream of the classical AUG codon used for the translation of the LMW form [Pasumarthi *et al*, 1997]. The sequence of the mRNA where ribosomes dock, the internal ribosome entry site (IRES), actually acts as a tissue-specific regulator of FGF-2 expression [Creancier *et al*, 2000].

The production of the FGF-2 antisense mRNA, transcribed from the same gene, may also contribute to FGF-2 regulation. It has been shown in glioma cells that introduction of FGF-2 antisense mRNA decreases intracellular FGF-2 protein levels [Li and Murphy, 2000].

Post-translational modifications. Some methods of post-translational modifications that FGF-2 may undergo include the methylation of HMW FGF-2, which has been shown to increase its localization to the nucleus [Pintucci *et al*, 1996], and the enzymatic transfer of ADP-ribose from nicotinamide adenine dinucleotide [Boulle *et al*, 1995]. Such modifications allow for further regulation of the FGF-2 protein, helping to influence its cellular distribution and function.

E.7. Stress triggers FGF-2 synthesis in the heart

Stressful events have been shown to trigger FGF-2 expression/synthesis [Erdos *et al*, 1995; Le and Corry, 1999]. As previously mentioned, in a normal physiological situation, FGF-2 has been shown to be released from adult rat ventricular myocytes upon mechanical activity through transient non-lethal sarcolemmal disruptions [Muthukrishnan *et al*, 1991; Clarke *et al*, 1995; Kaye *et al*, 1996]. Increasing mechanical activity of rat heart *ex vivo* through the

administration of the β-adrenergic agonist, isoproterenol (IsP), was shown to not only release FGF-2, but also increase extracellular FGF-2 protein levels [Padua and Kardami, 1993; Clarke *et al*, 1996]. The administration of cytokines known to be associated with physiological stress (FGF-2, angiotensin II, endothelin-1, and interleukin-1) has also been shown to increase FGF-2 transcript levels in cardiac myocytes in culture [Fischer *et al*, 1997], raising the possibility that FGF-2 may regulate its own synthesis (autoregulation) at the transcriptional level. Although the increase in mechanical activity above resulted in hypertrophy concomitant with the increase in FGF-2, pulmonary artery banding (which leads to right ventricular hypertrophy) did not induce an increase in FGF-2 mRNA levels [Bauer *et al*, 1997]. Overall regulation of FGF-2 synthesis is not yet well-understood. The possibility that FGF-2 autoregulation occurs at the transcriptional level is pursued in this thesis.

E.8. Schematic: What may be occurring

Non-injury scenario. The heart releases FGF-2 on a beat-to-beat basis. The gene, found on chromosome 4 (humans) or 3 (mice), is transcribed in the nucleus after certain transcription factors trigger promoter stimulation. (Some transcription factors associated with FGF-2 expression in other systems include: Egr-1 (glia cells) [Biesiada *et al*, 1996; Wang *et al*, 1997] and Jak-2 (endothelial cells) [Zeng *et al*, 2002].) FGF-2 mRNA is released from the nucleus, and is bound by multiple ribosomes in the cytosol, binding at the classical AUG start site, and several alternate sites (CUG) upstream, resulting in the synthesis of

LMW and HMW FGF-2 protein. FGF-2 protein may undergo post-translational modifications, and remains in the cytosol or nuclear localization sequences may direct their import into the nucleus. It has been proposed that the post-translational methylation of HMW FGF-2 causes an increase in its localization in the nucleus [Pintucci *et al*, 1996]. HMW FGF-2, which has been shown to be elevated after injury through β-adrenergic stimulation [Padua and Kardami, 1993], may then act in the nucleus [Pasumarthi *et al*, 1994; Pasumarthi *et al*, 1996], to affect chromatin condensation and cell death [Hirst *et al*, 2003]. LMW FGF-2 near the plasma membrane is released upon contraction. Released FGF-2 molecules can then bind to its low-affinity cell surface receptors or heparin in the extracellular matrix for storage. FGF-2 on cell surface heparan sulfate proteoglycans is available to bind to cell surface high affinity FGF receptors (FGFR-1 or FGFR-3), which then, upon autophosphorylation of its intracellular tyrosine kinase residues, triggers signal transduction cascades that lead to the translocation of transcription factors into the nucleus, causing an effect on the gene expression.

Upon injury. Upon injury, internal FGF-2 stores would be released from cells, and proteases and other enzymes "dislodge" FGF-2 bound to the extracellular matrix, increasing FGF-2 availability. Released intracellular and extracellular FGF-2 may then attach to high affinity receptors (FGFR-1) of nearby cells, and possibly to its lower affinity sites (FGFR-4 and FGFR-2). Saturation of cell surface receptors might trigger additional signal transduction cascades, thus

causing nuclear import of transcription factors, and ultimately resulting in the potential for a greater effect on the cell.

Whether under normal physiological circumstances, or even in an injury scenario, FGF-2 stores must be replenished (Figure 1).

F. HYPOTHESIS

In summary, a cardioprotective substance, FGF-2, is released from the heart upon contraction, and is also stored in the extracellular matrix. Its constant release and presence in the heart, and its upregulation upon injury [Padua and Kardami, 1993; Wen *et al*, 1995; Li *et al*, 2002; Madiai *et al*, 2003] suggests that FGF-2 may be involved in both the maintenance of a healthy myocardium [Detillieux *et al*, 1999] and in providing additional protection and/or contribute to repair processes (for example, revascularization). Certainly, increasing FGF-2 levels genetically through transgene expression in the heart increases cardiac myocyte membrane integrity, an indicator of cardioprotection [Sheikh *et al*, 2001].

Given that FGF-2 is released from the heart on a beat-to-beat basis, replacement of both intracellular and extracellular matrix stores by FGF-2 synthesis at the transcriptional level is a necessary component of this process. The hypothesis to be tested is that FGF-2 is autoregulated at the transcriptional level in cardiac myocytes (Figure 1).

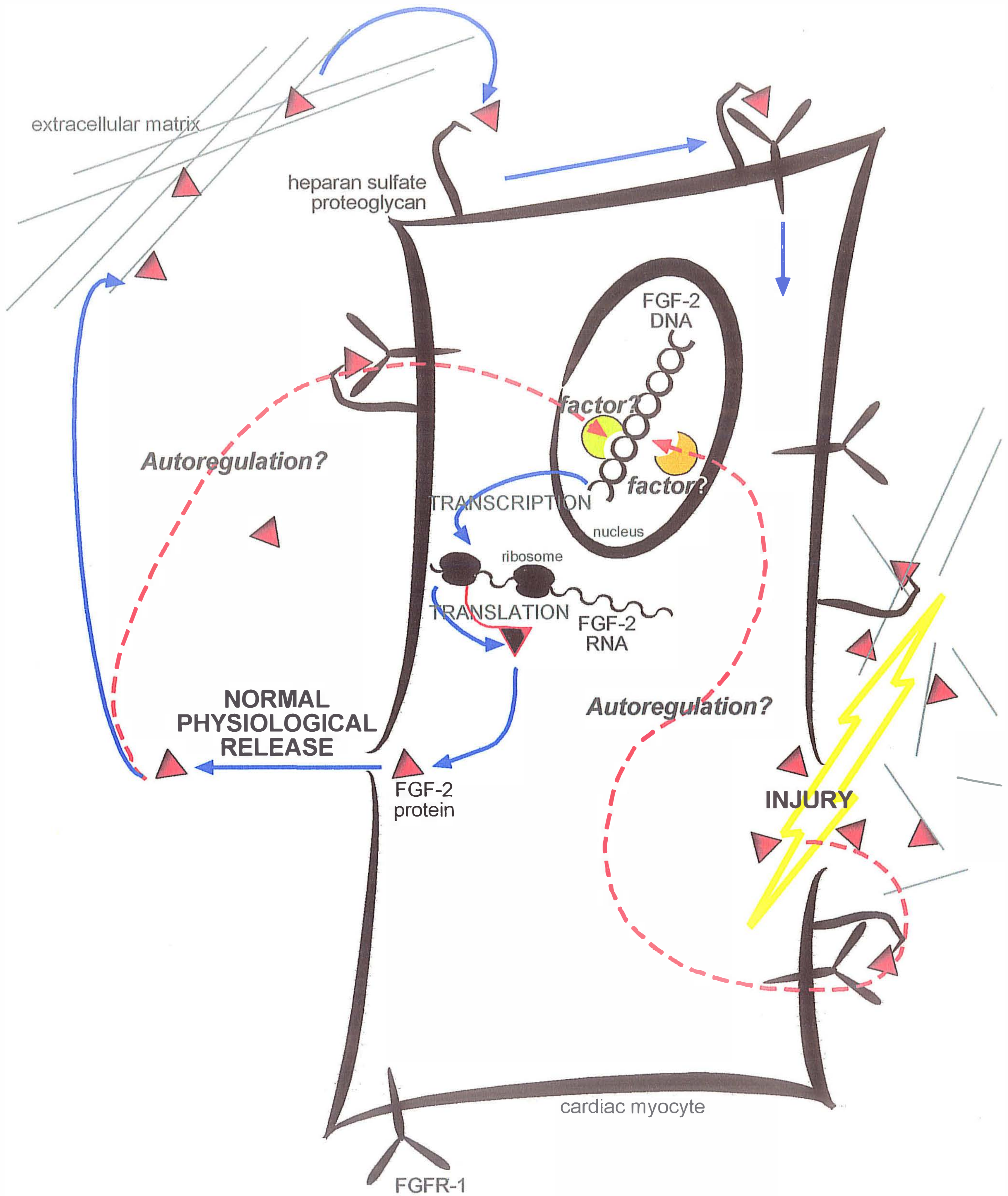

Figure 1. Schematic of FGF-2 regulation and possible autoregulation in cardiac myocytes.

G. MODELS/APPROACH

G.1. Primary neonatal rat cardiac myocyte cultures

To determine mechanisms of FGF-2 regulation, primary cultures of neonatal rat cardiac myocytes were utilized, which have been well-characterized, and are a common tool in the field of cardiovascular research [Reviewed in Chlopcikova *et al*, 2001]. It allows for relatively easy introduction of foreign DNA into cardiac myocytes, facilitating the study of the effects of certain sequences that may play a role in FGF-2 gene regulation.

Cardiac myocytes are more difficult to transfect with DNA, when compared to cell lines. Unfortunately, due to the highly differentiated property of cardiac myocytes, there are currently no stable cell lines that reflect cardiac myocyte characteristics *in vivo.* Fresh neonatal rat cardiac myocytes were therefore isolated for each experiment, a useful tool widely used for cardiovascular research studies *in vitro.*

G.2. Transgenic mouse model

To examine the effects on FGF-2 promoter activity *in vivo*, a previously generated transgenic mouse model expressing the firefly luciferase gene under the control of approximately 1000 bp of the rat FGF-2 promoter was utilized (-1058FGFp.*luc*). As the FGF-2 transcript is quite unstable, this transgenic mouse model was generated for the easy detection of FGF-2 promoter activity in the heart and other tissues [Detillieux *et al*, 1999]. Two mouse lines were generated (P66 and P300), expressing approximately 800 and 3000 relative light

units/μg (rlu/μg) protein of the -1058FGFp.*luc* transgene in the heart, respectively [Detillieux *et al*, 1999]. The levels of -1058FGFp.*luc* were also assessed in the brain, where P66 expresses approximately 5000 rlu/μg protein and P300 expresses 700 rlu/μg protein. Administration of α1-adrenergic agonist, phenylephrine, induced increased luciferase activity in mouse hearts from both transgenic mouse lines when compared to saline-treated controls.

G.3. FGF-2 release through β-adrenergic stimulation

FGF-2 has previously been reported to increase upon β-adrenergic stimulation of cardiac myocytes [Padua and Kardami, 1993], and its release from is also increased [Clarke *et al*, 1995]. This observation was exploited for *in vivo* studies in this thesis. Specifically, this was done to induce an increase in endogenous FGF-2 release in the heart through an increase in heart rate and force of contraction (inotropy) in cardiac myocytes.

G.4. β-adrenergic response

Function. The adrenergic response is initially described as the effect of the neurotransmitter, epinephrine (adrenalin). The mechanism of action is mediated by Phospholipase C (PLC) or cAMP, and works on the cell through transmembrane receptors found on several different cell type [Sherwood, 1993].

β-adrenergic receptors. There are two types of β-adrenergic receptors, β_1 and β_2, both of which are found in the heart and are known for their role in control of inotropy, or the heart's force of contraction [Alquist, 1966].

Structurally similar, the β-adrenergic receptors are composed of at least seven distinct gene products that act together to stimulate the response [Reviewed in Bristow *et al*, 1990]. The first gene product involved is the hydrophilic extracellular receptor subunit, known to bind neurotransmitters, hormones, or other molecules that can trigger an adrenergic response. β-adrenergic agonists bind to the extracellular receptor in a low affinity capacity. These receptors are anchored into the membrane through a multi-spanning transmembrane domain which also has an intracellular domain including several tyrosine and serine residues that could be phosphorylated for further signal transduction. Ligand binding at the extracellular domain triggers a response from the following two subunits [Reviewed in Bristow, 1988; Bristow *et al*, 1990].

The next protein involved is the guanine nucleotide regulatory region. These have been classified into 3 main types: (1) stimulatory (G_s), (2) inhibitory (G_i), and "other" (G_o), of which both G_s and G_i are present in the heart. The G proteins consist of several subunits: α, β, and γ. The main role of the G proteins is the conversion of guanosine triphosphate moieties (GTP) into guanosine diphosphates (GDP), a process which releases energy to trigger the action of the catalytic subunit, physically situated nearby. This mechanism is mediated in the most part, by the alpha subunits mediating GTP to GDP cleavage. The inhibitory subunits act to absorb the alpha subunits to prevent its binding to GTP, and thus cleavage [Reviewed in Harris and Harding, 1986; Bristow, 1988].

The third protein involved in the tripartite system is the adenylate cyclase catalytic subunit. This component acts, in response to activity of the previous

two parts, to convert ATP molecules into the cyclic adenosine monophosphate (cAMP) molecules. cAMP can then act to trigger further signal transduction cascades within the cell, causing the β-adrenergic response, which is to increase heart rate and inotropy. cAMP stimulation causes a change in permeability of the sarcolemma to calcium ions. Increased calcium influx can cause an increase in cardiac myocyte force of contraction, thus, increasing inotropy [Reviewed in Harris and Harding, 1986; Bristow, 1988; Ross Jr., 1998].

H. SUMMARY

In summary, FGF-2 is a cardioprotective agent. To be able to maximally exploit this property, it is necessary to understand more clearly the method in which endogenous FGF-2 gene expression is regulated. To this end, *in vitro* models were used to determine the direct effects of FGF-2 on its promoter through transient gene transfer in cardiac myocyte cultures and reporter gene assays. In an attempt to assess the effect of FGF-2 release on FGF-2 gene regulation *in vivo*, transgenic mouse models expressing a hybrid FGF-2 promoter-luciferase reporter gene and β-adrenergic stimulation were used to increase cardiac inotropy and heart rate, which presumably increases the release of endogenous FGF-2 protein from cardiac myocytes.

Chapter II

MATERIALS AND METHODS

A. CELL CULTURE

All procedures performed in this thesis were conducted in accordance with the current *Guide to the Care and Use of Experimental Animals*, published by the Canadian Council on Animal Care.

The protocol for generating neonatal rat cardiac myocyte cultures was based on a method used by Dr. E. Kardami's laboratory [Doble *et al*, 1996], and modified by the laboratories of Drs. E. Kardami and P.A. Cattini at the University of Manitoba, Canada.

Per myocyte preparation, approximately 36 rat pups (Sprague-Dawley, 1 to 24 hours old) were euthanized by decapitation. Ventricles were harvested and placed in a dish containing pre-warmed (to 35°C) phosphate buffered saline (PBS) with glucose (137 mM NaCl, 2.68 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 55.5 mM glucose; pH=7.3). Hearts were washed twice using more PBS-glucose. The ventricles were then transferred to fresh PBS-glucose and minced using scissors into pieces small enough to fit through a 25 ml pipette. The pieces were transferred to a 50 ml sterile polypropylene tube and washed further using PBS-glucose until the supernatant starts to clear. Tissue pieces were subsequently transferred to a water-jacketed (35°C) spinner flask containing 8.5 ml PBS-glucose, 0.5 ml (1480 U/ml) collagenase Type 2, 0.5 ml (740 U/ml) (Worthington Biochemical Corporation, New Jersey), crystallized

trypsin-3x, 0.5 ml (5760 U/ml) deoxyribonuclease I. The mixture was gently agitated in the flask for 10 minutes to dissociate cells, then the supernatant collected into a 100 ml vial containing 10 ml fetal bovine serum (FBS; Gibco, Grand Island, NY). The previous step was repeated for a total of 7 times; all supernatant was collected in the same 100 ml vial. The cells in FBS solution were then passed through a Nytex nylon membrane. Cells were washed once with 1x Ads buffer (116 mM NaCl, 20 mM HEPES, 10 mM $NaH_2PO_4 \cdot H_2O$, 5.5 mM glucose, 5.36 mM KCl, 0.8 mM $MgSO4 \cdot 7H_2O$; pH=7.35), and passed through another Nytex filter. The myocytes were separated through a discontinuous Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient (1.059 g/ml: 1.110 g/ml) in 15 ml tubes, and centrifuged at 2068 x *g* for 30 minutes. The upper fibroblast-enriched layer was discarded, and the myocyte layer from all tubes was collected into a fresh tube. The cells were then washed with Ham's Nutrient Mixture F-10 (Sigma-Aldrich, St. Louis, MO). The pellet was resuspended in 30 ml Myocyte Feeding Medium (10% Ham's Nutrient Mixture F-10, 10% horse serum (Gibco, Grand Island, NY), antibiotics (1000 U/ml penicillin, 1 mg/ml streptomycin). Cells were counted using a haemocytometer and plated at a density of 0.85 x 10^6 cells per 35 mm well. Wells in 6-well dishes (Corning, Inc., Corning, NY) were coated previously with 1 ml of 0.05% rat tail collagen Type I (Upstate Cell Signaling Solutions, Lake Placid, NY) in sterile water and left to try under ultraviolet (UV) light overnight.

B. PLASMIDS AND CONSTRUCTS

The hybrid genes -1058FGFp.*luc* and -110FGFp.*luc*, containing 1112 (nucleotides -1058/+54) and 152 (nucleotides -110/+42) base pairs of the rat FGF-2 gene, respectively, fused upstream of a promoterless firefly luciferase gene in the vector pxp1 [Nordeen, 1988], were described previously [Pasumarthi *et al*, 1997; Jin *et al*, 2000]. A modified -110FGFp.*luc* gene in which three early growth response protein-1 (Egr-1) like elements were mutated (-110mFGFp.*luc*) was reported [Jin *et al*, 2000]. The expression vectors for Egr-1 protein (CMVp.Egr-1) and stimulating protein 1 (CMVp.Sp1) were generous gifts from Dr. V.P. Sukhatme (Beth Israel Hospital, Boston, MA), and Dr. R Tjian (UC Berkely, CA), respectively. The "empty" expression vector pcDNA3 (Invitrogen, San Diego, CA), promoterless luciferase gene vector pxp1 [Nordeen, 1988] and the firefly luciferase gene directed by a minimal (-81/+53) thymidine kinase (TK) promoter [Nordeen, 1988] were used as controls.

C. TRANSIENT GENE TRANSFER

Neonatal rat cardiac ventricular myocytes were transfected using the cationic lipid reagent Lipofectamine PLUS (Invitrogen Life Technologies, Calrsbad, CA; FGF-2 and isoproterenol effect studies), or by calcium phosphate-DNA precipitation (transcription factor overexpression studies).

C.1. Lipofectamine experiments

Lipofection. Briefly, cells at 70 to 90% confluency (16-20 hours after plating) were transferred to Dulbecco's Modified Eagle Medium (DMEM)-F12 with 10% FBS and antibiotics (1000 U/ml penicillin, 1 mg/ml streptomycin) for 4 hours. For every 35 mm well, 2.25 μg DNA was added to 1.98 ml of DMEM in a fresh 50 ml polypropylene centrifuge tube (Corning), and incubated for 15 minutes with 8.5 μl of PLUS reagent. Meanwhile, 8.75 μl Lipofectamine reagent was added to 1.98 ml of DMEM per 35mm well of cells. The Lipofectamine solution was added to the PLUS solution and incubated for another 15 minutes. Two ml of the mix was added to each well after washing twice with calcium- and magnesium-free PBS (PBS-CMF; 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 8.1 mM Na_2HPO_4), and incubated at 37°C with 5% CO_2 for approximately 20 hours.

Gene regulation studies. For gene regulation studies, cells were treated with or without 1 ng FGF-2 per ml of 10% FBS-DMEM-F12 after washing at least two times with PBS-CMF warmed up to 37°C. Cells were washed with PBS-CMF every 24 hours after treatment.

Effect of isoproterenol studies. To determine whether (-)-isoproterenol hydrochloride (IsP; Sigma-Aldrich, St. Louis, MO) has a direct effect on FGF-2 promoter activity, cultured ventricular myocytes were treated with 10 nM IsP as described for the gene regulation studies performed.

C.2. Calcium phosphate-DNA precipitation experiments

Calcium phosphate-DNA precipitation (Overexpression Studies). The calcium phosphate-DNA precipitation method of transfection was done as previously prescribed, with slight modifications [Jin *et al*, 2000]. Briefly, 60 µg of -1058FGFp.*luc* and 30 µg of CMVp.Egr-1, CMVp.Sp1, or pcDNA3 plasmids were made up to a volume of 1.5 ml in 252 mM $CaCl_2$ and added gradually to an equal volume of aerated 2x N-{2-Hydroxyethyl}piperazine-N'-{2-ethanesulfonic acid} (HEPES) buffer, and allowed to precipitate for 30 minutes, and 0.5 ml was added to each of 6 culture dishes (60 mm) containing 5 ml of 10% FBS-DMEM. After 16 hours, the cells were washed with PBS-CMF, and re-fed with 10% FBS-DMEM-F12 with antibiotics, and harvested 24 hours later.

D. CELL HARVESTING; PREPARATION FOR ASSAY

After transfection (lipofection or calcium phosphate-DNA precipitation) and treatment, myocytes were harvested using a rubber policeman spatula in 1.5 ml PBS-CMF, then centrifuged at 2000 x *g* at 4°C for 15 minutes. Pellets were resuspended in 50 to 70 µl 1% Tris-triton (0.1 M Tris pH=7.8, 0.1% Triton X-100), and incubated on ice for another 15 minutes before centrifuging at 15,800 x *g* at 4°C for 15 minutes. Twenty µl of the supernatant was assayed for luciferase activity.

E. LUCIFERASE ASSAY

The Dual Luciferase Kit (Promega Corporation, Madison, WI) was used to assay for luciferase activity essentially according to manufacturer's instructions. Twenty μl of cell or tissue extract was added to a luminometer test tube for assay. One hundred μl of luciferase assay substrate in buffer was added to the tube just prior to luciferase reading, then inserted into the luminometer (EG&G Berthold Lumat LB 9506). The automated luminometer adds 100 μl of Stop & Glo substrate mix prior to luciferase activity reading. Values are given in relative light units (rlu) for both firefly luciferase and renilla luciferase activities. For data presented, firefly luciferase rlu's is corrected for amount of protein (μg) using the Bradford method of protein assay.

F. BRADFORD PROTEIN ASSAY

Cell extract (5 μl) is diluted using water (795 ul). Bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) is diluted into 1, 2, 4, 6, 8, and 10 μg/ml water in two sets of tubes. A blank tube (1 ml water) is also set up for calibration (blank). Two hundred μl of Bradford Protein Assay Dye Reagent Concentrate (Bio-rad Corporation, Hercules, CA) was added to all tubes, and let sit at room temperature for 10 minutes. Concentration values were read using a spectrophotometer (Hitachi U-1100) at 595 nm wavelength. Cell extract values were plotted against the standard curve determined from the BSA values, and are given in micrograms.

G. TRANSGENIC MICE

The generation and characterization of two independent transgenic mouse lines (P66 and P300) expressing -1058FGFp.*luc* was described elsewhere [Detillieux *et al*, 1999].

Determination of isoproterenol dosage. Adult animals (9-12 weeks) were injected intraperitoneally (i.p.) with vehicle (saline), or 80 mg/kg and 160 mg/kg isoproterenol (in saline). After 24 hours, the animals were euthanized and hearts were harvested, and placed in formalin for 24 hours. Fixed hearts were then paraffin embedded and cut into 7 μm sections onto glass slides.

Effect of isoproterenol on FGF-2 regulation. Adult animals (9-12 weeks) were grouped into two, and injected i.p. with vehicle (saline) or 80 mg/kg isoproterenol in saline. The animals were euthanized after 6 or 96 hours, and hearts were harvested. The tissues were fast-frozen on dry ice, and homogenized prior to assay in 1x Lysis buffer (Promega, Fisher Scientific, Mississauga, ON). Insoluble material was removed by centrifugation and luciferase activity was assessed as described above.

Effect of isoproterenol (RNA studies). Adult animals were treated as described above (*Effect of isoproterenol on FGF-2 regulation*). RNA isolation from these hearts, denaturing formaldehyde-gel electrophoresis, and RNA blotting were done as described previously [Detillieux *et al*, 1998]. Atrial natriuretic factor (ANF) [Lytras and Cattini, 1994] , FGF-2 [Biesiada *et al*, 1996], and FGFR-1 [Detillieux *et al*, 1998] cDNAs were used to probe RNA (50 μg). Autoradiographs from RNA blots were assessed by densitometry.

H. HAEMATOXYLIN & EOSIN STAINING

Deparaffination. Hearts were harvested and sectioned as described above in preparation for haematoxylin and eosin staining. Sections were deparaffinized in xylene for a total of 15 minutes, and gradually rehydrated in decreasing concentrations of ethanol in water (95% - 4 mins, 90% - 10 mins, 80% - 10 mins). Rehydrated heart sections were then submerged in PBS prior to unmasking of antigen. Slides were then submerged in citrate buffer (18 ml of 0.1 M citric acid, 82 ml of 0.1 M sodium citrate, 900 ml water; pH=6.0) in a beaker, and placed in the microwave on high for 30 minutes. To keep citrate buffer molarity constant, a beaker of boiling water is kept on hand to replenish any evaporated liquid. Once cool (at least 30 minutes at room temperature), slides were washed with PBS for 10 minutes each, 3 times.

Staining. Slides are removed from PBS and placed in a container of haematoxylin (Harris Modified Haematoxylin with Acetic Acid, Fisher, New Jersey) for 5 minutes, then washed with tap water for 5 minutes, before dipping in acid alcohol (1% hydrochloric acid in 70% ethanol) 8 times. After washing with water for 5 minutes, slides are immersed in ammonia water (3 ml ammonia hydroxide/1 l water) for 2 minutes. Slides were again washed with water, then counterstained with eosin (Sigma, Missouri) for 1 minute, then washed again with water until run off water is clear. Slides were then slowly dehydrated by immersion in increasing concentrations of ethanol in water (70% - 10 dips, 95% - 10 dips, 100% - 4 minutes). Slides were then submerged in xylene for 4 minutes, prior to coverslip mounting using Permount (Fisher, NJ).

I. ELECTROPHORETIC GEL MOBILITY SHIFT ASSAY (EMSA)

Nuclear protein was isolated from whole mouse hearts by a method of hypotonic cell lysis followed by high-salt protein extraction as previously described [Detillieux *et al*, 1998]. The EMSA was done using an established protocol [Lytras and Cattini, 1994; Detillieux *et al*, 1998; Jin *et al*, 2000]. For competition with DNA elements, competitor double stranded oligonucleotides (25-750x mass excess) were added with nuclear extract for 10 minutes at room temperature and then radiolabelled probe for a further 20 minutes. The sequence of one strand from each of the DNA fragments, wild type (WT), and mutant (m), which were used as probes and/or competitors are shown in Table 1.

J. STATISTICAL ANALYSIS

Data presented in the text and figures are mean values plus or minus standard error of the mean. Statistical analysis of the results was carried out using one-way analysis of variance and Tukey-Kramer Multiple Comparisons (post hoc) test, as well as the Student t (parametric) or Mann-Whitney (non parametric) tests (InStat 3.0, GraphPad Software). In all cases a value was considered statistically significant if p was determined to be 0.05 (*). Note: $p < 0.01$ (**).

Chapter III

RESULTS

A. FGF-2 PROMOTER (NUCLEOTIDES -1058/+54) STUDIES

A.1. Addition of exogenous FGF-2 on cardiac myocytes increases FGF-2 promoter activity

To test the hypothesis that FGF-2 may play a part in the regulation of its own synthesis at the transcriptional level, we tested whether the exogenous addition of FGF-2 to cardiac myocytes *in vitro* would stimulate activity of the FGF-2 promoter.

Determination of FGF-2 dose. Neonatal rat cardiac myocytes were isolated and then transfected with a hybrid firefly luciferase gene directed by 1112 bp of rat FGF-2 5' flanking DNA (-1058FGFp.*luc*). Transfected myocytes were then treated with different doses of recombinant human FGF-2 (0.001, 0.01, 0.1, 1, and 10 ng/ml; n=6-13) or without FGF-2 as a control. The cells were incubated in treatment media for 48 hours, then harvested and assayed to determine: (i) whether there is a response to FGF-2, (ii) whether the effect is dose-dependent, and (iii) the optimal FGF-2 dose for the study (Figure 2). Luciferase assay of the cell extracts showed a dose-dependent increase in transfected FGF-2 promoter activity, with a significant difference detected after the 0.1 ng/ml FGF-2 treatment (approximately 1.7-fold when compared to non-treated myocytes where the actual vehicle value is 287±25).

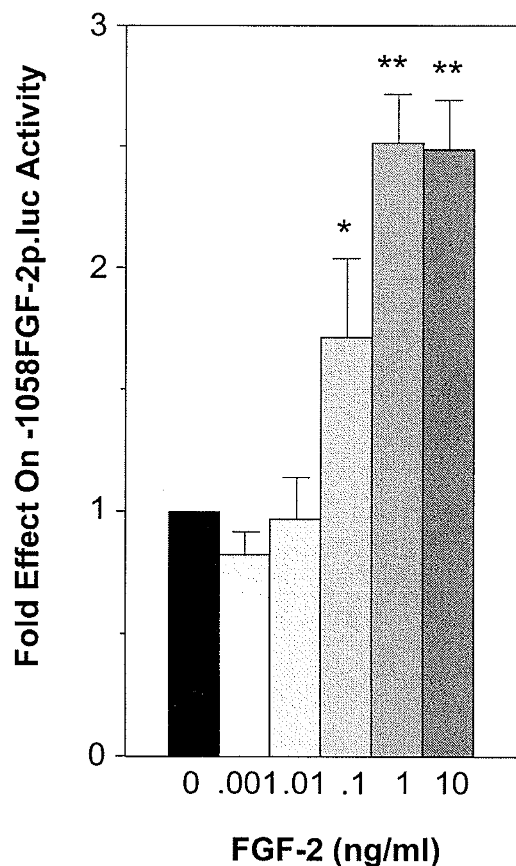

Figure 2. Administration of varying amounts of FGF-2 resulted in the dose-dependent increase in FGF-2 promoter activity in myocytes. Cultured neonatal rat cardiac myocytes were transfected with -1058FGFp.*luc* then treated with different FGF-2 concentrations (0.001, 0.01, 0.1, 1, and 10 ng/ml). Luciferase activity was determined and compared to control plates with medium alone (DMEM-F12). Values are presented as fold effect on promoter activity when compared to controls; measured in rlu/μl protein. Error bars indicate standard error from the mean (SEM).

This dose-dependent increase in activity plateaus at the 1 and 10 ng/ml FGF-2 treatment doses, at approximately 2.5-fold of the promoter activity seen in control transfected myocytes in the absence of FGF-2 treatment. From the profile observed in this experiment, 1 ng/ml FGF-2 was subsequently used in the *in vitro* experiments conducted. All luciferase assay results were corrected with the amount of protein in the extract and are thus expressed as rlu/μg protein.

Determination of FGF-2 effects on its own promoter. To assess whether the treatment time affects the response of the FGF-2 promoter to exogenous FGF-2 addition, neonatal rat cardiac myocytes were isolated and transfected with -1058FGFp.*luc*, then treated with or without (control) 1 ng/ml FGF-2, and harvested at different time points (6, 24, and 48 hours; n=3-18) post treatment (Figure 3). No significant difference in promoter activity was detected 6 hours after treatment when compared to myocytes not treated with FGF-2 (arbitrarily set to 1; actual value is 220±54). Although a 1.4-fold increase in FGF-2 promoter activity was detected at 24 hours (control value = 1484±341), a significant increase in activity is seen only at 48 hours after treatment, where an increase of approximately 2.5-fold in FGF-2 promoter activity was observed, when compared to non-treated control myocytes (control value = 447±58).

The results suggest that exogenous addition of FGF-2 increases transfected FGF-2 promoter activity in cardiac myocytes *in vitro*.

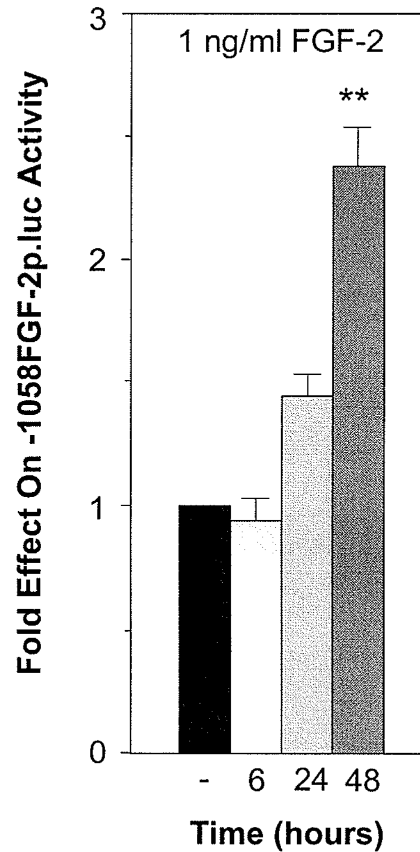

Figure 3. Addition of 1 ng/ml FGF-2 to cardiac myocytes significantly increased transfected FGF-2 promoter activity 48 hours after treatment. Neonatal rat cardiac myocyte cultures were treated with FGF-2 and harvested 6, 24, and 48 hours later. Fold effect of promoter activity when compared to non-treated control (set to 1) is presented in rlu/μl. Error bars indicate SEM.

A.2. **FGF-2 promoter activity is significantly increased in mouse hearts after increased FGF-2 release through β-adrenergic stimulation *in vivo***

In an attempt to examine whether extracellular FGF-2 might stimulate FGF-2 promoter activity *in vivo*, use was made of a transgenic mouse model expressing -1058FGFp.*luc* as a transgene in the heart. This transgenic mouse model allows easy detection of FGF-2 promoter activity (and thus transcriptional activity) in the heart and other tissues [Detillieux *et al*, 1999]. It has been reported previously that the administration of the β-adrenergic agonist IsP to cardiac myocytes induces FGF-2 release both *in vitro* and *ex vivo*. [Clarke *et al*, 1995; Padua and Kardami, 1993] (see Sections G.3 and G.4). This observation was exploited to induce an increase in mouse heart rate, thus increasing FGF-2 release upon contraction.

Isoproterenol dose determination. To determine an appropriate dose of IsP to use for an increase in heart rate, but induce minimal damage to the heart cells (visible through lesions under the microscope), two different doses were tested: 80 mg/kg and 160 mg/kg. Normal CD-1 mice were injected intraperitoneally with either 80 mg/kg or 160 mg/kg IsP, or saline for controls. Hearts were harvested 24 hours after treatment, formalin-fixed, then sectioned into 7 μm slices. Heart sections were stained with haematoxylin and eosin to detect its histology (Figure 4). Pathological examination of the heart sections revealed

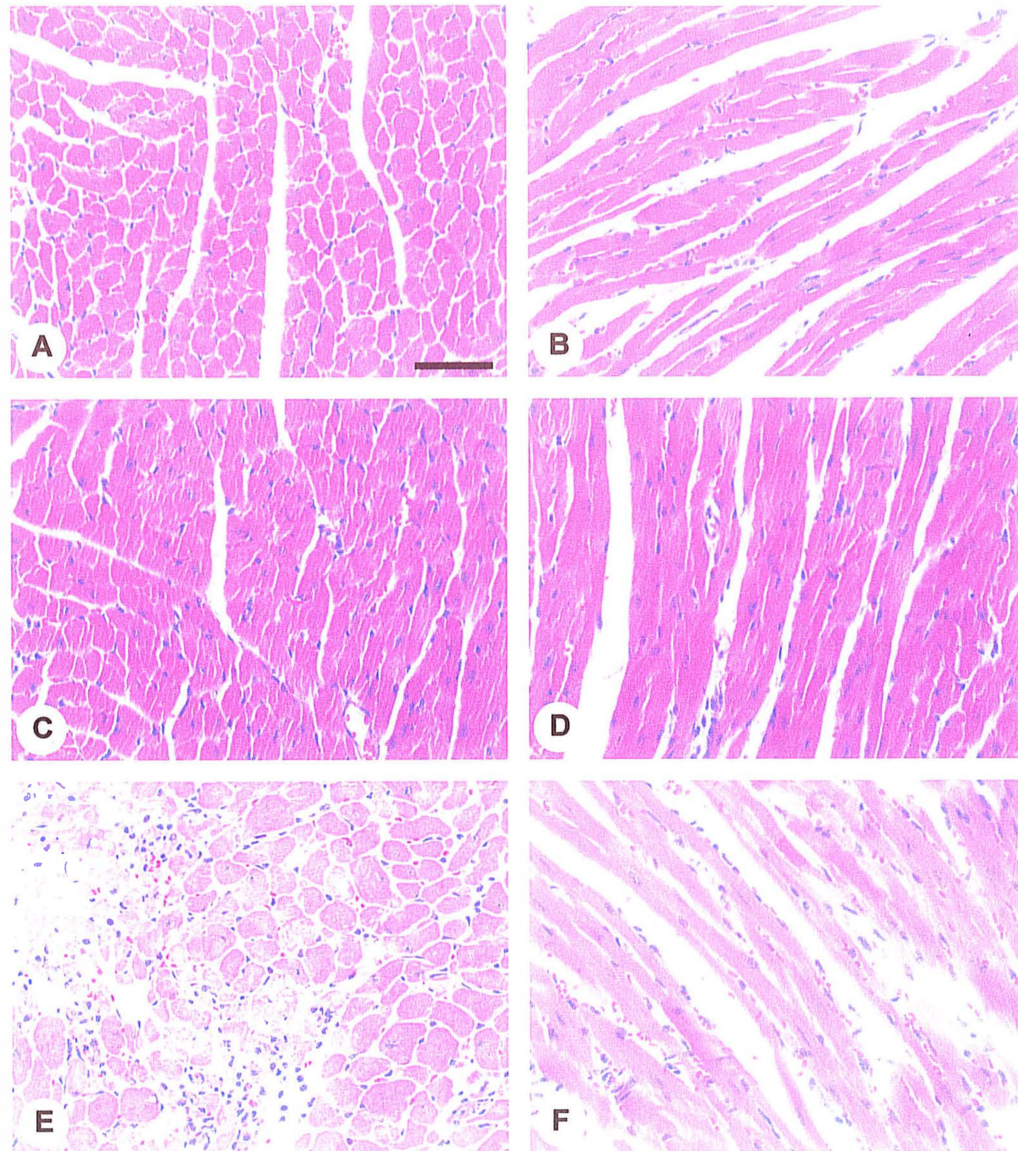

Figure 4. Different doses of isoproterenol (80 or 160 mg/kg) resulted in different histological patterns in adult mouse hearts. Adult mice (9-12 weeks) were injected with 80 (C, D) or 160 (E, F) mg/kg IsP, or saline (A, B). Hearts were harvested 24 hours later, sectioned to 7 μm slices, and stained with haematoxylin and eosin. The first column (A, C, E) show myocytes in cross section, while the second column (B, D, F) show myocytes in longitudinal sections. Note the increased immune cell infiltration in hearts treated with 160 mg/kg isoptroterenol (E, F). Bar is equivalent to 0.1 mm.

lesions in mouse hearts after 160 mg/kg IsP administration whereas the 80 mg/kg tissues did not have lesions visible at 20x magnification. Damage and lesions were characterized by detection of increased infiltration of immune cells,myocyte disarray, and fibrosis [Meij *et al*, 2002]. We therefore used an 80 mg/kg IsP dose for the subsequent *in vitro* experiments.

Determination of isoproterenol effects: heart weight-to-body weight ratio. To determine whether the IsP dose administered (80 mg/kg) is enough to stimulate a response in mouse hearts, we searched for classic markers of hypertrophy, a known effect resulting from β-adrenergic stimulation. Mouse heart weights were measured and compared to their body weights (n=9; Figure 5). Heart weight to body weight ratios were significantly increased 96 hours after IsP treatment. Although a slight increase was detected in heart weight to body weight ratio 6 hours after treatment, this was not significantly different from saline treated mice. However, the heart weight to body weight ratio at 6 hours was also significantly lower than that of the 96 hour samples. This suggests that hypertrophy does result after 80 mg/kg IsP administration in CD-1 mice.

Determination of isoproterenol effects: production of ANF. Levels of ANF mRNA, another classic marker of hypertrophy from β-adrenergic stimulation [Reviewed in Sagnella, 1998], was also measured (Figure 6). Mirroring the results from heart weight to body weight ratios, ANF mRNA levels increased significantly only at 96 hours after treatment. The 0.9 kb ANF band on the

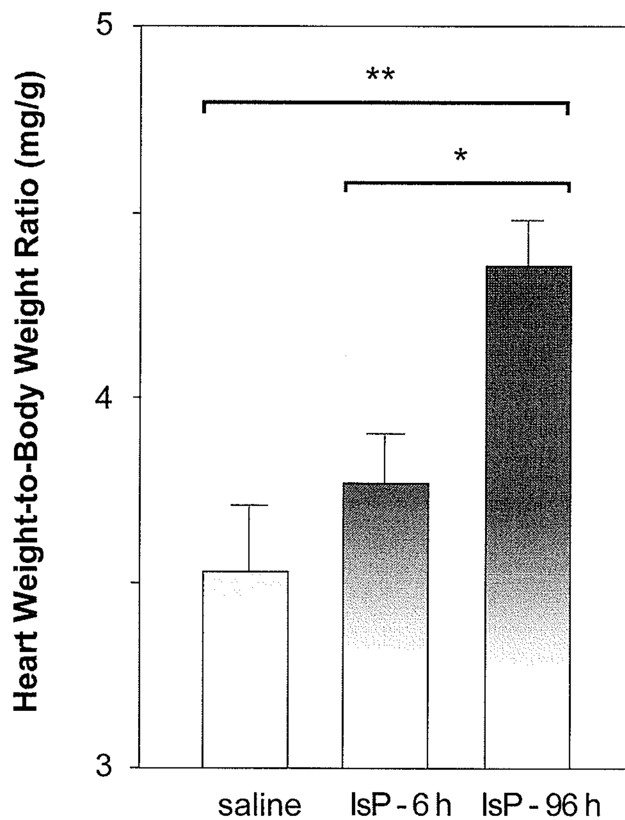

Figure 5. Heart weight-to-body weight ratio significantly increased in mice treated with 80 mg/kg isoproterenol 96 hours after treatment. Adult mice (9-12 weeks) were injected with 80 mg/kg IsP or vehicle (saline) and euthanized 6 or 96 hours later. Error bars indicate SEM. The result shown was provided by Dr. F. Sheikh.

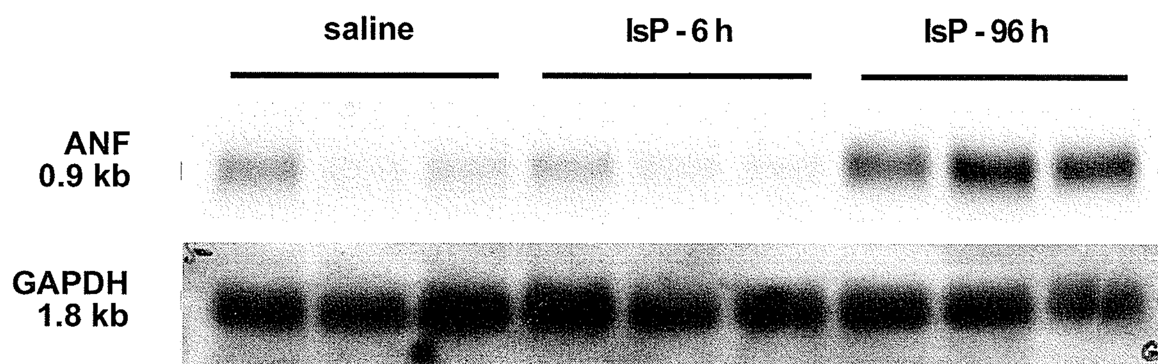

Figure 6. ANF mRNA levels significantly increased in mouse hearts 96 hours after isoproterenol treatment. Adult mice (9-12 weeks) were injected with 80 mg/kg IsP or vehicle (saline) and euthanized 6 or 96 hours later. Total RNA was isolated from ventricles, electrophoresed, blotted, probed for ANF and GAPDH mRNA using radiolabeled cDNAs, and subjected to autoradiography.

northern blot was normalized using the 1.8 kb GAPDH band detected; this assumes that GAPDH is constitutively expressed.

The assessment of heart weight to body weight ratio and detection of increased ANF mRNA levels in mouse hearts after treatment revealed that80 mg/kg of IsP is adequate to stress the heart (presumably increasing heart rate and force of contraction and, thus FGF-2 release) as evidenced by signs of compensatory hypertrophy for the increased workload in CD-1 mouse hearts.

Effects of isoproterenol administration on FGF-2 promoter activity. To indirectly examine whether FGF-2 affects the FGF-2 promoter *in vivo*, transgenic CD-1 mice engineered with the -1058FGFp.*luc* construct were injected intraperitoneally with 80 mg/kg IsP. Transgenic mice were injected with saline for controls. Hearts were harvested 6 and 96 hours after treatment, then assessed for luciferase activity (signifying transgenic rat FGF-2 promoter activity) and normalized to amount of protein (n=4-6; Figure 7). A significant 1.8-fold and 3.3-fold increase was seen in the two transgenic mouse lines (lines P300 and P66) 6 hours after treatment when compared to saline treated mouse hearts (arbitrarily set to 1, where actual value = 339±44). P300 mouse lines were also assessed 96 hours after IsP administration, and although still 1.4-fold higher than controls, this difference is no longer significantly different.

These results suggest that IsP treatment, and thus -adrenergic stimulation, directly or indirectly (possibly through FGF-2 release) increases FGF-2 promoter activity *in vivo*.

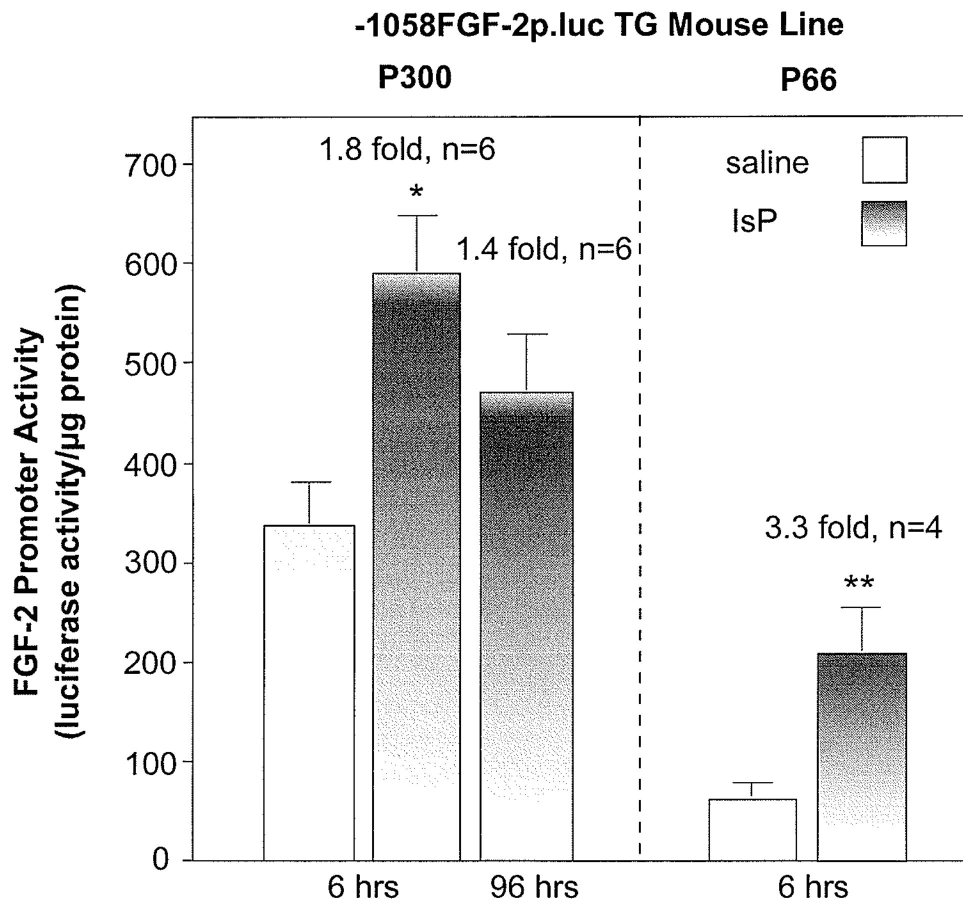

Figure 7. Administration of isoproterenol significantly increased FGF-2 promoter activity in -1058FGFp.*luc* transgenic mouse hearts. Adult transgenic mice from lines P300 and P66 were injected intraperitoneally with saline or 80 mg/kg isoproterenol and euthanized 6 or 96 hours later. Hearts were removed and luciferase activity (rlu/µg protein) was determined. Both lines showed a significant increase in luciferase activity 6 hours after treatment. Error bars indicate SEM. Results shown are a combination of those provided by S.K. Jimenez and K.A. Detillieux.

A.3. Increased endogenous FGF-2 release induced augmented FGF-2 and FGFR-1 mRNA levels in vivo

To determine whether FGF-2 affects its own regulation in the transgenic FGF-2/luciferase mice, we also assessed for endogenous FGF-2 and FGFR-1 mRNA levels in the heart after treatment with IsP (n=3). In contrast to FGF-2 promoter activity (-1058FGFp.*luc*) at 96 hours post-treatment, endogenous 6.1 kb FGF-2 mRNA levels showed a significant 2-fold increase by 96 hours of treatment (Figure 8). Endogenous FGFR-1 mRNA levels was significantly increased in treated hearts 6 hours after administration of IsP, while at 96 hours, levels were comparable to that of vehicle (Figure 9). The endogenous FGFR-1 mRNA assessment reflected the activity pattern of the FGF-2 transgene promoter where an increase at 6 hours is observed, but is closer to control by 96 hours.

These results suggest that IsP treatment, and thus -adrenergic stimulation, directly or indirectly (possibly through FGF-2 release) increases endogenous FGF-2 and FGFR-1 mRNA levels, although accumulation to detectable levels occurred at different time points.

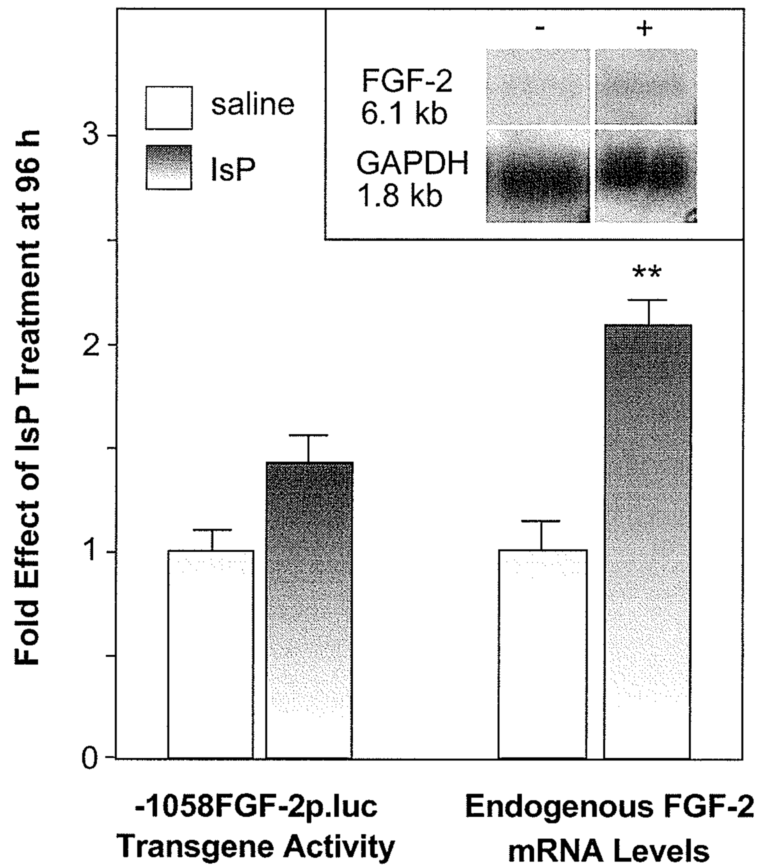

Figure 8. Isoproterenol administration increased endogenous FGF-2 mRNA in the mouse heart 96 hours after treatment. Adult mice were treated with 80 mg/kg isoproterenol and euthanized 96 hours later. Endogenous FGF-2 mRNA accumulation in the heart was determined by northern blotting, and expressed as the mean fold effect of isoproterenol compared to saline levels of the 6.1 kb FGF-2 transcript as determined by densitometry. Values are standardized to GAPDH levels. For FGF-2 promoter activity (as also shown in Fig 7), the results (rlu/μg protein) are expressed as the mean fold effect of isoproterenol, when compared to controls. Error bars indicate SEM.

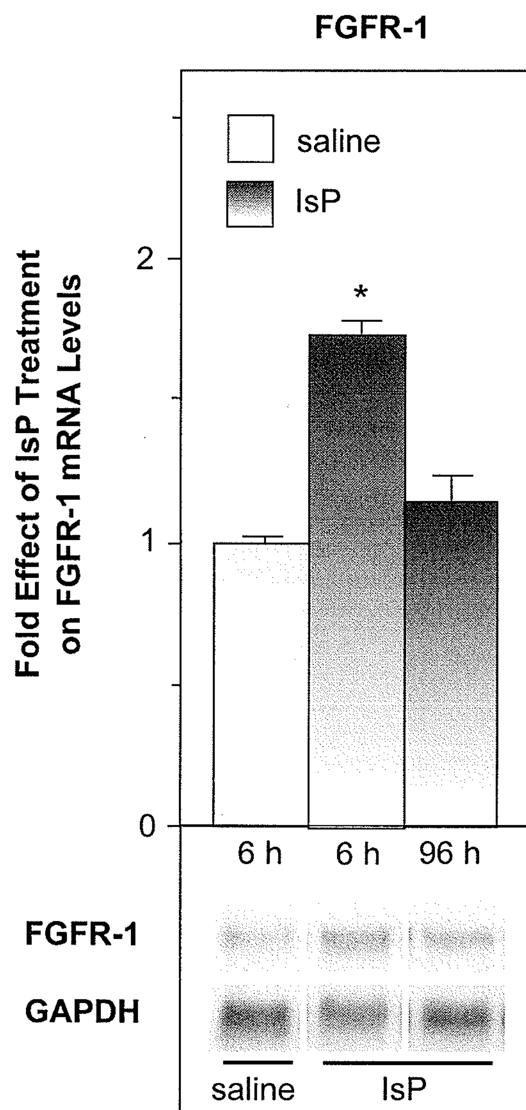

Figure 9. FGFR-1 levels in the adult mouse heart significantly increased 6 hours after isoproterenol treatment. Adult mice (8-10 weeks) were treated with saline or 80 mg/kg isoproterenol and euthanized 6 or 96 hours later. Hearts were excised and total RNA was isolated, electrophoresed, blotted, and probed for FGFR-1 and GAPDH mRNA using radiolabeled cDNAs. Results are shown as the fold effect of isoproterenol administration compared to saline-treated. Autoradiograph is shown inset. Error bars indicate SEM.

A.4. Direct treatment of cultured neonatal rat cardiac myocytes with isoproterenol shows no effect on FGF-2 promoter activity

In an attempt to examine whether there is a direct effect of IsP on FGF-2 promoter activity, cardiac myocytes in culture were transiently transfected with the -1058FGFp.*luc* gene and treated with or without IsP.

Specifically, neonatal rat cardiac myocytes were isolated and transfected with the -1058FGFp.*luc* construct, then treated with 10 μM IsP or vehicle as a control. The cultures were examined under a light microscope (100x magnification) approximately 5 minutes after addition. A higher frequency in contractions/vibrations in myocyte plates with IsP treatment was observed when compared to controls. Cells were harvested 6, 24, and 48 hours after treatment, and luciferase activity was assessed (n=3-6). Luciferase activity was normalized for amount of protein (Figure 10). In contrast to the *in vivo* stimulation with IsP, there was no significant effect of IsP on FGF-2 promoter activity at any of the time points checked. Vehicle values in each experiment are 343±36 for 6 hours, 1820±160 for 24 hours, and 285±43 for 48 hours.

IsP treatment of neonatal cardiac myocytes transfected with a FGF-2/luciferase reporter gene [Nordeen, 1988] results in (i) a visual increase in the incidence of myocyte contractions, but (ii) no effect on transfected FGF-2 promoter activity up to 48 hours after treatment.

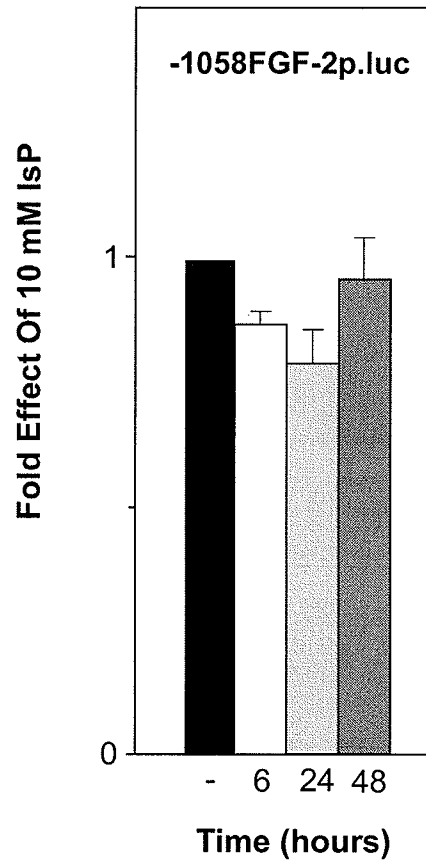

Figure 10. Direct administration of isoproterenol to cardiac myocytes *in vitro* showed no significant difference in FGF-2 promoter activity from control plates. Cultured neonatal rat cardiac myocytes were transfected with -1058FGFp.*luc* then treated with 10 μM isoproterenol. Cells were harvested 6, 24, or 48 hours later, and luciferase values were determined. Values presented are fold effect of isoproterenol treatment when compared to vehicle, measured in rlu/μg protein. Error bars indicate SEM.

B. FGF-2 PROMOTER (NUCLEOTIDES -110/+42 OR -7/+42) STUDIES

B.1. Stimulation of -110FGFp.*luc* expression in neonatal rat cardiac myocytes by addition of FGF-2

Previous characterizations of the FGF-2 promoter region in both human and rat genes revealed the absence of a TATA box (associated with accurate initiation of transcription) and the presence of GC rich sequence [Shibata *et al*, 1991; Pasumarthi *et al*, 1997]. In an attempt to further localize FGF-2 responsiveness, a region of about 150 bp of the rat FGF-2 promoter (nucleotides -110/+42) was isolated and used to generate the hybrid luciferase gene -110FGFp.*luc*. This includes the sub-region of nucleotides -7/+42 that contains several potential binding sites for transcription factors, for example, stimulatory protein-1 (Sp1; implicated in the regulation of several "housekeeping" genes), and the early growth response-1 factor (Egr-1; stress induced). To test for FGF-2 responsiveness, the -110FGFp.*luc* gene was used to transfect neonatal rat cardiac myocytes and tested for the effect of FGF-2 on promoter (luciferase) activity.

Neonatal rat cardiac myocytes were isolated, transfected with -110FGF-2p.*luc*, and then treated with or without 1 ng/ml FGF-2 (n=4-6). Myocytes were harvested 48 hours after treatment and assessed for FGF-2 transgene promoter activity (luciferase activity) (Figure 11). Basal luciferase activity levels were decreased in the myocytes transfected with the -110FGFp.*luc* when compared to that of the -1058FGFp.*luc*. However, treatment with FGF-2

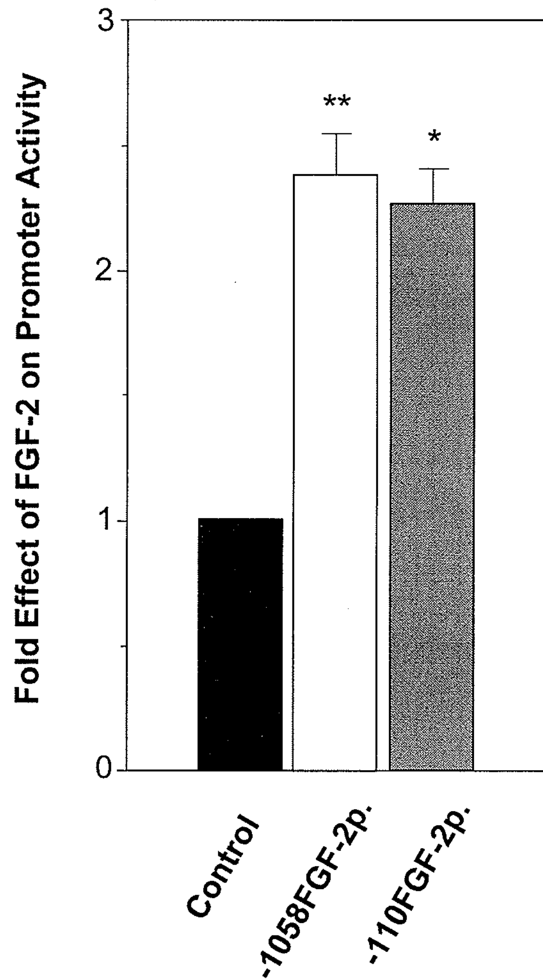

Figure 11. Addition of FGF-2 increases -110FGFp.*luc* promoter activity in cardiac myocytes. Cultured neonatal rat cardiac myocytes were transfected with -110FGFp.*luc* then treated with 1 ng/ml human recombinant FGF-2. Cells were harvested and luciferase activity assessed. Values presented are fold effect of direct FGF-2 administration on transfected promoter activity, presented in rlu/μg protein. -110FGFp.*luc* promoter activity was increased significantly when compared to control (set to 1), but not significantly different from -1058FGFp.*luc* promoter activity. Error bars indicate SEM.

increased -110FGF-2p.*luc* activity approximately 2-fold (where control value is arbitrarily set to 1 and actual control value is 433±39), which is not significantly different from the effect seen in treated -1058FGFp.*luc*-transfected cardiac myocytes (Figure 2).

These results suggest that sufficient sequences for FGF-2 autoregulation are located in the region -110/+42 of the FGF-2 promoter.

B.2. Direct treatment of transfected (–110FGFp.*luc*) cultured neonatal rat cardiac myocytes with isoproterenol shows no effect on FGF-2 promoter activity

To assess whether IsP treatment results in a direct effect on the 152 bp region of the FGF-2 promoter, neonatal rat cardiac myocytes were transfected with –110FGF-2p.*luc*, and treated with or without 10 μM IsP for 6, 24 and 48 hours (Figure 12). Just as with the –1058FGF-2p.*luc*-transfected cells treated with IsP (Figure 10), no significant increases in FGF-2 promoter activity were detected at any of the time points tested.

B.3. Evidence for Sp1 as well as Egr-1 binding to the proximal promoter region of rat FGF-2

The 150 bp proximal promoter region of rat FGF-2 contains putative elements for transcription factors including Egr-1. Coincidentally, studies in other laboratories have previously implicated Egr-1 in the autoregulation of FGF-2 in

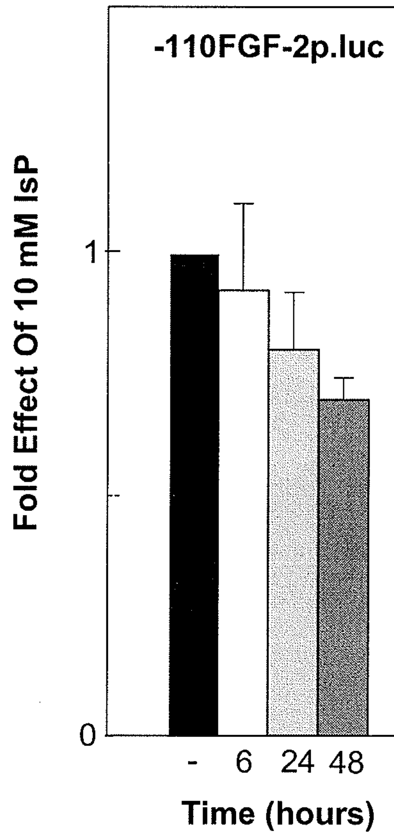

Figure 12. Cardiac myocyte cultures transfected with -110FGFp.*luc* did not increase promoter activity after isoproterenol administration. Cultured neonatal rat cardiac myocytes were transfected with -110FGFp.*luc* and treated with 10 μM isoproterenol in DMEM-F12 and harvested 6, 24, and 48 hours later. No increase is seen in transfected promoter activity. Values are shown as fold effect of isoproterenol on promoter activity, where control values was arbitrarily set to 1. Error bars indicate SEM.

glial cells [Wang *et al*, 1997]. Examination of the putative Egr-1 sites (5'-GCGGGGGCG-3') in the proximal promoter region of rat FGF-2 (nucleotides -7/+42) also reveals overlapping, potential sites, for Sp1 binding (5'-GGCGG-3') (Figure 13). Given the FGF-2 responsiveness of -110FGFp.*luc*, as well as the presence of Egr-1-like and Sp1 elements in the region -7/+42, the capacity of this region to bind Sp1 as well as Egr-1 was assessed by electrophoretic mobility shift assay (EMSA).

Specific Egr-1 and Sp1 protein/DNA complexes were identified by EMSA using consensus DNA elements for Egr-1 and Sp1 (Figure 14) as probes with mouse heart nuclear extracts, and competition by wild type (Egr-1 and Sp1) versus mutant (Egr-1m and Sp1m) oligonucleotide competitors (Figure 13). To assess the relative binding of the putative Egr-1 and Sp1 elements located in the FGF-2 proximal promoter region, double-stranded oligonucleotides containing the putative sites (fragments WT1-3 in Figure 13) were used as competitors of the radiolabeled consensus Egr-1 or Sp1 DNA elements. WT1 but not WT2 or WT3 was able to compete effectively for Egr-1 binding (closed arrowhead, Figure 14). Two base pair mutant forms of WT1 and WT3 (WT1m and WT3m), which would be expected to interfere with both overlapping Egr-1/Sp1 sites, were generated (Table 1). When these were used as competitors, no efficient competition of the specific Egr-1 band was detected. In the case of Sp1, WT1 and to a lesser extent WT3 but not WT2 were able to compete effectively for the specific Sp1 complex. In contrast WT1m and WT3m were not efficient competitors of the specific Sp1 complex.

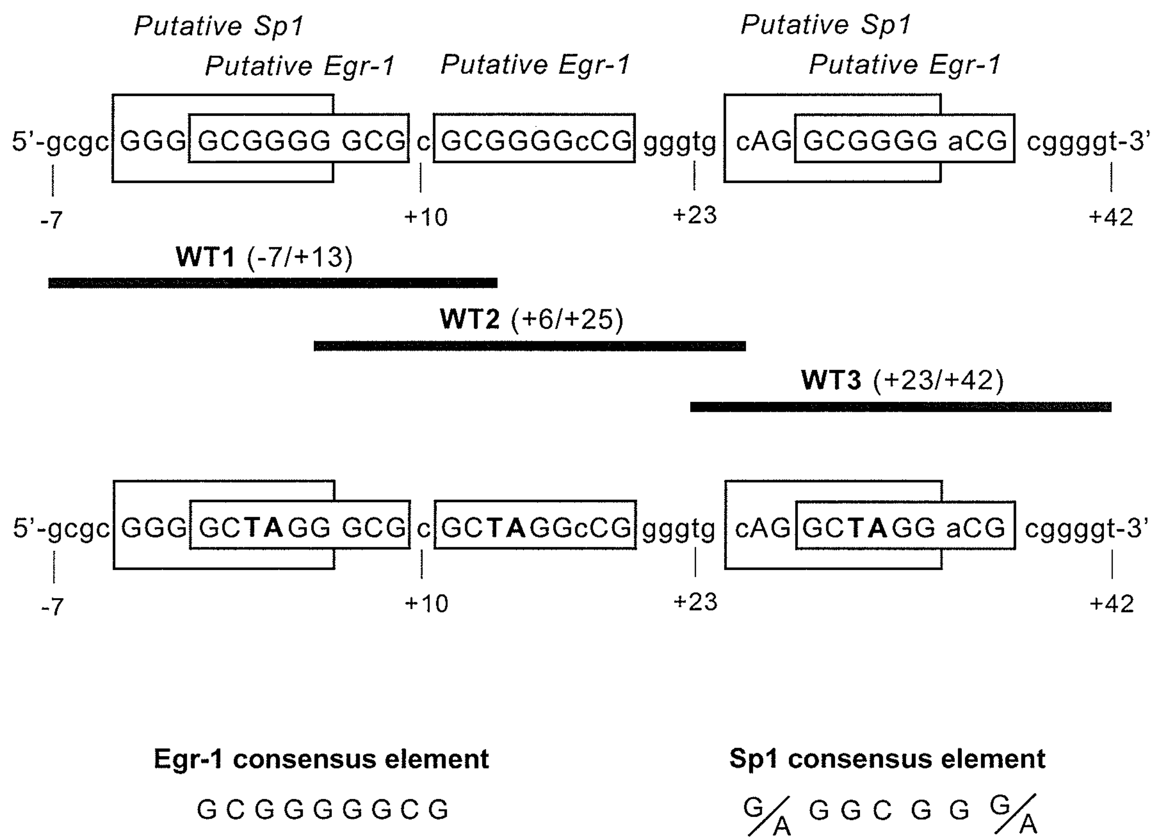

Figure 13. Sequence of the proximal promoter region of rat FGF-2 corresponding to nucleotides -7/+42. Disrupted sequences are indicated by bold type (TA). Putative Egr-1 (small boxes) and overlapping Sp1 (large boxes) sites are indicated. The subfragments WT1, WT2, and WT3 used to assess Egr-1 and Sp1 binding and their consensus sequence are shown.

Table 1. Sequences of oligonucleotides or primers used for experiments. Mutated nucleotides are indicated by lower case letters.

Name	Probe (P) / Competitor (C)	Oligonucleotide or Primer Sequence
Egr-1	P / C	5´-GGATCCAGCGGGGGCGAGCGGGGGCGA-3´
Egr-1m	C	5´-GGATCCAGCtaGGGCGAGCtaGGGCGA-3´
Sp1	P / C	5´-ATTCGATCGGGGCGGGGCGAGC-3´
Sp1m	C	5´-ATTCGATCGGttCGGGGCGAGC-3´
WT1	C	5´-GCGCGGGGCGGGGGCGCGCG-3´
WT1m	C	5´-GCGCGGaGaGGGtGCGCGCG-3´
WT2	C	5´-GGCGCGCGGGGCCGGGGTGC-3´
WT3	C	5´-TGCAGGCGGGGACGCGGGGT-3´
WT3m	C	5´-TGCAGtCGaGGACGCGaGGT-3´

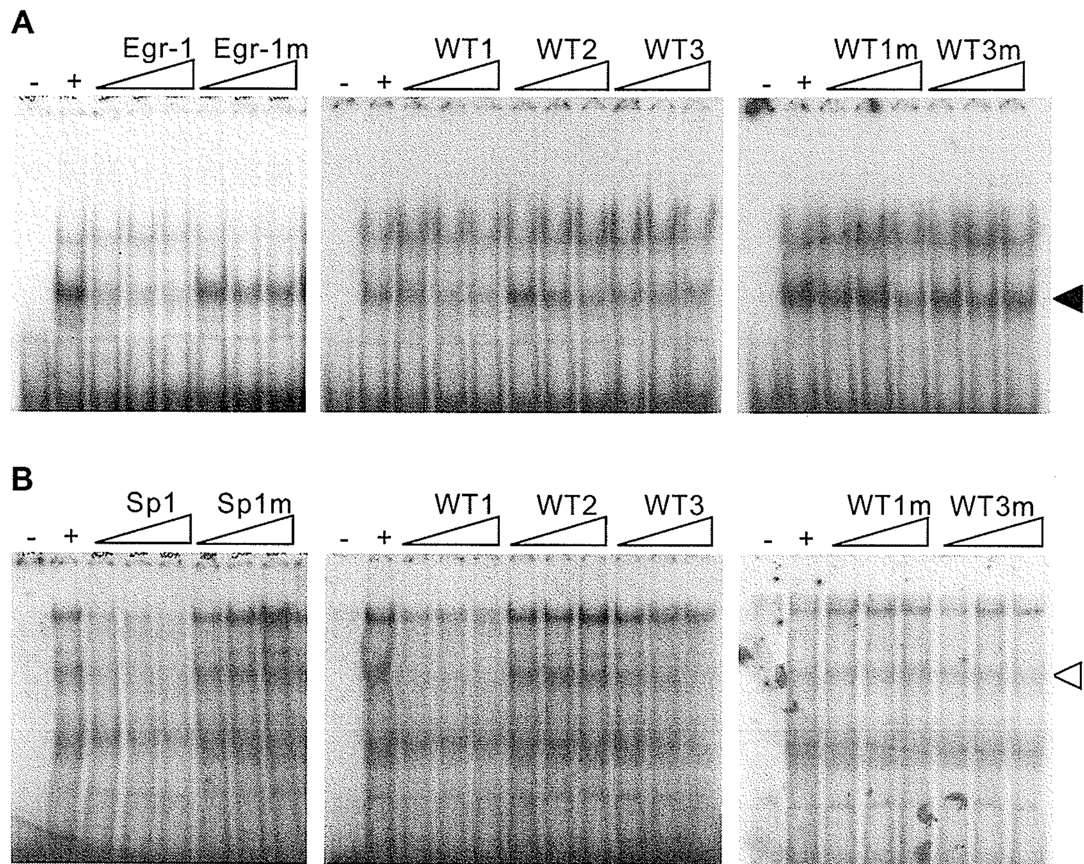

Figure 14. DNA binding studies showed competition for Egr-1 by WT1 and Sp1 by WT1 and WT3. Nuclear extract was isolated from adult mouse hearts and used in combination with a radiolabeled (A) Egr-1 or (B) Sp1 consensus DNA element as probes for EMSA. Multiple complexes are seen in the presence (+) versus absence (-) of nuclear protein after non-denaturing gel electrophoresis and autoradiography. Specific Egr-1 and Sp1 complexes were identified by competition with increasing amounts of consensus Egr-1 (100, 500, and 750x mass excess) and Sp1 (25, 50, and 100x mass excess) versus mutant (Egr-1m and Sp1m) DNA elements. Closed and open arrowheads indicate the mobility of major high affinity/specific Egr-1 (A) and Sp1 (B) complexes, respectively. The relative ability of oligonucleotides corresponding to WT1, WT2, and WT3, or mutated variants WT1m and WT3m, to compete (25, 50, and 100 mass excess) for these specific complexes is shown. Results provided by J. Dhaliwal.

B.4. Overexpression of Egr-1 or Sp-1 increases FGF-2 promoter activity *in vitro*

The effect of Sp1 overexpression on FGF-2 promoter activity in transfected neonatal rat cardiac myocytes was assessed, based on the binding of Sp1 (Figure 14) and the potential for regulation of FGF-2 by Sp1.

Neonatal rat cardiac myocytes were isolated and then co-transfected with the -1058FGFp.*luc* construct and CMVp.Sp1 (Sp1 expression vector). Separate plates of cardiac myocytes were also co-transfected with CMVp vector (cytomegalovirus promoter alone) for a negative control, or CMVp.Egr-1 (Egr-1 expression vector) for a positive control. Assessment of luciferase activity showed a significant increase in transfected FGF-2 promoter activity in cardiac myocytes overexpressing either Sp1 or Egr-1 when compared to vector control (vector value = 369±53). Sp1 overexpressing myocytes showed approximately an 8.8-fold increase when compared to vector control while Egr-1 overexpressing myocytes showed a significant 4.5-fold increase (n=3; Figure 15).

These results suggest that an increase in either Sp1 or Egr-1 expression in cardiac myocytes stimulates a significant increase in FGF-2 promoter activity when compared to controls.

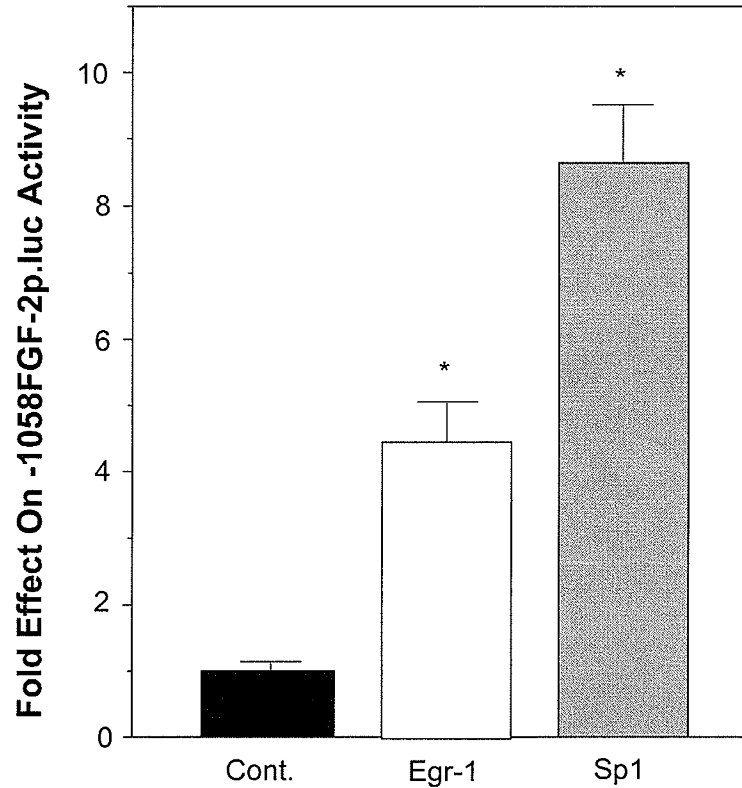

Figure 15. Overexpression of Egr-1 or Sp1 significantly increased -1058FGFp.*luc* promoter activity. Cultured neonatal rat cardiac myocyte were transfected with -1058FGFp.*luc* and co-transfected with empty expression vector (Ctrl) or expression vectors for Egr-1 or Sp1. Cells were harvested 48 hours later, and luciferase activity was determined. Values presented are mean fold effect on transfected promoter activity (rlu/μg protein) when compared to control values. Error bars indicate SEM.

B.5. Mutation of Egr-1 and Sp1 sites in the FGF-2 proximal promoter region decreases basal expression but has no effect on FGF-2 autoregulation in cardiac myocytes

The presence of Egr-1 (WT-1) and Egr-1-like (WT-2 and WT-3) DNA elements in the proximal promoter region of rat FGF-2 (nucleotides -7/+42; Figure 13) as well as the involvement of Egr-1 in FGF-2 autoregulation of glial cells [Wang *et al*, 1997], made them, and specifically the site represented by WT-1, likely candidates for similar regulation in cardiac myocytes. In a previous study, the Egr-1 sites (in WT-1, WT-2, and WT-3) in the -110FGFp.*luc* construct were mutated to form -110mFGFp.*luc*, and this was shown to abrogate the effects of α-adrenergic stimulation on the transfected FGF-2 promoter in neonatal rat cardiac myocytes [Jin *et al*, 2000]. Interestingly, the mutations made in -110mFGFp.*luc* would also be predicted to disrupt two consensus Sp1 sites, which overlap the Egr-1/like elements in WT-1 and WT-3, and thus the WT-1-related Sp1 site identified by this study (section B.3., Figure 14). Therefore, this same mutant construct was used in an attempt to examine the involvement of the Egr-1/Sp1 elements located in the proximal promoter region of FGF-2 (nucleotides -7/+42) in FGF-2 autoregulation in neonatal cardiac myocytes in the context of -110FGFp.*luc*. In addition, an unrelated minimal Herpes Simplex thymidine kinase promoter (nucleotides -81/+53) fused to the luciferase gene

(TKp.*luc*), as well as a promoterless luciferase gene (-p.*luc*) were also used for comparison.

Neonatal rat cardiac myocytes were transiently transfected with the mutant -110FGFp.*luc* construct, or TKp.*luc*, or (promoterless) –p.*luc* then treated as before, with 1 ng/ml FGF-2 for 48 hours. Myocytes were then assessed for basal FGF-2 promoter activity as well as relative response to FGF-2 treatment (n=4-6; Figure 16). The basal FGF-2 promoter activity for the "mutant" construct was 283±28 and was significantly lower (approximately 35%) than that observed with the "wild type" -110FGFp.*luc* gene (433±39) in untreated myocytes. However, mutant -110FGFp.*luc* gene expression was still increased significantly in response to FGF-2 treatment. A significant 1.7-fold increase is also observed in TKp.*luc*-transfected myoctes (basal activity 142±15), and no increase was observed in the activity of the –p.*luc* (basal activity 51±15).

In summary, the significant decrease in basal promoter activity observed on mutation of the -110FGFp.*luc* suggests that important regulatory information is contained in the proximal promoter region of rat FGF-2. However, the effects of FGF-2 treatment on FGF-2 promoter activity is not abolished by the sequence mutation. The results also suggest that Egr-1 and Sp1 sites in the region -7/+42, and particularly those characterized and represented by WT-1 sequences, are not required for FGF-2 responsiveness under the conditions tested.

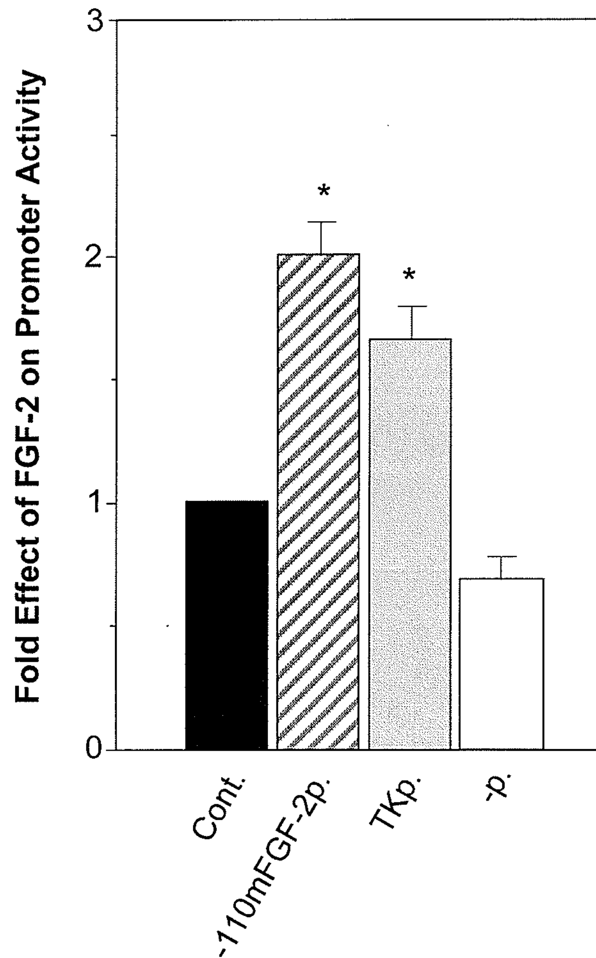

Figure 16. Addition of FGF-2 to cardiac myocytes significantly increased transfected FGF-2 promoter activity (-110FGFp.*luc*) 48 hours after treatment. Neonatal rat cardiac myocyte cultures were transfected with either vector (-p.), TKp., or mutant -110FGFp.*luc*, then treated with FGF-2 and harvested 6, 24, and 48 hours later. Fold effect of promoter activity when compared to non-treated control is presented in rlu/μl. Error bars indicate SEM.

Chapter IV

DISCUSSION

A. RATIONALE

Cardiovascular disease is becoming the most frequent cause of death worldwide, and is currently responsible for approximately 36% of all deaths in Canada [Heart and Stroke Foundation of Ontario, 1999[c]]. In the majority of the cases, deaths from cardiovascular disease are caused by coronary artery disease and other diseases that affect the vasculature, which supplies blood supply to the myocardium. The cessation of blood flow to the myocardium results in ischemia, and myocyte cell death, which leads to other pathologies (including arrhythmia and angina) and may lead to cardiac failure. Survivors of a myocardial infarction are reported to experience a poorer quality of life [Heart and Stroke Foundation of Ontario, 2003[d]]. This may be due to the multiple restrictions placed on these patients due to the inferior functional capacity of their hearts coupled with increased workload post-MI. The most obvious method of preventing heart disease is through the prevention of vasculature problems, and is currently being extensively promoted by the Heart and Stroke Foundation of Canada and the American Heart Association of the United States through healthy lifestyle living. There are also in place several methods of reinstating blood flow

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[c] Heart and Stroke – Just the facts 2002/2003 edition. Retrieved November 20, 2003, from http://www.heartandstroke.ca

[d] Heart and Stroke – Just the facts 2002/2003 edition. Retrieved November 20, 2003, from http://www.heartandstroke.ca

to the myocardium post-ischemia that involves surgery (including PTCA and CABG) and although improving, are still quite invasive. Another less invasive approach, is the idea of protecting the myocardium (cardioprotection) making the cells less susceptible to injury in the case an insult does occur.

A substance naturally produced and released in the heart, FGF-2, is known to be a potent angiogenic agent, and is currently being used in clinical trials to alleviate chest angina and to improve collateral blood flow in ischemic patients [Sellke *et al*, 1998; Laham *et al*, 1999; Unger *et al*, 2000; Ruel *et al*, 2002]. In addition, FGF-2 has been shown to be a cardioprotective molecule [Kardami *et al*, 1993; Padua and Kardami, 1993; Sheikh *et al*, 2001; Jiang *et al*, 2002]. Addition of FGF-2 to cardiac myocytes *in vitro* and *ex vivo* have been shown to result in the decreased release of cardiac markers of injury, after ischemia and reperfusion, presumably, in part, through maintenance of cardiac myocyte membrane integrity [Kardami *et al*, 1993; Padua and Kardami, 1993; Sheikh *et al*, 2001; Jiang *et al*, 2002]. *In vivo*, improvement of left ventricular ejection fraction was also noted when compared to non-FGF-2-treated rat hearts after ischemia-reperfusion [Jiang *et al*, 2002]. Clearly, FGF-2 cardioprotective property presents a plausible method for the prevention of heart disease that can be developed for humans. Given that FGF-2 is naturally-produced and released in the heart, there is a potential for the development of methods in which cardioprotective properties may be exploited by non-invasive means. Certainly, it is important to understand the mechanisms that govern FGF-2 gene regulation in the heart before we can fully exploit its endogenous cardioprotective properties.

B. FGF-2 AUTOREGULATION

B.1. Findings and previous reports

The possibility of autoregulation was previously hypothesized based upon the observation that FGF-2 is synthesized and stored in the same cells that it acts upon. It was then shown that FGF-2 can induce an increase in its own mRNA in capillary endothelial cells. [Weich *et al*, 1991]. Furthermore, it has been reported that FGF-2 can regulate its own gene expression in glia cells [Biesiada *et al*, 1996, Wang *et al*, 1997]. In this thesis, a combination of *in vitro* and *in vivo* gene transfer studies were used to examine the regulation of FGF-2 synthesis at the transcriptional level in cardiac myocytes. Specifically, an indirect mechanism of control is suggested by the ability of the β-agonist isoproterenol to stimulate FGF-2 promoter activity in transgenic mouse hearts (*in vivo*) but not neonatal rat cardiac myocyte cultures (*in vitro*). FGF-2 autoregulation may provide this mechanism, based on the reported increase in FGF-2 release in response to increased heart rate and inotropy with β-adrenergic stimulation *in vivo* [Padua and Kardami, 1993; Clarke *et al*, 1995], and our ability to increase FGF-2 promoter activity in response to direct FGF-2 treatment of transfected neonatal rat cardiac myocyte cultures. However, unlike the report of autoregulation of FGF-2 in astrocytes [Biesiada *et al*, 1996; Wang *et al*, 1997], this does not appear to require intact GC-rich Egr-1 elements in the proximal promoter region (-7/+42). Nonetheless, increased expression of both Egr-1 and Sp1, which also binds GC-rich DNA, were able to increase FGF-2 promoter activity. Thus, while the capacity for autoregulation of FGF-2 synthesis exists, our

results are also consistent with the presence of multiple mechanisms to ensure constitutive FGF-2 promoter activity in cardiac myocytes (Figure 17).

B.2. At the level of transcription: possible mechanisms

Egr-1 involvement. The transcription factor Egr-1, also known as NGFI-A, krox24 and TIS8, has been implicated in the regulation of FGF-2 promoter activity, through α-adrenergic stimulation of cardiac myocytes and, autoregulation in astroglial cells [Detillieux *et al*, 1999; Biesiada *et al*, 1996]. Previously it was observed that the proximal promoter region of the rat FGF-2 gene (nucleotides –7/+42), contains three putative Egr-1 elements, and demonstrated that this region can bind Egr-1 [Jin *et al*, 2000]. However, it was not determined as to which of the three, or whether a combination, of the sites was capable of binding Egr-1 from a nuclear protein extract from adult mouse hearts. The data presented here indicate that the site located at nucleotides +1/+9 binds Egr-1 by virtue of its ability to compete with a high affinity Egr-1 binding site (Fig. 7A). Competition with WT2 and WT3 was not observed. This suggests that if Egr-1 does bind these regions, it does so with low affinity, which may be explained by the base mismatch with a consensus Egr-1 DNA element (Figure 13). Nonetheless, upon mutation of the Egr-1 site, continued FGF-2 responsiveness was observed in WT1 as well as the Egr-1 like sites in WT2 and WT3, using "GG" to "TA" dinucleotide mutations shown previously to interfere with binding [Jin *et al*, 2000]. Sequence analysis using the Transcription Factor Database (TRANSFAC) has shown no further consensus Egr-1 sites within nucleotides -110/+42

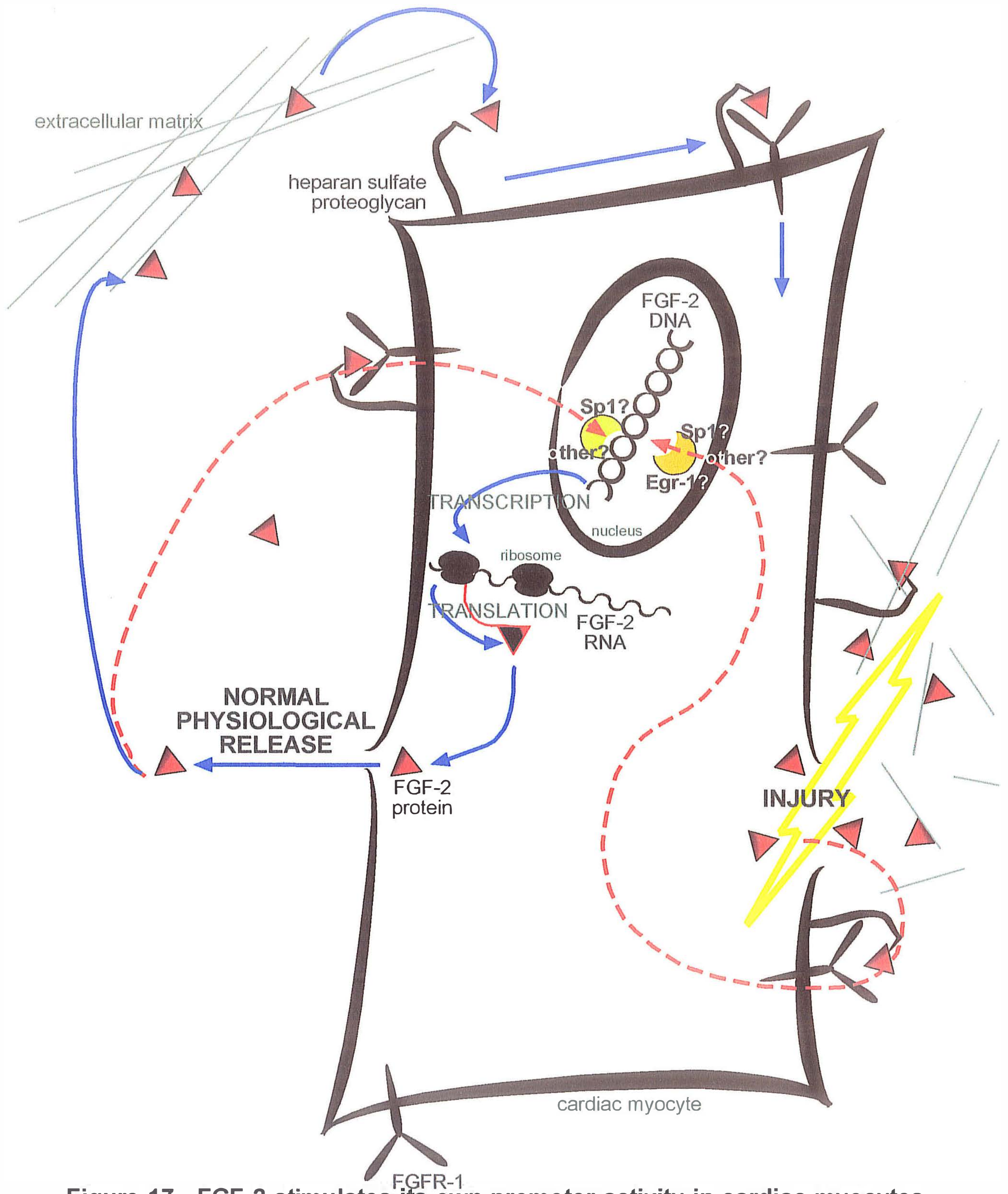

Figure 17. FGF-2 stimulates its own promoter activity in cardiac myocytes. Binding and overexpression studies suggest Egr-1 and Sp1 can regulate FGF-2 promoter activity, however, their role in FGF-2 autoregulation may not be essential and/or require additional, as yet unidentified, factors.

(or –1058/+54) of the rat FGF-2 promoter region. Thus, these data suggest that Egr-1 binding is not essential for FGF-2 regulation of its own promoter in cardiac myocytes, using the methods employed. Competition studies may help elucidate further the role that Egr-1 may play in the regulation of FGF-2 in the cardiac myocyte, as will be discussed in Section D.1.

Sp1 involvement. The FGF-2 gene promoter regions (human or rat) do not contain the conventional TATA sequences, but are rich in GC sequences, which could bind, besides Egr-1, the transcription factor Sp1 and are also characteristic of so-called "house-keeping" or constitutively active genes [Laniel *et al*, 1997; Philipsen and Suske, 1999; Song *et al*, 2001; Torigoe *et al*, 2003]. The Sp family of transcription factors has previously been linked to expression of genes in the heart [Takizawa *et al*, 2003]. Examples of consensus Sp1 binding sequences, as determined from TRANSFAC, can overlap with the Egr-1 sites observed in the rat FGF-2 promoter (Figure 13) [Lin and Leonard, 1997; Molander *et al*, 2001]. This allows for the possibility of a complex response based on displacement of one factor by the other, as has been proposed previously [Wang *et al*, 1997]. Given the previous link between Egr-1 and FGF-2 autoregulation, the possibility that Sp1 is involved in regulation of the FGF-2 promoter was investigated, specifically through any potential overlapping Egr-1/Sp1 sites. The results show that the overexpression of Sp1 in transfected neonatal rat cardiac myocytes results in a significant stimulation of the FGF-2 promoter, suggesting the possibility that this transcription factor indeed plays a role in the regulation of FGF-2 in cardiac myocytes. In addition, there was

evidence for two Sp1 binding sites (nucleotides -3/+6 and +25/+33) overlapping two putative Egr-1 sites in oligonucleotides WT1 and WT3, respectively. However, the mutation of these Sp1 sites was not able to inhibit FGF-2 responsiveness of the -110FGF-2p.*luc* gene. The core of the consensus Sp1 element (5′-GGGCGG-3′) is shorter than that of the related Egr-1 element (5′-GCGGGGGCG-3′) and overall is associated with more variability in terms of alternative binding sites, including the related CACC-box [Philipsen and Suske, 1999; Flesch, 2001]. Thus, given the GC-rich nature of the FGF-2 promoter region (-110/+42 is 71% GC) and the ability of Sp1 to bind alternative sequences, it is not clear whether an additional site or sites might have contributed to FGF-2 responsiveness. Evidence that a single Sp1 sequence may be sufficient for a functional response to FGF-2 treatment is indicated by the results with the minimal thymidine kinase (TK) promoter in TKp.*luc*. Sequence analysis reveals that the TK promoter corresponding to nucleotides -81/+52 contains a single consensus Sp1 element (5′-GGGCGGG-3′) [McKnight *et al*, 1981]. FGF-2 treatment in transfected cardiac myocytes significantly increased its own promoter activity, suggesting that this one binding site is capable of regulating the FGF-2 gene. In addition, there are at least 11 potential Sp1 and CACC-box-related sequences within nucleotides –1058/+54 of the rat FGF-2 gene, where five are located in the proximal promoter region (nucleotides –110/+42). These sequences might be sufficient to support factors requiring GC-rich motifs to bind with, perhaps, low affinity and possibly regulate FGF-2 transcription. However, it would be difficult to determine the relative importance of these GC-rich

sequences for regulation given their role in basal promoter activity, particularly in the absence of a TATA sequence. FGF-2 promoter activity was reduced ~35% with disruption of Sp1 elements in WT1 and WT3 alone. This value did not change significantly (~32%) when firefly luciferase values for each construct were corrected for DNA uptake using values obtained from co-transfection with a Renilla luciferase gene (data not shown).

B.3. At the level of the cardiac myocyte: autoregulation of FGF-2

Direct administration of recombinant human FGF-2 on cardiac myocytes in culture, the results presented support the hypothesis that FGF-2 can regulate its own gene expression at the level of transcription (assessment of promoter activity). This observation is consistent with other studies that have reported the increase of FGF-2 mRNA after addition of FGF-2 in endothelial cells [Weich *et al*, 1991] and in cardiac myocytes [Fischer *et al*, 1997] *in vitro*. The increased accumulation of FGF-2 mRNA after FGF-2 administration supports the hypothesis that FGF-2 is autoregulated at the transcriptional level.

Although it has been reported that FGF-2 mRNA increases after addition of FGF-2 to culture media, FGF-2 autoregulation at the transcriptional level has not been shown *in vivo*. It is, however, difficult at present to induce the release of a specific protein *in vivo*. In the case of FGF-2, which is not released through the classical ER/Golgi pathway, reported options for release include many mechanisms (Chapter I, Section E.2), mostly occurring through the "controlled leakage" from transient sarcolemmal disruptions [Floege *et al*, 1992; Clarke *et al*,

1995; Kaye *et al*, 1996]. Beta-adrenergic stimulation (using IsP) was used to stimulate muscle contraction and thus FGF-2 release [Clarke *et al*, 1995].

An 80 mg/kg dose of IsP was used to treat transgenic mice and was sufficient to increase heart rate, force of contraction and, presumably, FGF-2 export. The effectiveness of this IsP treatment in inducing adrenergic stimulation is supported by the resulting hypertrophy by 96 hours post-treatment. Specifically, a significant increase in heart weight to body weight ratio and ANF gene expression were observed (Fig. 6). This dose, unlike the higher 160 mg/kg IsP used previously [Meij *et al*, 2002; Detillieux *et al*, 2003], did not generate obvious evidence of lesions (number or size) as determined by sectioning, histochemical and microscopy analyses (data not shown).

Stimulation of cardiac myocytes with IsP results in the release of FGF-2 [Clarke *et al*, 1995]. Increased FGF-2 accumulation in rat hearts after systemic administration of injury-inducing doses of IsP is also reported [Padua and Kardami, 1993]. The data presented here suggest that β-adrenergic stimulation can cause a stimulating effect on FGF-2 transcription in the heart, whether directly or indirectly. More importantly, these results suggest that the effect on FGF-2 promoter activity through -1058FGF-2p.*luc* transgene expression reflects a "normal" response.

B.4. Response to IsP: *in vitro* versus *in vivo*

B.4.a. *Endogenous and transgene expression*

There was a difference between endogenous FGF-2 transcription and that of the introduced gene as to the timing of the response after IsP treatment. The increase in endogenous FGF-2 transcription was detected at 96 hours whereas transgene expression (-1058FGF-2p.*luc*) was detected by six hours. This observation presumably reflects differences in the stage of the synthetic pathway assessed, specifically, mRNA accumulation versus promoter activity, as well as the stability of the gene products concerned and sensitivity of the respective assays employed.

B.4.b. *Direct or indirect IsP effect*

To distinguish whether a direct effect of IsP caused the effect on FGF-2 promoter activity and transcript accumulation, transfected cardiac myocytes were treated with the agonist, and then assessed. Although transfected promoter activity was assessed at all the time points used in the FGF-2 administration culture experiments (where an effect from 24 hours onwards was evident), no significant changes were detected in transfected FGF-2 promoter activity in IsP-treated cardiac myocytes at any time point.

Because of the nature of the two models used (*in vitro* versus *in vivo*) in these experiments, it is difficult to conclude that what is seen in the results of one is an accurate representation of the other. Some explanations for the observed

difference in response include the differences in neonates versus adult heart properties, species, and protein concentrations.

Adrenergic system maturity. If neonatal rat cardiac myocytes are a good model, then the results may imply that IsP does not cause a direct effect on the FGF-2 promoter in cardiac myocytes. Nonetheless, there remains the difference in the developmental stage difference between the neonatal model and the adult transgenic model. The maturity of the β-adrenergic system may be a factor in the response. An immature system may not trigger a normal adrenergic response. Characterization of the β-adrenergic receptors in rabbit hearts showed a significantly higher receptor affinity and density in the adults when compared to neonates [Schumacher *et al*, 1984]. If the adrenergic system in the neonatal rat is not mature enough, it is possible that the response seen *in vivo* may be a direct effect of IsP on FGF-2 regulation beyond the effects of the increased FGF-2 release. However, cardiac myocytes had increased rate of contraction after treatment with IsP, suggesting an adrenergic stimulus. In addition, it requires dosages of IsP higher than 10 μM to induce hypertrophy [Tomita *et al*, 2003]. There are also many studies that have utilized primary cultures of neonatal rat cardiac myocytes and observed a response to adrenergic stimulation [Ramos *et al*, 1984; Ungureanu-Longrois *et al*, 1995].

Cell developmental stage. As previously mentioned, cardiac myocytes used in culture were harvested from neonatal hearts, in contrast to the adult cardiac myocytes from transgenic mouse hearts used for *in vivo* experiments. Besides the possible difference in β-adrenergic system maturity, there are many

different properties between neonatal and adult cardiac myocytes. Some differences include contraction properties [Reviewed in Anderson, 1989], expression of cell surface heparan sulfate proteoglycans [Asundi *et al*, 1997], and expression of certain genes such as Troponin T (isoform switch) [Anderson *et al*, 1995]. Any of these differences may contribute to the difference in response observed between *in vitro* and *in vivo* studies.

Difference in species. In addition to the different properties between neonates and adults as mentioned above, there are also differences in properties due to the animal model used that may affect response to IsP. Some differences between mice and rats include myosin heavy chain content [Franco *et al*, 2002], velocity of myocyte shortening and relaxation [Harding *et al*, 1990], and metabolism of carbohydrates and lipids [Menahan and Sobocinski, 1983].

Inhibitory peptides. It is, therefore, a very real possibility that the changes seen upon IsP-administration *in vivo* may be the result of the increased release of FGF-2 both from the cardiac myocytes and the extracellular matrix stores. IsP has been reported to upregulate the synthesis of some genes [Zhou *et al*, 1997] including FGF-2 in the rat central nervous system [Follesa and Mocchetti, 1993]. Increased FGF-2 expression in astrocytes after IsP stimulation has been suggested to be due to the increased stability of FGF-2 mRNA [Riva *et al*, 1996]. There also exists the possibility that there are no effects of direct β-agonist stimulation on FGF-2 promoter activity, due to effects being counteracted by opposing regulatory mechanisms. It has been reported that ANF represses FGF-2 synthesis in astrocytes [Biesiada *et al*, 1996]. The results presented here

suggest that IsP administration *in vivo* results in the significant increase in ANF mRNA in mouse hearts 96 hours after treatment. If ANF does have an effect on FGF-2 synthesis, then it is possible that the direct effect of IsP on FGF-2 promoter activity (if any) is being counteracted by the inhibitory effects of ANF.

Amount of FGF-2. The increased promoter activity after FGF-2 administration in culture points towards a direct effect of FGF-2 on its own promoter. Since β-adrenergic stimulation triggers increased FGF-2 release [Clarke *et al*, 1995], it is possible that the increase in promoter activity in the *in vivo* experiments is a result of the increased release of FGF-2 from cardiac myocytes. This is supported by data that show an increase in both promoter activity and FGF-2 mRNA, even in the presence of increased ANF transcript levels (Figures 5-7). The difference in effects observed after IsP administration *in vitro* may also be the result of a difference in FGF-2 levels in the medium when compared to the amount of FGF-2 released in the *in vivo* experiments. Although amounts of FGF-2 release *in vivo* after β-adrenergic stimulation were not measured, many studies have reported that the majority of FGF-2 stores reside within the extracellular matrix [Reviewed in Ornitz, 2000]. The ECM is not present in culture. Furthermore, it is assumed that some injury is caused by the administration of high dose β-agonist *in vivo*, which may have resulted in even more FGF-2 release. This may also contribute to the 48 hours of FGF-2 treatment versus 6 hours of IsP treatment required to detect a significant stimulation of FGF-2 promoter activity in transfected cultures versus transgenic mice, respectively (Figures 1 and 7).

B.5. FGFR-1 and FGF-2 autoregulation in cardioprotection

FGFR-1 is the dominant FGF receptor in the heart [Liu *et al*, 1995]. FGF-2 engineered with a diminished affinity for FGFR-1 is no longer cardioprotective [Jiang *et al*, 2002], showing that cardioprotection requires binding and activation of FGFR-1. FGF-2 autoregulation is also likely to be mediated by binding to FGFR-1. The significant increase in FGFR-1 mRNA levels detected in IsP-treated hearts (Figure 9) may reflect a direct or indirect response to β-adrenergic stimulation, and may be a component of the response to stress and, more specifically, of cardioprotection by FGF-2.

B.6. FGF-2 autoregulation in non-injury situations

The possibility exists that FGF-2 autoregulation may be more pronounced or important in an injury scenario than in normal physiological situations, linking it to cardioprotection. The results presented here suggest that direct administration of FGF-2 *in vitro* and through indirect means *in vivo* significantly increases FGF-2 promoter activity. Increasing FGF-2 release by increasing heart rate and inotropy (β-adrenergic stimulation) using non-injury-inducing IsP doses *in vitro* did not result in any changes in transfected FGF-2 promoter activity. In a pilot study separate from this thesis project, it was observed that exercise did not influence FGF-2 expression. This also suggests that FGF-2 autoregulation in normal physiological situations (exercise, fear) may not be as pronounced as that of injury scenarios.

C. IN CONCLUSION

FGF-2 is cardioprotective [Reviewed in Detillieux *et al*, 2003] and can be released from cardiac myocytes in the mammalian heart on a beat-to-beat basis under normal physiological conditions [Clarke *et al*, 1995]. This would be consistent with a role for FGF-2 in the normal maintenance of a healthy myocardium, perhaps by providing the heart with an increased capacity to function under a greater range of stressful conditions, through its cardioprotective properties. The results of this thesis support this idea by providing an association between FGF-2 synthesis and release in the heart through possible autoregulation and/or control through redundant transcription factor complexes in cardiac myocytes. Sp1 might play a role in FGF-2 autoregulation, as has been shown in other TATA-less housekeeping genes [Laniel *et al*, 1997; Philipsen and Suske, 1999; Song *et al*, 2001; Butta *et al*, 2001]. Egr-1 is a product of primary response or intermediate early genes, which might allow for additional control under periods of stress or in response to a growth stimulus [Biesiada *et al*, 1996; Stula *et al*, 2000], as has been suggested for other systems. Cardioprotection by FGF-2 is receptor-mediated [Jiang *et al*, 2002] and functional high affinity FGF-2 receptors, specifically FGFR-1, are present in the adult myocardium [Liu *et al*, 1995]. The data presented here indicate that IsP treatment increases FGFR-1 mRNA levels in the mouse heart (Figure 9), and may be a factor in the stress response and, possibly, FGF-2 cardioprotection. Assessment of isolated hearts from transgenic mice overexpressing FGF-2 revealed an increased capacity for FGF-2 accumulation, both intracellular and in the extracellular matrix, as well as

cardioprotection in response to ischemic injury [Sheikh *et al*, 2001]. Thus, stimulation of FGF-2 synthesis on a beat-to-beat basis may play a role in the maintenance of a healthy myocardium. Meanwhile, FGF-2 release upon injury, where evidence for autoregulation is more pronounced, may be a strategy for maximal cardioprotection (Figures 6-8).

D. FUTURE DIRECTIONS

D.1. Mechanism for FGF-2 autoregulation in cardiac myocytes

To fully determine the mechanisms governing autoregulation of FGF-2 in cardiac myocytes, it is necessary to identify factors that may be involved in the process. In previous studies, it was shown that the mutation of three specific binding sites for the transcription factor Egr-1 within the FGF-2 promoter abrogated the effects of α-adrenergic stimulation on FGF-2 promoter activity [Detillieux *et al*, 1999]. This is supported by previous reports that FGF-2 regulation by endothelin-1 in astrocytes [Biesiada *et al*, 1996] and autoregulation in glia cells [Wang *et al*, 1997] involves Egr-1. The data presented here indicated that Egr-1 can regulate the FGF-2 promoter (overexpression studies *in vitro*), however, transfection of constructs with mutated putative Egr-1 sites within the FGF-2 promoter did not abrogate FGF-2 effects on its own promoter. To show involvement of Egr-1, changes in its levels (if any) will have to be measured in cardiac myocytes after addition of FGF-2 (*in vitro*) and after increased FGF-2 release (*in vivo*) through protein analysis (Western blotting or ELISA). Data are also shown that allow the possibility for Sp1 regulation of FGF-2 in cardiac

myocytes. To determine whether Sp1 is associated with the autoregulation of FGF-2, its levels will also have to be determined in similar experiments to those of Egr-1 just mentioned. In addition, to determine whether any of the putative Egr-1 or Sp1 sites directly play a role in FGF-2 autoregulation, DNA sequences used in the EMSA studies can be used to compete with nuclear extracts of both neonatal rat cardiac myocytes and of adult rat hearts in competition EMSA studies. Competition would suggest that all or any of WT1, WT2, and WT3 is (are) involved in binding of either or both Egr-1 and Sp1 in cardiac myocytes.

D.2. FGF-2 autoregulation in injury versus non-injury scenarios

To study the effect of injury on FGF-2 regulation, another method of injury may be used to support existing data that show injured hearts have a more pronounced FGF-2 promoter response. The transgenic -1058FGFp.*luc* mice may be utilized with another model of injury such as coronary ligation, then promoter activity and other parameters may be assessed. A different model of injury would allow the determination of effects on the FGF-2 promoter by increased FGF-2 release through injury without adrenergic stimulation. If FGF-2 promoter activity does increase, this would strengthen the evidence presented in this thesis that FGF-2 is autoregulated at the transcriptional level.

Chapter IV

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