REGULATION OF THE FIBROBLAST GROWTH FACTOR-2 AXIS IN CARDIAC CELLS: EFFECTS ON CARDIOPROTECTION AND CARDIAC MUSCLE CELL GROWTH

BY

FARAH SHEIKH

A THESIS
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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DEDICATED TO

MY LOVING PARENTS

Akbar M. Sheikh and Naseem R. Sheikh

ABSTRACT

Cardiovascular disease is the leading cause of death worldwide. Treatments have primarily focussed on the vascular system and shown only moderate 10-20% reductions on major outcomes such as death or myocardial infarction. The research described in this thesis is focussed on developing and testing multiple strategies to regulate cardiac myocyte protection and regeneration in an effort to provide the basis for novel treatments and/or prevention of cardiac disease.

FGF-2 is a growth factor that is naturally present in the heart as well as cardiac myocytes and possesses growth promoting and cardioprotective properties, which make it an important therapeutic tool for reducing or preventing damage by ischemia and/or improving cardiac prognosis subsequent to cardiac injury. Although, the protective effects of FGF-2 and the development of exogenous delivery systems are currently being exploited, there has been little effort to exploit these effects by controlling endogenous production of FGF-2. Using gain-of-function transgenics, my research has demonstrated a role for endogenous FGF-2 in the adult heart *in vivo*. Chronic FGF-2 overexpression was associated with increased "local" FGF-2 release resulting in augmentation of kinases linked with ischemic preconditioning, angiogenesis and cardioprotection. This likely contributed to the increased cardiac myocyte viability observed after ischemia-reperfusion injury in isolated FGF-2 transgenic mouse hearts, representing the first account of the cardioprotective potential of endogenous FGF-2. In addition, my results also demonstrate that endogenous production of FGF-2 can be targetted and is

significantly increased in adult mouse cardiac myocytes using the natural catecholamine, norepinephrine.

FGF-2 signaling also plays a major role in embryonic and neonatal cardiac myocyte proliferation *in vitro* and as a result, is implicated in cardiac regeneration. However, the lack of a measurable proliferative response by FGF-2 in the postnatal heart suggested that FGF-2 signaling may be limited and/or antagonized. My results have provided the first indication that levels of the high affinity receptor for FGF-2, FGFR-1, may limit cardiac cell proliferation. Overexpression of FGFR-1 resulted in FGF-2 mediated mitogenic response in FGFR-1 deficient cardiac H9c2 cells as well as primary neonatal cardiac myocytes. This response may involve ERK1/2 MAPK activation and promotion of FGF-2 release. My results also provide direct evidence that transforming growth factor (TGF)-β plays an important role in antagonizing FGF-2 mediated cardiac myocyte entry into the cell cycle. Overexpression of the kinase-deficient TGF-β receptors (TGF-βRII) resulted in serum-induced cardiac myocyte cell cycle entry as well as an amplification of FGF-2 induced S phase entry (13 fold versus 3 fold with FGF-2 alone). Most importantly, these data support a "multiple hit" approach as a therapeutic strategy to stimulate cardiac myocyte proliferation/regeneration following cardiac injury *in vivo*.

In conclusion, my doctoral studies have provided substantial evidence that regulating the FGF-2 axis can be used as a means to exploit the effects of FGF-2 on cardioprotection and cardiac myocyte regeneration in an effort to provide the basis for novel treatments and prevention strategies for cardiac disease.

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A	adenosine
ACE	angiotensin-converting enzyme
Ad	adenovirus
ADP	adenosine-5′ -diphosphate
aFGF	acidic fibroblast growth factor
AIDS	acquired immunodeficiency syndrome
AMP	adenosine-5′ -monophosphate
α-MHC	alpha-myosin heavy chain
ANF	atrial natriuretic factor
AngII	angiotensin II
aPKC	atypical protein kinase C
AR	adrenergic receptor
ATP	adenosine-5′ -triphosphate
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
bpm	beats per minute
β-gal	beta-galactosidase
β-MHC	beta-myosin heavy chain
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C	cytosine

°C	degrees centigrade
Ca^{2+}	calcium
CaMKI	Ca^{2+}/calmodulin-dependent protein kinase I
CaMKIV	Ca^{2+}/calmodulin-dependent protein kinase IV
cdk	cyclin dependent kinase
cDNA	complementary DNA
cPKC	conventional protein kinase C
cpm	counts per minute
CK	creatine phosphokinase
CMF	calcium-magnesium free
CMV	cytomegalovirus
CO_2	carbon dioxide
d	day(s)
DAG	1,2-diacyl-sn-glycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DSS	disuccinimidyl suberate
EDTA	ethylenediaminetetraacetic acid
EDP	end-diastolic pressure
EM	electron microscopy
e.g.	for example
Egr-1	early growth reponse-1 protein
EGTA	ethylene glycol-bis[b-aminoethyl ether]-N,N,N′,N′-tetraacetic acid

ELISA	enzyme-linked immunoabsorbant assay
ERK	extracellular-signal-regulated protein kinase
ET-1	endothelin-1
FBS	fetal bovine serum
FAST-1	forkhead activin signal transducer protein-1
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor(s)
FGFR-1 (L)	long form of the FGFR
FGFR-1(S)	short form of the FGFR
Fig.	Figure
FRS2	FGF receptor substrate 2
g	gram(s) OR gravity equivalents
G	guanosine
G1	gap 1 phase of the cell cycle
G2	gap 2 phase of the cell cycle
G protein	GTP-binding protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GPR	G protein coupled receptors
GTP	guanosine 5′-triphosphate
h	hour(s)
H^+	hydrogen
H	histochemistry

^3H	tritiated thymidine
HCl	hydrochloric acid
5-HD	5-hydroxydecanoate
HEPES	N-[2-hydroxyethyl]piperazine-N´[2-ethanesulfonic acid]
HIV	human immunodeficiency virus type I
HMW	high molecular weight
hsp70	heat shock protein 70
HSPG	heparan sulfate proteoglycan
Hz	hertz
I	immune histology
i.e.	that is
Ig	immunoglobulin
IGF-1	insulin growth factor-1
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
IP_3	1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
K^+	potassium
kb	kilobase(s)
kD	kilodalton(s)
KH	Krebs-Henseleit
ΔkTGF-βRII	kinase-deficient TGF-beta receptor type II
LDH	lactate dehydrogenase

LI	labeling index
LM	light microscopy
LMW	low molecular weight
luc	luciferase
mRNA	messenger ribonucleic acid
pg/µg/mg	picogram/microgram/milligram
ml/µl	millilitres/microlitres
mm	millimetre
mM	millimolar
mmHg	millimetres mercury
mol/L;umol/L	moles per litre; micromole per litre
M	mitotic phase of cell cycle OR molar (units of measurement)
MAPK	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2
MEK-1	MAP kinase kinase
MHC	myosin heavy chain
min	minute(s)
MLC-2	myosin light chain-2
MOI	multiplicity of infection
N	nuclear staining
Na^{2+}	sodium
NFAT3	nuclear factor of activated T cells 3
NF-κB	nuclear factor-kappa B

NLS	nuclear localization sequence
NO	nitric oxide
non-TG	non-transgenic
NOS	nitric oxide synthase
O_2	oxygen
%	percent
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PH	phase-contrast microscopy
phospho-H1	phosphorylated histone H1
PIP_2	phosphotidylinositol biphosphate
PKA	protein kinase A
PKC	protein kinase C
nPKC	novel protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
R	receptor(s)
Rb	retinoblastoma protein

RSV	Rous sarcoma virus
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
s	second(s)
sm	smooth muscle
S	synthetic phase of cell cycle
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulphate
Ser	serine
SEK-1	SAPK/ERK kinase-1
SERCA2a	sarcoplasmic reticulum calcium ATPase pump 2a
SH2	Src-homology 2
SNIP1	Smad nuclear interacting protein-1
SV40	Simian virus 40
SVenh	SV40 enhancer sequences
TAB1	TAK1 binding protein-1
TAK1	TGF-β activated MAP kinase protein 1
TBS-T	Tris buffered saline with Tween
TCA	trichloroacetic acid
TG	transgenic
TGF-β	transforming growth factor-β
TGF-βR	transforming growth factor-β receptor
Thr	threonine

Tyr	tyrosine
T	thymidine
TK	tyrosine kinases
tPA	tissue plasminogen activator
U	units
uPA	urokinase type plasminogen activator
VEGF	vascular endothelial growth factor
w/v	weight/volume

CHAPTER 1

INTRODUCTION

1.1 Perspectives on Cardiovascular Disease

Research advances during recent years in the field of molecular and clinical cardiology have led to significant breakthroughs in the medical treatment and management of various cardiovascular diseases some of which include atherosclerosis, ischemic and congenital heart disorders, stroke, thrombosis and hypertension {Kauffmann-Zeh and Dhand, 2000}. However, despite these recent advances, cardiovascular diseases remain the leading cause of death worldwide. In North America, mortality rates from cardiovascular diseases account for an astounding 38 and 41.2 % of all deaths in Canada and the United States, respectively {Health Statistics Division, Statistics Canada, 1995; American Heart Association, 1997}. Compared to other causes of death, cardiovascular diseases claim approximately two, ten and sixty times more lives than those reported by cancer, accidents or human immunodeficiency virus type I (HIV) diseases such as acquired immunodeficiency syndrome (AIDS), respectively, in a year {American Heart Association, 1997}. Table 1 presents an overview of the percentage breakdown of deaths from the various cardiovascular diseases in the United States in 1997 {American Heart Association, 1997}. The dominant etiology for heart failure is coronary/ischemic heart disease (resulting in myocardial infarction or angina pectoris) which accounts for 49% of all deaths (Table 1). Current strategies to treat cardiovascular diseases have limitations because treatments have (i) primarily focussed on the vascular system and (ii) shown at best only moderate reductions (10-20%) on major outcomes such as death or myocardial infarction {Yusuf et al., 1996}. In fact, more than 40% of

Table 1. Deaths from cardiovascular diseases in United States in 1997.

Cardiovascular Diseases	**Percentage Breakdown of Deaths**
Coronary/Ischemic Heart Disease (Myocardial Infarction & Angina)	49 %
Stroke	17 %
High Blood Pressure	5 %
Congestive Heart Failure	5 %
Atherosclerosis	2 %
Rheumatic Fever/ Heart Disease	1 %
Congenital Cardiovascular Defects	1 %
Other	20 %

Source: American Heart Association

people experiencing new or recurrent heart attacks will likely die of them this year {American Heart Association, 1997}. Clearly, progress in the treatment of this disease will depend on the development of new therapeutic strategies as well as multiple approaches. Furthermore, since cardiac myocytes are a target of this disease, therapeutic strategies must also be directed towards cardiac myocytes as well as vascular cells. Thus, it is my view that there must not only be a fundamental understanding of the basic regulatory mechanisms underlying the pathogenesis of heart failure but also an expansion of knowledge in understanding the mechanisms regulating cardiac muscle cell (myocyte) biology.

1.2 Heart Failure

Heart failure is defined as the inability of cardiac output to keep pace with the body's demands for supplies/nutrients and removal of wastes {Sherwood, 1993}. The two most common causes of heart failure are: (i) myocardial infarction, which causes damage to cardiac myocytes as a result of impaired circulation and (ii) prolonged pumping of the myocardium against a chronically increased afterload (e.g., high blood pressure, valvular disorders) {Sherwood, 1993}. The following section will briefly discuss the events in the heart following acute myocardial infarction.

1.3 Acute Myocardial Infarction

Acute myocardial infarction is defined as the actual death or necrosis of cardiac myocytes, usually caused by the blockage (i.e., occlusive thrombi) of a blood vessel supplying an area of the heart {Henderson, 1996; Sherwood, 1993}. This event results in a total or marked reduction of blood flow to the heart tissue, which is termed myocardial

ischemia {Henderson, 1996}. Infarction is caused by prolonged ischemia which leads to irreversible changes to the affected cardiac myocytes {Henderson, 1996}. Unlike skeletal muscle cells which can regenerate muscle upon injury (due to populations of stem/satellite cells), adult cardiac myocytes are limited in their ability to regenerate heart muscle through proliferation following injury as a result of myocardial infarction {Chien, 1995; Karsner et al., 1925; Parker and Schneider, 1991; Rumyantsev, 1977}. Instead, the compensatory response involves replacing damaged cardiac myocytes with proliferative non-muscle cells that form a non-contractile scar, while forcing the remaining viable cardiac myocytes to adapt to the increased workload by regional cardiac hypertrophy to meet the functional demands {Henderson, 1996}. The adaptive response of the heart is referred to as "ventricular remodelling" and is characterized by not only the development of cardiac hypertrophy, but also includes interstitial fibrosis, chamber dilatation and sphericalization of the myocardium {Pfeffer and Braunwald, 1990}. The major determinants of heart failure and life expectancy hinge on (i) the severity and duration of reduction of coronary blood flow, (ii) the pre-existence of collaterals (microvascular vessels from vascular beds) which may increase tissue perfusion, (iii) any pre-existing state of preconditioning (protective effect of brief periods of ischemia) which may protect cardiac myocytes from severe damage, and (iv) the degree of compensation by cardiac hypertrophy {Henderson, 1996}. However, beyond a certain capacity for cardiac myocytes to compensate, cardiac performance will be severely compromised and congestive heart failure will ensue {Sherwood, 1993}.

1.4 Growth Factors and Cardiovascular Disease

The limited ability of adult cardiac myocytes to divide/regenerate or 'shield' themselves from injury has a major impact on the compensatory response of the heart subsequent to an acute myocardial infarction, and ultimately on a patient's prognosis. To date, there is limited information on the mechanisms that control cardiac myocyte growth and protection. Knowledge of these mechanisms and their regulators would be essential in facilitating a better understanding of the limitations imposed on adult cardiac myocytes. Considerable attention has focussed on the use of growth factors for treatment of cardiovascular disease {Waltenberger, 1997}. Growth factors are also considered major regulators of myocardial and vascular growth and differentiation {Parker et al., 1990}. Clearly, the identification of a factor or combination of factors that could (i) reduce the size of the non-muscle scar by stimulating the proliferative potential of cardiac myocytes to actively divide (regeneration), (ii) promote the formation of "new" blood vessels (angiogenesis) and/or (iii) protect cardiac myocytes from the effects of ischemia (cardioprotection) could circumvent the deleterious effects of cardiac injury following acute myocardial infarction and as a result, prolong cardiac lifespan.

The following chapter will review basic mechanisms regulating cardiac myocyte growth and protection as well as the roles of specific growth factors in controlling cardiac myocyte growth, potential regenerative response and protection. The review will focus primarily on the growth factor, fibroblast growth factor (FGF)-2 and its axis and, to a lesser extent, on the role of transforming growth factor (TGF)-β in controlling cardiac myocyte growth.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Cardiac Myocyte Protection

Cardiac myocyte protection in the event of injury such as myocardial infarction is dependent on the extent of damage caused by ischemia as well as reperfusion (establishment of flow after period of no flow) {Ip and Levin, 1988}. The duration of ischemia is the primary determinant of the severity and reversibility of cardiac myocyte damage {Ip and Levin, 1988}. Studies involving the use of an *in vivo* temporary coronary occlusion model in dogs demonstrated that a short period of cardiac ischemia (10-20 minutes (min)) was associated with a reduction of high-energy phosphates and glycogen stores, peripheral aggregation of nuclear chromatin, mild tissue and mitochondrial swelling, however, the integrity of the cardiac myocyte sarcolemmal membrane was maintained {Jennings and Ganote, 1974; Jennings and Reimer, 1983; Jennings et al., 1985}. As a result, restoration of high-energy phosphates after this period allows for the reversal of cardiac myocyte structural changes associated with ischemia {Jennings et al., 1985}. On the other hand, reperfusion after prolonged periods of ischemia (>20 min) causes irreversible injury to cardiac myocytes {Ip and Levin, 1988}. Irreversible injury is characterized by the disruption of the cardiac myocyte sarcolemma {Ip and Levin, 1988}. As a result, reperfusion further accelerates the destruction of cells since cell volume regulation is lost {Ip and Levin, 1988}. In addition, if ischemia is further prolonged, reperfusion may not occur at all, resulting in the "no-reflow phenomenon" {Engler et al., 1983; Kloner et al., 1974}. This event involves capillary compression by endothelial and parenchymal cell swelling, interstitial edema and aggregation of blood cellular

components {Ip and Levin, 1988}. The result is vascular dysfunction, ischemic contracture and/or increased basal tone {Engler, 1987; Engler et al., 1983; Jennings and Reimer, 1983; Kloner et al., 1974}. The "no-reflow phenomenon" has been described following reperfusion in patients with acute myocardial infarction {Birnbaum et al., 1997}.

There are several ultrastructural changes which characterize irreversibly injured ischemic cardiac myocytes. Following the loss of high-energy phosphates and glycogen stores, cardiac myocytes undergo cellular swelling, peripheral aggregation of nuclear chromatin and vesiculation of sarcoplasmic reticulum (disrupting cellular calcium homeostasis) {Ip and Levin, 1988}. This leads to hypercontracture, weakened cell membranes as well as marked mitochondrial and sarcolemmal damage {Ip and Levin, 1988; Jennings et al., 1990}. Sarcomeric changes include distortion of Z, M and A bands, loss of lateral Z-band-sarcolemmal attachments as well as detachment of sarcomeres from intercalated discs {Ganote and Vander Heide, 1987}. These events result in "sarcolemmal blebbing" {Sage and Jennings, 1988}. In addition, cardiac myocytes show a reduction in the levels of the cytoskeletal-associated proteins vinculin and α-actinin {Ganote and Vander Heide, 1987} as well as loss of vinculin association with the plasma membrane {Steenbergen et al., 1987}.

In terms of energy sources, ischemic cardiac myocytes shift energy production from fatty acid production in mitochondria towards anaerobic glycolysis, leading to a reduction in the production of adenosine-5′ -triphosphate (ATP) {Opie, 1976; Ip and

Levin, 1988}. As ischemia progresses, glycolytic metabolites accumulate in cardiac myocytes leading to acidosis {Rouslin et al., 1986}. The reduced ATP production and acidosis leads to the reduced myocardial contractility (which is ATP dependent) observed following ischemia {Harrison et al., 1992}. It is thought that the increased hydrogen (H^+) ions (acidosis), inhibit actin-myosin cross-bridge cycling and thus, contractility by interfering with calcium binding to actin {Karmazyn, 1991; Sobel., 1974}. In addition, decreased ATP production also leads to an accumulation of extracellular potassium (K^+) {Janse and Kleber, 1981} as well as increased intracellular levels of sodium (Na^{2+}) and calcium (Ca^{2+}) {Shen and Jennings, 1972}. These events are due to alterations in the functions of energy-dependent ion pumps/channels involved in maintaining intracellular and extracellular potassium, sodium, and calcium levels {Janse and Kleber, 1981; Kleber, 1984, Steenbergen et al., 1993}. Changes in ionic maintenance as a result of ischemia also lead to changes in cardiac myocyte action potential durations {McDonald and McLeod, 1973}. The opening of mitochondrial K^+ATP channels, however, has been postulated to be a part of the endogenous protective response in the heart during ischemia to conserve ATP {Liu et al., 1998}.

The second determinant of the extent of myocardial damage subsequent to acute myocardial infarction is reperfusion {Ip and Levin, 1988}. Reperfusion injury is described as cellular death or cardiac dysfunction caused by the restoration of blood flow to previously ischemic tissue {Birnbaum et al., 1997}. Although recovery from reperfusion is dependent on the severity and duration of cardiac ischemia and the extent of collateral flow {Ip and Levin, 1988}, there is continued debate as to whether

reperfusion by itself can kill viable cardiac myocytes, which have otherwise survived ischemic insult (lethal reperfusion injury) {Birnhaum et al., 1997}. Other types of reperfusion injury include: (i) nonlethal reperfusion injury (myocardial stunning), which refers to postischemic ventricular dysfunction of viable myocytes; (ii) reperfusion arrhythmias which include ventricular tachycardia and fibrillation that occur after restoration of coronary flow following brief episodes of ischemia; and (iii) vascular reperfusion injury which refers to the progressive damage to the vasculature over time during the phase of reperfusion and includes the "no-reflow phenomenon" {Kloner, 1993; Birnbaum, 1997}. Nonlethal and vascular reperfusion injury as well as reperfusion arrhythmias have been documented in animal models and humans {Kloner, 1993; Birnhaum, 1997}.

Reperfusion injury is associated with a series of events including generation of free radicals, complement system activation (part of the inflammatory response), neutrophil activation, increased eicosanoid synthesis, activation of the Na^+/H^+ exchanger, increased calcium influx as well as apoptosis {Birnbaum et al., 1997; Benitz-Bribiesca et al., 2000; Karmazyn, 1991; Levitt, 1999; Pierce and Czubryt, 1995; Scholz and Albus, 1993}. The major determinants of reperfusion injury include free radical generation and an increased influx of calcium {Karmazyn, 1991}. The free radical mediated injury causes significant cardiac myocyte damage, in part by perioxidative injury to intracellular membranes, including mitochondria {Levitt, 1999}. Protective enzyme systems for mitochondria such as superoxide dismutase, catalase and selenium-dependent glutathione peroxidase are scant in cardiac muscle compared to other tissues such as liver {Levitt,

1999}. Oxygen free radicals also damage the sarcoplasmic reticulum and the sarcolemma which would contribute to an increase in membrane permeability, decreased sensitivity of contractile proteins to calcium as well as calcium overload {Poole-Wilson et al., 1997}. The increased levels of intracellular calcium in cardiac myocytes and increased damage during reperfusion is described as the calcium paradox {Zimmerman and Hulsmann, 1966}. The maintenance of physiological levels of intracellular calcium is critical for cardiac myocyte integrity and function; however, with reperfusion, restoration of physiological levels of calcium to ischemic tissue results in increased cardiac myocyte damage {Zimmerman and Hulsmann, 1966}. The alterations in calcium homeostasis lead to further mechanical or contractile dysfunction in the heart including reduced myocyte viability {Poole-Wilson et al., 1997}.

Cardiac myocyte necrosis, as a consequence of ischemia-reperfusion injury, also results in the leakage of intracellular proteins and enzymes, which then enter the systemic circulation {Donnelly and Millar-Craig, 1998}. As a result, assessment of alterations in plasma levels of cardiac proteins are used as sensitive biochemical markers to diagnose or prognose acute myocardial infarction in patients {Adams and Miracle, 1998}. These proteins originate either directly from injured cardiac myocytes, connective tissue or blood elements from the ischemic heart, or other organs (e.g., skeletal muscle) subject to diminished perfusion {Sobel, 1974}. Their appearance in peripheral blood is dependent on their molecular weight, location within the cell, release characteristics as well as the rate of drainage and clearance in patients {Donnelly and Millar-Craig, 1998}. Assessment of mitochondrial enzymes, creatine phosphokinase (CK) and lactate

dehydrogenase (LDH) have been used effectively for detection of myocardial infarction in patients {O'Brien et al., 1997}. Other non-enzymatic, cardiac biomarkers include myoglobin, myosin light chain, cardiac troponin-I and T, enolase and myosin heavy chain (MHC) {Donnelly and Millar-Craig, 1998}. Cardiac troponin I and T appear to have significant advantages over other markers and may become the assays of choice, based on their cardiac specificity, early detectability (3-12 hours (h)), sustained release (5-14 days (d)) and their assessment as predictors of mortality and myocardial infarction in patients {Donnelly and Millar-Craig, 1998; Jaffe et al., 2000}. Release of these cardiac proteins and enzymes can be easily quantitated in serum and/or cardiac tissue from animals and/or patients in diseased or non-diseased states, perfusates from isolated heart model systems subject to ischemia-reperfusion injury as well as conditioned medium from cardiac myocyte cultures undergoing stress or injury.

2.1.1 Models of cardiac ischemia-reperfusion injury

There are several models and methods which can be used to mimic ischemia and/or reperfusion injury {Ytrehus, 2000}. However, it should be noted that in most cases the effects of ischemic injury cannot be completely separated from reperfusion injury. *In vivo* procedures include subjecting animals to either permanent coronary artery ligation (ischemia) or coronary ligation for various periods followed by reflow (ischemia-reperfusion) {Fliss and Gattinger, 1996}. In terms of *ex vivo* procedures, isolated heart preparations are commonly used to subject hearts to global ischemia by total cessation or partial reduction of coronary flow and then reflow (ischemia-reperfusion) {Karmazyn, 1991}. Hypoxia can also be used to simulate ischemic conditions in isolated heart

preparations {Ho et al., 2000}. Stunning (non-lethal reperfusion injury) can also be mimicked in isolated heart preparations {Klainguti et al., 2000; Seiler et al., 1996}. In terms of *in vitro* procedures, cardiac myocyte cultures are subjected to ischemic injury through anoxia, hypoxia, metabolic inhibition, glucose and serum deprivation {Bialik et al., 1999; Bond et al., 1991; Delcamp et al., 1998; Yamauchi-Takihara et al., 1995}. Hydrogen peroxide (free radicals) can also be used to induce reperfusion injury in cultured cardiac myocytes {Josephson et al., 1991}. In addition, a recent study has described an *in vitro* experimental model which mimics reperfusion-induced arrhythmias {Arutunyan et al., 2001}. All of the heart models described above result in severe depletion of ATP, which is classically associated with ischemia and are characterized by cardiac myocyte ultrastructural and biochemical features which parallel those observed in cardiac myocytes during ischemia-reperfusion injury as stated in section *2.1.* Disadvantages and advantages of some of these various experimental models have been extensively discussed in a recent review by Ytrehus {Ytrehus, 2000}.

Ischemia-reperfusion injury can also be observed in a variety of clinical settings, in addition to acute myocardial infarction. Most cardiac surgical procedures require the use of prolonged induced myocardial ischemia {Gardner, 1988}. Cross-clamping the aorta in isolated hearts or cold cardioplegic arrest of hearts during surgical coronary artery bypass or heart transplants results in global ischemia {Ip and Levin, 1988}. As a result, heart models (*in vivo* and *ex vivo*) which induce global ischemia have been deemed important for the study of ischemia-reperfusion injury. There are four clinical conditions which result in reperfusion injury. These include: (i) coronary artery

vasospasm where reperfusion is achieved by relaxation of vasospastic vessels (spontaneous or drug-induced), (ii) balloon angioplasty where removal of a clot using a balloon catheter restores perfusion to cardiac tissue previously deprived of oxygen, (iii) cardiac bypass surgery where the heart is subjected to low-flow ischemia during surgery prior to the connection of an arterial graft which restores reperfusion, and (iv) thrombolytic therapy where anticoagulants are used subsequent to ischemia to reperfuse ischemic tissue {Pierce and Czubryt, 1995; Braunwald, 1985; Bulkelley and Hutchins, 1977; Whalen et al., 1974}.

2.1.2. Preconditioning and cardioprotection signaling pathways

Ischemic preconditioning is a phenomenon that was first described by Murry and colleagues as the endogenous cardiac myocyte protection which results from brief transient episodes of nonlethal myocardial ischemia, rendering the heart resistant to a subsequent more sustained ischemic insult {Murry et al., 1986; Przyklenk and Kloner, 1998}. Reduction of infarct size is considered the "gold standard" of ischemic preconditioning {Przyklenk and Kloner, 1998}. Other surrogate endpoints and secondary consequences of ischemic preconditioning include: (i) slowing the rate of ATP depletion, (ii) recovery of contractile function, (iii) reduction in incidence of ischemia-reperfusion induced arrhythmias, (iv) reduction of cardiac myocyte apoptosis, (v) preservation of coronary vasodilatory reserve, and (vi) attenuation of platelet-mediated thrombosis {Przyklenk and Kloner, 1998}. Ischemic preconditioning has since been demonstrated in other organs as well as patients with cardiovascular disease {Jerome et al., 1995; Korthuis et al., 1998; Downey et al., 1994}.

There is limited information on the signaling mechanisms underlying preconditioning, however, recent evidence suggests that there are two temporally and mechanistically distinct types of protection afforded by preconditioning {Carden and Granger, 2000}. Acute or classical preconditioning is effective as long as the time frame between preconditioning (short period of ischemia) and longer ischemic insult is less than two hours {Carden and Granger, 2000}. On the other hand, delayed preconditioning ("second window of protection") occurs when the protection is apparent when the prolonged ischemia occurs 24 h following the initial preconditioning event {Korthius et al., 1998}. Given the time frames of protection, it is postulated that the effects of delayed preconditioning are protein synthesis-dependent whereas the effects of acute preconditioning are protein-synthesis independent {Carden and Granger, 2000}. Evidence suggests that the signals initiating both types of protection are similar; however, nitric oxide (NO) seems to be a prime mediator of delayed preconditioning {Carden and Granger, 2000}.

Ischemic preconditioning has been shown to be receptor-mediated {Baines et al., 1999a}. Adenosine or α_1-adrenergic receptor activation of G proteins appear to be crucial activators of a preconditioning response through stimulation of phospholipase C (PLC) or D (PLD) {Korthius et al., 1998; Downey et al., 1994}. For example, adenosine receptor agonists and antagonists can augment or block the cardioprotective effects of ischemic preconditioning in isolated hearts and cardiac myocyte cultures {Armstrong et al., 1994; Armstrong and Ganote, 1994; Tsuchda et al., 1994}. Studies have suggested that adenosine-receptor stimulated hydrolysis of PLC enhances intracellular levels of 1,2-

diacyl-sn-glycerol (DAG), which activates protein kinase C (PKC), thus translocating specific PKC isoforms to the cell membrane {Korthius et al., 1998; Downey et al., 1994}. Oxygen free radicals generated during the preconditioning ischemic episode may also contribute to the protective effect by directly activating PKC {Baines et al., 1999a}. As a result, the preconditioning event via adenosine receptors primes an increase in DAG levels to subsequently activate the translocated membrane-associated PKC {Cardan and Granger, 2000}. PKC activation as a result of preconditioning can also increase the levels of 5′-nucleotidase, the enzyme responsible for producing adenosine from adenosine-5′ –monophosphate (AMP) {Baines et al., 1999a}. An indirect pathway to increase adenosine levels is thought to be important since it may help maintain tissue adenosine levels (i.e., protection) in the event that endogenous adenosine levels are decreased during more prolonged ischemia {Baines et al., 1999a}. Bradykinin and opioid receptors which act through G proteins and PKC have also been shown to contribute to the preconditioning response {Baines et al., 1999a}.

PKC is an integral signaling molecule in ischemic preconditioning {Simkhovich et al., 1998}. The PKC family consists of at least 12 serine/threonine kinases which can be split into three broad categories: conventional (cPKC), novel (nPKC) and atypical (aPKC) {Baines et al., 1999a}. The cPKCs (α, β_I, β_{II}, γ) require calcium, DAG and phospholipid for activation {Baines et al., 1999a}. The nPKCs (δ, ε, η, θ) are not dependent on calcium for activation but require DAG and phospholipid for activation {Baines et al., 1999a}. The aPKCs (ζ, ι, λ, μ) are independent of calcium and DAG but require 3′-phosphoinositides for activation {Baines et al., 1999a}. In the heart, the use of

specific inhibitors and activators to PKC have demonstrated a direct role for this molecule in ischemic preconditioning and thus cardioprotection {Qi et al., 1998}. Both αPKC and εPKC have been implicated in playing a major role in ischemic preconditioning in the heart {Li et al., 2000; Lu et al., 2001; Qi et al., 1998}.

A potential role for tyrosine kinases in ischemic preconditioning has also been implicated {Baines et al., 1999a}. Recent studies have revealed that tyrosine kinase inhibitors can block the phorbol 12-myristate 13-acetate (PMA; activator of PKC)-induced cardioprotection in the rabbit myocardium {Baines et al., 1999a}. These results suggested that in terms of ischemic preconditioning/cardioprotection, tyrosine kinases are downstream effectors of PKC {Baines et al., 1999a}. Other studies have corroborated this notion, by demonstrating that PKC dependent activation of Src and Lck tyrosine kinases is required for ischemic preconditioning in rabbit hearts {Ping et al., 1999}. However, this does not exclude the possibility that receptor tyrosine kinases could act in parallel to other activators of PKC during preconditioning to induce cardioprotection {Baines et al., 1999a}. Stress activated mitogen activated protein kinases (MAPK; p38 and c-Jun N-terminal kinase (JNK)) have also been shown to play an important role in ischemic preconditioning, and have been shown to be coupled to tyrosine kinase receptors {Baines et al., 1999a}. There are at least five isoforms of p38 MAPK, however p38α and β isoforms are expressed in the heart {Sugden and Clerk, 1998). There are two isoforms of JNK (JNK1 and JNK2) and both are expressed in the heart {Clerk et al., 1998}. Ischemic preconditioning can increase phosphorylation of p38 and JNK in the heart, via direct and indirect pathways {Baines et al., 1999a}. Direct activation of both p38 and JNK via

anisomycin was able to reduce infarct size in isolated rabbit hearts subjected to cardiac injury {Baines et al., 1998}.

There is evidence to suggest that mitochondrial K_{ATP} channels are the end-effector in ischemic preconditioning {Grover et al., 1997}. Traditionally, it was thought that sarcolemmal K_{ATP} channels were the prime target, however, recent assessment of the specificity of K_{ATP} channel inhibitors revealed that they were more specific for mitochondrial as opposed to sarcolemmal channels {Baines et al., 1999a}. Studies revealed that the K_{ATP} channel inhibitor, 5-hydroxydecanoate (5-HD), could block diazoxide-induced mitochondrial K^+ flux and cardioprotection, while having little effect on cardiac sarcolemmal channels {Garlid et al., 1997}. Diazoxide was demonstrated to be approximately 2,000 fold more selective for opening mitochondrial channels than sarcolemmal K_{ATP} channels {Garlid et al., 1997}. Similar results implicating a role for mitochondrial K_{ATP} channel in cardioprotection were obtained in isolated rabbit myocytes {Liu et al., 1998}. Opening of mitochondrial K_{ATP} channels could be beneficial in ischemia as it may prevent wasteful ATP hydrolysis {Garlid et al., 1997} or reduce the electrical gradient favoring calcium influx into the mitochondria {Liu et al., 1998}. Furthermore, the protective effect of the p38 and JNK activator, anisomycin can be reversed by 5-HD {Baines et al., 1999b}, suggesting that the kinase cascade activated during preconditioning terminates in the opening of mitochondrial K_{ATP} channels {Baines et al., 1999a}.

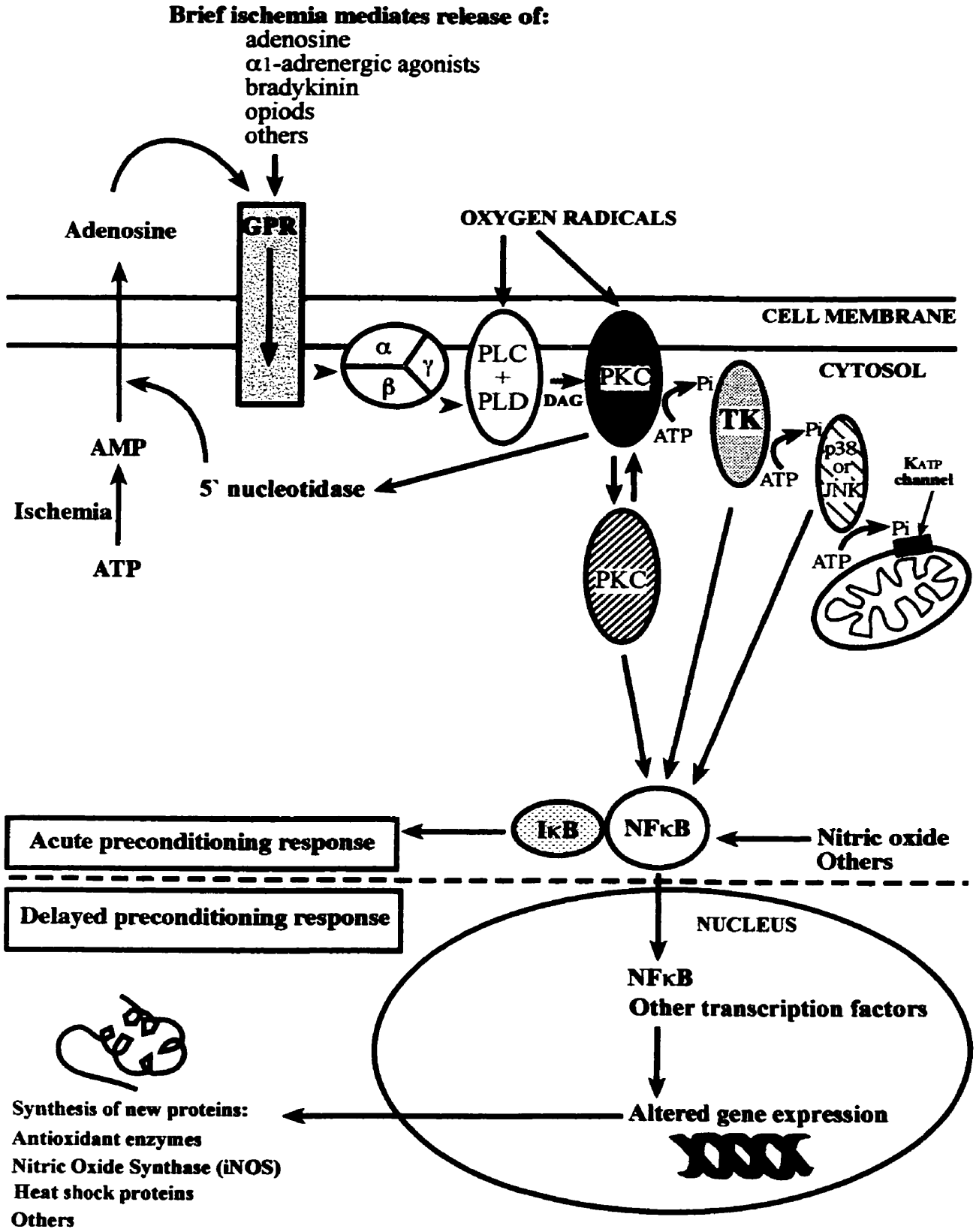
Figure 1 summarizes the signaling pathways mediating the acute and delayed preconditioning response. In terms of the protective effects of the acute preconditioning response, activation of translocated PKC during prolonged ischemia can phosphorylate tyrosine kinases (Src, Lck and others) which in turn activate one or both stress-activated MAPK {Baines et al., 1999a; Carden and Granger, 2000}. As a result, through mechanisms unknown, stress activated MAPK would open mitochondrial K_{ATP} channels which are implicated as the end effectors of cardioprotection {Baines et al., 1999a}. Other mechanisms for the acute preconditioning response include the induction of PKC-dependent translocation of 5′-nucleotidase to the cell surface to increase cellular adenosine production during prolonged ischemia to augment cellular energy stores and/or inhibit leukocyte adherence {Kitakaze et al., 1994}. The events involved in delayed preconditioning response are similar to those of the acute preconditioning response, however, activation of PKC as well as other kinase systems may be involved in this process {Kitakaze et al., 1994}. This response, unlike acute preconditioning, is dependent on altered gene expression as well as synthesis of new proteins {Korthius et al., 1998; Rizvi et al., 1999}. The cytoplasmic transcription factor, nuclear factor (NF)-κB has been implicated in playing an essential role in the late phase of preconditioning in hearts of conscious rabbits {Xuan et al., 1999}. Activation and nuclear transclocation of NF-κB would initiate transcription of genes involved in the postischemic inflammatory response, like antioxidant enzymes, heat shock proteins and nitric oxide synthase (NOS) {Carden and Granger, 2000}. The effects of NF-κB in delayed ischemic preconditioning were shown to be mediated by tyrosine kinases, PKC, reactive oxygen species, and NO {Xuan et al., 1999}. There is evidence to suggest that p38 may be upstream of NF-κB in

mediating its effects on ischemic preconditioning {Maulik et al., 1998}. Augmented NO production contributes to the delayed preconditioning response, since it appears to be associated with attenuated oxidative stress and diminished reperfusion-induced leukocyte adhesion {Korthius et al., 1998; Osborne et al., 1994}. Additional evidence for a role for NO in the delayed preconditioning was demonstrated when targeted disruption of the inducible NOS (iNOS) gene in mice was shown to completely ablate the infarct-sparing effect of the delayed preconditioning in hearts {Guo et al., 1999}. The protective effects of delayed preconditioning are also associated with induction of heat shock proteins {Gray, 1999}, and this would serve to alter cellular metabolism to protect the structure and function of critical proteins during stress {Marber and Yellon, 1996}. Clearly, unraveling the mechanisms of ischemic preconditioning in the heart and understanding endogenous myocardial protection may provide novel avenues to mediate cardiac myocyte protection in the event of prolonged ischemia such as in instances of acute myocardial infarction or cardiac surgery.

Figure 1.

Schematic representation of the proposed mechanisms thought to be responsible for the acute and delayed ischemic preconditioning response.

During preconditioning ischemia, adenosine, α_1-adrenergic agonists, bradykinin, opioids, as well as other agonists are released and in turn activate G protein coupled receptors (GPR), which then activate pertussis-sensitive G proteins to initiate the preconditioning response through stimulation of phospholipase C (PLC) or D (PLD). Hydrolysis of PLC enhances intracellular 1,2-diacyl-sn-glycerol (DAG) concentrations, thereby inducing translocation and activation of protein kinase C (PKC). Free radicals can also directly activate PLC or PLD as well as PKC. During prolonged ischemia, PKC can then either translocate 5' nucleotidase to the cell surface or activate tyrosine kinases (TK) that in turn activate one or both of the stress activated mitogen activated protein kinases (MAPK). As a result, through mechanisms unknown, this induces the opening of mitochondrial K_{ATP} channels which may be the end effectors of cardioprotection. The events initiating the delayed preconditioning response are similar to those for the acute preconditioning response, but include the enhanced production of NO and was shown to be highly dependent on altered gene expression and protein synthesis elicited by kinase activated transcription factors, such as nuclear factor (NF)-κB. This figure is adapted from Baines et al., 1999a and Carden and Granger, 2000, and includes additional information from Guo et al., 1999, Maulik et al., 1998 and Xuan et al., 1999.


Brief ischemia mediates release of:
adenosine
α1-adrenergic agonists
bradykinin
opiods
others
Adenosine
GPR
OXYGEN RADICALS
CELL MEMBRANE
CYTOSOL
α
β
γ
PLC
+
PLD
DAG
PKC
Pi
ATP
TK
Pi
ATP
p38
or
JNK
KATP
channel
Pi
ATP
AMP
5` nucleotidase
Ischemia
ATP
PKC
IκB
NFκB
Nitric oxide
Others
Acute preconditioning response
Delayed preconditioning response
NUCLEUS
NFκB
Other transcription factors
Altered gene expression
Synthesis of new proteins:
Antioxidant enzymes
Nitric Oxide Synthase (iNOS)
Heat shock proteins
Others

2.1.3 Treatments for cardiac ischemia-reperfusion injury

Several pharmacological interventions have been evaluated in the management of acute myocardial infarction {Yusuf et al., 1996}. Extensive research has been dedicated towards this area, employing the use of *in vitro* and *in vivo* cardiac models of ischemia-reperfusion injury (described in section ***2.1.1***) to assess the effects of various cardioprotective agents. There is already evidence in the literature to suggest that endogenous mechanisms which promote preconditioning (brief periods of ischemia), adenosine, PKC activation, opening of mitochondrial K_{ATP} channel, NO, antioxidants and heat shock proteins can be potentially used to treat cardiac ischemia-reperfusion injury (reviewed in section ***2.1.1***). The following section will discuss therapeutic strategies, which are currently in use to treat patients following acute myocardial infarction as well as future directions for therapies in patients suffering from congestive heart failure.

Thrombolytic therapy (i.e., reperfusion therapy) using the serine protease, tissue plasminogen activator (tPA), is used to dissolve both pathological thrombi as well as fibrin deposits at the sites of vascular injury in patients with uncomplicated acute myocardial infarction {Yusuf et al., 1996}. In addition to reperfusion therapy, all patients are treated with aspirin and heparin {Poole-Wilson et al., 1997}. Aspirin (antithrombotic therapy) inhibits the production of prostaglandins which reduces platelet aggregation and vascular dysfunction in patients {Poole-Wilson et al., 1997}. Heparin (anti-coagulant) also inhibits platelet aggregation, reducing the risk of clots and thromboembolic complications in patients {Yusuf et al., 1996}. Beta-blockers (i.e., metoprolol) are also used in the presence or absence of thrombolytic therapy to: (i) reduce myocardial oxygen

consumption, by lowering heart rate, blood pressure and myocardial contractility, (ii) block the adverse effects of catecholamines which have arrhythmogenic and direct toxic effects on myocardial metabolism, (iii) increase threshold for ventricular fibrillation, and (iv) increase distribution of blood flow {Yusuf et al., 1996}. As a result, β-blockers have been shown to reduce infarct size, decrease myocardial wall stress, prevent cardiac rupture and lower risk of recurrent ischemia {Yusuf et al., 1996}. Nitroglycerine, a vasodilator, is also routinely used in patients to relieve ischemic pain and decrease pulmonary venous pressure in patients with pulmonary venous congestion {Poole-Wilson et al., 1997}. In addition, angiotensin-converting enzyme (ACE) inhibitors (i.e., captopril) are used to treat patients with acute myocardial infarction {Yusuf et al., 1996}. They have been shown to: (i) increase peripheral vasodilation by blocking formation of angiotensin II (AngII; vasoconstrictor), (ii) improve diastolic function by inhibiting adverse effects of AngII on ventricular relaxation, (iii) prevent progress loss of cells (via apoptosis), and (iv) positively influence the effects on remodeling {Poole-Wilson et al., 1997}. ACE inhibition not only blocks formation of AngII but also enhances the positive actions of kinins on blood vessels {Poole-Wilson et al., 1997}. Randomized clinical trials have demonstrated that thrombolytic therapy, aspirin, β-blockers and ACE inhibitors have all proven to reduce mortality risk and the latter three shown to reduce morbidity {Yusuf et al., 1996}. Calcium channel blockers have also been shown to reduce myocardial oxygen demands, by lowering blood pressure, myocardial contractility, dilating coronary arteries and preventing calcium overload in ischemic cardiac myocytes {Yusuf et al., 1996}. However, the results of randomized trials have been variable since some calcium antagonists have been shown to aggravate myocardial ischemia by, for

example, causing reflex tachycardia {Yusuf et al., 1996}. In terms of congestive heart failure, more aggressive approaches are taken to treat this condition {Poole-Wilson et al., 1997}. Future directions in the treatment of this disease include the use of: (i) diuretic agents (i.e., natriuretic peptides and endopeptidase inhibitors) which prevent fluid retention, (ii) positive ionotropic agents (i.e., receptor agonists, ion channel mediators, modifiers of signal transduction, calcium sensitizers) which increase myocardial contractility (iii) vasodilatory agents (i.e., calcium antagonists, peptide vasodilators) which "unload" the ventricle, (iv) neurohormonal agents (i.e., ACE inhibitors) which delay progression of ventricular dysfunction, (v) agents that alter myocardial energetics (i.e., heart rate depressants, modifiers of substrate utilization) to improve cardiac myocyte metabolism and (vi) gene therapy to modify gene expression in the heart to increase cardioprotection {Poole-Wilson et al., 1997}. Alternative strategies would be to exploit "endogenous" protective systems, which already exist in the heart, in an effort to increase cardiac myocyte protection subsequent to ischemia-reperfusion injury.

2.2 Cardiac Myocyte Growth

Cardiac myocytes undergo two major stages of growth to increase cardiac ventricular mass during development {Zak, 1974}. The first type of growth, which is predominant from early to late embryonic stages of development, occurs through an increase in cardiac myocyte number or hyperplasia {Zak, 1974; MacLellan and Schneider, 2000}. The second type of growth, which is predominant from neonatal development to adulthood, occurs through an increase in cardiac myocyte size or cardiac myocyte hypertrophy {Zak, 1974; MacLellan and Schneider, 2000}. As a result, it is thought that hyperplastic growth is completely replaced by hypertrophic growth in adult cardiac myocytes {MacLellan and Schneider, 2000}. Although there is limited information regarding the regulation of this transition, however, given its timing it is possible that the switch in growth may be triggered by the increased functional demands placed on the heart after birth {MacLellan and Schneider, 2000}. The following sections will provide reviews on cardiac myocyte proliferation and cardiac myocyte hypertrophy during cardiac development and/or disease.

2.2.1 Cardiac myocyte proliferation

Cardiac myocyte proliferation is most prevalent during early embryonic stages of cardiac myocyte growth and results in an increase in the number of mononucleated cardiac myocytes through mitotic cell division {Zak, 1974; MacLellan and Schneider, 2000}. A second essential process that occurs during prenatal development involves the structural differentiation of the myocyte cytoplasm into organized myofibrils and other cytoplasmic components {Anversa et al., 1975; Anversa et al., 1981}. The ability of

cardiac myocytes to undergo proliferation and differentiation simultaneously is one of the features which makes cardiac muscle unique from any other muscle type {Claycomb, 1992}.

Numerous studies have focussed on quantitating the level of cardiac myocyte proliferation (mainly through DNA synthesis) in the heart during development, however, the conclusions drawn from these results have been controversial. One of the major controversies in the literature has been the interpretation of an increase in DNA synthesis as an increase in proliferation {Anversa and Kajstura, 1998}. Cell proliferation, by its true definition, can only be determined by counting the number of cells or presence of mitotic figures {Anversa and Kajstura, 1998}. However, determining cardiac myocyte proliferation using this approach has not always been feasible or practical. This is mainly due to the short duration of mitotic (M) phase (1 h), low proliferative capacity of cardiac myocytes and difficulties in using mitotic figures to distinguish multinucleated cells undergoing cytokinesis (cell division) versus karyokinesis (nuclear division) in cardiac tissue {Soonpaa and Field, 1998}. On the other hand, DNA synthesis or entry into the synthetic (S) phase of the cell cycle is a prerequisite for cell proliferation, and albeit not an absolute indicator of proliferation, has been considered a reliable marker of cardiac myocyte growth {Soonpaa and Field, 1998}. Table 2 provides a compilation of studies assessing the levels of cardiac myocyte DNA synthesis which are associated with the three key development periods (embryonic, neonatal, adult) in rats and mice. As shown in Table 2, the cardiac myocyte DNA synthesis labeling index, which is defined as the percentage of cardiac myocytes undergoing DNA synthesis (as assessed by

bromodeoxyuridine (BrdU) incorporation or tritiated thymidine (^{3}H) uptake) over the total number of cardiac myocytes, during mid-gestation (10 days post-coitum) is 46% in the mouse heart {Erokhina and Rumyantsev, 1986}. At the end of gestation (18 days post-coitum), however, the DNA labeling indices decrease to values of 13.3 % in the rat heart {Marino et al., 1991} and between 10% and 19.1% in the mouse heart {Erokhina and Rumyantsev, 1986; Soonpaa et al., 1996}, suggesting a decrease in the proliferative capacity of cardiac myocytes with age {Soonpaa and Field, 1998}.

During the early postnatal period of growth, cardiac myocytes enter a transitional stage, where they continue to progressively decrease in their ability to divide. Cardiac ventricular mass instead, increases predominantly through an increase in cardiac myocyte size or hypertrophy {Zak, 1974; MacLellan and Schneider, 2000}. A more thorough discussion on cardiac hypertrophy will follow (see section ***2.2.3***). As shown in Table 2, labeling indices, although variable, decrease significantly from 13% and 11% at postnatal day 1 {Cheng et al., 1995; Machida et al., 1997} to 4.2% as well as 4.5-7.5% at postnatal day 7 {Erokhina and Rumyantsev, 1988; Nakagawa et al., 1988; Sasaki et al., 1970; Soonpaa et al., 1996} in the rat and mouse heart, respectively {Soonpaa and Field, 1998}. In fact, it has been documented in the rat heart that cardiac myocytes completely cease to undergo proliferation as early as three to four days postnatally {Claycomb, 1992} to as late as twelve days postnatally {Li et al., 1996c}. In the mouse heart, cardiac myocyte proliferation has been reported to cease by birth {Soonpaa et al., 1996}. It has been suggested that any DNA synthesis or mitotic division that occurs after birth in the

rat or mouse heart has been characterized by an increase in cardiac myocyte binucleation {Brodsky et al., 1980; Clubb and Bishop, 1984; Soonpaa et al., 1996}.

Binucleation is a process whereby cells undergo nuclear division (karyokinesis) in the absence of cytoplasm division (cytokinesis), resulting in no overall increase in cell number {MacLellan and Schneider, 2000}. It has been documented that about 85% of cardiac myocytes become binucleated by the third week after birth in the rat heart {Clubb and Bishop, 1984}. Completion of myocyte binucleation is claimed to occur at postnatal day twelve in the rat heart {Li et al., 1996c}. The functional significance of this uncoupling in cardiac myocytes is currently unknown. However, similar processes in insects, plants and mammals have been documented in events such as meiosis, where two successive events of chromosome segregation occur without an intervening DNA synthesis, and endoreduplication, where there are multiple rounds of DNA replication in the absence of intervening mitoses {Grafi, 1998}. Regardless, the presence of binucleated cardiac myocytes in and of itself suggests that an attempt at proliferation or cell division, albeit unsuccessful, is still present in postnatal cardiac myocytes.

Adult cardiac myocytes, having completed this transitional stage, are generally considered to be permanently withdrawn from the cell cycle with an inability to divide or undergo mitosis *in vivo* and their growth is traditionally viewed to occur exclusively through an increase in size {Zak, 1974; MacLellan and Schneider, 2000}. As a result, it was thought that in the event of cardiac injury adult mammalian cardiac myocytes do not regenerate or proliferate, but instead undergo cardiac hypertrophy as part of the

compensatory response {MacLellan and Schneider, 2000}. Despite these traditional views it has long been known that ventricular cardiac myocytes in other species such as the adult newt have the ability to undergo regeneration in the event of cardiac injury {Oberpriller et al., 1988; Soonpaa et al., 1994}. In addition, cardiac myocyte DNA synthesis is observed in the normal adult mouse and rat ventricle (Table 2). There is now convincing evidence, however, that there are 'proliferative activities' in the cardiac myocytes of the adult mammalian heart, which although low, do suggest that withdrawal from the cell cycle, even in the mammalian heart, is not irreversible {Anversa et al., 1995; Kajstura et al., 1998; Kajstura et al., 1994; Rumyantsev and Kassem, 1976}. This evidence mainly stems from studies that assess cardiac myocyte cell number, mitotic figures and cell death during cardiac development and disease in human hearts {Anversa and Kajstura, 1998}. For instance, an assessment of the total number of ventricular myocytes in human hearts at birth revealed approximately 1 x 10^9 cells {Adler and Costabel, 1975; Adler and Costabel., 1980; Anversa and Kajstura, 1998}. A similar assessment of the total number of cardiac myocytes in hearts from women and men at 20 years of age, revealed a significant 6 and 8 fold increase in cardiac myocyte number compared to number of myocytes at birth {Olivetti et al., 1991; Olivetti et al., 1995}, suggesting proliferative activity during postnatal myocardial growth {Anversa and Kajstura, 1998}. Furthermore, using confocal microscopy it was shown that normal adult human cardiac myocytes have the ability to undergo mitotic cell division through the observations of mitotic figures (14 cardiac myocytes per million in mitosis) {Kajstura et al., 1998}. In addition, it has been documented that the number of mitotic figures significantly increase by approximately 10-fold in human hearts undergoing various

cardiac pathologies, which provides additional evidence that adult cardiac myocytes are not terminally differentiated and may be capable of a regenerative response upon injury {Kajstura et al., 1998}. Finally, myocyte cell death increases with age and cardiac disease {Anversa et al., 1990; Kajstura et al., 1996} yet it has been demonstrated that both the absolute numbers of cardiac myocytes as well as number of cardiac myocytes in mitosis are significantly increased {Kajstura et al., 1998}. These contrasting observations of increased cardiac myocyte death with an increase in cardiac myocyte number have been reconciled with evidence showing that myocyte cell loss in the aging human heart is replaced by myocyte cellular hyperplasia {Anversa et al., 1990; Anversa et al., 1991}. Altogether these data suggest that the heart is governed by a balance between ongoing cell loss and cell proliferation/regeneration during the entire life of a human being or an animal {Anversa and Kajstura, 1998}. These data also offer strong support to the notion that cardiac myocyte proliferation is a necessary and active process in the adult heart.

Although the proliferative activity in adult cardiac myocytes is low, the significance might only be appreciated over time. Based on the level of mitosis observed in failing human hearts of 45 year old males (11 mitotic myocyte nuclei per million which results in 59,000 nuclei in the left ventricle) {Olivetti et al., 1995; Quaini et al., 1994} and assuming a mitotic time of 1 hour, it has been calculated that this would result in a 10% increase in the generation of new cardiac myocytes in a year and almost a doubling in the total number of ventricular myocytes over a period of 10 years {Anversa and Kajstura, 1998}. These results are consistent with quantitative results in severely hypertrophied human hearts {Anversa and Kajstura, 1998}, however, this may be an

underestimate since they are based on the assumption that myocytes divide only once. Therefore, the identification of pathways, which result in relatively small effects on DNA synthesis and/or cell number, can have profound effects on the relative rate of S and/or M phase entry and potential generation of new myocytes. The next section will review some of the most widely recognized models of cardiac myocyte proliferation. Given the growing use of transgenic mouse models for the study of cardiac growth, this review will preferentially focus on *in vivo* models of cardiac myocyte proliferation.

Table 2. Level of cardiac myocyte DNA synthesis in the ventricle during normal development in rats and mice.

Species	Age	Synthetic Cardiac Myocytes (%) and their Identification	DNA Synthesis Assay	Reference
Mouse	10ed	46.0 LM/H	^{3}H	Erokhina and Rumyantsev, 1986
Mouse	18ed	12.1 and 19.1 LM/H and EM, resp.	^{3}H	Erokhina and Rumyantsev, 1986
Mouse	18ed	10.0 Isolated Myocytes	^{3}H	Soonpaa et al., 1986
Rat	18ed	13.3 LM/H	^{3}H	Marino et al., 1991
Mouse	1d	11 LM/H	BrdU	Machida et al., 1997
Mouse	1d	2.8 Isolated Myocytes	^{3}H	Soonpaa et al., 1996
Rat	1d	13 LM/I	BrdU	Cheng et al., 1995
Mouse	7d	7 LM/H	BrdU	Nakagawa et al., 1988
Mouse	7d	4.5 LM	^{3}H	Erokhina and Rumyantsev, 1986
Mouse	7d	12 LM/H	BrdU	Machida et al., 1997
Mouse	7d	5.5 Isolated Myocytes	^{3}H	Soonpaa et al., 1996
Rat	7d	4.2 LM/H	^{3}H	Sasaki et al., 1970
Mouse	Adult	0 Isolated Myocytes LM/H	^{3}H	Soonpaa and Field, 1994 Soonpaa et al., 1996 Soonpaa et al., 1997 Soonpaa and Field, 1997 Petersen and Baserga, 1965
Mouse	Adult	0.0004 - 0.0006 Nuclear Marker	^{3}H	Soonpaa et al., 1997 Soonpaa and Field, 1997
Mouse	Adult	<0.01 LM/H	^{3}H	Rumyantsev, 1966
Rat	56d	0.35 LM/I	BrdU	Baba et al., 1996
		Table 2. continued on next page		

Table 2. Continued

Species	Age	Synthetic Cardiac Myocytes (%) and their Identification	DNA Synthesis Assay	Reference
Rat	60d	0.2 LM/I	BrdU	Cheng et al., 1995
Rat	60d	0.2 LM/H	BrdU	Cheng et al., 1995
Rat	60d	0.13 - 0.16 PH/N	BrdU	Kajstura et al., 1994
Rat	75d	0.15 - 0.25 PH/N	BrdU	Reiss et al., 1993
Rat	>70d	0-0.004 LM/H	^{3}H	Kuhn et al., 1974
Rat	Adult	0.45 LM/H	^{3}H	Marino et al., 1991
Rat	Adult	0.005 LM/H	^{3}H	Rumyantsev and Mirakjan, 1968
Rat	Adult	0 LM/H LM/I	PCNA ^{3}H	Marino et al., 1996 Rumyantsev, 1970 Reiss et al., 1994 Heron and Rakusan, 1995

Source: Adapted from Soonpaa and Field, 1998.

EM, electron microscopy; LM, light microscopy; I, immune histology; H, histochemistry; Isolated myocytes, dispersed cell analysis; PH, phase-contrast microscopy; N, nuclear staining; and Nuclear Marker, transgenic mice which express the nLAC reporter gene in cardiac myocyte nuclei. The DNA synthesis assays include: tritiated thymidine incorporation (^{3}H), bromodeoxyurdine labeling (BrdU) and proliferating cell nuclear antigen (PCNA) detection.

2.2.2 *In vivo models of cardiac myocyte proliferation*

One of the earliest *in vivo* models to demonstrate an increase in postnatal cardiac myocyte proliferation was a mouse model overexpressing the large T antigen of the simian virus (SV40), under the control of the atrial natriuretic factor (ANF) promoter {Field, 1988}. Transgenic mice were characterized with large tumors composed of differentiated and dividing cardiac myocytes in the right atria {Field, 1988}. In an effort to induce cardiac ventricular myocyte proliferation, expression of the large T antigen was targeted to the ventricle using the rat α-myosin heavy chain (α-MHC) gene promoter {Katz et al., 1992}. Overexpression resulted in both atrial and ventricular myocyte hyperplasia in adult transgenic mice {Katz et al., 1992}. Myocytes retained the ability to beat spontaneously and maintain their differentiated state; however, cardiac myocytes underwent a limited number of passages when cultured {Katz et al., 1992}. These data suggest that cardiac myocytes can be stimulated to divide by SV40 large T antigen without the loss of differentiation {Claycomb, 1992}. Although the mechanisms remain to be determined, it has been proposed that the large T antigen interacts with cell cycle tumor suppressor proteins such as retinoblastoma protein (Rb), p107 and p53, to neutralize the anti-proliferative effects of these suppressor proteins *in vivo* {Claycomb, 1992}.

Proto-oncogenes such as c-myc have also been shown to play a pivotal role in cardiac myocyte proliferation in the postnatal heart *in vivo*. An assessment of c-myc RNA levels during cardiac development demonstrates that a decrease in c-myc levels is synchronous with the transition of cardiac myocytes from hyperplastic to hypertrophic

growth {Schneider et al., 1986}. To increase cardiac ventricular proliferation, transgenic mice were generated overexpressing the c-myc oncogene in cardiac myocytes, under the control of the Rous sarcoma virus (RSV) promoter {Jackson et al., 1990}. These transgenic mice displayed hyperplastic growth of both atrial and ventricular cardiac myocytes during fetal development {Jackson et al., 1990}. Although c-myc transgene expression persisted in the postnatal heart, cardiac myocyte proliferation was reported to cease during this time and instead resulted in premature entry of myocytes into the hypertrophic phase of growth {Machida et al., 1997}. Since it has been shown that the interaction of normal cells with v-myc transformed cells can suppress its transformed phenotype {La Rocca et al., 1989}, it was postulated that non-myocytes in the c-myc transgenic mouse hearts could perform a similar function to suppress proliferation postnatally {Jackson et al., 1990}.

Certain cell cycle proteins such as p27, have also been shown to play an important role in regulating cardiac myocyte proliferation *in vivo*. p27 is a cyclin dependent kinase (cdk) inhibitor that is a member of the KIP/CIP family which negatively regulates the cell cycle during gap 1(G1) and S phases {Brooks et al., 1998}. Protein levels of p27 have been shown to significantly increase during cardiac development, to a point where they are highest in the adult heart {Flink et al., 1998}. To increase cardiac myocyte proliferation, mice were generated lacking p27 {Poolman et al., 1999}. Loss of p27 in the mouse heart resulted in both an increase in cardiac myocyte number and a prolongation of cardiac myocyte proliferation {Poolman et al., 1999}. Parallels have been drawn between the c-myc overexpression model and the p27

knockout model, as cardiac myocytes from both models cease to proliferate postnatally {Poolman et al., 1999; Machida et al., 1997}. This is not surprising given the links between both and by the demonstration that p27 activity can be sequestered by myc-induced proteins {Vlach et al., 1996; Poolman et al., 1999}. The only difference observed between the models is that in the case of the p27 knockout model, the onset of binucleation is delayed by approximately two days whereas in the c-myc model there is an acceleration towards a differentiated state {Poolman et al., 1999}.

Growth factors such as insulin growth factor (IGF)-1 have also been shown to play an essential role in cardiac myocyte proliferation *in vivo* {Anversa and Kajstura, 1998}. An attenuation of the autocrine IGF-1/IGF-1 receptor system is synchronous with the decline in myocyte proliferation during cardiac development {Cheng et al., 1995; Engelmann et al., 1989}. With a view towards increasing cardiac myocyte ventricular proliferation, transgenic mice were generating overexpressing the human IGF-1B, under the control of the rat α-MHC promoter {Reiss et al., 1996}. It was shown that although the aggregate number of myocytes in the hearts of transgenic mice were identical to non-transgenic littermates at birth, a significant increase in aggregate number of myocytes was detected during the late stages of postnatal development {Anversa and Kajstura, 1998; Reiss et al., 1996}. However, this increase was mainly due to an increase in cardiac myocyte binucleation {Anversa and Kajstura, 1998}. In addition, it was shown that subsequent to cardiac injury, transgenic mice revealed a dramatic decrease in ventricular dilation and myocardial loading, which is consistent with a protective response {Li et al.,

1997c}. However, it remains to be determined whether this protective response involves the generation of new myocytes in the area of infarction.

In summary, many approaches have succeeded in "initiating" cardiac myocyte proliferation *in vivo*; however, it remains to be determined whether the cardiac myocyte "hyperplasia" observed (i) is dependent on early developmental expression of the candidate genes; due to the nature of the promoters used in these studies, (ii) can be recapitulated exclusively in the postnatal heart using an inducible system, or (iii) translates into the generation of "new" myocytes in the event of cardiac injury. It is also clear from these models that the candidate genes studied and their respective pathways are sufficient but not necessary on their own, to increase cardiac myocyte proliferation throughout the postnatal developmental period of an animal. Therefore, strategies that assess the contribution of specific pathways in controlling cardiac myocyte proliferation are of vital importance to define this complex process.

2.2.3 Cardiac myocyte hypertrophy

Cardiac hypertrophy is characterized by an increase in cardiac myocyte size and mass and represents an adaptive response to hemodynamic load caused by various stimuli {Hefti et al., 1997; MacLellan and Schneider, 2000}. Hypertrophic growth can be classified into two types, namely physiological and pathological. Physiological hypertrophic growth occurs during normal cardiac development and is first observed immediately after birth when the heart is adapting to the increase in functional demands {Zak, 1974}. Physiological hypertrophy also occurs in the adult heart, and can most

prominently be observed in the athletic heart {Schannwell et al., 2001}. On the other hand, pathological hypertrophic growth occurs as part of a compensatory response in the adult heart following a variety of pathological stimuli which include myocardial infarction, hypertension, endocrine disorders and perturbations in sarcomeric function due to altered expression or mutations of contractile proteins {Passier et al., 2000}. In the case, of physiological hypertrophy normal heart function is not compromised, however, in the case of pathological hypertrophy normal heart function is compromised (e.g., pathological diastolic filling pattern) {Schannwell et al., 2001}. Hypertrophic growth in both cases, however, is associated with a number of phenotypic changes and alterations in gene expression in cardiac myocytes {Hefti et al., 1997}.

The molecular events that are involved during the process of cardiac hypertrophy (pressure overload) are summarized in Figure 2B. The early response to hypertrophic stimuli occurs within 30 min of exposure and involves the induction of immediate early genes, which include early growth response-1 protein (Egr-1), heat shock protein 70 (hsp70), c-fos, c-jun, and c-myc {Hefti et al., 1997}. In the adult heart, this transient response represents a pattern of growth induction in cells with a limited ability to undergo DNA synthesis {Izumo et al., 1988}. The second response, which occurs 6 to 12 hours from stimulation involves re-expression of "fetal" genes, which are genes not normally expressed in the adult ventricle but highly expressed in the embryonic heart {Hefti et al., 1997; MacLellan and Schneider, 2000}. These include induction of genes such as the β-myosin heavy chain (β-MHC) {Nag and Cheng, 1986; Eppenberger et al., 1988}, α-skeletal actin {Schwartz et al., 1986}, ANF {Vikstom et al., 1998; Hefti et al., 1997}, Na^+/K^+ ATPase α subunit {Charlemagne et al., 1994}, Na^+/Ca^{2+} exchanger

{Kent et al., 1993; Studer et al., 1994}, β-tropomyosin {Izumo et al., 1988} and atrial myosin light chain-1 {MacLellan and Schneider, 2000}. In addition, genes normally expressed at higher levels in adult ventricles, such as α-MHC and the sarcoplasmic reticulum calcium ATPase pump 2a (SERCA2a), become downregulated during the hypertrophic response {MacLellan and Schneider, 2000}. Third, there is an upregulation of constitutively expressed contractile genes such as the ventricular myosin light chain-2 {Lee et al., 1988} and α-cardiac actin {Long et al., 1989}, which occurs at 12 to 24 hours. In cultured adult cardiac myocytes, re-expression of some fetal genes such as the β-MHC, α-smooth muscle actin and ANF can take up to several days {Hefti et al., 1997}. As a result, in terms of a final response, cardiac myocytes are subject to increased protein synthesis, a two to three fold increase in their original cell volume as well as the breakdown and reassembly of myofibrils {Hefti et al., 1997}. Myocyte loss through apoptosis and replacement fibrosis are also associated with cardiac hypertrophy and are postulated to mediate the decline in myocardial function that occurs with the transition from hypertrophy to failure {MacLellan and Schneider, 2000}.

A variety of stimuli which can trigger hypertrophic growth including mitogens, cardiac agonists as well as mechanical stress which are summarized in Figure 2A {MacLellan and Schneider, 2000}. Although changes in wall stress (i.e., mediated by pressure overload) can act as the primary mechanical sensor for the cascade of events leading to cardiac hypertrophy *in vivo*, it has been demonstrated that hypertrophy can also be induced *in vitro* {MacLellan and Schneider, 2000}. Hypertrophic stimuli can be categorized into various groups based on their intracellular signaling pathways and these include: (i) catecholamines such as the α-1 adrenergic agonists (i.e., phenylephrine) as

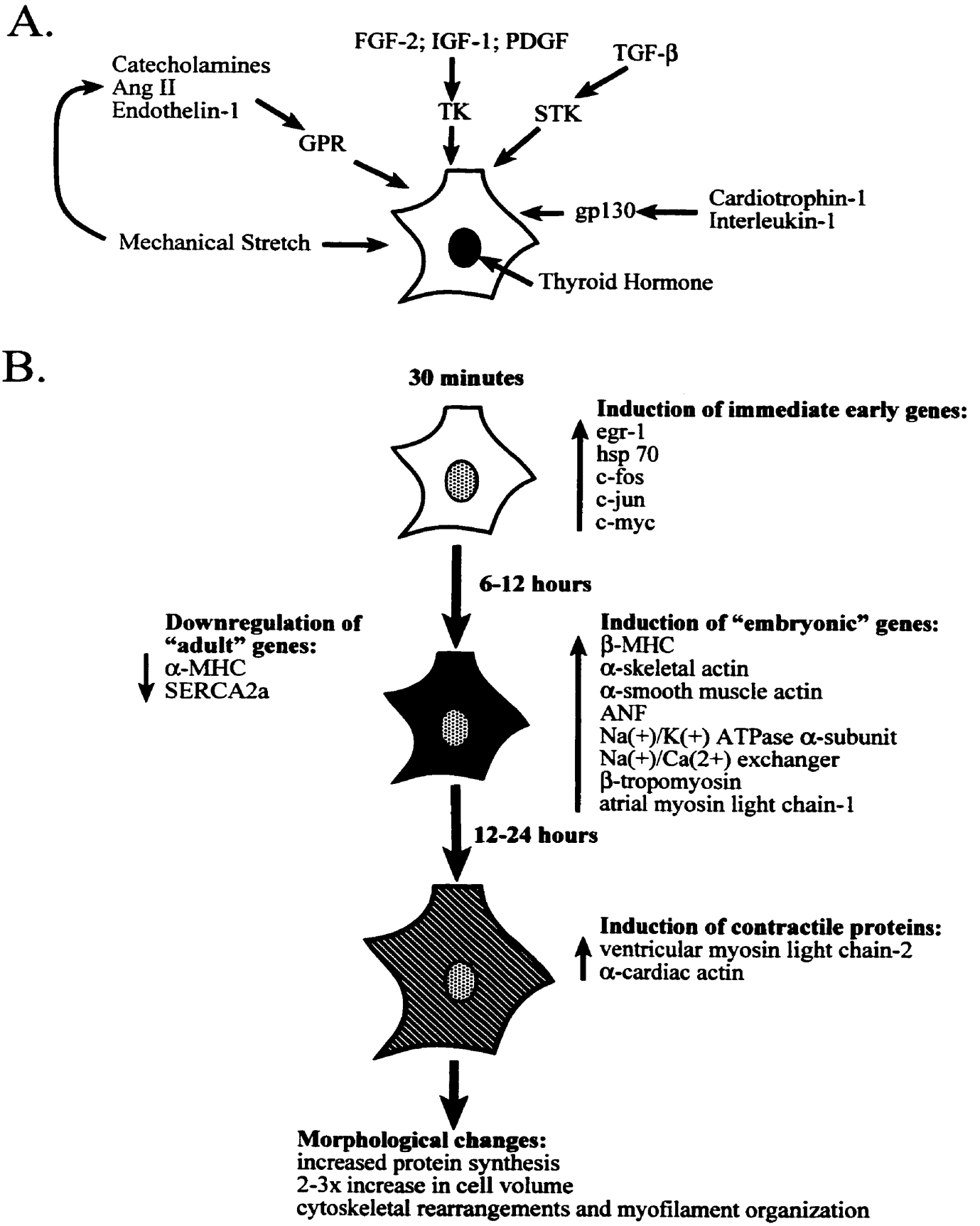
well as vasoactive peptides; AngII and endothelin-1 (ET-1); which are all coupled to the G protein receptor signaling pathway (ii) growth factors such as FGF-2, IGF-1, platelet derived growth factor (PDGF) as well as TGF-β which are coupled to receptor tyrosine or receptor serine-threonine kinase signaling pathways, respectively (iii) cytokines such as cardiotrophin-1 and interleukin-1 (IL-1) which all share the common signal transducer gp130 in their receptor signaling pathway and (iv) thyroid hormone which acts via nuclear membrane receptors {Hefti et al., 1997}. Mechanical stretch of cardiac myocytes can also elicit a hypertrophic response {Sodoshima et al., 1993; Schneider et al., 1991}. Since AngII is reportedly secreted from cardiac myocytes under mechanical stress, it has been postulated that one of the intracellular signaling pathways mediated by mechanical stretch is associated with the activation of G protein coupled receptors {Hefti et al., 1997; Sadoshima et al., 1993}. It should also be noted that that not all "hypertrophic" stimuli assessed *in vitro* express the same subset of molecular genetic markers observed in cardiac hypertrophies *in vivo* (i.e., pressure overload hypertrophy). In fact, stimulation of cardiac myocyte cultures with either FGF-2 or TGF-β are amongst the only stimuli which exhibited molecular features that paralleled pressure overload hypertrophy *in vivo* {Hefti et al., 1997}. Given the complexities of these pathways, it is imperative that efforts be directed towards unraveling the signaling molecules mediating cardiac hypertrophy in addition to cardiac myocyte proliferation, to prevent and protect the heart from failure. The next section will review some of the most widely recognized *in vivo* models of cardiac hypertrophy.

Figure 2.

Schematic representation of the intracellular signaling pathways and molecular events that are linked to the induction of cardiac myocyte hypertrophy.

A. The four major intracellular signaling pathways which mediate hypertrophy are (i) G protein coupled receptors (GPR), (ii) tyrosine (TK) and serine-threonine kinase (STK) receptors, (iii) cytoplasmic intermediary pathway involving gp130 and (iv) thyroid hormone which acts via nuclear membrane receptors. Mechanical stretch and wall stress are also known to induce hypertrophic growth.

B. The sequential molecular events which lead to cardiac hypertrophy following stimulation are summarized. egr-1, early growth response-1 protein; β-MHC, β-myosin heavy chain; ANF, atrial natriuretic factor; α-MHC, α-myosin heavy chain; SERCA2a, sarcoplasmic reticulum calcium ATPase pump 2a.


A.
FGF-2; IGF-1; PDGF
TGF-β
Catecholamines
Ang II
Endothelin-1
TK
STK
GPR
gp130
Cardiotrophin-1
Interleukin-1
Mechanical Stretch
Thyroid Hormone
B.
30 minutes
Induction of immediate early genes:
egr-1
hsp 70
c-fos
c-jun
c-myc
6-12 hours
Downregulation of "adult" genes:
α-MHC
SERCA2a
Induction of "embryonic" genes:
β-MHC
α-skeletal actin
α-smooth muscle actin
ANF
Na(+)/K(+) ATPase α-subunit
Na(+)/Ca(2+) exchanger
β-tropomyosin
atrial myosin light chain-1
12-24 hours
Induction of contractile proteins:
ventricular myosin light chain-2
α-cardiac actin
Morphological changes:
increased protein synthesis
2-3x increase in cell volume
cytoskeletal rearrangements and myofilament organization

2.2.4 In vivo models of cardiac hypertrophy

The process of cardiac hypertrophy has been best characterized through the assessment of the G protein coupled receptor pathway {Jalili et al., 1999}. G protein coupled receptors are heterotrimeric, comprised of three subunits (α, β, γ) and have a role in mediating extracellular biochemical signals to intracellular effectors {Jalili et al., 1999}. Overexpression of $G\alpha_q$ in transgenic mice is characterized by whole heart hypertrophy, recapitulation of the fetal gene program and premature death {D'Angelo et al., 1997}. Superimposing hemodynamic stress on this model, results in the classical features of decompensated heart failure which is characterized by the preferential development of left ventricular hypertrophy {Sakata et al., 1998}. The $G\alpha_q$ family of proteins are considered activators of the intracellular protein, PLCβ, with sensitivity to PLCβ1 in the heart {Jalili et al., 1999}. Activation of PLCβ leads to phosphotidylinositol biphosphate (PIP_2) hydrolysis and its byproducts, DAG and inositol 1,4,5-triphosphate (IP_3), which are potent activators of PKC {Jalili et al., 1999}. PKCs have also been implicated in cardiac myocyte hypertrophy {Jalili et al., 1999}. Overexpression of $G\alpha_q$ in transgenic mouse hearts was shown to activate PKCε {D'Angelo et al., 1997}. In addition, cardiac-specific overexpression of PKCβII in transgenic mice was able to produce a hypertrophic phenotype, upregulation of the fetal gene program and myocyte dysfunction in mouse hearts {Wakasaki et al., 1997}. Taken together these results suggest a major role for the G protein coupled receptor pathway in cardiac hypertrophy and failure.

Calcium-dependent signaling pathways have recently been recognized as major regulators of cardiac hypertrophy. In search for factors capable of interacting with the cardiac-specific transcription factor, GATA-4, a novel cardiac growth pathway was identified {Molkentin et al., 1998}. The calcineurin-nuclear factor of activated T cells 3 (NFAT3) pathway has been shown to be dependent on calcium signaling and transgenic mice expressing constitutively activated forms of calcineurin, the Ca^{2+}/calmodulin-dependent phosphatase, and NFAT3, the GATA-4 binding factor, which is dephosphorylated by calcineurin in the event of increased cytosolic calcium levels, each provoked cardiac hypertrophy {Molkentin et al., 1998}. In addition it was shown that well established inhibitors of the calcineurin pathway, cyclosporine A and FK506, are not only effective in blocking hypertrophy in calcineurin and NFAT3 overexpressing mice but also in several, but not all, genetic models of cardiac hypertrophy {Ding et al., 1999; Luo et al., 1998; Meguro et al., 1999; Mende et al., 1998; Olson and Williams, 2000; Sussman et al., 1998; Walsh, 1990}. Recently it has also been shown that the overexpression of the activated Ca^{2+}/calmodulin-dependent protein kinases-I and –IV (CaMKI and CaMKIV) also induced cardiac hypertrophy *in vivo* via a myocyte enhancer factor 2 (MEF2)-mediated pathway {Passier et al., 2000}. Crossing of transgenic lines expressing constitutively activated form of CaMK with those expressing NFAT3 resulted in a synergistic response on cardiac hypertrophy, suggesting that CaMK and calcineurin act cooperatively and in parallel to preferentially activate distinct transcriptional targets in the heart in the response towards hypertrophy {Passier et al., 2000}.

The proto-oncogene p21ras has also been shown to play an important role in cardiac hypertrophy. Transgenic mice overexpressing oncogenic H-Ras, under the control

of the ventricular form of myosin light chain-2 promoter, resulted in increased ventricular mass in the postnatal heart which was reflective of cardiac hypertrophy and not cardiac hyperplasia {Hunter et al., 1995}. This response is surprising since p21ras downregulates the cyclin-dependent kinase inhibitor, p27, whose expression when lost in the postnatal heart results in cardiac myocyte hyperplasia {Takuwa and Takuwa, 1997; Poolman et al., 1999}.

Another intracellular pathway implicated in cardiac hypertrophy is the mitogen-activated protein (MAP) kinase pathway. TGF-β activated MAP kinase protein 1 (TAK1) is a member of the MAP kinase kinase kinase family which was found to be upregulated subsequent to cardiac hypertrophy induced by aortic banding {Zhang et al., 2000}. To define the role of TAK1 in the adult myocardium, transgenic mice were generated overexpressing an activated form of TAK1 under the control of the mouse α-MHC promoter {Zhang et al., 2000}. TAK1 overexpression in transgenic mice was sufficient to induce cardiac hypertrophy, interstitial fibrosis, severe myocardial dysfunction, induction of fetal genes, apoptosis and premature death as a result of heart failure {Zhang et al., 2000}. Cardiac specific overexpression of the MAPK signaling pathway, MEK1-ERK1/2, in mice also resulted in concentric hypertrophy, however this was followed by increased cardiac function and partial resistance to apoptotic stimuli {Bueno et al., 2000}.

Genetic mutations in sarcomeric proteins have also been considered major players in the development hypertrophic cardiomyopathy {Redwood et al., 1999}. To date, there are at least seven different disease-genes which have been linked with hypertrophic cardiomyopathy {Redwood et al., 1999}. These include proteins encoding

components of either the thick filament (β-MHC), regulatory myosin light chain, essential myosin light chain, myosin binding protein-C, or the thin filament (i.e., cardiac troponin T and I as well as α-tropomyosin) of striated muscle {Redwood et al., 1999}. The mouse model of familial hypertrophic cardiomyopathy, which resulted from a mutation in the β-MHC gene was the first characterized sarcomeric mutation {Geisterfer-Lowrance et al., 1996}. Transgenic mice heterozygous for the mutation demonstrated cardiac dysfunction preceded by myocyte disarray, hypertrophy and fibrosis with increased age {Geisterfer-Lowrance et al., 1996}. Transgenic mice homozygous for the mutation, however, died within seven days after birth {Geisterfer-Lowrance et al., 1996}.

2.3 Cardioprotection and Cardiac Myocyte Growth Control Pathways

Limited information is known about the key molecular triggers that control cardiac myocyte protection and growth, however, there is some evidence suggesting that selective local polypeptide growth factors, present in the heart, can act in a paracrine and autocrine manner to target cardiac myocytes in these normal processes {Parker et al., 1990}. In fact, two classes of growth factors, namely the fibroblast and transforming growth factors, have been strongly implicated in targeting and modulating signaling events in cardiac myocytes {Schneider and Parker, 1991}. The following sections will review characteristic features, gene regulation, signaling and biological activities of FGF-2 as well as its high affinity receptor, fibroblast growth factor receptor-1 (FGFR-1) in the myocardium and cardiac myocytes. In addition, the role of a negative regulator of FGF-2, TGF-β, will also be discussed with respect to cardiac myocyte growth. Important questions, which remain unresolved in these areas, will also be highlighted in this review.

2.4 The Fibroblast Growth Factor (FGF) Family

The FGF family consists of at least 23 structurally related polypeptide growth factors, termed FGF-1 to FGF-23 {Yamashita et al., 2000}. FGFs were first identified in brain and pituitary extracts, and named for their ability to stimulate proliferation of BALB-C 3T3 fibroblasts {Trowell et al., 1939; Hoffman, 1940}. This activity was later shown to be due to two proteins, the first being FGF-1, also known as acidic FGF (aFGF), which has an acidic pI (5.6) and the second being FGF-2, also known as basic FGF (bFGF), which has a basic pI (>9.0) {Esch et al., 1985; Maciag et al., 1984; Thomas et al., 1984}. A defining feature of FGFs is their high affinity for heparin and this has facilitated their isolation, purification and use for structural studies {Gospadarowicz et al., 1987}. As a result, FGFs are also considered members of the larger heparin binding growth factor family, which also include other members such as vascular endothelial growth factor (VEGF) and heparin-binding epidermal growth factor-like growth factor {Ferrara et al., 1991; Peipkorn et al., 1998; Szebenyi and Fallon, 1999}. Phylogenetic analysis of the FGF family also suggests that all members derive from a common ancestral gene which has gone through at least two phases of gene duplication to give rise to the present diversity in FGFs {Coulier et al., 1997}. It is thought that the first series of duplications occurred at the time of emergence of the vertebrates and the second phase at the time of fin-to-limb transition {Coulier et al., 1997}. Despite its name and initial reported biological effects, this diverse family can modulate numerous cellular functions in multiple cell types, including cardiac myocytes {Galzie et al., 1997; Kardami et al., 2001}. Their functions range from cell proliferation, differentiation, survival, adhesion, migration, motility and apoptosis, to processes such as limb formation, wound healing,

tumorigenesis, angiogenesis, embryogenesis and blood vessel remodeling {reviewed in Szebenyi and Fallon, 1999}. The biological functions of FGFs are known to be mediated primarily by specific cell surface receptors of the tyrosine kinase family {Szebenyi and Fallon, 1999}. A more detailed discussion of these FGF receptors (FGFR) will occur in the following sections. Although FGFs play multiple roles, some FGFs and FGFRs exhibit a restricted yet overlapping spatial and temporal expression pattern in various organ systems and cell types {Szebenyi and Fallon, 1999}. In terms of the heart, there is considerable attention focussed on the role of FGF-2 because of its mitogenic, angiogenic and cardioprotective effects {Kardami et al., 2001}. This review will focus on discussing FGF-2 gene regulation at the level of transcription, mRNA structure, mRNA stability, translation, post-translational modifications, trafficking, release as well as the interactions of FGF-2 with its cell surface receptors and its various biological activities in the myocardium, with emphasis placed on its role in cardiac myocytes.

2.4.1 FGF-2 gene structure and transcriptional regulation in the myocardium

FGF-2 is one of two prototypic members of the FGF family (the other being FGF-1) which was originally purified from the brain and pituitary as a 16-18 kD (kilodalton) monomeric peptide {Gospadarowicz et al., 1985}. Assessment of the three dimensional structure of crystalline 18 kD FGF-2 revealed that it is organized in a trigonal pyramidal structure composed of 12 anti-parallel β sheets {Eriksson et al., 1991; Zhu et al., 1991}. The FGF-2 gene has been cloned from many species including human, bovine, rat, mouse, chick, opposum, sheep, *Xenopus* and newt {Szebenyi and Fallon, 1999}. It shares 40-50% sequence homology to other FGF family members and is highly conserved amongst species {Basilico and Moscatelli, 1992; Kardami et al., 2001}. The

human FGF-2 gene exists as a single copy, is mapped to chromosome 4 and spans over 40 kb of the genome {Abraham et al., 1986a,b; Mergia et al., 1986}. It contains three exons interrupted by two 16 kb introns and possesses large 5′ and 3′ non-coding regions {Abraham et al., 1986a; Shibata et al., 1991}. Human and rat FGF-2 promoter regions have been cloned {Shibata et al., 1991; Pasumarthi et al., 1997} and were shown to lack conventional TATA or CCAAT elements responsible for transcriptional initiation {Shibata et al., 1991; Pasumarthi et al., 1997}. Instead, the human FGF-2 gene was shown to rely on GC rich sequences for transcriptional initiation at a single start site {Shibata et al., 1991}, whereas the rat FGF-2 gene was shown to contain four transcriptional start sites (GC-rich) which were differentially regulated by phorbol esters {Pasumarthi et al., 1997}. The lack of conventional TATA or CCAAT elements, high GC content around transcriptional start site(s) and multiple transcription sites (in the case of the rat) in the promoter {Pasumarthi et al., 1997; Shibata et al., 1991}, as well as its ubiquitous pattern of expression {Bikfalvi et al., 1997}, suggests that FGF-2 has characteristics associated with so-called "housekeeping" genes of the cell. "Housekeeping" genes are usually expressed constitutively at low levels, making use of ubiquitous transcription factors, like Sp1 for their regulation and their products are often important for cell maintenance {Gallagher et al., 2000}. However, the term "housekeeping" gene is used loosely, since even well recognized low-level constitutively expressed "housekeeping" genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a potent enzyme which is essential for cell energetics, can be upregulated by certain stimuli, such as in the case of pathological conditions {McNulty and Toscano, 1995}.

Although transcription is an important level of gene regulation and both human and rat FGF-2 promoters have been cloned, there is limited information on transcriptional regulation of FGF-2, and even less with respect to its regulation in the mammalian heart. In fact, in the period of 1995 to early 2001 less than 5% of publications in the area of FGF-2 in the myocardium have reported on the transcriptional regulation of FGF-2. Studies using the human FGF-2 promoter have been predominantly carried out in non-cardiac cell types, and have implicated a role for p53, cell density, growth factors and PKC as regulators of FGF-2 transcription {Moffett et al., 1996; Moffett et al., 1998; Ueba et al., 1994}. The phorbol ester PMA, a potent PKC activator, was also shown to stimulate rat FGF-2 promoter activity in glioma cells {Pasumarthi et al., 1997}. Recent studies using the rat FGF-2 promoter region have demonstrated that FGF-2 transcription can be regulated in neonatal cardiac myocytes *in vitro* as well as in the adult myocardium *in vivo* by adrenergic agonists {Detillieux et al., 1999}. Specifically, the α_1-adrenergic agonist phenylephrine was shown to significantly increase FGF-2 promoter activity in neonatal rat cardiac myocytes {Detillieux et al., 1999}. Intraperitoneal administration of phenylephrine to adult transgenic mice expressing a hybrid firefly luciferase reporter gene directed by approximately 1 kilobase (kb) of upstream rat FGF-2 gene sequences (–1058FGFp.luc), also resulted in a significant increase in FGF-2 promoter activity in adult hearts *in vivo* {Detillieux et al., 1999}. This increase in FGF-2 promoter activity coincided with an increase in endogenous FGF-2 mRNA levels in adult hearts *in vivo* {Detillieux et al., 1999}. This suggested that the genetic information contained within the 1 kb fragment of rat FGF-2 promoter region was sufficient to drive FGF-2 gene expression in adult hearts *in vivo* {Detillieux et al., 1999}. The –1058FGFp.luc

transgenic model has significantly increased our understanding of FGF-2 transcription in the mammalian heart. However, since FGF-2 promoter activity was assessed in whole hearts, it remains to be determined whether FGF-2 can be regulated, at the transcriptional level, in adult cardiac myocytes.

Studies which addressed the mechanism of α_1-adrenergic regulation of FGF-2 transcription have been limited to neonatal cardiac myocytes, have ruled out a role for A/G rich sequences {Detillieux et al., 1998; Detillieux et al., 1999}, and implicated a direct role for the transcription factor Egr-1 in this mechanism {Jin et al., 2000}. Egr-1 is a member of the (three) zinc finger family of transcription factors, which binds to G/C rich motifs in DNA {Nakagama et al., 1995; Swirnoff et al., 1995}. It often constitutes the first step in the sequential expression of growth regulatory proteins, and as such is considered an immediate early gene {Biesiada et al., 1996}. Egr-1 is induced by mitogenic stimuli, such as serum and phorbol esters (i.e., PKC activators) {Biesiada et al., 1996} as well as hypertrophic agents (see section ***2.2.3***). Studies using the human FGF-2 promoter have already implicated a central role for Egr-1 in FGF-2 transcription in astrocytes {Biesiada et al., 1996}. The rat, like the human, FGF-2 gene contains consensus Egr-1 DNA elements in its upstream coding sequences {Biesiada et al., 1996; Jin et al., 2000; Pasumarthi et al., 1997, Wang et al., 1997}. Egr-1 synthesis was also shown to increase as a result of α_1-adrenergic stimulation in neonatal cardiac myocytes {Iwaki et al., 1990}. Studies from our laboratory using neonatal cardiac myocytes have demonstrated that (i) the α_1-adrenergic agonist phenylephrine increases Egr-1 levels and binding to the rat FGF-2 promoter region, (ii) an increase in Egr-1 levels stimulates rat

FGF-2 promoter activity, and (iii) mutation of Egr-1 sites can ablate α_1- adrenergic stimulation of a minimal rat FGF-2 promoter {Jin et al., 2000}. Altogether these studies suggests an important role for Egr-1 in the regulation of FGF-2 transcription.

Several studies suggest that PKC may be the cytoplasmic intermediate involved in mediating the effects of Egr-1 on FGF-2 gene transcription in cardiac myocytes. PKC activators, such as PMA and AngII were also shown to increase Egr-1 binding to the FGF-2 promoter region in neonatal cardiac myocytes {Jin et al., 2000} and preliminary studies using the –1058FGFp.luc transgenic mouse model, suggest that PMA can significantly increase FGF-2 promoter activity in adult cardiac myocytes {Sheikh and Cattini, unpublished observations}. Although a role for PKC was not demonstrated in the mechanism regulating α_1-adrenergic stimulation of FGF-2 transcription by Egr-1, it is conceivable that this would be a likely path, since catecholamines like phenylephrine which act through G protein coupled receptors are known to signal through PKC in cardiac myocytes {Hefti et al., 1997}. In addition, studies using adult cardiac myocytes have revealed that Ang II, ET-1 and IL-1β can increase FGF-2 mRNA levels (i.e., accumulation) {Fischer et al., 1997} and these effectors are all known to signal via PKC in cardiac myocytes {Grohe et al., 1994; He et al., 2000; Hefti et al., 1997; Jin et al., 2000; Neyses et al., 1993}. It is conceivable that in each case Egr-1 is a downstream target that can mediate an increase in FGF-2 transcription. In fact, a central role for PKC in the AngII and ET-1 mediated induction of immediate/early gene expression (i.e., inclusive of Egr-1) in adult cardiac myocytes was demonstrated when Egr-1 induction could be blocked by the calcium channel inhibitor, nisoldipine, which inhibits PKC

signaling pathways {Grohe et al., 1994}. A central role for PKC in Egr-1 induction by IL-1β has been established, when it was demonstrated that this induction could be blocked by PKC inhibitors in osteoblastic cells {Chaudhary et al., 1996}. FGF-2 mRNA levels, as assessed by reverse transcriptase polymerase chain reaction (RT-PCR), can also be increased in human ventricles following cardiac transplantation {Ationu and Carter, 1994}. It is also conceivable, in this case, that PKC may be involved since mechanical stress or preconditioning (i.e., brief period of ischemia) occuring during cardiac transplantation are known to stimulate PKC activity {Baines et al., 1999a; Hefti et al., 1997}. As a result, PKC activation could lead to increased Egr-1 levels {Hefti et al., 1997; Jin et al., 2000} which could then stimulate FGF-2 transcription. Mechanical stress has also been shown to induce AngII {Hefti et al., 1997}, which could then as a result regulate Egr-1 levels and binding to the FGF-2 promoter region {Jin et al., 2000}. It is possible that the effects of AngII, ET-1, IL-1β as well as cardiac transplantation in adult cardiac myocytes or hearts, respectively, could also be linked to effects on FGF-2 RNA stability. However, in all cases where PKC is activated, studies have implicated a direct role for this pathway in increasing Egr-1 levels and binding to the FGF-2 promoter region in cardiac myocytes {Jin et al., 2000}.

The ubiquitously expressed zinc finger transcription factor, Sp1, was also implicated in playing a role in FGF-2 transcription in astrocytes {Biesiada et al., 1996}. However, the functional effects of this interaction have not been elucidated in terms of FGF-2 expression in these cells, since these studies were limited to binding assays {Biesiada et al., 1996}. Sp1 sites have been identified in both rat and human FGF-2 gene

sequences, and were found to overlap with Egr-1 sites {Biesiasda et al., 1996; Pasumarthi et al., 1997}. The interplay between Sp1 and Egr-1 has been documented in various cell types, including human epithelial and vascular endothelial cells, but not cardiac myocytes {Cui et al., 1996; Khachigian et al., 1995}. Specifically, studies involving the tissue factor gene promoter in human epithelial cells demonstrated that Sp1 was required for basal expression whereas both Sp1 and Egr-1 mediated inducible expression by phorbol esters and serum, implicating cooperativity between these factors as well as temporal regulation in directing gene expression {Cui et al., 1996}. In addition, studies involving the platelet-derived growth factor-A promoter demonstrated that PKC-mediated induction of Egr-1 levels resulted in the displacement of Sp1 to increase promoter activity and gene expression in vascular endothelial cells {Khachigian et al., 1995; Khachigian and Collins, 1997}. A role for Sp1 in α1-adrenergic or PKC mediated transcriptional regulation of FGF-2 in cardiac myocytes remains to be determined.

Autoregulation is also an important component of transcriptional regulation of FGF-2 {Fischer et al., 1997}. In this case, secreted FGF-2 would act back through its cell surface receptors to regulate FGF-2 gene expression. Autoregulation of FGF-2 gene expression has been observed in adult cardiac myocytes, endothelial cells and vascular smooth muscle cells {Alberts et al., 1994; Fischer et al., 1997; Wang et al., 1997; Weich et al., 1991}. FGF-2 lacks a conventional signal peptide for secretion via conventional pathways, however, in the postnatal heart, FGF-2 was shown to be released from cardiac myocytes with every contraction in the myocardium under normal physiological conditions {Clarke et al., 1995; Kaye et al., 1996; see section ***2.4.5***}. The β-adrenergic

agonist, isoproterenol, through an increase in force and rate of contraction, can increase release of FGF-2 from the adult myocardium *in vivo* {Clarke et al., 1995}. Preliminary studies from our laboratory demonstrated that the β-adrenergic agonist, isoproterenol, can also increase FGF-2 mRNA levels and promoter activity in adult hearts of -1058FGFp.luc mice *in vivo*. Although the mechanism of this increase remains to be determined, together these studies suggest that regulation of FGF-2 transcription by β-adrenergic agonists could be dependent on released FGF-2 and autoregulation (i.e., β-adrenergic stimulation increases FGF-2 release which then acts through cell surface receptors to increase FGF-2 transcription), although direct regulation of FGF-2 transcription by β-adrenergic stimulation cannot be excluded {Riva et al., 1996}. Clearly, with the development of appropriate cell model systems, the -1058FGFp.luc transgenic mouse model could be potentially exploited to address the role of FGF-2 transcription in adult cardiac myocytes, as well as its role in β-adrenergic stimulation.

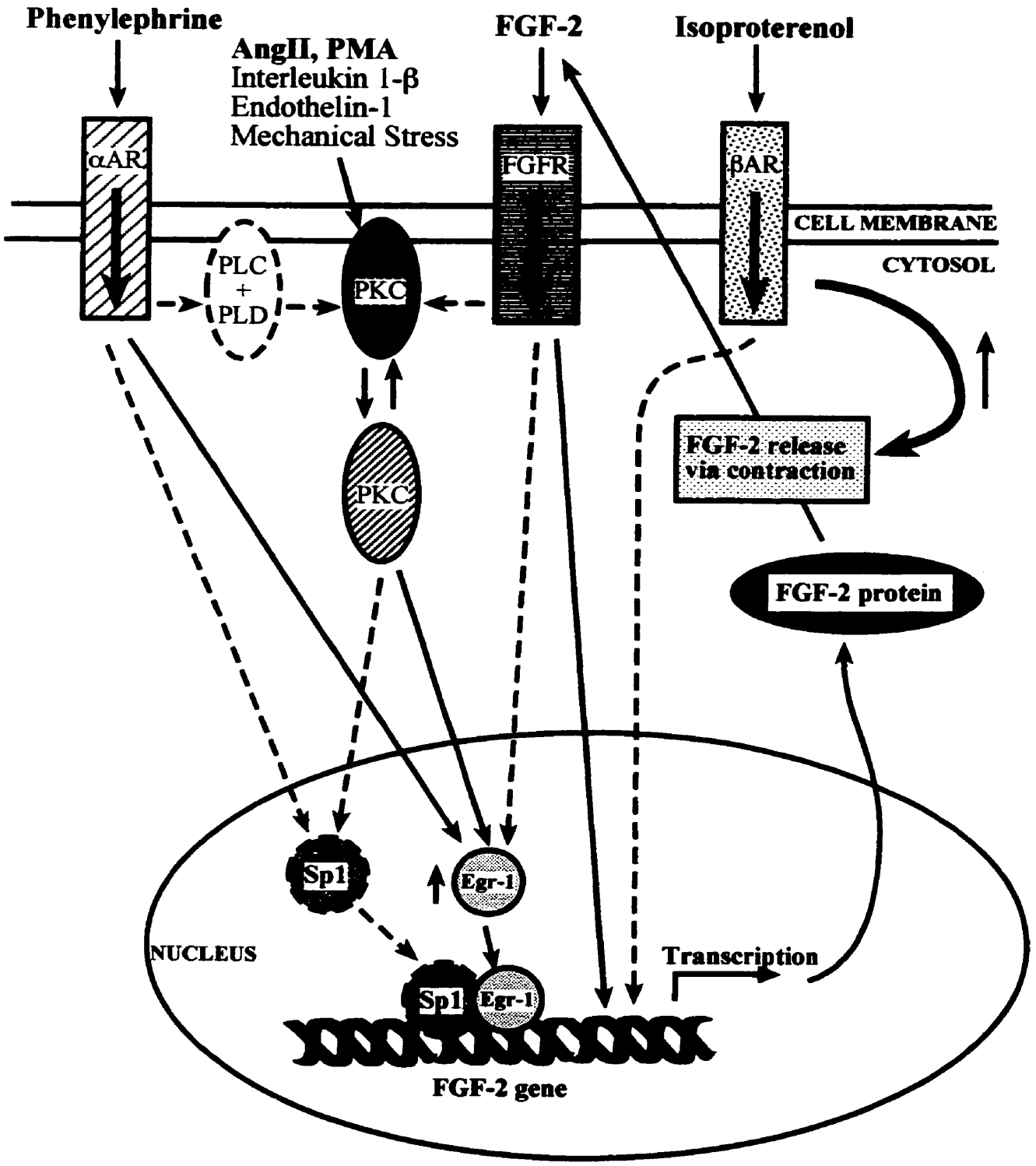
The mechanisms involved in autoregulation of FGF-2 gene expression in cardiac myocytes as well as other cell types are unclear. Studies using the human FGF-2 promoter in human heptacellular carcinoma cells have implicated a central role for Egr-1 in autoregulation of FGF-2 transcription {Wang et al., 1997}. In addition, endogenous release of FGF-2 as a result of injury, was also shown to act in a paracrine manner to activate Egr-1 promoter activity in endothelial cells in a mitogen activated protein kinase kinase (MEK) and extracellular-signal-regulated protein kinase (ERK) dependent manner, implicating an indirect mechanism of autoregulation of FGF-2 transcription {Santiago et al., 1999}. In this case, increased FGF-2 release as a result of injury would

directly increase Egr-1 promoter activity and thus Egr-1 protein levels, which could in turn increase FGF-2 transcription. Although Egr-1 is implicated as a central mediator of α1-adrenergic regulation of FGF-2 transcription in cardiac myocytes {Jin et al., 2000}, there is no direct evidence to support autoregulation of FGF-2 as a component of this mechanism. Studies have ruled out a role for calcium influx and cell contraction (which would presumably increase FGF-2 release) in mediating the effects of α_1-adrenergic regulation of FGF-2 transcription in neonatal cardiac myocytes {Detillieux et al., 1998; Detillieux et al., 1999}. Although autoregulation is postulated to play an important role in β-adrenergic regulation of FGF-2 transcription in cardiac myocytes (see above), Egr-1 may not be involved directly, since a study has demonstrated that Egr-1 synthesis cannot be directly increased by β-adrenergic stimulation {Iwaki et al., 1990}. Clearly, the use of appropriate model systems and promoter studies would facilitate determining the mechanisms which mediate FGF-2 transcription under various levels of regulation. Figure 3 provides a summary of the pathways implicated in mediating transcriptional regulation of FGF-2 in the myocardium.

Figure 3.

Schematic representation of the signaling pathways involved in transcriptional regulation of FGF-2 in cardiac myocytes.

Both known (solid arrows) and speculated (dashed arrows) pathways which mediate FGF-2 transcription in cardiac myocytes are summarized. The three major receptor pathways involved in regulating FGF-2 transcription in cardiac myocytes include: (i) alpha (αAR) adrenergic receptor pathway, (ii) β (βAR) aderenergic receptor pathway and (iii) FGF-2 receptor (FGFR) pathway. In terms of α-adrenergic regulation, a direct mechanism involving Egr-1 was demonstrated {Jin et al., 2000}. In terms of β-adrenergic regulation of FGF-2, an indirect mechanism was proposed which was dependent on released FGF-2 acting back through its' cell surface receptors to increase FGF-2 transcription. Autoregulation of FGF-2 transcription in cardiac myocytes has been demonstrated in many cell types including cardiac myocytes {Alberts et al., 1994; Fischer et al., 1997; Wang et al., 1997; Weich et al., 1991}. Egr-1 was implicated in autoregulation of FGF-2 {Wang et al., 1997}, this remains to be determined in cardiac myocytes. PKC (via PMA and AngII stimulation) has also been proposed to play a central role in FGF-2 transcription, and was demonstrated to increase Egr-1 binding to the FGF-2 promoter region in cardiac myocytes {Jin et al., 2000}. Although interplay between Sp1 and Egr-1 was shown in other cell types {Biesiada et al., 1996; Cui et al., 1996; Khachigian et al., 1995; Khachigan and Collins, 1997}, this remains to be determined in cardiac myocytes.


Phenylephrine
AngII, PMA
Interleukin 1-β
Endothelin-1
Mechanical Stress
FGF-2
Isoproterenol
αAR
FGFR
βAR
CELL MEMBRANE
CYTOSOL
PLC
+
PLD
PKC
PKC
FGF-2 release
via contraction
FGF-2 protein
Sp1
Egr-1
NUCLEUS
Transcription
Sp1
Egr-1
FGF-2 gene

2.4.2 Regulation of FGF-2 mRNA structure and stability in the myocardium

Multiple sizes of FGF-2 transcripts have been reported in *Xenopus* (4.5, 2.3 and 1.5 kb), chicken (10.2, 7.8, 4.9, 2.8, 2.6, 2.3 and 1.5 kb), rat (7.0, 6.0, 4.7, 3.7, 2.5, 2.2, 1.8, 1.6 and 1.0 kb), mouse (6.1, 4.7 and 3.6 kb), bovine (7.1 and 4.6 kb), and human (7.5, 5.0, 3.7, 3.5 and 2.1-1.8 kb) cells and/or tissues {Ationu and Carter, 1994; Borja et al., 1993; Cattini et al., 1998; Detillieux et al., 1999; Goldsmith et al., 1991; Hurley et al., 1994; Kimelman and Kirschner, 1989; Murphy et al., 1988; Pasumarthi et al., 1997; Powell et al., 1991; Logan et al., 1992; Sheikh et al., 1999; Stachowiak et al., 1994}. However, a recent study from our laboratory demonstrated that the rat and mouse 4.7 kb mRNA is not a "bona fide" FGF-2 transcript, but likely represents cross-hybridization of the "intact" FGF-2 cDNA to abundant 28S ribosomal RNA through G/C rich non-coding sequences {Cattini et al., 1998}. In addition, with the discovery of antisense FGF-2 transcripts (1.5, 1.3 and 1.1 kb) {Knee et al., 1997; Li et al., 1996a; Murphy and Knee, 1994}, it is conceivable that some FGF-2 transcripts, previously detected by RNA blotting using cDNAs (detects both sense and antisense transcripts), thought to be 'sense' could in fact represent products of antisense transcripts. This could be true, for example, for the 1.5 kb FGF-2 transcript detected in *Xenopus* and human tissues {Kimelman and Kirschner, 1989; Knee et al., 1994; Murphy and Knee, 1994}. The 6.1 kb FGF-2 was considered as the predominant endogenous FGF-2 transcript in the mouse heart {Cattini et al., 1998} as well as rat cardiac myocytes {Detillieux et al., 1999; Sheikh et al., 1999}. However, induction of both 6.1 and 3.6 kb FGF-2 transcripts was detected in phenylephrine-stimulated mouse hearts {Detillieux et al., 1999} and 7.5, 3.5 as well as 2.0 kb FGF-2 transcripts were detected and upregulated in the human ventricle following

transplantation {Ationu and Carter, 1994}. The reason for the variations in FGF-2 transcript size in the myocardium is largely unknown. However, it was demonstrated that FGF-2 transcript sizes can be regulated by: (i) the use of alternative polyadenylation sites within large 3′ untranslated regions {Kurokawa et al., 1987}, (ii) varying lengths of 5′ and 3′ untranslated sequences {Prats et al., 1989}, (iii) alternative splicing (Borja et al. 1993; Borja et al., 1996; el-Husseini et al., 1992}, as well as (iv) RNA degradation or stability {Abraham et al., 1986a; el-Husseini et al., 1992; Knee and Murphy, 1997}.

Transcription of antisense FGF-2 mRNA from the opposite strand of the FGF-2 gene, or antisense transcripts, were originally reported in *Xenopus* {Kimelman and Kirschner, 1989}, and subsequently found in the chicken {Borja et al., 1993; Salvage and Fallon, 1995}, human {Murphy and Knee, 1994} and rat {Knee et al., 1997}. In *Xenopus*, natural antisense transcripts were demonstrated to regulate FGF-2 mRNA stability {Kimelman and Kirschner, 1989}. Since the antisense transcript is complementary to regions of the 3′ untranslated region of the FGF-2 mRNA, it was postulated that formation of double-stranded RNA helices in these regions would regulate FGF-2 mRNA stability {Knee et al., 1997}. Antisense transcripts modulate FGF-2 mRNA stability by inhibiting transcript, processing and/or translation of 'sense' FGF-2 {Knee et al., 1997; Knee and Murphy, 1997; Li and Murphy, 2000}. Recent studies, have demonstrated that expression of alternatively spliced FGF-2 antisense RNA transcripts in rat brain and C6 glioma cells increase cellular FGF receptor content but inhibit cell proliferation as a result of suppression of cellular FGF-2 protein not mRNA levels (i.e., accumulation), indicating disruption of the FGF-2 autocrine pathway at the level of

translation {Li and Murphy, 2000}. To date the physiological significance of antisense FGF-2 transcripts in the myocardium or cardiac myocytes is largely unknown. Antisense FGF-2 transcripts have been shown to translate into proteins resembling members of the MutT-like family enzymes, which play a "house-cleaning" role in the hydrolysis of potentially hazardous compounds or metabolites {Bessman et al., 1996; Li et al., 1996b; Li et al., 1997a; Li and Murphy, 2000}. Thus, it is tempting to speculate that antisense FGF-2 transcripts play a role in postnatal cardiac growth and/or injury when there is increased metabolic activity. In addition, antisense FGF-2 transcripts were shown to be under tissue-specific and developmental control {Li et al., 1996a}. In the rat heart, it was demonstrated that the levels of antisense FGF-2 transcripts were low in embryonic hearts (days 15-19), increased 5-10 fold postnatally (day 10) over fetal levels, and then slowly declined to new steady-state levels in the adult heart {Li et al., 1996a}. It was noted that the levels of antisense FGF-2 RNA in several tissues, including heart, were much higher than sense FGF-2 RNA, which was undetectable by RNA blotting {Li et al., 1996a}. Clearly the reciprocal relationship between antisense and sense FGF-2 transcripts in the myocardium during development, along with the effects of antisense FGF-2 transcripts on inhibiting FGF-2 translation (which is important in autoregulation), point to an important level of control in regulating FGF-2 bioavailability and biological activity in the postnatal heart. This could play a major role in opposing or limiting the actions of "endogenous" FGF-2 in the adult myocardium in processes such as cardiac myocyte regeneration/proliferation upon injury *in vivo*. This notion is supported by recent evidence which demonstrated that antisense FGF-2 transcripts were involved in restraining pituitary tumor cell growth while promoting hormonal activity {Asa et al.,

2001}. Clearly, defining the role of antisense FGF-2 transcripts in the myocardium could be important in regulating the biological activities of FGF-2 in cardiac myocytes, including its proposed effects on growth and/or protection.

2.4.3 Translational regulation of FGF-2 in the myocardium

Both high and low molecular weight forms of FGF-2 have been identified in the myocardium as well as cardiac myocytes {Kardami et al., 1995; Liu et al., 1993}. FGF-2 isoforms arise predominantly through alternative translation initiation from leucine (CUG) versus methionine (AUG) codons from a single mRNA {Florkiewicz and Sommer, 1989}. High molecular weight (HMW) forms of FGF-2 (21-25 kD) result from translation initiation from unconventional, upstream CUG sites, whereas the widely studied 18 kD low molecular weight (LMW) form of FGF-2 results from translation initiation from a conventional, downstream, AUG site from the same mRNA {Florkiewicz and Sommer, 1989}. Alternative translation of human FGF-2 mRNA was shown to be controlled by internal ribosomal entry sequences in the 5′ untranslated region in a cap-independent manner {Vagner et al., 1995}. Preferential CUG versus AUG codon usage was shown to be modulated by cis-acting elements (in the 5′ leader sequence of FGF-2) corresponding to secondary or tertiary RNA structures, which could be the targets of cell-specific trans-acting factors {Prats et al., 1992}. Recently, a larger 34 kD HMW form of FGF-2 was identified in human HeLa cells arising from translation initiation at a more distal CUG site, involving a cap-dependent process {Arnaud et al., 1999}. Translational regulation of FGF-2 is also dependent on the species examined. In fact, it is interesting to note that there is considerable diversity in amino-terminal

extensions found in HMW FGF-2 forms between different species which have evolved with time {Detillieux, 1999}. For example, human HMW FGF-2 exist as 34, 24, 23 and 22.5 kD forms {Arnaud et al., 1999; Florkiewicz and Sommer, 1989}, rat HMW FGF-2 exist as 22 and 21.5 kD forms {Pasumarthi et al., 1997}, while in some species like fish, HMW forms of FGF-2 do not exist at all {Hata et al., 1997}. The shorter HMW forms of FGF-2 in the rat and the lack of HMW forms in the fish are due to the presence of a translation stop codon either upstream from the two CUG start codons in the rat FGF-2 mRNA {Pasumarthi et al., 1997} or immediately 5′ of the AUG site in the fish FGF-2 mRNA {Hata et al., 1997}. Translational regulation of FGF-2 forms can also be dependent on the tissue examined {Coffin et al., 1995; Liu et al., 1993}. Specifically, a study showed that overexpression of the full-length human FGF-2 cDNA (i.e., containing CUG and AUG sites) under the ubiquitous phosphoglycerate kinase promoter in transgenic mice *in vivo* resulted in different molar ratios of FGF-2 protein isoforms between different tissues examined {Coffin et al., 1995}. Multiple isoforms of FGF-2 can also arise from alternative splicing of mRNA {Borja et al., 1993} or proteolysis of HMW forms {Doble et al., 1990; Gualandris et al., 1993; Klagsbrun et al., 1987}.

The mechanisms directing translational regulation of FGF-2 in the myocardium and cardiac myocytes are largely unknown. However, there is evidence to suggest that FGF-2 translation may be regulated by factors or events involved in modulating cardiac development and/or injury. For example, studies have demonstrated that HMW forms of FGF-2 dominate in the immature myocardium, whereas LMW forms of FGF-2 dominate in the mature adult myocardium {Liu et al., 1993}. In addition, a transient increase in

HMW FGF-2 protein was observed in rat heart extracts following catecholamine-induced injury {Padua and Kardami, 1993}. Appearance of HMW forms of FGF-2 were shown to be similarly controlled in the liver during development and following injury {Presta et al., 1989}. Stress and factors present during injury (i.e., cytokines) can also regulate translation of HMW FGF-2 in other cell types, including astrocytes and fibroblasts {Kamiguchi et al., 1996; Vagner et al., 1996}. Furthermore, thyroid hormone could also be involved in translational regulation of FGF-2 as it has been shown to significantly reduce the accumulation of HMW forms of FGF-2 in cardiac myocytes *in vitro* and hearts *in vivo* {Kardami et al., 1995; Liu et al., 1993}. Similar effects were observed in skeletal muscle, but not other non-muscle organs such as brain or spleen, suggesting a muscle specific effect of thyroid hormone on FGF-2 translational regulation {Anderson et al., 1994; Kardami et al., 1995}. We cannot, however, exclude the possibility that thyroid hormone could affect FGF-2 gene regulation at the level of transcription, mRNA stability and/or post-translational processing {Kardami et al., 1995}. The functional differences and significance of HMW and LMW FGF-2 in terms of their expression during cardiac development and following injury will be discussed in section ***2.4.7***.

2.4.4 Post-translational modifications and trafficking of FGF-2 in the myocardium

FGF-2 protein can undergo various post-translational modifications, which include glycosylation, methylation, phosphorylation, ribosylation and nucleotidylation {Mason, 1994}. However, the significance of these modifications has not been fully elucidated in the myocardium or cardiac myocytes. Adenosine-5′-diphosphate (ADP) ribosylation is postulated to play an important role in signal transduction, DNA repair,

control of the cell cycle and cell differentiation {Boulle et al., 1995}. Since endogenous FGF-2 was demonstrated to be a substrate for post-translational ADP-ribosylation at arginine residues, it was postulated that the role played by ADP ribosylation in the various processes described above may involve its ability to target FGF-2 {Boulle et al., 1995}. It is therefore conceivable that ribosylation (and thus, "post-translation state" of FGF-2) could play an important role in regulating the biological effects of FGF-2 in the myocardium, by mediating signal transduction pathways involved in cardiac myocyte growth (e.g., cell cycle entry versus differentiation) and/or protection (e.g., DNA repair). Phosphorylation of FGF-2 was also thought to be important in regulating biological activities of FGF-2 {Feige et al., 1989}. Specifically, it was demonstrated that phosphorylation of serine (Ser-64) and threonine (Thr-112; receptor binding domain) residues of FGF-2 by PKC and protein kinase A (PKA), respectively, were differentially regulated by heparin as well as other extracellular matrix proteins including fibronectin and laminin {Feige et al., 1989}. Although the physiological significance of this phosphorylation is unknown it was suggested that the complex array of biochemical interactions between FGF-2 and proteins, proteoglycans, and glycosaminoglycans present in the extracellular matrix and cytoplasm, may regulate FGF-2's bioavailability by influencing its affinity for high affinity receptors {Feige and Baird, 1989; Feige et al., 1989}. Clearly, this would have an impact in modulating the biological effects of FGF-2 in any cell type, including cardiac myocytes.

There is limited information on the mechanisms mediating trafficking and/or compartmentalization of FGF-2 isoforms specifically in cardiac myocytes. However, it is

generally accepted that HMW forms of FGF-2 are localized to the nucleus, whereas the LMW form of FGF-2, are found predominantly in both cytoplasmic and nuclear sites in most cell types, including cardiac myocytes {Bugler et al., 1991; Pasumarthi et al., 1994; Pasumarthi et al., 1996}. Post-translational modification of FGF-2 via methylation may play a role in the nuclear compartmentalization of HMW forms of FGF-2 {Pintucci et al., 1996}. The amino terminal extensions of HMW forms of FGF-2 contain highly methylated glycine-arginine long term repeats which were demonstrated to be responsible for their intracellular distribution {Pintucci et al., 1996}. HMW forms of FGF-2 also contain nuclear localization sequence (NLS)-like signals responsible for nuclear targeting, which are found within a 37 amino acid sequence located between the second CUG and AUG start codons of human FGF-2 mRNA {Delrieu, 2000}. However, the NLS-signal is not a requirement for nuclear localization of FGF-2, since LMW FGF-2, which does not contain a NLS-like signal, can also localize to the nucleus in several cell types including cardiac myocytes {Hawker and Granger, 1992; Kardami et al., 2001; Patry et al., 1994}. Fusion of the NLS signal from SV40 large T antigen to LMW FGF-2 did not allow nuclear accumulation of this fusion protein {Patry et al., 1994}, suggesting that (i) LMW FGF-2 accumulates in the nucleus via an NLS-independent pathway of nuclear import and (ii) inhibitory sequences for nuclear import (via NLS) contained within the LMW FGF-2 can be overcome by amino terminal extensions in HMW forms {Bikfalvi et al., 1997}. The functional differences between HMW and LMW forms of FGF-2 in terms of their differential subcellular localization in cardiac myocytes will be discussed in section ***2.4.7***.

2.4.5 Regulation of FGF-2 release from the myocardium

There has been considerable focus on uncovering the mechanisms involved in regulating FGF-2 release since FGF-2, like various other members of the FGF family, lack a "classic" hydrophobic export signal for release through traditional pathways {Szebenyi and Fallon, 1999}. Various studies suggest that the mechanisms regulating FGF-2 release are in place since: (i) FGF-2 receptors are present on the cell surface of many cell types including cardiac myocytes (see section ***2.4.6***), (ii) both autocrine and paracrine effects of FGF-2 have been described in many cell types {Bikfalvi et al., 1997} including cardiac myocytes {Kardami et al., 2001, Pasumarthi et al., 1996; see section ***2.4.1***}, and (iii) FGF-2 protein was detected in the extracellular spaces/matrix surrounding various cells including cardiac myocytes, as well as in serum at low levels {Bikfalvi et al., 1997; Galzie et al., 1997; Kardami et al., 2001; Kurobe et al., 1992 }.

There are currently two views to explain the mechanism of FGF-2 release, and this includes release through exocytosis via non-classical pathways and/or release via passive processes {Bikfalvi et al., 1997}. In terms of non-classical pathways, exocytosis of FGF-2 via a mechanism independent of the endoplasmic reticulum (ER)-Golgi system has been described {Mignatti et al., 1992}. It was demonstrated in COS-1 cells that this pathway was energy dependent {Florkiewicz et al., 1995} and could be blocked with ouabain and other cardenolides, implicating a role for the Na^+/K^+ ATPase pump {Florkiewicz et al., 1998}. Although a functional interaction between FGF-2 and the catalytic subunit of the ATPase was demonstrated through co-immunoprecipitation studies {Florkiewicz et al., 1998}, it has subsequently been demonstrated using the yeast-

two hybrid system that this interaction may be indirect and involve other proteins {Oh and Lee, 1998}. In terms of passive processes, FGF-2 can be released as a result of cell lysis during tissue injury and cell death {Gajdusek and Carbon, 1989; Kaye et al., 1996}, complement-mediated injury {Floege et al., 1992}, matrix-associated release via heparin, heparan sulfate and heparinase {D'Amore, 1990; Thompson et al., 1990} as well as plasminogen activator-mediated proteolysis {Saskela and Rifkin, 1990}. In the postnatal heart, there is evidence to support a passive mechanism of FGF-2 release, as endogenous FGF-2 appears to be released from adult cardiac myocytes on a beat-to-beat basis through contraction-induced transient remodelling or "wounding" of the myocyte plasma membrane under normal physiological conditions {Clarke et al., 1995; Kaye et al., 1996}. FGF-2 is also released from endothelial and vascular smooth muscle cells through a similar mechanism involving plasma membrane disruptions {Cheng et al., 1997; Ku and D'Amore, 1995; McNeil et al., 1989}. Interestingly, a separate study has demonstrated that FGF-2 release from endothelial cells can be increased by estrogens through a mechanism involving the estrogen receptor and PKC activity but not requiring new protein synthesis, ER/Golgi secretion or protein tyrosine phosphorylation {Albuquerque et al., 1998}. Although this study claimed that these experiments were done in the absence of "wounding", as measured by markers of cell damage (i.e., LDH) {Albuquerque et al., 1998}, clearly a mechanism of FGF-2 release via "survivable" non-lethal membrane disruptions, which would result in no cellular damage, cannot be excluded.

Survivable plasma membrane disruptions have been proposed to be a biologically important process in many tissues, including the myocardium, and correlate with the level of mechanical stress imposed {McNeil and Steinhardt, 1997}. Tissues experiencing higher levels of stress were shown to increase disruption-mediated release of factors {McNeil and Steinhardt, 1997}. Indeed, increasing the level of "wounding frequency", as achieved through gentle mechanical disruption of neonatal cardiac myocyte in culture {Kardami et al., 1991}, mechanical pacing of adult cardiac myocytes in culture {Kaye et al., 1996}, or β-adrenergic stimulation (i.e. increased force and rate of contraction) of the isolated adult myocardium {Clarke et al., 1995} resulted in an increase in FGF-2 release. In addition, stress as achieved by expression of heat shock proteins in endothelial cells was shown to double the rate of estrogen-induced FGF-2 secretion, preferentially inducing release of the "intracellular" HMW FGF-2 {Piotrowicz et al., 1997}. Furthermore, catecholamine-induced injury in rat hearts *in vivo*, resulted in an accumulation of endogenous FGF-2 protein around cardiac myocytes in infarcted regions, which was also suggestive of its release {Padua and Kardami, 1995}. However, in cases of irreversible injury we cannot exclude the possibility that intracellular release of FGF-2 is also as a result of cell lysis or death {Kaye et al., 1996}.

Factors released through "wounding" are then proposed to mediate cellular events related to both normal homeostasis (e.g., tissue remodeling) as well as adaptation to increased workload (i.e., hypertrophy) {McNeil and Steinhardt, 1997}. Indeed, in adult cardiac myocytes cultures, it was demonstrated that paced (i.e., increased FGF-2 release) but not non-paced control myocytes exhibited a "hypertrophic response" which could be

mimicked by exogenously administered FGF-2 and blocked by neutralizing antibodies to FGF-2 {Kaye et al., 1996}. Studies utilizing FGF-2 knockout mice, reported that hearts ablated of endogenous FGF-2 resulted in a statistically smaller degree of hypertrophy during pressure overload injury *in vivo* {Schultz et al., 1998}. Although these studies did not assess FGF-2 release in hearts, it is conceivable that knockout of endogenous FGF-2 in mouse hearts, would eliminate FGF-2 release and could play an important role in the reduced hypertrophy observed. FGF-2 release may also play an important role in collateral vessel development observed 21 days after repetitive coronary artery occlusions in dogs {Weihrauch et al., 1998}. In this case, the formation of collaterals was blocked by neutralizing antibodies to FGF-2 at 12-14 days {Weihrauch et al., 1998}. In terms of estrogen-mediated FGF-2 release, it was postulated that this could contribute to the cardioprotective effects of estrogen {Albuquerque et al., 1998}. Altogether these studies suggest that endogenous FGF-2 which is released via transient plasma membrane disruptions can: (i) act in an autocrine (i.e., back through its cell surface receptors) and/or paracrine manner to mediate various biological effects of FGF-2 in the myocardium and/or cardiac myocytes, and (ii) be potentially exploited to regulate known biological effects of FGF-2 in various cell types including cardiac myocytes. There is currently limited information regarding the physiological relevance or role of endogenous FGF-2 in the adult heart *in vivo* as well as the role of this proposed natural mechanism of FGF-2 release. Clearly, exploiting any strategy (via genetic or other means) to stimulate endogenous FGF-2 levels in the myocardium might allow for prolonged and increased FGF-2 release from intracellular pools during contractions, which could be important for

regulating the effects of FGF-2 in terms of growth, protection and/or the maintenance of a healthy myocardium.

2.4.6 FGF-2 cell surface receptors in the myocardium

There is significant evidence to suggest that cellular FGF receptors (i.e., ligand/receptor interactions) play a major role in regulating FGF-2 bioavailability and thus, its biological activity. The biological effects of FGF-2, like other members of the FGF family, are regulated by binding to two classes of cell surface receptors, namely high and low affinity types {Galzie et al., 1997}. The high affinity, low capacity FGFR bind FGFs with a K_d of 20-600 pM {Partanen et al., 1992}, whereas the low affinity, high capacity receptors (i.e., heparan sulfate proteoglycans) bind FGFs with a K_d of 2-20 nM {Burgess and Maciag, 1989}. In fact, all FGFs, including FGF-2, contain both FGFR and heparin binding sites in a conserved core region within their structures {Szebenyi and Fallon, 1999}. Recent crystallization of a fragment of FGFR (specifically FGFR-1) with FGF-2 identified the location of a FGF binding site in a region of FGFR-1 as well as a potential heparin binding cleft in FGFR-1 that was contiguous with the heparin binding site on FGF-2 {Plotniknov et al., 1999}. This study further substantiated, at a structural level, the importance of FGF-2's interactions with its cell surface receptors in regulating FGF-2 bioavailability and signaling. The following sections will primarily focus on the role of FGFR-1 as well as heparan sulfate proteoglycan (HSPG) receptors in regulating FGF-2 bioavailability, signaling and biological activities in the myocardium as well as cardiac myocytes.

2.4.6.1 The FGFR Family and the role of FGFR-1 in the myocardium

FGFs transduce signals to the cytoplasm largely through their high affinity receptors {Johnson and Williams, 1993}. FGFRs are members of the tyrosine kinase family and consist of four major types, each encoded by separate genes {Galzie et al., 1997}. They can be divided as: FGFR-1 (flg or fms-like gene) {Lee et al., 1989; Pasquale and Singer, 1989}, FGFR-2 (bek or bacterial expressed kinase) {Houssaint et al., 1990; Kornbluth et al., 1988}, FGFR-3 (cek or chicken embryonic kinase) {Pasquale, 1990; Keegan et al., 1991} and FGFR-4 {Partanen et al., 1991; Stark et al., 1991}. Structurally, FGFRs are monomeric proteins which consist of three domains including: (i) an extracellular ligand binding domain, consisting of immunoglobulin (Ig)-like loops I-III, (ii) a single transmembrane spanning domain, and (iii) a functional intracellular tyrosine kinase domain {Klint and Claesson-Welsh, 1999}. This form is most commonly referred to as the 'long' or 'alpha' FGFR {Ornitz et al., 1996; Wang et al., 1995}. The FGFR protein is glycosylated and studies have reported molecular weights ranging from 110 to 165 kD {Gospodarowicz et al., 1986; Klagsbrun, 1989; Neufeld and Gospodarowicz, 1985; Neufeld and Gospodarowicz, 1986}. The overall amino acid sequence identity between the four receptors is 48-69%, however sub-regions, primarily within the intracellular (i.e., tyrosine kinase) domains, were shown to be nearly 100% conserved {Chellaiah et al., 1999; Szebenyi and Fallon, 1999}. The overall structure of the FGFR protein as well as various unique features defined within its structure are indicated in Figure 4. A feature which distinguishes FGFRs from other tyrosine kinase receptors includes the presence of an acidic rich region (acid box) in the extracellular domain which shares homology to cell adhesion molecules {Doherty and Walsh, 1996}.

Different FGFs bind to various FGF receptors in an overlapping and complex manner and these interactions are further complicated by the occurrence of multiple forms of FGFRs. Although the significance of this variability is not understood, it was shown that these multiple forms arise predominantly through alternative splicing events in the same RNA {Powers et al., 2000}. Different exon usage allows the translation of FGFR proteins which may be prematurely truncated, lack Ig-like domains, or utilize different coding regions for the same Ig-like domains {Powers et al., 2000}. The most common splicing event that occurs in FGFR-1 and FGFR-2 involves the exclusion of the exon encoding the IgI-like domain, resulting in a 'short' (two Ig-like domain form) of the receptor (splicing site indicated in Figure 4) {Johnson et al., 1991; Powers et al., 2000}. The binding affinities of FGFs to 'short' (Ig-like loops II and III) and 'long' (Ig-like loops I-III) FGFRs do not appear to change {Jaye et al., 1992; Johnson and Williams, 1993; Ornitz et al., 1996}, and their significance remains to be determined. Another RNA splicing event which occurs in FGFR-1, FGFR-2, and FGFR-3, results in the utilization of one of two unique exons causing three alternative versions of Ig-like domain III (referred to as domains IIIa, IIIb, IIIc) {Chellaiah et al., 1994; Johnson et al., 1991; Werner et al., 1992}. IgIII receptor forms appear to be expressed in a tissue-specific manner, with the IIIb and IIIc exon splice variant being exclusively expressed in epithelial and mesenchymal lineages, respectively {Alarid et al., 1994; Orr-Urteger et al., 1993; Yan et al., 1993}. On the other hand, the IgIIIa splicing variant, as well as some IgI splice variants result in secreted FGFR forms, due to the introduction of early stop codons or use of differential polyadenylation sites {Duan et al., 1992; Johnson and Williams, 1993}.

The specificity of different FGFs (1-9) for various FGFR splice variants are shown in Table 3. FGF-2 was shown to have a high affinity for FGFR-1IIIc, FGFR-3IIIc as well as 'short' FGFR-4 when compared to FGF-1 {Ornitz et al., 1996}. These studies were done in the presence of heparin (shown not to demonstrate inhibitory activity toward FGF-2) and it should be noted that FGFR-3 as well as FGFR-4 forms were engineered to carry the tyrosine kinase domain for FGFR-1 to enhance signaling capacity in BaF3 cells (a bone marrow cell line devoid of FGFR). Since some FGFs can bind to subsets of FGFRs, whereas others have a higher degree of specificity this suggests that a cell determines its susceptibility to FGFs by the receptor or receptor isoforms it expresses {Galzie et al., 1997}. Clearly, assessing the expression pattern and role of FGF receptors in the myocardium and in cardiac myocytes would be essential towards defining the biological role of FGF-2 in the myocardium and in cardiac myocytes.

Figure 4.

Schematic representation of the structure of the high affinity receptor for fibroblast growth factor (FGFR).

The structural features of FGFR as well as common exon splicing sites (Ig-like domains I and III) and stop codons are indicated. Modified from Johnson and Williams, 1993 as well as Powers et al., 2000.

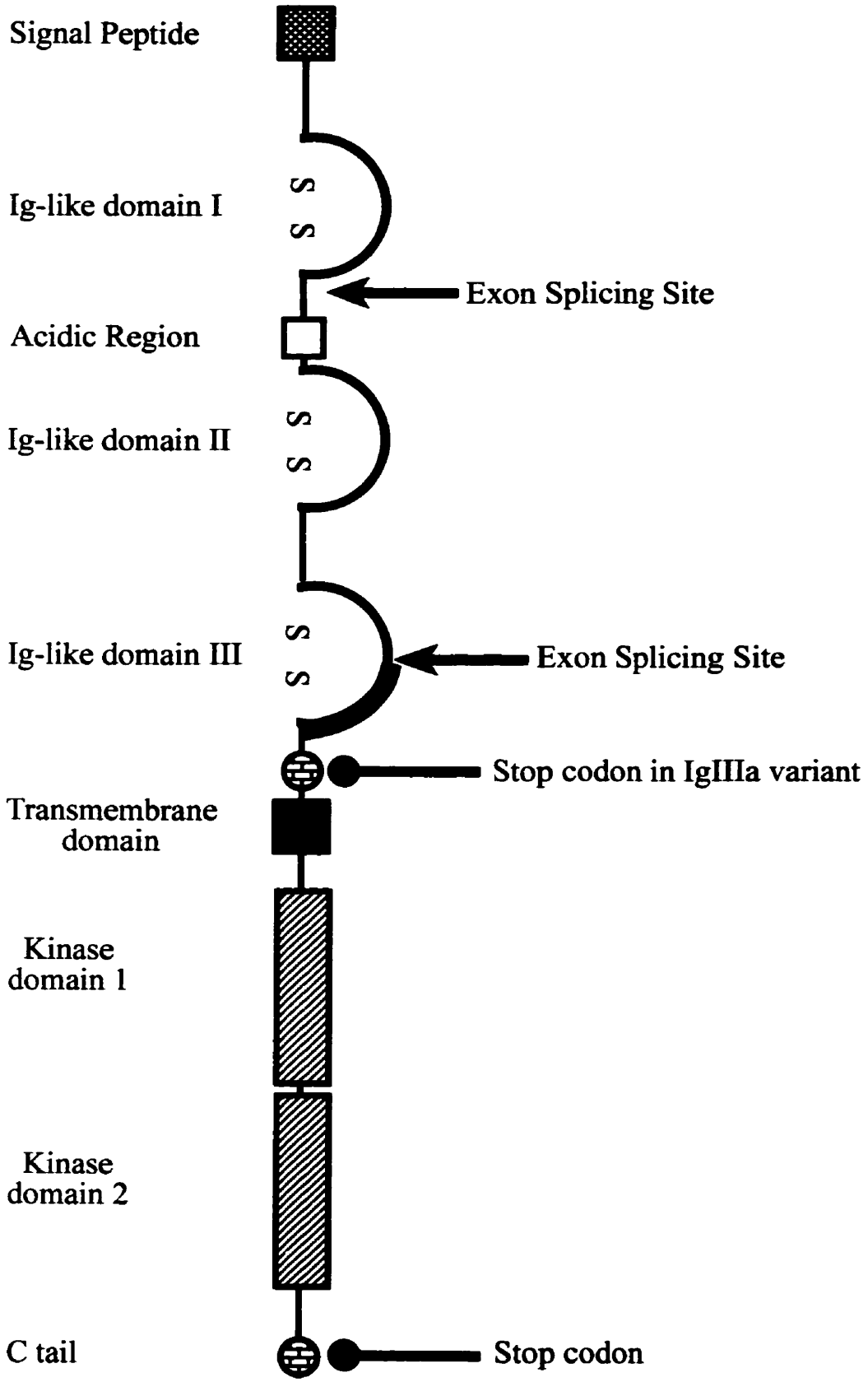

Signal Peptide
Ig-like domain I
S S
S S
Exon Splicing Site
Acidic Region
Ig-like domain II
S S
S S
Ig-like domain III
S S
Exon Splicing Site
Stop codon in IgIIIa variant
Transmembrane domain
Kinase domain 1
Kinase domain 2
C tail
Stop codon

Table 3. Specificity of FGFs 1-9 with various FGFR isoforms as determined by relative mitogenic activity in BaF3 cells expressing FGFR variants.

FGFR	FGF-1	FGF-2	FGF-3	FGF-4	FGF-5	FGF-6	FGF-7	FGF-8	FGF-9
1 IIIb	100	59.9	34.4	15.6	3.8	4.6	6.3	3.5	3.5
1 IIIc	100	**103.9**	0.3	**102.3**	59.0	54.9	0.3	0.7	21.1
2 IIIb	100	9.0	44.6	14.9	5.0	5.4	80.6	3.8	7.3
2 IIIc	100	64.0	4.2	**94.3**	25.0	60.7	2.5	16.1	89.2
3 IIIb	100	1.2	1.5	1.0	1.0	0.9	1.2	0.9	41.5
3 IIIc	100	**107.2**	0.6	69.1	11.8	**8.8**	1.0	40.5	**95.6**
4 'short'	100	**113.4**	5.8	**108.0**	7.0	79.4	1.9	76.1	75.4

Source: Modified from Ornitz et al., 1996

Bolded values indicate mitogenic activity of FGF-FGFR interactions that are above or in the range of those values obtained with FGF-1, which are indicative of a high affinity interaction.

The predominant high affinity receptor for cardiomyocytes in the embryonic or neonatal heart is FGFR-1, shown to exist as 'long' (102 kD) and 'short' (86 kD) forms {Bernard et al., 1991; Engelmann et al., 1993; Jin et al., 1994; Liu et al., 1995; Partanen et al., 1991; Peters et al., 1992b; Patstone et al., 1993; Pasumarthi et al., 1995; Speir et al., 1992; Wanaka et al., 1991; Yazaki et al., 1993}. The majority of FGFR-1 transcripts (4.2/4.3 kb) in the embryo as well as adult heart contain exon IIIc, which is associated with isoforms that display the highest affinity for FGF-2 (see Table 3) {Jin et al., 1994; Johnson and Williams, 1993; Pasumarthi et al., 1995}. Signaling via FGFR-1 was shown to play a vital role in the early stages of growth and development of the heart and vasculature {Amaya et al., 1991; Leconte et al., 1998; Lee et al., 2000; Mima et al., 1995; Sugi et al., 1995; Wannaka et al., 1991; Zhu et al., 1999}. This was supported by studies in *Drosophila* which demonstrated that a mutant form of FGFR-1 (*heartless*) was unable to induce formation of cardiac muscle from mesoderm {Gisselbrecht et al., 1996}. Although these studies have established an essential role for FGFR-1 in early cardiac development, its role specifically in cardiac cells (myocytes) as well as during postnatal cardiac development is less clear. Adult myocytes were shown to contain functional high affinity FGF-2 receptors (i.e., FGFR-1) {Liu et al., 1995}. However, an assessment of FGFR-1 mRNA levels during rat cardiac development demonstrated that FGFR-1 mRNA levels are significantly decreased and there is a switch in relative levels of 'long' versus 'short' FGFR-1 isoforms in embryonic versus postnatal hearts {Engelmann et al., 1993; Jin et al., 1994; Liu et al., 1995}. The 'long' and 'short' FGFR-1 mRNAs were shown to predominate in the embryonic and adult heart, respectively {Jin et al., 1994; Pasumarthi et al., 1995}, pointing to an association of 'long' FGFR-1 form with "hyperplastic"

phenotype. In contrast, 'long' FGFR was shown to correlate with restriction of growth, malignancy and enhanced differentiated function in view of its reduced expression in various tumors {Duan et al., 1992; Kobrin et al., 1993; Yamaguchi et al., 1994}. Defining the role of 'long' and 'short' FGFR isoforms in cardiac cells would be important in understanding the role of FGF-2 and its receptors in postnatal cardiac myocyte growth. Furthermore, since the decrease in FGFR-1 levels and the isoform switch {Jin et al., 1994; Liu et al., 1995; Pasumarthi et al., 1995} correlate with the transition of cardiac myocytes from a hyperplastic (high level of proliferation) to a hypertrophic (limited level of proliferation) phenotype. Both changes may be required to limit cardiac myocyte proliferation. The importance of FGFR-1 for cardiac myocyte proliferation has been illustrated by studies which demonstrated that knockout of FGFR-1 function *in vivo* during early avian cardiac development resulted in an inhibition of cardiac myocyte proliferation {Mima et al., 1995}. In addition, chronic FGFR activation has been implicated to play an important role in tumorigenesis (i.e., increased proliferation) {Klint and Claesson-Welsh, 1999}. Increasing FGFR-1 levels, and/or changing the relative levels of the 'long' versus 'short' isoform, could therefore increase the proliferative potential of postnatal cardiac myocytes, as well as the ability of FGF-2 to stimulate a proliferative/regenerative response in the event of cardiac injury *in vivo*.

A recent study has also suggested that FGFR-4 may play a role in the myocardium, since "intense" staining for FGFR-4 protein, comparable to FGFR-1, was observed in human adult cardiac myocytes using immunohistochemistry {Hughes, 1997}. Separate studies, however, have demonstrated that FGFR-4 RNA could not be detected in

mouse cardiac muscle at any developmental stage or in the human fetal heart {Stark et al., 1991; Partanen et al., 1991}. In fact, the expression pattern for FGFR-4 in human fetal tissues was found to be distinct from the expression pattern for FGFR-1 (i.e., not in the heart) {Partanen et al., 1991}. However, this may reflect differences between an assessment of FGFR-4 at the RNA versus protein level, changes observed during cardiac development or species differences. To date, there is limited information on the relevance of FGFR-4 in the myocardium, however, in view of the importance of FGF-2 which can also signal via FGFR-4 (see Table 3), there is a need to define its role. This could also reveal a biological role for other FGFs in the myocardium which depend on FGFR-4 and not FGFR-1 signaling, such as the recently discovered member of the FGF family, FGF-16, shown to be exclusively expressed in the postnatal heart {Miyake et al., 1998; Konishi et al., 2000}.

2.4.6.2 Heparan sulfate proteoglycans (HSPG) and other receptors for FGF-2 in the myocardium

FGF-2 bioavailability and biological activities can also be modulated by its low affinity binding to heparin and HSPGs {Ornitz and Leder, 1992; Roghani et al., 1994}. HSPGs are integral components of the basement membranes and are categorized into two major classes which include proteoglycans that are either attached to the plasma membrane by: (i) transmembrane core proteins (e.g., syndecans, perlecans) or (ii) covalently linked glycosylphosphatidylinositol (GPI) anchors (e.g., glypicans) {Asundi et al., 1997}. Perlecans, syndecans and glypicans have all been shown to bind FGF-2 via their covalently attached heparan sulfate chains, and thus, act as low affinity binding sites for FGF-2 {Brunner et al., 1991; Chernousov et al., 1993; Filla et al., 1998; Guillonneau

et al., 1996; Kiefer et al., 1990}. The number of sulfate groups present on heparan sulfate proteoglycans also alters its' affinity for FGF-2 {Miao et al., 1996}. Two major physiological functions for interactions between HSPGs and FGF-2 are proposed: (i) to protect FGF-2 from degradation from circulating proteases *in vivo*, thus, increasing its half life and (ii) to sequester FGF-2, creating a local reservoir for storage, which allows for strict spatial regulation of FGF-2 signaling as well as mobilization of large supplies when needed {Powers et al., 2000}. Although HSPGs are not absolutely required for binding of FGF-2 to cells, their presence on the cell surface and mobilization from the ECM were shown to increase the affinity of FGF-2 for its high affinity receptor {Fannon and Nugent, 1996; Powers et al., 2000; Roghani et al., 1994}. This notion is further supported by the three current FGF-FGFR dimerization models which advocate that HSPGs play an important role in the stabilization of this complex {Plotnikov et al., 1999; Stauber et al., 2000; Venkataraman et al., 1999}. Thus, the extracellular matrix plays an added dynamic role in regulating FGF-2 bioavailability. In fact, binding as well as release and diffusion of FGF-2 from the extracellular matrix was shown to be rapid and dependent on several factors which include FGF-2 concentration and/or basement membrane integrity {Dabin and Courtois, 1991, Dowd et al., 1999}. However, matrix degradation is not an absolute requirement to 'free' FGF-2 {Nugent and Edelman, 1992}. HSPGs were also suggested to act as direct transducers of FGF-2 signaling by internalization of FGF-2 into the cell independent of the FGFR {Quarto and Amalric, 1994}. Recent studies have implicated a direct role for syndecans as signal transducers as they were shown to have a direct interaction with PKC {Horowitz and Simons, 1998; Oh

et al., 1998}, with PIP_2 {Lee et al., 1998} and signal via the Src kinase-cortactin pathway {Kinnunen et al., 1998}.

There is limited information on the role and regulation of HSPGs in the myocardium and cardiac myocytes. Low affinity binding sites for FGF-2 have been identified on cardiac sarcolemmal membranes, and in some cases were purified, however specific forms were not identified {Liu et al., 1995; Ross and Hale, 1990; Ross et al., 1993a}. Both glypican and syndecan-3 (N-syndecan) were implicated as the major forms of membrane-associated HSPGs expressed in the neonatal and adult rat heart {Asundi et al., 1997; Liu et al., 1995}. However, recent studies using perlecan-null mice have also demonstrated an essential role for perlecan in the developing myocardium {Costell et al., 1999}. Although perlecan was not crucial for the assembly of basement membranes on early contracting cardiac myocytes it was essential in maintaining myocardial basement membrane integrity when subjected to mechanical stress {Costell et al., 1999}. The role of perlecan in the postnatal myocardium as well as the role of FGF-2 in perlecan-deficient mice remains to be determined. On the other hand, glypicans were shown to be expressed specifically in cardiac myocytes and to co-localize with FGF-2 binding sites in adult heart tissue {Asundi et al., 1997}. There is conflicting information regarding the localization of expression of syndecan-3 in the myocardium. Although, syndecan-3 (N-syndecan) expression was restricted to cardiac myocytes in the adult myocardium {Liu et al., 1995} a separate study demonstrated that expression of syndecan-3 was restricted to non-myocytes in the neonatal myocardium {Asundi et al., 1997}. However, this difference could reflect developmental differences (neonatal versus adult cardiac

myocytes cultures) or the specificity of the probe used. Assessment of expression of glypican and syndecan-3 during development revealed that their expression was low in late embryonic hearts, significantly increased in neonatal hearts and sustained at high levels in the adult heart {Asundi et al., 1997}. Clearly, the increase in HSPGs during cardiac development {Asundi et al., 1997} could reflect the progressive increase in overall FGF-2 levels observed during cardiac development {Kardami et al., 1995}. However, the impact of the imbalance between HSPGs and FGFR-1 (i.e., high HSPGs and low FGFR-1 in the adult heart) in the myocardium and in cardiac myocytes remains to be determined. Thus, defining the role of HSPGs and its relationship to FGFR-1 in the myocardium could be important in regulating FGF-2 bioavailability and biological activities in cardiac myocytes including its' proposed effects on growth and/or protection.

Other possible receptors for FGF-2 include the high affinity cysteine rich FGF-2 receptors which may play an important role in intracellular FGF-2 trafficking and secretion of FGFs {Burrus et al., 1992; Kohl et al., 2000; Zuber et al., 1997} as well as the vitronectin receptor which was shown to influence FGF-2's ability to induce cell adhesion, mitogenesis, and urokinase type plasminogen activator (uPA) upregulation in endothelial cells {Rusnati et al., 1997}. However, the roles of these receptors in the myocardium and cardiac myocytes remains to be determined.

2. 4.7 FGFR mediated signal transduction pathways in cardiac myocytes

Although most studies on FGF-2-mediated signal transduction pathways via FGFR have been carried out using FGFR-1 {Powers et al., 2000}, these studies were

limited to non-cardiac cell types. Thus, the role of FGFR-1 in FGF-2 mediated signal transduction pathways in cardiac myocytes remains to be determined. The following section, will first review known FGFR-1 mediated signal transduction pathways elucidated in diverse cell types and secondly, address signaling pathways which are activated by FGF-2 in cardiac myocytes, which are postulated to occur via high affinity receptors. Figure 5 presents a schematic diagram of the known signal transduction pathways for FGF-2 in cardiac myocytes, as well as speculated pathways based on those elucidated in other cell types.

Following FGF ligand binding, high affinity receptor dimerization occurs {Powers et al., 2000}. The mechanism of FGFR receptor dimerization has been recently defined. Three recent independent models have proposed a 2 FGF:2 FGFR dimer model which is stabilized by HSPGs as a means to explain the trimolecular complex of HSPGs, FGF and FGFR {Plotnikov et al., 1999; Stauber et al., 2000; Venkataran et al., 1999}. In these models each FGFR in the dimer binds one FGF, and the complex itself is stabilized by HSPGs binding across a canyon formed by the FGF-FGFR pairs {Powers et al., 2000}. The IgII and IgIII-like domains of FGFR wrap around a single FGF molecule at sites which pertain to the high and low affinity binding sites, respectively, within their core regions {Plotnikov et al., 1999; Stauber et al., 2000; Venkataran et al., 1999}. Together these pairs form a highly positive canyon at the IgII-FGF interface into which a single HSPG can bind, placing minimal size restrictions to the HSPG that must span both pairs in order to stabilize dimerization of FGFR {Powers et al., 2000}. Flanking structural domains in FGFR are postulated to modify FGF affinity and determine

specificity {Powers et al., 2000; Wang et al., 1997}. Following ligand binding and receptor dimerization, FGFRs are capable of phosphorylating specific tyrosine kinase residues (seven residues) on their own and each other's cytoplasmic tails {Lemmon and Schlessinger, 1994}. Transphosphorylation at tyrosine residues was shown to be essential for the catalytic activity for FGFR-1 {Mohammadi et al., 1996}. The tyrosine phosphorylation sites on FGFR-1 serve as high affinity binding sites for Src Homology 2 (SH2) domain-containing molecules as well as other adapter proteins, which then transduce downstream signaling events {Klint and Claesson-Welsh, 1999}.

Ligand-plasma membrane interactions lead to at least two independent FGFR mediated signal transduction pathways. One pathway involves the traditional SH2-linked pathway joining FGFR directly to PLC-γ1 (via Tyr^{766}) and Crk (via Tyr^{463}) as well as indirectly to Src (via PLCγ1) to activate downstream signaling pathways {Powers et al., 2000}. Activated PLCγ cleaves PIP_2 to IP_3 and DAG, which in turn facilitates calcium release via the endoplasmic reticulum via IP_3 and PKC activation via DAG and calcium {Powers et al., 2000}. Mutation of Tyr^{766} in FGFR-1 was shown to be essential for PIP_2 hydrolysis {Mohammadi et al., 1992; Peters et al., 1992a} but not for FGFR-mediated mitogenesis, neuronal differentiation or mesoderm induction in *Xenopus* {Muslin et al., 1994; Spivak-Kroizman et al., 1994}. On the other hand, interaction of the SH2/SH3-containing adapter protein, Crk, at Tyr^{463} was shown to essential for FGFR-1 mediated DNA synthesis in endothelial cells {Larsson et al., 1999}. Although, the non-receptor kinase, Src, was postulated to play an important role in FGFR-mediated cytoskeletal alterations (via cortactin), there is continued debate of whether there is a direct interaction

between FGFR and Src {Powers et al., 2000}. However, there is evidence to suggest that mutation of the PLCγ pathway inhibits Src phosphorylation {Langren et al., 1995}. The second FGFR-mediated pathway, involves linking FGFR to the SNT-1/FGF receptor substrate 2 (FRS2) adapter protein through an interaction at the juxtamembrane domain to mediate downstream signaling pathways {Langren et al., 1995; Powers et al., 2000; Zhan et al., 1994; Zhan et al., 1993}. FGFR activation of SNT-1/FRS2 was shown to recruit the adapter protein Grb-2/Sos, that then recruits Ras, thereby activating the MAPK signaling pathway which was shown to be important for FGF-2-induced cell cycle progression {Kouhara et al., 1997; Wang et al., 1996}. FGFR-1 activation of FRS2 has also been linked with the protein tyrosine phosphatase Shp2 as well as atypical PKCs {Lim et al., 1999; Ong et al., 2000}. In addition, recent studies have demonstrated that FRS2 is constitutively associated with FGFR-1, which suggests a function independent of receptor phosphorylation {Ong et al., 2000; Powers et al., 2000}. Some FGF target genes activated by FGFR signaling include immediate early genes such as c-myc, c-fos, c-jun and egr-1, cell cycle proteins such as cyclins A and E, as well as matrix proteases such as uPA and collagenase {Szenbyi and Fallon, 1999; Tomono et al., 1998}.

The direct intracellular action of FGF-2 and/or FGFR-1 in the nucleus has also been reported. Mobilization and accumulation of FGFR-1 to a region surrounding the nucleus has been described as nuclear trafficking in several systems, although not cardiac myocytes {Prudovsky et al., 1996; Feng et al., 1996; Stachowiak et al., 1996; Stachowiak et al., 1997}. Translocation of FGFR-1, with its ligand (FGF-1 or FGF-2), to the nucleus during the G1 phase of the cell cycle {Kilkenny and Hill, 1996; Prudovsky et al., 1996}

has been associated with a transition from quiescent to a proliferative cellular state {Stachowiak et al., 1997}. Recent evidence in mouse fibroblast cells demonstrated that nuclear transport of FGFR-1 was mediated by importin β, which is a component of multiple nuclear import pathways {Reilly and Maher, 2001}. These data suggested a model whereby activation of FGFR-1 (via FGF-2) at the cell surface initiated a set of signals required for proliferation, and that these events are followed by translocation of FGFR-1 to the nucleus and initiation of a sequence of events, including c-jun induction and increased expression of cyclin D1, that was also required for proliferation {Reilly and Maher, 2001}. It remains to be determined whether nuclear translocation of FGFR-1 is a component of FGF-2 mediated signal transduction pathway in cardiac myocytes.

The two predominant signal transduction pathways, which mediate the effects of FGF-2 in cardiac myocytes are ERK1/2 MAPK and PKC {Kardami et al., 2001}. The adapter proteins which mediate these responses and the role of FGFR-1 have not been fully established in cardiac myocytes. There is some evidence, however, suggesting a role for FGFR in FGF-2 signaling in cardiac myocytes as increased tyrosine phosphorylation staining was observed in cardiac tissue sections corresponding to increased pericellular accumulation of exogenous FGF-2 in isolated hearts {Liu et al., 1995; Padua et al., 1995a}. In addition, increased tyrosine phosphorylation in response to FGF-2 stimulation was also observed in proliferating and nonmitotic myocytes {Pasumarthi et al., 1995}. The mitogenic effects of FGF-2 in neonatal cardiac myocytes are predominantly mediated by ERK1/2 MAPK {Kardami et al., 2001}. However, since 30 % of this effect could not be blocked by MAPK inhibitors, it was postulated that FGF-2 mediated cardiac

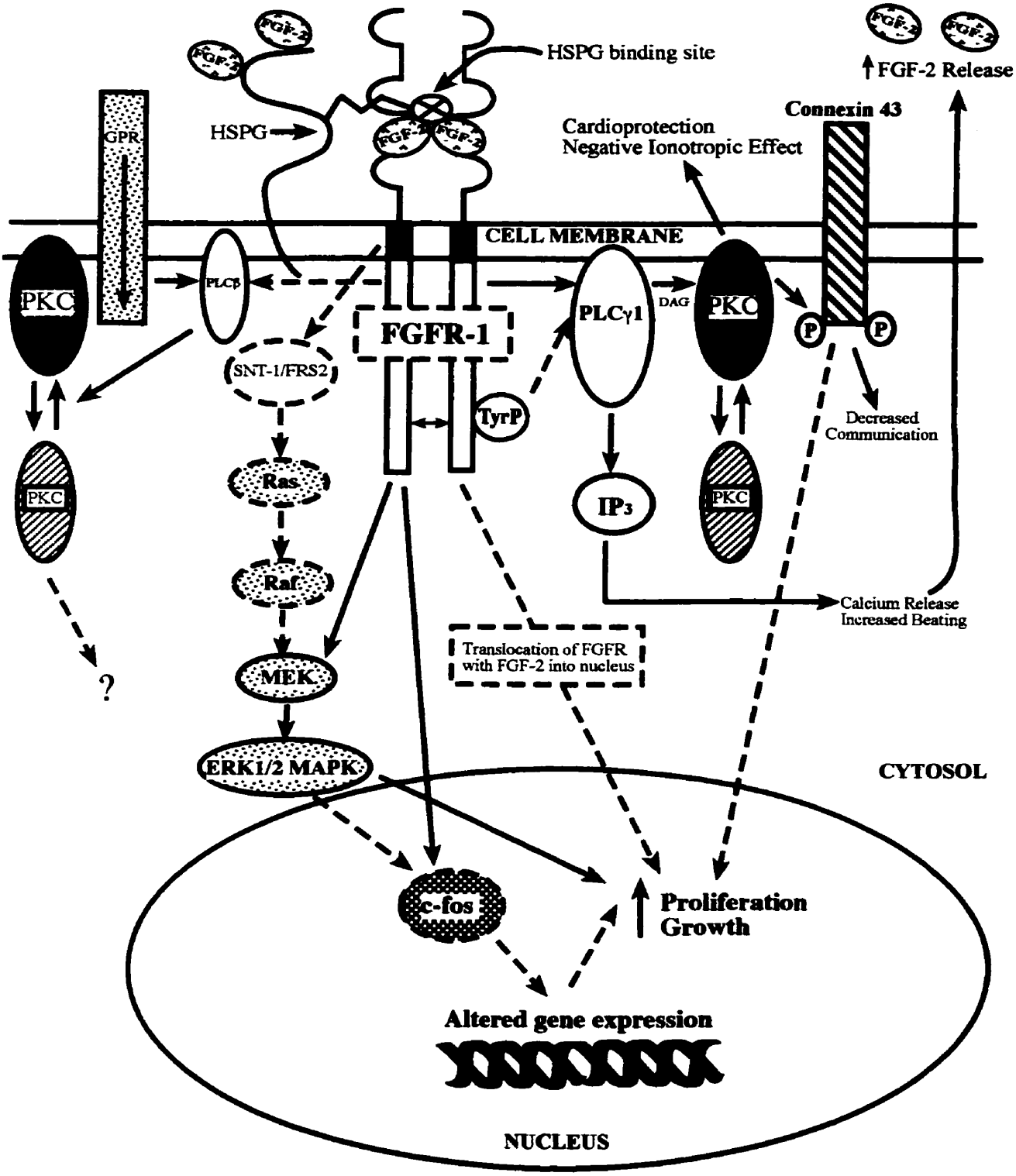
myocyte proliferation could occur through MAPK-independent pathways such as nuclear targeting of FGF-2 or PKC {Kardami et al., 2001}. FGF-2 cell surface receptors have been implicated to play a role in the mitogenic effects of FGF-2 in neonatal cardiac myocytes since these effects could be blocked by neutralizing antibodies to FGF-2 {Pasumarthi et al., 1994; Pasumarthi et al., 1996}. FGF-2 mediated ERK1/2 MAPK activation was also observed in adult cardiac myocytes and isolated whole hearts, however the significance of their activation remains to be determined {Liu et al., 1995; Padua et al., 1998}. Recent evidence demonstrated that FGF-2 can induce translocation of c-fos to the nucleus of cardiac myocytes in a PKC independent fashion, and suggested that c-fos activation may instead be a product of MAPK activation which is important for changes in gene expression for promoting growth {Kardami et al., 2001}. On the other hand, the decrease in cardiac myocyte communication observed with FGF-2, or FGF-2 induced connexin 43 phosphorylation in neonatal cardiac myocytes was shown to be mediated by PKC (i.e., εPKC) and not MAPK {Doble et al., 2000}. It was postulated that this may lead to an increase in proliferation or growth since there is growing evidence of a correlation between decreased intercellular communication and stimulation of cell division {Kardami et al., 2001; Xie et al., 1997; Yamasaki and Naus, 1996}. Thus, this could be an example of a MAPK independent pathway, which could mediate FGF-2's effects on cardiac myocyte proliferation. Although a specific role for FGFR-1 in these effects has not been established, it was demonstrated that the tyrosine phosphorylation inhibitor, genistein, was able to block the effects of FGF-2 on loss of cardiac myocyte communication {Doble et al., 1996}. The effects of FGF-2 on cardioprotection were also recently shown to be mediated via PKC (i.e., εPKC) {Padua et al., 1998}. FGF-2 was

shown to stimulate PLCγ1 in adult whole hearts as well as cardiac myocytes {Tappia et al., 1999}. In addition, the PKC inhibitor chelerythrine was shown to block FGF-2 mediated: (i) activation of PKC isoforms in cardiac sarcolemmal membranes (ii) negative ionotropic effect before ischemia and (iii) improvement of contractile recovery after ischemia-reperfusion injury in isolated hearts {Padua et al., 1998}. FGF-2 also increased membrane association of εPKC, and presumably activation, in cultured adult cardiac myocytes, suggesting that the cardioprotective effects of FGF-2 via PKC signaling were attributed to direct effects on cardiac myocytes {Padua et al., 1998}. Although the role of FGFR-1 in FGF-2 induced cardioprotection has not been determined, cross-talk between G protein coupled receptors and FGF-2 receptors (i.e., FGFR-1) was implicated since FGF-2 was shown to activate PLCβ isoforms in the adult rat heart and cardiac myocytes {Tappia et al., 1999}. The significance of this cross-talk in cardiac myocytes remains to be determined. FGF-2 has also been demonstrated to increase the activity of voltage-dependent calcium channels in neonatal cardiac myocyte cultures, which resulted in increased beating frequencies {Merle et al., 1995}. IP_3 was implicated as one of the cytoplasmic intermediates involved in this response {Merle et al., 1995}, and this would then in turn increase calcium (via calcium channels) from the endoplasmic reticulum. However, since PLCγ would be likely upstream of IP_3, it is conceivable that PKC activation by DAG would also occur as a result.

Figure 5.

Schematic representation of the signal transduction pathways for FGF-2 in cardiac myocytes.

ERK1/2 MAPK and PKC are the primary intracellular signaling pathways implicated in mediating the effects of FGF-2 on cardiac myocyte growth, cardioprotection and cardiac myocyte communication. Known pathways are indicate by solid arrows or outlines, whereas, speculated pathways are indicated as dashed arrows or outlines. Although the role of FGFR-1 in FGF-2 mediated signal transduction pathways in cardiac myocytes remains to be determined, there has been suggestion of cross-talk between FGFR and G protein coupled (GPR) receptors. Modified from Kardami et al., 2001.


FGF-2
HSPG binding site
FGF-2 Release
Connexin 43
HSPG
GPR
Cardioprotection
Negative Ionotropic Effect
CELL MEMBRANE
PKC
PLCβ
FGFR-1
PLCγ1
DAG
PKC
SNT-1/FRS2
TyrP
Decreased
Communication
PKC
Ras
IP3
PKC
Raf
Calcium Release
Increased Beating
?
Translocation of FGFR
with FGF-2 into nucleus
MEK
ERK1/2 MAPK
CYTOSOL
c-fos
Proliferation
Growth
Altered gene expression
NUCLEUS

2.4.8 Biological activities of FGF-2 in the myocardium

2.4.8.1 Distribution of FGF-2

In the myocardium, FGF-2 is widely distributed in various cell types, including cardiac myocytes and vascular cells, at all developmental stages {Cummins, 1993; Kardami et al., 1995}. In cardiac myocytes, FGF-2 was shown to be associated with the basement membrane, cell membrane, gap junctions, Z-lines, cytoplasm, as well as nucleus {Kardami et al., 1995}. As stated previously, HMW FGF-2 is expressed in the nucleus whereas LMW FGF-2 is predominantly cytoplasmic, however, also found in the nucleus of cardiac myocytes {Pasumarthi et al., 1994; Pasumarthi et al., 1996}. The extracellular, intracellular and nuclear localization of FGF-2 in cardiac myocytes suggests an important role for FGF-2 in various aspects of physiological function in cardiac myocytes and the myocardium.

2.4.8.2 FGF-2 in the developing myocardium

Various members of the FGF family are expressed in the developing heart and these include FGF-1, FGF-2, FGF-4, FGF-6, FGF-7, FGF-8, FGF-10, FGF-12, FGF-13, FGF-16 and FGF-18, however, FGF-2 has been the most widely characterized to date {Beer et al., 1997; Engelmann et al., 1993; Hartung et al., 1997; Hu et al., 1998; Kardami and Fandrich, 1989; de Lapeyreiere et al., 1990; Mason et al., 1994; Miyake et al., 1998; Schmitt et al., 1996; Zhu et al., 1996}. During development, FGF-2 expression first appears restricted to myocardial cells (stage 9), prior to the onset of contraction, and is then subsequently found in the extracellular matrix (stage 15) in the embryonic chick heart {Parlow et al., 1991}. A vital role for FGF-2 in early growth and differentiation of

cardiac myocytes was demonstrated when it was shown to induce mesoderm formation in chick embryo explants, which exhibited synchronous contractions and expression of cardiac α-actin mRNA {Sugi and Lough, 1995; Sugi et al., 1993}. Similarly, FGF-2 induced mesoderm formation in *Xenopus* embryos, which was characterized in most cases by the expression of the cardiac α-actin gene {Kimelman et al., 1988; Logan and Mohun, 1993; Slack et al., 1987}. FGF-2 was also shown to mediate the transformation of epithelial cells to mesenchyme during the formation of the cardiac cushions {Markwald et al., 1996}, which is a process involving loss of cell-cell interactions and increased migratory potential. In addition, since FGF-2 can signal via FGFR-1, and FGFR-1 was also shown to play a vital role in the early stages of growth and development of the heart and vasculature {Amaya et al., 1991; Leconte et al., 1998; Lee et al., 2000; Mima et al., 1995; Sugi et al., 1995; Wannaka et al., 1991; Zhu et al., 1999}, together these studies further suggested an important role for the FGF-2 axis in cardiac development. Recent studies, however, using FGF-2 knockout mice have questioned a role for FGF-2 in cardiac development. Specifically, several groups have demonstrated that mice ablated of the endogenous FGF-2 gene were not only viable, but were phenotypically indistinguishable from wild-type animals by gross examination {Dono et al., 1998; Ortega et al., 1998; Zhou et al., 1998}. Although mild phenotypes including effects on vascular tone, cortical development, bone development and wound healing have been observed {Dono et al., 1998; Montero et al., 2000; Ortega et al., 1998; Zhou et al., 1998}, these studies showed that FGF-2 was not essential for cardiac development. While other FGF family members could conceivably compensate for the phenotype observed, FGF-1 does not seem to be the active factor involved {Miller et al., 2000}.

Despite these observations, recent studies in mice have demonstrated that a reduction in FGF-2 levels after a period of embryogenesis (gestation day 7.5) prevented further cardiac development {Leconte et al., 1998}, suggesting the possibility that other members of the FGF family do not fully compensate for the lack of FGF-2 during later stages of cardiac development. This notion is further corroborated by studies which demonstrate that treatment of stage 6 chick embryos with antisense oligonucleotides complementary to FGF-2 could inhibit precardiac mesoderm cell proliferation and contractility {Sugi et al., 1993}. In addition, late cardiovascular anomalies as evidenced by increased myocardial cell proliferation, were observed in chick embryos when FGF-2 soaked beads were placed adjacent to the developing ventricle during later stages of development {Franciosi et al., 2000; Watkins et al., 1998}. To date there is limited information on the role of endogenous FGF-2 in the postnatal myocardium *in vivo*. Evaluating the role of FGF-2 at various stages of cardiac muscle development, by overexpression or underexpression of FGF-2 specifically in cardiac myocytes, could be an important strategy towards elucidating its role in the developing heart *in vivo*.

2.4.8.3 Cardiac myocyte proliferation and regenerative potential

The dynamic pattern of FGF-2 staining observed in chicken cardiac myocytes during the cell cycle suggests an important role for FGF-2 in cardiac myocyte proliferation/mitosis {Liu et al., 1997}. Specifically this study demonstrated that FGF-2 was localized at all stages of the cell cycle including: (i) interphase nuclei in the S and G_2 phase of the cell cycle, (ii) chromosomes in prophase and metaphase, (iii) cleavage furrow in anaphase, as well as (iv) midbody sites during late telophase and cytokinesis

{Liu et al., 1997}. In addition, since higher concentrations of FGF-2 were observed in adult atrial myocytes (still capable of DNA synthesis and possibly cell division) compared to ventricles, this suggested that an increase in FGF-2 correlates with an increased proliferative/regenerative capacity {Kardami and Fandrich, 1989; Kardami et al., 1993; Rumyantsev, 1977}. Studies have also demonstrated that an increase in FGF-2 accumulation is observed in cardiac muscles in the mdx mouse model of injury and regeneration, suggestive of a potential role in regeneration/proliferation {Anderson et al., 1991}. Indeed, increasing LMW FGF-2 levels (via gene transfer or exogenous addition) resulted in an increase in embryonic and neonatal cardiac myocyte proliferation *in vitro* {Kardami, 1990; Kardami et al., 1995; Pasumarthi et al., 1994; Pasumarthi et al., 1996}. The effects of LMW FGF-2 (via gene transfer) on cardiac myocyte proliferation could be blocked by neutralizing antibodies to FGF-2, suggestive of a paracrine (cell surface receptor mediated) pathway {Pasumarthi et al., 1996}. On the basis of levels of FGF-2, since LMW FGF-2 can increase cardiac myocyte proliferation *in vitro* and an increase in overall FGF-2 levels is observed during development *in vivo* (predominantly LMW FGF-2 in the adult heart) {Kardami et al., 1995; Liu et al., 1993}, one would expect that the adult heart may have an increased proliferative/regenerative capacity in response to injury. However, despite the relatively high abundance of FGF-2 in the adult versus embryonic heart {Kardami et al., 1995}, and the fact that endogenous FGF-2 accumulates around infarcted regions in the event of cardiac injury *in vivo*, which is suggestive of a role for FGF-2 in myocardial healing {Padua and Kardami, 1993}, adult cardiac myocytes still retain a limited ability to regenerate/proliferate in response to cardiac injury *in vivo* {Field and Soonpaa, 1998; Kardami et al., 1995}. Exogenous addition of

FGF-2 can stimulate adult cardiac myocyte DNA synthesis *in vitro*, however, this response occurred only in long term cultures and in one study this response was demonstrated to be coincident with re-expression of FGFR-1 {Claycomb and Moses, 1988; Speir et al., 1992}. FGF-2 mediated cardiac myocyte proliferation via exogenous addition (LMW form) can also be inhibited by thyroid hormone and TGF-β1 {Engelmann et al., 1992; Kardami, 1990; Kardami et al., 1993}, which suggests that inhibitors of FGF-2 may play a role in limiting postnatal cardiac myocyte proliferation/regeneration. A more detailed review on the relationship between FGF-2 and TGF-β in terms of cardiac myocyte growth will follow in section ***2.5***. There is limited information on the physiological relevance of HMW FGF-2 in cardiac myocytes. It is the predominant FGF-2 species in the immature myocardium, suggesting involvement with hyperplastic growth {Liu et al., 1993}. Indeed, overexpression of HMW FGF-2 also resulted in an increase in embryonic and neonatal cardiac myocyte proliferation *in vitro* {Pasumarthi et al., 1994; Pasumarthi et al., 1996}. The effects of HMW FGF-2 on cardiac myocyte proliferation could also be blocked by neutralizing antibodies to FGF-2, suggestive of a paracrine (cell surface receptor mediated) pathway similar to that of LMW FGF-2 {Pasumarthi et al., 1996}. In terms of cardiac injury, it was shown that a transient increase in HMW FGF-2 was observed in adult hearts following cardiac injury *in vivo*, coincident with a time frame of intense cellular infiltration and presumably attempt at proliferation in sites of myocyte degeneration {Padua and Kardami, 1993}. However, the role of HMW FGF-2 in adult cardiac myocytes and potential regenerative/proliferative response remains to be determined. Studies have also demonstrated that thyroid hormone can block the accumulation of

HMW FGF-2 in adult hearts *in vivo* and cardiac myocytes *in vitro* {Kardami et al., 1995; Liu et al., 1993}. Together these studies along with others suggest at least five possible reasons, which may contribute to the limited ability of adult cardiac myocytes to respond to FGF-2 by proliferation after injury *in vivo*. These include: (i) levels of FGF-2 in postnatal ventricular cardiac myocytes may not be high enough to induce a proliferative response (ii) levels or activity of FGFR-1 (i.e., reduction during cardiac development) in postnatal heart which could limit a proliferative response, (iii) presence of inhibitors of FGF-2 activity which prevent proliferation, such as TGF-β and thyroid hormone {Engelmann et al., 1992; Kardami, 1990; Kardami et al., 1993; Liu et al., 1993}, (iv) HSPGs in the postnatal heart which may interfere with proliferation, as an increase in HSPGs is observed during cardiac development {Asundi et al., 1997}, and (v) form of FGF-2 (i.e., HMW versus LMW FGF-2) and/or subcellular localization (i.e., nuclear versus cytoplasmic) of FGF-2 in the postnatal heart which may be important in increasing proliferative/regenerative response. Clearly, targeting strategies to address the issues outlined above, alone or in combination, could serve as a means to increase FGF-2 mediated adult cardiac myocyte proliferation/ regeneration in response to cardiac injury *in vivo*.

2.4.8.4 Intracrine effects of FGF-2: High molecular weight FGF-2

Although LMW and HMW FGF-2 were shown to have similar effects on cardiac myocyte proliferation {Pasumarthi et al., 1994; Pasumarthi et al., 1996}, HMW FGF-2 was found to have distinct effects in the cardiac myocyte nucleus. An increase in binucleation was observed in HMW but not LMW FGF-2 overexpressing myocytes; this

phenotype could not be blocked by neutralizing antibodies to FGF-2, consistent with an intracrine mechanism of action {Pasumarthi et al., 1996}. It was postulated that binucleation could be a product of amitosis since symmetric and asymmetric nuclear cleavage was observed in 5% of cardiac myocytes overexpressing HMW FGF-2 {Pasumarthi et al., 1996}. Binucleation has been considered an early marker of cardiac myocyte hypertrophy {Clubb and Bishop, 1984}, and thus it was postulated that some of these binucleated cardiac myocytes may serve as potential sources of "new" cells in pathological hypertrophy in the event of cardiac injury {Pasumarthi et al., 1996}. The apparent chromatin condensation, nuclear fragmentation or "DNA clumping" observed in cardiac myocytes was thought to resemble nuclei in the prophase of mitosis or apoptosis {Pasumarthi et al., 1994; Pasumarthi et al., 1996}. Recent studies, however, have demonstrated that the nuclear phenotype induced by HMW FGF-2 in cardiac myocytes likely reflects a direct effect of HMW FGF-2 on chromatin structure which does not require mitosis or apoptosis, but instead affects chromatin compaction which is important in the regulation of gene expression {Sun et al., 2001}. This data supports studies which have demonstrated that FGF-2 can bind to chromatin and directly modify gene transcription *in vitro* {Gualandris et al., 1993; Nakanishi et al., 1992}. Although the physiological relevance of this phenotype in cardiac myocytes remains to be determined, it was postulated that the effects of HMW FGF-2 on chromatin compaction may be important in modulating gene expression during instances of "stress" or cardiac injury {Sun et al., 2001}.

2.4.8.5 Cardiac hypertrophy

There is growing evidence that FGF-2 plays an important role in cardiac hypertrophy. Exogenous addition of FGF-2 (i.e., LMW) to cultured neonatal cardiac myocytes alters the gene profile of contractile proteins from an "adult" to "fetal" program, which is characteristic of pressure overload-induced cardiac hypertrophy *in vivo* {Parker et al., 1990}. This study also suggests that FGF-2 may prevent differentiation of cardiac myocytes. Indeed, FGF-2 was also shown to decrease overall myosin accumulation in embryonic cardiac myocytes *in vitro* {Kardami et al., 1995; Pasumarthi et al., 1994} and increases in local FGF-2 release were associated with re-expression of vimentin in cardiac myocytes surrounding the fibrotic region subsequent to cardiac injury *in vivo* {Padua and Kardami, 1993}. It was also demonstrated that paced adult cardiac myocytes (i.e., increased FGF-2 release) but not non-paced control myocytes exhibited a "hypertrophic response" which could be mimicked by exogenously administered FGF-2 and blocked by neutralizing antibodies to FGF-2 {Kaye et al., 1996}. Furthermore, both exogenous addition of FGF-2 and human pericardial fluid containing high levels of FGF-2 were able to induce adult cardiac myocyte hypertrophy *in vitro* {Corda et al., 1997}. Although studies using FGF-2 knockout mice demonstrated reduced cardiac hypertrophy in response to pressure overload hypertrophy *in vivo* {Schultz et al., 1998}, to date, there are no gain-of-function models (i.e., overexpressing FGF-2) which have been used to directly address the effects of increased FGF-2 in the heart on the possible stimulation of cardiac myocyte hypertrophy. Gain-of-function models could provide an important tool to address the role of FGF-2 and mechanisms involved in both physiological and pathological cardiac hypertrophy *in vivo*.

2.4.8.6 Cell adhesion and cardiac myocyte communication

FGF-2 has been implicated in playing a role in cardiac myocyte cell adhesion and cardiac myocyte communication. Specifically, FGF-2 (at doses greater than 20ng/ml) was shown to inhibit attachment of chicken embryonic cardiac myocytes from fibronectin-coated dishes {Kardami et al., 1993}. It was suggested that this response may be due to FGF-2's ability to compete with other adhesive proteins for cell binding sites (i.e., integrin interaction) {Kardami et al., 1993}. Although the physiological relevance of FGF-2's role in decreasing cardiac myocyte adhesion remains to be determined, it is tempting to speculate that this may play a role during cardiac development for migration of cells or in the event of cardiac injury for possibly removal of cells from injured site. There is strong evidence implicating a role for FGF-2 in cardiac myocyte communication as FGF-2 was demonstrated to (i) localize to cardiac gap junctions {Kardami et al., 1991} and (ii) decrease cardiac myocyte communication through a mechanism involving serine phosphorylation of connexin 43 by PKCε {Doble et al., 1996; Doble et al., 2000}. Since a decrease in cardiac myocyte communication has been demonstrated to correlate with increased proliferation, it was suggested that this pathway may contribute to FGF-2's ability to increase cardiac myocyte proliferation via a MAPK independent mechanism, during instances of injury {Kardami et al., 2001}. In addition, it is conceivable that a decrease in communication could be cardioprotective in instances of injury as it may "localize" cardiac myocyte damage to the affected myocytes.

2.4.8.7 Cardioprotection

There is significant evidence implicating FGF-2 with cardioprotection. Addition of FGF-2 to neonatal rat cardiac myocyte cultures treated with hydrogen peroxide or serum-starved resulted in improved cell survival and decreased cardiac myocyte injury as evidenced by preservation of nuclear morphology and myofibrillar structure {Kardami et al., 1993}. In addition, administration of exogenous FGF-2 prior to ischemic injury in various heart ischemia/reperfusion models resulted in an increase in myocyte viability and/or functional recovery in the rat heart {Cuevas et al., 1997; Padua et al., 1998; Padua et al., 1995b}. Furthermore, increasing FGF-2 levels also stimulated improved myocardial function and/or reduced infarct size in ischemic porcine, canine and human hearts through increased angiogenesis and systolic function {Harada et al., 1994; Horrigan et al., 1999; Laham et al., 1999; Unger et al., 1994; Yanagisawa-Miwa et al., 1992}. It is not known, however, if FGF-2 affects myocyte viability, specifically, in the mouse heart. This information is essential given the importance that genetically altered mice now play in studies of heart function. Furthermore, this would facilitate the use of genetic approaches to assess whether stimulating endogenous FGF-2 levels would allow prolonged and increased FGF-2 release form intracellular pools during contractions. Assessment for a role for endogenous FGF-2 in the adult myocardium could be of vital importance given that the adult cardiac myocytes have been shown to contain functional FGFR {Liu et al., 1995} and release FGF-2 during contractions under normal physiological conditions {Clarke et al., 1995; Kaye et al., 1996}, suggestive of a role for endogenous FGF-2 in the normal maintenance of a healthy myocardium as well as possibly limiting the extent of injury. This notion is supported by a recent study which

reported that transcoronary arterial gene transfer of a secreted form of FGF-2 was beneficial for recovery of LV systolic function and development of collaterals in the microembolized rabbit heart {Iwatate et al., 2001}. FGF-2 was also implicated in the cardioprotective effects of estrogen, as estrogen was shown to increase FGF-2 release from endothelial cells {Albuquerque et al., 1998}. Although the mechanisms remain to be determined it was proposed that estrogen enhances endothelial cell-basement membrane interactions leading to the release of 'trapped' FGF-2 from the extracellular matrix {Albuquerque et al., 1998} and/or by mechanisms involving the chaperoning of intracellular FGF-2 via heat-shock protein 27 {Piotrowicz et al., 1997}. It would be important to determine whether estrogen increases the release of endogenous FGF-2 from cardiac myocytes, either as an ongoing process involved in maintaining a healthy myocardium, or as a response to cardiac injury.

Despite strong evidence for the a role of FGF-2 (via exogenous addition) in cardioprotection, there is limited information on the mechanisms which dictate this response. FGF-2 may act as an antioxidant, based on its structure. FGF-2 contains four cysteine residues whose thiol groups exist in the reduced state which may act as a free radical scavenger {Padua et al., 1995a}. Other thiol containing antioxidants such as glutathione and captopril have already been shown to protect the heart against ischemia-reperfusion injury {Janier et al., 1993; Menasche et al., 1992}. Recent studies in the rat heart have also demonstrated that FGF-2 can induce a negative ionotropic effect, which may contribute to cardioprotection by acting to preserve energy stores or suppressing the energy requirement for contraction {Padua et al., 1998}. A negative ionotropic effect of

FGF-2 was also observed in adult cardiac myocytes {Ishibashi et al., 1997}. FGF-2 is also known to have vasodilator effects {Cuevas et al., 1991; Unger et al., 1994} and vasodilators are cardioprotective in ischemia-reperfusion injury {Aiello et al., 1995; Sommerchild and Kirkeboen, 2000}. However, since FGF-2 is also associated with the cardiac myocytes, a direct shielding effect on cardiac myocytes has also been proposed to be a part of the mechanism of FGF-2 mediated cardioprotection which was demonstrated to be independent of the effects on the vasculature {Padua et al., 1998}. In fact, several pieces of evidence have suggested links between pathways which mediate ischemic preconditioning (endogenous mechanism of cardioprotection) and pathways which are involved in FGF-2 mediated cardioprotection. The cardioprotective effects of FGFs in the pig myocardium were proposed to mimic ischemic preconditioning since they could be blocked by genistein (tyrosine kinase inhibitor) {Htun et al., 1998}. The effects of ischemic preconditioning are known to be receptor mediated primarily G protein coupled receptor but also tyrosine kinase receptors {Baines et al., 1999a}. However, it remains to be determined whether the cardioprotective effects of FGF-2 can also be blocked by genistein. Cross-talk between G protein coupled receptors and FGF-2 tyrosine kinase receptors has been suggested, since FGF-2 was able to induce PLCβ isoforms in adult hearts and cardiac myocytes {Tappia et al., 1999}. Recent studies in the rat heart have also implicated PKCεas the cytoplasmic intermediate involved in FGF-2 mediated cardioprotection {Padua et al., 1998}. Extensive work by Ping and colleagues, and other investigators has established a central role for PKCε in ischemic preconditioning {Ping et al., 1997}. K^+ ATP channels may also be involved in the cardioprotective effects of FGFs, as glibenclamide, a specific K^+ATP channel blocker, could block the

cardioprotective effects of FGF-1 {Cuevas et al., 2000}. Mitochondrial K+ATP channels are proposed to be the end-effector of ischemic preconditioning {Baines et al., 1999a}. However, it remains to be determined whether mitochondrial K^+ATP channels play a role in the cardioprotective effects of FGF-2. Finally, NO has also been proposed to be a prime mediator of delayed ischemic precondtioning {Carden and Granger, 2000}. Indeed, FGF-2 was demonstrated to block myocardial stunning by stimulating NO production via an NOS_2-dependent pathway {Hampton et al., 2000}. FGF-2 has also been shown to induce NO release into the coronary milieu, as part of the vasodilatory response {Cuevas et al., 1991}. In conclusion, these data suggest that FGF-2 may play a role in ischemic preconditioning.

2.4.8.8 Vasculature

FGF-2 is most notably known for its effects on the vasculature. In fact, through the use of antisense strategies, FGF-2 was demonstrated to be essential for embryonic mouse vascular development {Leconte et al., 1998}. FGF-2 also stimulates proliferation of three principal vascular cell types (endothelial cells, smooth muscle cells and fibroblasts) and is chemotactic for endothelial cells *in vitro*, inducing capillary tube formation {Lazarous et al., 1995}. In fact, overexpression of human FGF-2 in transgenic mice led to increased DNA synthesis in isolated vascular smooth muscle cells *in vitro* {Davis et al., 1997}. Major vessels form by the process of vasculogenesis, whereas small vessels including capillaries arise from angiogenesis {Poole et al., 2001}. FGF-2 has been demonstrated to be important for both processes {Cox and Poole, 2000; Parsons-Wingerter et al., 2000; Poole et al., 2001}. Thus, it is not surprising that FGF-2 is one of

the most extensively studied angiogenic growth factors to date {Simons et al., 2000}. True angiogenesis is described as the growth of thin-walled intramyocardial collateral-like vessels lacking the development of media {Simons et al., 2000}. The ability of FGF-2 to induce angiogenesis in mature ischemic tissues was demonstrated in various animal injury models {Simons et al., 2000}. These results are further supported by a study which demonstrated that transgenic mice overexpressing FGF-2 were predisposed to accelerated tumor angiogenesis, once provided with an angiogenic substrate {Fulgham et al., 1999}. Although the mechanisms involved in FGF-2 mediated angiogenesis have not been fully elucidated, the p38 MAP kinase signaling pathway has been implicated {Tanaka et al., 1999}, and recent studies have demonstrated that FGF-2 induces VEGF expression in vascular endothelial cells in an autocrine and paracrine manner to mediate angiogenesis {Seghezzi et al., 1998}. As might be expected, FGF-2 cell surface receptors are essential in FGF-2 mediated angiogenesis {Parsons-Wingerter et al., 2000; Seghezzi et al., 1998}. FGF-2 can also modulate vascular function. Ablation of the endogenous FGF-2 gene in a genetic mouse model resulted in impaired vascular smooth muscle contractility and a hypotensive phenotype (Dono et al., 1998; Zhou et al., 1998). Consistent with an effect of FGF-2 on vascular function, neutralizing antibodies to FGF-2 inhibited lumen narrowing and negative remodeling during intimal lesion formation in a coronary ligation model in mice {Bryant et al., 1999}. In addition, earlier studies demonstrated that FGFR-1 was essential in mediating vascular smooth muscle cell proliferation *in vitro* and could be important in mediating restenosis {Yukawa et al., 1998}. A recent study using a rat model of experimental peripheral arterial insufficiency demonstrated that normal NO production is essential for the enhanced vascular remodeling induced by FGF-2, along

with another angiogenic factor {Yang et al., 2001}. In addition, FGF-2 has also been implicated as a survival factor for vascular smooth muscle cells and endothelial cells {Fox and Shanley, 1996; Gospodarowicz et al., 1981; Karsan et al., 1997; Ku and D'Amore, 1995; Schweigerer et al., 1987}. Thus, regulating FGF-2 levels in the vasculature could have potential therapeutic effects in the myocardium either by promoting angiogenesis to reperfuse ischemic myocardium through FGF-2 addition {Simons et al., 2000} or reducing restonosis (characterized by smooth muscle cell proliferation) to prevent blockage of a vessel by inhibiting FGF-2 activity {Yukawa et al., 1998}.

2.4.8.9 Clinical trials in the myocardium

In the last five years, therapeutic angiogenesis has been advocated as a promising treatment strategy for patients with advanced ischemic heart disease who are not candidates for standard revascularization, since it results in the generation of a new blood supply in the diseased heart {Simons et al., 2000}. The two most widely studied proangiogenic growth factors, to date, which show promise in clinical trials are VEGF and FGF-2 {Post et al., 2001}. In terms of FGF-2, several strategies have been used to deliver FGF-2 into the human heart {Simons et al., 2000}. Initial clinical studies used a heparin alginate FGF-2 delivery system in patients with coronary disease undergoing coronary artery bypass surgery {Sellke et al., 1998; Laham et al., 1999}. Both studies demonstrated the safety and feasibility of this mode of therapy and patients in the FGF-2 group were found to be symptom free (i.e., no angina) after three months of surgery {Sellke et al., 1998; Laham et al., 1999}. These effects were shown to be dose-dependent

in the presence of a placebo-controlled group {Laham et al., 1999}. Patients in the 100 μg FGF-2 group at the three month time point also showed a trend towards a reduction in the target ischemic area while placebo-control groups did not {Laham et al., 1999}. Intracoronary and intravenous FGF-2 delivery were also found to be feasible and tolerable in patients with severe coronary disease {Laham et al., 2000; Udelson et al., 2000}. Delivery of FGF-2 in patients with advanced coronary artery disease resulted in attenuation of stress-induced ischemia and an improvement in resting myocardial perfusion up to 180 days after treatment, consistent with a favourable effect of angiogenesis {Udelson et al., 2000}. In addition, intracoronary delivery of FGF-2 significantly improved symptom assessment, as assessed by angina frequency and exertional capacity (i.e., exercise tolerance) {Laham et al., 2000} and was well tolerated in patients with stable angina {Unger et al., 2000}. Overall FGF-2 appears to be well tolerated producing functionally significant benefits in the ischemic myocardium {Simons et al., 2000}. Nevertheless adverse effects (i.e., blood pressure, platelet count, hypertrophy, fibrosis, arrhythmias) and toxicity are also issues which must be addressed when considering long term therapies with FGF-2. These claims are currently being evaluated in phase II clinical trials {Simons et al., 2000}. Finally, recent studies on engineering human cardiovascular structures for transplantation purposes demonstrated that FGF-2 (supplemented with ascorbate), resulted in enhanced early human vascular myofibroblast proliferation on biodegradable polymers and structurally more mature tissue formation {Hoerstrup et al., 2000}, thus, providing evidence for an additional clinical use for FGF-2 in the myocardium.

2.5 *Transforming Growth Factor (TGF)-β in the Myocardium*

Polypeptides of the TGF-β superfamily have received considerable attention for their potential role in the myocardium {Brand and Schneider, 1995}. There are more than 25 members in the TGF-β superfamily, however, there are only three recognized mammalian isoforms, which are named TGF-β1, TGF-β2 and TGF-β3 {Brand and Schneider, 1996; Dünker and Krieglstein, 2000}. The three mammalian isoforms are considered prototypical members of the TGF-β family, and are expressed in the myocardium in various cell types including cardiac myocytes {Thompson et al., 1988; Thompson et al., 1989; Brand and Schneider, 1995}. Each isoform, however, displays a distinct expression pattern in both embryo and adult {Akhurst et al., 1990; Millan et al., 1991; Dickson et al., 1993}. Targeted mutations of TGF-β genes in the mouse reveal distinct and largely non-overlapping phenotypes (Table 4). These mutant mice demonstrated that TGF-β isoforms are important for various developmental processes in several organs, including the heart {Dünker and Krieglstein, 2000}. However, since some of the phenotypes resulted in non-cardiac defects as well as embryonic lethality, the physiological role of endogenous TGF-β signaling in the postnatal myocardium and in particular postnatal cardiac myocytes remains unclear.

TGF-β are secreted factors, however, once secreted they must be activated by extreme pH, heat or proteolytic enzymes to form the mature 25 kD dimeric protein {Lembo et al., 1995}. Once biologically active, TGF-β can elicit a wide range of effects on various cell types in the myocardium {Brand and Schneider, 1995}. Although this review will focus on the role of TGF-β signaling in cardiac myocyte growth, it is

important to note that TGF-β is implicated in many processes in the myocardium including cell adhesion and migration, cardiac myocyte contractility, angiogenesis, cardioprotection, fibrosis, and disease {Brand and Schneider, 1995}.

Table 4. Phenotypes of TGF-β ligand, TGF-β receptor* and Smad knockout mice.

TGF-β Pathway	Gene Targeting	Mutant Phenotype
Ligand	TGF-β1	50% are embryonic lethal at E10.5 from defective yolk sac vasculogenesis and haematopoiesis Rest die postnatally (4 weeks old) from multi-focal inflammatory disease, platelet aggregation defect
	TGF-β2	Die from congenital cyanosis before or during birth Craniofacial (e.g., cleft palate), eye, spinal column, innerear, cardiac, lung, limb, and urogenital malformations
	TGF-β3	Die within 24h of birth Defect in palatogenesis (cleft palate) and delay in pulmonary development/lung maturation
Receptor	TGF-βRI* (ALK5)	*Cardiac specific expression of a constitutively active form of TGF-βRI in mice Arrest in cardiac looping, causing linear, dilated hypoplastic heart tube at E9.5 No change in Nkx2.5 or dHAND expression but increased p21
	TGF-βRII	Embryonic lethal at E10.5 Defect in yolk sac hematopoiesis and vasculogenesis
Intracellular Mediators	Smad2	Embryonic lethal at E7.5-8.5 Defect in egg cylinder elongation, mesoderm formation and gastrulation Primary defect in extraembryonic tissues Chimeras (with wild type morula) exhibit defect in left/right assymetry and anterior development
	Smad3	Viable and fertile at birth Metastatic colorectal cancer at 4-6 months of age Impaired immunity and chronic infection Accelerated wound healing
	Smad4	Embryonic lethal at E7.5-8.5 Growth retardation, no mesoderm formed, no gastrulation Abnormal visceral endoderm Primary defect in extraembryonic tissues Chimeras (with wild type morula) show anterior defects
	Smad5	Embryonic lethal at E9.5-10.5 Defect in angiogenesis, left/right asymmetry, cranial-facial abnormalities Increased mesenchymal apoptosis
	Smad6	Cardiovascular abnormalities Defect in endocardial cushion transformation

Source: Modified from Itoh et al., 2000 and Dünker and Krieglstein, 2000, with additional information from Charng et al., 1998. References for various mutant phenotypes are within the listed sources.

2.5.1 TGF-β receptors and signaling in cardiac myocytes

TGF-βs bind to at least three receptors (Rs), which are found on most mammalian cell types that are termed type I (TGF-βRI, 53 kD), type II (TGF-βRII, 70-80 kD) and type III (betaglycan; 200-400 kD) {Brand and Schneider, 1995}. Crosslinking studies have demonstrated that all three mammalian TGF-β receptors are present on cardiac myocyte membranes {Roberts et al., 1992}. Cardiac myocytes contain approximately 2000 and 5000 TGF-βRI and TGF-βRII binding sites, respectively {Ross et al., 1993b, Ross et al., 1993c}. There is currently limited information on the physiological role of TGF-βRs in cardiac myocytes, however, recent studies in mice revealed that mutation of TGF-βRII resulted in embryonic lethality due to defects in yolk sac hematopoiesis and vasculogenesis, whereas, constitutive expression of TGF-βRI (ALK5) resulted in defects in cardiac looping (Table 4). The constitutive expression of ALK5 signaling in mouse hearts was associated with increased p21 {Charng et al., 1998}, a cdk inhibitor associated with terminally differentiated muscle {Parker et al., 1995}. As a result, it was postulated that ALK5 mediates cardiac looping through the control of cardiac myocyte proliferation {Charng et al., 1998}.

All TGF-β isoforms elicit their cellular responses through formation of a heteromeric receptor complex, consisting of specific type I and type II serine/threonine kinase receptors {Itoh et al., 2000}. Type II receptors determine the specificity of ligand binding, whereas type I receptors specify the signaling response within the cell {Bassing et al., 1994a; Bassing et al., 1994b}. The role of type III receptors are not clear, however, they have been implicated in facilitating TGF-β binding to the type II and type I receptors

{Brand and Schneider, 1995}. TGF-β signaling via TGF-βRs occurs as follows: TGF-β binds to the type II receptor, which then recruits the type I receptor to form a heteromeric complex, and this in turn causes phosphorylation of serine/threonine residues and 'activation' of the type I receptor, resulting in downstream signaling events within a cell {Itoh et al., 2000}. TGF-β receptor heterodimerization and phosphorylation is essential for TGF-β signaling in various cell types, including cardiac myocytes {Brand et al., 1993; Massague, 1998; Brand and Schneider, 1995}. Studies in neonatal cardiac myocytes demonstrated that overexpression of a kinase-deficient TGF-βRII, which would effectively block heterodimerization and phosphorylation of TGF-βRI by competing for endogenous receptor complex, could block the upregulation of fetal cardiac gene expression by TGF-β {Brand et al., 1993}.

Smad proteins are the major intracellular regulators of TGF-β signaling {Massague, 1998} however their role in cardiac myocytes is largely unknown. The name Smad originates from a fusion between *C. elegans Sma* and *Drosophila mothers against dpp* (Mad) {Derynck et al., 1996}. The intracellular domain of TGF-βRI was shown to specifically and transiently interact with and phosphorylate receptor-regulated Smad proteins (Smad 1, 2, 3, 5 or 8) {Itoh et al., 2000}. Subsequent activation, receptor-regulated Smads recruit the common-partner Smad (Smad 4) to form a heteromeric complex which then translocates into the nucleus {Itoh et al., 2000}. Nuclear Smad complexes regulate gene transcription of TGF-β target genes by binding to DNA directly or indirectly through other DNA-binding proteins {Itoh et al., 2000}. The transcription factor, *Xenopus* forkhead activin signal transducer (FAST)-1 was identified as the first

binding partner for Smad {Chen et al., 1996}. Subsequently, a large number of Smad-binding partners have been identified which regulate transcriptional activity of TGF-β responsive genes, some of which include; ALK5, β-catenin, calmodulin, TAK-1, p300, Smad nuclear interacting protein-1 (SNIP1), c-fos and c-jun {Itoh et al., 2000}. In cardiac myocytes, TGF-β can effect the transcriptional regulation of the skeletal α-actin and α-MHC genes {Brand et al., 1993}, however it remains to be determined whether their regulation is directly Smad-dependent. Other TGF-β target genes include cyclin dependent kinase inhibitors p21, p27 and p15 {Reynisdottir et al., 1995}. TGF-βRI interactions with cytoplasmic Smad proteins can also be negatively influenced by inhibitory Smads (Smad 6 or 7) {Massague, 1998}. Targeted mutations of Smads in mice reveal distinct roles for these proteins during mouse development (Table 4). Smad 2, 4 and 5 are essential for early development, however, Smad 3 is implicated in tumorigenesis and Smad 6 is implicated in playing an essential role in cardiac development (Table 4). TGF-β family members can also signal via the TAK1 binding protein (TAB1)/TAK1 pathway {Shibuya et al., 1996; Yamaguchi et al., 1995}. TAK1 was shown to activate the SEK1/MKK4 and MKK3, which then activates the SAPK/JNK and p38/RK pathway, respectively {Moriguchi et al., 1996}. Cross-talk exists between TAK1 and Smads {Kimura et al., 2000; Sano et al., 1999; Yue et al., 1999}. In addition, TGF-β family members can also activate small GTP-binding proteins (i.e., Rho and Ras) in certain cells {Atfi et al., 1997; Hocevar et al., 1999}.

2.5.2 TGFβ signaling and cell growth

TGF-β can exert both positive and negative effects on cell growth {Sporn et al., 1987}, however, recent studies tend to agree that TGF-β acts as an endogenous inhibitor

of growth *in vivo* {Gold, 1999; Dünker and Krieglestein, 2000}. The anti-proliferative effects of TGF-β have been well established in various cell types including epithelial cells, T- and B-lymphocytes as well as mesenchymal cells such as skeletal muscle cells, fibroblasts, endothelial cells and hepatocytes {Sporn and Roberts, 1990}. In fact, TGF-β has been shown to arrest growth at the late G1 phase of the cell cycle {Pietenpol et al., 1990; Laiho et al., 1990}. Regulation of TGF-β cell cycle arrest by the Cdk/pocket protein/E2F pathway was implicated to occur via at least two mechanisms including the induction of Cdk inhibitors including p15, p21, p27 {Reynisdottir et al., 1995} and downregulation of tumor suppressor protein E2F-1 through hypophosphorylation of Rb{Li et al., 1997b}. A decline of Cdk2 and Cdk4 levels was also observed, however, this is postulated to occur as a secondary event representative of a program of cell adaptation to the quiescent state {Reynisdottir et al., 1995}. In hepatocytes, TGF-β expression is upregulated during liver regeneration after the first major wave of hepatocyte DNA synthesis and mitosis has taken place, and it has been suggested that this acts as a signal to prevent uncontrolled hepatocyte growth as regeneration is completed {Braun et al., 1988}. In addition, targeted mutation of TGF-β1 in mice results in diffuse and lethal inflammation characterized by infiltration (proliferation) of lymphocytes and macrophages in the postnatal heart and lungs {Kulkarni et al., 1993; Shull et al.,1992}. Furthermore, targeted mutations of TGF-βRII to specific cell types in mice *in vivo* resulted in various proliferative conditions including skin tumorigenesis {Go et al., 2000}, T cell lymphoproliferative disorder {Lucas et al., 2000}, and mammary epithelial hyperplasia {Gorska et al., 1998}. Mutations in TGF-βRII, Smad 2, Smad 3 and Smad 4, which abrogate TGF-β signaling are also associated with a variety of human tumors,

including colorectal cancers {Eppert et al., 1996; Markowitz et al., 1995; Lu et al., 1998; Polyak, 1996; Thiagalingam et al., 1996}. Although, Smad 3 knockout mice were the only mice to develop tumors after birth (Table 4), it has been postulated that the early lethality of other mutant phenotypes may have precluded the formation of tumors {Itoh et al., 2000}.

2.5.3 TGF-β signaling and cardiac myocyte growth

TGF-β has been implicated as a negative regulator of cardiac myocyte proliferation {Kardami, 1990; Roberts and Sporn, 1993}. Infusion of TGF-β1 in rat hearts *in vivo* resulted in an inhibition of cardiac cell DNA synthesis {Sigel et al., 1994}. Furthermore, an assessment of TGF-β1 expression during cardiac development, reveals that TGF-β1 levels increase with myocardial maturation {Engelmann et al., 1992}. Cardiac myocyte TGF-βR levels were also shown to increase during cardiac development {Engelmann and Grutoski, 1994}. The increase in TGF-β1 and TGF-βR levels during cardiac development correlates with the transition of cardiac myocytes from a hyperplastic state (high proliferation) to a hypertrophic state (limited proliferation). In addition, there is evidence to suggest that TGF-β directly interferes with myocyte proliferation as neutralization of endogenous and/or serum-derived TGF-β in neonatal cardiac myocytes kept in serum resulted in an increase in DNA synthesis {Kardami et al., 1993}. Furthermore, there is evidence to implicate TGF-β in cardiac myocyte hypertrophy (i.e., differentiated state) {Brand and Schneider, 1995}. TGF-β1 can alter gene expression in neonatal cardiac myocytes by stimulating the re-expression of "fetal" cardiac genes (β-MHC, skeletal α-actin, smooth muscle actin and ANF) and

downregulation of "adult" cardiac genes (α-MHC and sarcoplasmic reticulum ATPase) {Parker et al., 1990}. These changes are consistent with the pattern of gene expression observed in hearts following cardiac hypertrophy induced by mechanical overload {Schneider et al., 1992}. In addition, classical hypertrophic agonists, such as norepinephrine and AngII, have also been shown to induce the expression of TGF-β1 in cardiac myocytes {Bhambi and Eghbali, 1991; Sadoshima et al., 1993}. Neutralizing antibodies to TGF-β were also shown to inhibit isoproterenol-induced hypertrophy in adult rat cardiac myocytes in high serum conditions, while exogenous TGF-β was shown to induce hypertrophy in adult rat cardiac myocytes in low serum conditions {Schluter et al., 1995}. The increase in TGF-β expression in response to hypertrophic stimuli in adult cardiac myocytes was demonstrated to be mediated by an autocrine and/or paracrine mechanism {Takahashi et al., 1994}. An increase in cardiac TGF-β1 expression was also observed in animal models of cardiac hypertrophy {Komuro et al., 1991; Li and Brooks, 1997; Sakata, 1993; Villarreal and Dillmann, 1992} as well as human idiopathic cardiomyopathies {Li et al., 1997d}.

2.5.4 TGF-β and FGF-2 signaling in cardiac myocyte growth

Extracellular controls have been shown to exist between TGF-β and FGF-2. For example, TGF-β will promote extracellular matrix deposition {Butt et al., 1995}, which will mobilize FGF-2 on HSPGs and downregulate availability. On the other hand, FGF-2 will tend to mobilize, plasminogen activator and liberate FGF from the matrix {Rusnati et al., 1997}, but also liberate and activate TGF-β as a short negative feedback loop. In the myocardium, studies have demonstrated that TGF-β can inhibit FGF-2 mediated

proliferation in embryonic and neonatal cardiac myocytes {Engelmann et al., 1992; Kardami, 1990; Kardami et al., 1993}. TGF-β's inhibitory effects on FGF-2 mediated proliferation have been documented in other cell types, including smooth muscle cells, fibroblasts and endothelial cells {Baird and Durkin, 1986; Chambard and Pouyssegur, 1988; McCaffery and Falcone, 1993}. In addition, since the expression of both FGF-2 and TGF-β are increased after cardiac injury and localize to the margins of infarcted regions {Padua and Kardami, 1993; Thompson et al., 1988; Wunsch et al., 1991}, this raises the possibility that TGF-β may contribute to the limited ability of postnatal cardiac myocytes to regenerate/proliferate in response to FGF-2, despite its abundance, after injury *in vivo*. In terms of cardiac hypertrophy, a study has demonstrated that exogenously administered TGF-β inhibits adult cardiac myocyte hypertrophy induced by either exogenous FGF-2 or human pericardial fluid containing high levels of FGF-2 {Corda et al., 1997}.Taken together these studies clearly demonstrate that TGF-β influences the biological effects of FGF-2 in cardiac myocytes. In terms of proliferation, the ability of TGF-β to antagonize the actions of FGF-2 could have significant physiological consequences in terms of inhibiting a regenerative response in the myocardium subsequent to injury. "Neutralizing" the effects of TGF-β in cardiac myocytes could prove to be a useful strategy to stimulate or allow a regenerative/proliferative response by FGF-2 in postnatal cardiac myocytes. Previous studies have used a dominant negative approach to inhibit TGF-β signalling in cardiac myocytes {Brand et al., 1993; Schneider and Brand, 1995; Brand and Schneider, 1996}, however, to date this strategy has not been employed to influence effects on cardiac myocyte proliferation.

There is limited information on the cross-talk between FGF-2 and TGF-β signaling pathways in cardiac myocytes. PKC and ERK1/2 MAPK signaling pathways largely mediate the biological effects of FGF-2 in cardiac myocytes (Figure 5). PKC signaling was found to be important for FGF-2 mediated cardioprotection, whereas, ERK1/2 MAPK was shown to be important for FGF-2 mediated cardiac myocyte proliferation, although MAPK independent pathways have also been implicated to induce proliferation{see section ***2.4.7***}. Recent evidence, however, suggests that PKC is a likely target of this interaction, as TGF-β was shown to inhibit FGF-2 mediated translocation of PKC to the membrane and thus, presumably activation, but not activation of ERK1/2 MAPK in neonatal cardiac myocytes {Kardami et al., 2001}. TGF-β was also demonstrated to prevent FGF-2 induced connexin43 phosphorylation which is mediated by PKC {Doble et al., 2000; Kardami et al., 1997; Kardami et al., 2001} and implicated as a potential path to increase in cardiac myocyte proliferation {Kardami et al., 2001}. Together these studies suggest that proliferative pathways which are mediated by FGF-2 and linked to PKC could be the likely target of TGF-β. There is a clear need to define the interactions underlying TGF-β and FGF-2 signaling in order to define and exploit the pathways that are important to mediate cardiac myocyte proliferation or hypertrophy.

2.6 Rationale, Hypotheses and Specific Aims

Cardiovascular diseases are the leading cause of death worldwide. The research described in this thesis is focussed on developing and testing multiple strategies to

regulate cardiac myocyte growth and protection in an effort to provide the basis for treatment and/or prevention of cardiovascular diseases.

Considerable attention has focussed on the use of polypeptide growth factors and in particular FGF-2 in the treatment of cardiovascular disease {Waltenberger, 1997}. FGF-2 is a multifunctional protein, which exerts many of its biological effects by binding to high affinity cell surface receptors (FGFR-1) of the tyrosine kinase family (section ***2.4.6.1***). FGF-2 possesses properties, which could make it an important therapeutic tool for reducing or preventing damage by ischemia and/or improving cardiac prognosis subsequent to cardiac injury (section ***2.4.8***). An increase in FGF-2 (18 kD) levels (mainly through exogenous administration) is associated with: (i) an increase in cardiac myocyte proliferation in cultures (implicated in regeneration) (section ***2.4.8.3***), (ii) an increase in *de novo* angiogenesis (section ***2.4.8.8*** and ***2.4.8.9***}, (iii) an increase in cell survival (section ***2.4.8.7*** and ***2.4.8.8***), (iv) protection of cardiac myocytes and the myocardium from the effects of ischemia (section ***2.4.8.7***), and (iv) a decrease in cardiac myocyte communication which may be important in protecting adjacent myocytes from further injury (section ***2.4.8.6***). These properties as well as the observations that FGF-2 is released from adult cardiac myocytes with every contraction of the myocardium (section ***2.4.5***) and adult cardiac myocytes contain functional high affinity FGF-2 receptors (i.e., FGFR-1) {Liu et al., 1995}, raise the possibility that FGF-2 may play a role in maintaining a healthy myocardium. Although, considerable attention has been given to the protective effects of FGF-2 and the development of delivery systems when supplied exogenously (section ***2.4.8.9***), there has been little effort to exploit these effects by

controlling endogenous production of FGF-2. Clearly, if FGF-2 is released from cardiac myocytes upon contraction and FGF-2 plays a role in cardioprotection then:

Hypothesis 1:

Increasing endogenous FGF-2 levels in the heart *in vivo* will translate into an increase in FGF-2 release from intracellular stores and result in increased cardioprotection. This hypothesis is addressed through three specific aims and the results are presented in Chapter 4.

Specific Aims 1:

1.1 To generate and characterize transgenic mice overexpressing 18 kD FGF-2 in the heart.

1.2 To assess the effects of increased endogenous production of FGF-2 on levels of FGF-2 release in hearts.

1.3 To assess the effects of increased endogenous production of FGF-2 on cardioprotection, as measured by LDH release (myocyte integrity) and developed pressure (contractile recovery), in a cardiac injury model.

While intact animal preparations can mimic cardiac injury in man, it is difficult, if not impossible to define the exact role of factors involved in cellular injury in this system. As a result, adult cardiac myocyte cultures are an essential tool in understanding normal cardiac structure and function as well as disease, at the cellular level. Adult cardiac myocyte cultures are widely used by researchers in a variety of disciplines (i.e.,

molecular biology, cellular biology, electrophysiology, pharmacology, etc.) to gain insight into "therapeutic" strategies to "rehabilitate" these cells in the event of injury. Given the growing use of transgenic and gene "knockout" mouse models for the study of gene expression and regulation in the heart, there is now a special need for adult mouse cardiac myocytes. The ability to isolate and culture viable adult cardiac myocytes from mice would prove to be an invaluable system to complement studies in the whole animal (transgenic or otherwise).

Mouse cardiac myocyte cultures would provide an important system to study transcriptional regulation of FGF-2 in the heart. In the period of 1995 to early 2001, less than 5% of publications in the area of FGF-2 in the heart have reported on its transcriptional regulation. Transcription is an important component of FGF-2 gene regulation (see section ***2.4.1***), and discovery of regulators of FGF-2 transcription in cardiac myocytes could serve as an additional target to exploit the "therapeutic" effects of FGF-2. In an effort to increase knowledge of FGF-2 transcription, our laboratory has recently generated and characterized a transgenic mouse model expressing a hybrid FGF-2/luciferase gene (-1058FGFp.luc) in the heart containing approximately 1 kb of the rat FGF-2 promoter region which directs expression of the luciferase reporter gene {Detillieux et al., 1999}. Studies using these mice showed that FGF-2 promoter activity could be increased by adrenergic stimulation (use of catecholamines) in adult hearts *in vivo* (section ***2.4.1***). However, since these studies examined FGF-2 promoter activity at the whole heart level, it remained to be determined whether (i) the FGF-2 promoter is active in adult cardiac myocytes and (ii) if active, whether its activity can be increased by

adrenergic stimulation in adult cardiac myocytes, as observed in whole hearts *in vivo*. Addressing the first premise would be essential in exploiting the use of this transgenic model for future studies in the area of transcriptional regulation of FGF-2 in the heart. Hence:

Hypothesis 2:

Treatment of isolated and cultured adult cardiac myocytes from -1058FGFp.luc transgenic mice with the natural catecholamine, norepinephrine, will result in increased FGF-2 promoter activity. This hypothesis is addressed through two specific aims and the results are presented in Chapter 5.

Specific Aims 2:

2.1 To generate viable adult cardiac myocytes from mouse hearts.

2.2 To use adult cardiac myocyte cultures from -1058FGFp.luc mice to determine whether the natural catecholamine, norepinephrine, can stimulate FGF-2 promoter activity.

FGF-2 stimulates cardiac myocyte proliferation and as a result, is implicated in cardiac regeneration (section ***2.4.8.3***). There is however no evidence that FGF-2 can increase adult cardiac myocyte proliferation/regeneration in the postnatal heart. This raises the possibility that FGF-2 signaling may be limited and/or antagonized in the postnatal heart (section ***2.4.8.3***).

FGF-2 signaling is dependent on: (i) FGF-2 bioavailability and levels, (ii) FGF-2 receptor (i.e., FGFR-1) activity and levels as well as (iii) downstream signal transduction. In term of FGF-2 bioavailability and levels, it has been shown that (i) FGF-2 is released on a continuous basis from adult cardiac myocytes upon contraction from the myocardium (section ***2.4.5***) and (ii) FGF-2 is expressed in both embryonic and adult hearts (section ***2.4.6.1***), and in fact, "total" levels were shown to increase during development {Kardami et al., 1995}. On the other hand, analysis of FGFR-1 during development suggests that it may be a limiting factor in the postnatal heart (section ***2.4.6.1***). A decrease in FGFR-1 levels and the isoform switch correlate with and may regulate the transition of cardiac myocytes from a hyperplastic (high level of proliferation) to a hypertrophic (limited level of proliferation) phenotype. Clearly, if FGFR-1 levels are decreased and FGFR-1 isoforms are switched in postnatal cardiac myocytes, then:

Hypothesis 3:

An increase in FGFR-1 levels and/or change in relative levels of the 'long' versus 'short' FGFR-1 isoforms in cardiac cells will stimulate the proliferative potential of postnatal cardiac myocytes. This hypothesis is addressed through two multicomponent specific aims, which reflect the use of two model systems, a cardiac myoblast cell line and neonatal rat ventricular cardiac myocytes. The results are presented in Chapter 6.

Specific Aims 3:

3.1 Rat heart H9c2 myoblast cell line (cardiac cell line deficient in FGFR-1 RNA)

3.1.1 To generate and characterize H9c2 cells stably expressing FGFR-1 hybrid genes.

3.1.2 To assess whether FGFR-1 isoform expression results in an increase in H9c2 cell number.

3.2 Neonatal rat cardiac myocytes (postnatal cells with a limited ability to divide)

3.2.1 To overexpress FGFR-1 hybrid genes in neonatal cardiac myocyte cultures and assess expression as well as the effects on (i) FGF-2 binding on cardiac myocyte membranes and (ii) levels of active MAPK (known downstream target of FGF-2).

3.2.2 To assess the effects of overexpressing FGFR-1 isoforms on cardiac myocyte DNA synthesis and cardiac myocyte number.

FGFR-1 may not be the only variable which could limit the effects of FGF-2 on cardiac myocyte proliferation since there is evidence to suggest that negative factors (local or humoral factors) present in serum and/or expressed in cardiac cells can also antagonize the proliferative effects of FGF-2 in cardiac myocytes. In this context, TGF-β is abundantly expressed in the heart and cardiac myocytes and is implicated as a negative regulator of cardiac myocyte proliferation (section ***2.5.3***). TGF-β was also shown to inhibit the mitogenic effects of FGF-2 in cardiac myocytes {Engelmann et al., 1992; Kardami, 1990; Kardami et al., 1993}. To define the role of TGF-β signaling in cardiac myocytes, a hybrid gene expressing a kinase-deficient TGF-βRII was generated as a molecular tool to "neutralize" TGF-β signaling {Brand et al., 1993; Brand and Schneider, 1996; Schneider and Brand, 1995}. To date, there is no information on whether this

kinase-deficient TGF-βRII can be exploited to "neutralize" the "anti-proliferative" effects of TGF-β and FGF-2 on postnatal cardiac myocyte growth. Clearly, if TGF-β can "inhibit" the actions of FGF-2 on proliferation then:

Hypothesis 4:

Inhibition of TGF-β signaling, using the kinase-deficient TGF-βRII, will increase entry of cardiac myocytes into the cell cycle (S phase entry), as well as amplify the stimulatory effects of FGF-2. This hypothesis is addressed through three specific aims and the results are presented in Chapter 7.

Specific Aims 4:

4.1 To characterize overexpression of an adenovirus encoding a kinase-deficient TGF-βRII in neonatal rat cardiac myocytes.

4.2 To assess whether overexpression of the kinase-deficient TGF-βRII can affect the entry of neonatal cardiac myocytes into the cell cycle (S phase) in the presence of serum.

4.3 To assess whether overexpression of the kinase-deficient TGF-βRII can influence the effects of FGF-2 on cell cycle entry (S phase) in neonatal cardiac myocytes.

The results of these studies are presented in Chapters 4 to 7 and discussed in terms of a role for the FGF-2 axis in cardioprotection and cardiac muscle cell growth.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Culture

Rat heart myoblast H9c2 and human glioma C6 cells were obtained from the American Type Culture Collection and grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL; Burlington, ON, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco-BRL), antibiotics (Gibco-BRL, 1000 units/ml penicillin, 1 mg/ml streptomycin) and L-glutamine (Gibco-BRL, 0.4 mM) at 37 °C in the presence of 5% CO_2. Human embryonic kidney 293 cells, containing E1A and E1B Ad5 viral genes, were obtained from Quantum (Montreal, PQ, Canada), grown in monolayer culture and maintained in 5% FBS-DMEM, supplemented with antibiotics as described above, at 37 °C in the presence of 5% CO_2.

Neonatal rat ventricular cardiac myocytes were isolated from 1-2 day old Sprague Dawley rats by enzymatic digestion with 0.085-0.1% (w/v) trypsin (Gibco-BRL) using a temperature regulated (35 °C) spinner flask, followed by fractionation on a Percoll gradient using a protocol developed by the laboratory of Dr. K. Chien {Iwaki et al., 1990} and modified by the laboratories of Dr. E. Kardami and Dr. P.A. Cattini {Pasumarthi et al., 1996}. Briefly, 36 rat pups (1-2 day old) were sacrificed by decapitation and ventricles were dissected and submerged into Ham's F10 medium (Gibco-BRL) chelated with 1.33 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA) at room temperature. Once the dissection was

complete, ventricles were (i) transferred to fresh chelated F10 medium (Gibco-BRL), (ii) minced with scissors until tissue pieces could be easily aspirated with a 10 ml pipette and then (iii) transferred to a temperature regulated (35 °C) spinner flask, containing 9.5 ml of chelated F10 with trypsin (Gibco-BRL, 0.85-1.0 mg/ml) and DNase I (Sigma-Aldrich; Oakville, ON, Canada, 30 U/ml) for a 10 min digestion period. After the 10 min digestion period, the supernatant was removed and collected and subsequently an additional 9.5 ml of chelated F10 medium containing enzymes was added to cells for another 10 min digestion period. A total of 10 x 10 min digestion periods with chelated F10 medium containing enzymes is required for complete dissociation of tissue into cells. The first two supernatants were considered fibroblast-enriched and thus were collected separately or discarded, whereas the subsequent eight supernatants, were considered myocyte-enriched, and were collected in FBS (20 ml) at room temperature. It should also be noted that the amount of trypsin could vary between 0.085-0.1% (w/v), depending on the potency of the enzyme, as assessed by the viscosity of the supernatant (dissociated cells) after the third 10 min digestion time period. Dissociated cells were then centrifuged (250 g for 7 min) and resuspended in 1x Ads Buffer (20 mM N-[2-hydroxyethyl]piperazine-N′[2-ehanesulfonic acid] (HEPES), pH 7.35; 116 mM NaCl; 10 mM NaH_2PO_4; 5.5 mM glucose; 5.36 mM KCl., 0.8 mM $MgSO_4 \cdot 7H_20$) and treated with 300 U DNase I for 10 min before they were passed through a Nytex nylon membrane. The filtered cells were then layered on a discontinuous Percoll (Amersham Pharmacia Biotech; Baie d'Urfé, PQ, Canada) density gradient (1.059 g/ml: 1.110 g/ml) and centrifuged at 2500 g for 45 min at room temperature. The upper fibroblast enriched layer was discarded, and the myocyte enriched layer as well as pellet were collected, washed twice with 1x Ads buffer and then

resuspended in 30 ml of plating medium. Plating medium consisted of F10 medium containing 10% (w/v) FBS, 10% horse serum (Gibco-BRL) and antibiotics. Large round cells with continuous (smooth) membranes were counted using a hemacytometer and plated at densities of 1.3 x 10^6 or 0.7-0.9 x 10^6 cells per 60 mm or 35 mm culture dishes, respectively, and maintained at 37 °C in the presence of 5% CO_2. Cardiac myocytes are subsequently maintained in 10% FBS in F12-DMEM (Gibco-BRL) medium, unless otherwise specified. Culture dishes with or without coverslips (22 mm diameter) were coated using 0.1% (w/v) rat tail type I collagen stock solution (Upstate Biotechnology; Lake Placid, NY) and left to dry overnight under ultraviolet light. Coverslips were pretreated with 0.5 M sodium hydroxide (Sigma-Aldrich) overnight and the next day, washed extensively with sterile water and dried under ultraviolet light prior to collagen coating.

3.2 Hybrid Gene Constructions

The construction of hybrid genes containing the full-length cDNAs for both long and short mouse FGFR-1 {Jin et al., 1994} under the control of the cardiac specific rat myosin light chain-2 (MLC-2) promoter with simian virus 40 (SV40) enhancer sequences (SVenh), referred to as SVenhMLCp.*FGFR-1(L)* and SVenhMLCp.*FGFR-1(S)*, were generated by Ms. Yan Jin in the laboratory of Dr. P.A. Cattini and described in Sheikh et al., 1997. The expression vector containing the promoter, but with the firefly luciferase gene instead of FGFR-1 sequences (SVenhMLCp.*luc*) has been previously generated and characterized, and was used as a control for FGFR-1 transfection experiments {Jin et al., 1995; Sheikh et al., 1997}. The hybrid gene containing the cDNA for LMW FGF-2

directed by the RSV promoter, has been previously constructed and expression has been characterized *in vitro* in both embryonic chicken and neonatal rat ventricular cardiac myocytes {Pasumarthi et al., 1994; Pasumarthi et al., 1996}. The hybrid reporter gene, RSVp.*β-galactosidase* (β-gal) has been described previously {Pasumarthi et al., 1996}.

3.3 Adenoviral Gene Construction and Generation of Virus

The full length human TGF-βRII cDNA (H2-3FF) was obtained as a 4.7 kb *EcoR*I fragment in pcDNAI {Lin et al., 1992}. The kinase-deficient TGF-βRII (ΔkTGF-βRII) fragment was generated by polymerase chain reaction (PCR) using specific primers with unique restriction sites generating a stop codon (forward primer: 5′-AGCCA GGCCTGCCATGGGTCGGGGGCTGC-3′; reverse primer: 5′-TCTCTCTAGATTA TGTCTCAAACTGCTCTGAAGTGTTCTG-3′) and using PCR amplification conditions as described previously {Jin et al., 1994}. The PCR fragment was subsequently digested by *Stu*I/*Xba*I and subcloned into *Sma*I/*Xba*I sites of pBluescript II SK+ (pBS) to generate pBS.Δ*kTGF-βRII*. Generation of the pBS.Δ*kTGF-βRII* hybrid gene was done by Ms. Yan Jin in the laboratory of Dr. P.A. Cattini.

To generate an adenoviral vector containing the kinase-deficient TGF-βRII, first, the pBS.Δ*kTGF-βRII* was digested with *EcoR*I/*Xba*I to release the approximately 850 bp ΔkTGF-βRII fragment which was subsequently cloned into the *EcoR*I/*Xba*I sites of the pcDNA3.1 vector (Promega Incorp.; Madison, WI) to generate the CMVp.Δ*kTGF-βRII* hybrid gene. Secondly, the CMVp.Δ*kTGF-βRII* hybrid gene was digested with *Hind*III/*Xba*I to release the approximately 850 bp ΔkTGF-βRII fragment which was

subsequently cloned into the *Hind*III/*Xba*I sites of the pShuttle-CMV adenoviral transfer vector (Quantum) to generate pSCMV.Δ*kTGF-βRII*. Generation of recombinant adenovirus was performed as described by the manufacturer's instructions (AdEasy kit, Quantum). Briefly, the pSCMV.Δ*kTGF-βRII* was linearized with *Pme*I, and homologous recombination was performed in bacteria *in vivo* by cotransforming the linearized pSCMV.Δ*kTGF-βRII* with pAdEasy-1 (Quantum), which is the adenovirus serotype 5 genome deleted in the E1 and E3 regions, in RecA+ bacterial cells (BJ5183) via electroporation to generate the recombinant plasmid, Ad.Δ*kTGF-βRII*. Linearization of the Ad.Δ*kTGF-βRII* recombinant plasmid with *Pac*I, generated 35 kb and 4.5 kb sized fragments (data not shown), indicative of a recombination event between the origins and right arms. Subsequently, 5 μg of linearized Ad.Δ*kTGF-βRII* was transfected into QBI-293 cells (7.5×10^5 cells per 60 mm dish) in 5% FBS-DMEM using a calcium phosphate technique described by the manufacturer's instructions (Quantum). The next day, the medium was removed, the cells were washed very gently, once with 1 mM EGTA in phosphate buffered saline (PBS), twice with PBS only and then lifted with a small volume (2 ml) of PBS by repeatedly pipetting PBS onto cells, using a 10 ml pipette as this exerts more force and as a result doesn't require as many repeats (i.e., reducing damage to cells). Lifted cells are then supplemented with 5% FBS-DMEM and split onto 4 x 60 mm dishes, and left to attach overnight. The next day, the medium is removed and the cells are overlaid with 5 ml 1.25% Seaplaque agarose dissolved in 5% FBS-DMEM as described by the manufacturer's instructions (Quantum), and returned to the incubator for appearance of viral plaques, which takes from 10-21 days. Once viral plaques appeared, a small scale virus amplification was performed according to manufacturer's

instructions (Quantum). Screening of positive recombinant ΔkTGF-βRII viral clones was assessed through: (i) integration of the ΔkTGF-βRII gene in 293 cells via PCR, using a specific set of primers (forward: 5′-GCGGTAGGCGTGTACGGTGGAG-3′; reverse: 5′-CGTTAAGATACATTGATGAGTTTGGAC-3′) corresponding to regions of the adenoviral vector immediately adjacent to the ΔkTGF-βRII cDNA and using conditions as previously described (data not shown) {Jin et al., 1994} and (ii) expression of ΔkTGF-βRII protein in C6 cells via protein blotting, using specific TGF-βRII antibodies and procedures as described in section *3.7* (data not shown). An adenovirus expressing β-gal (AdCMV5.*β-gal*) was also generated using a recombinant AdCMV5.*β-gal* DNA supplied by the manufacturer (QBI-viral DNA) and following the manufacturer's instructions (Adenoquest system, Quantum).

3.4 Gene Transfer

3.4.1 Stable gene transfer in H9c2 cells

For stable gene transfer, H9c2 cells were plated at 2 x 10^6 per 100 mm dish and transfected with the calcium phosphate/DNA precipitation method essentially as previously described {Cattini et al., 1988}. Briefly, cells were transfected with 15 μg of hybrid SVenhMLCp plasmid DNA and 1.5 μg of pneodX {Cattini et al., 1986} per 100 mm plate in 10% FBS-DMEM. The lower amount (approximately 10%) of selection plasmid (pneodX) was used to bias the generation of stable transfectants carrying the experimental SVenhMLCp plasmid DNA. After 24 h, cells were refed with growth medium and selected with 0.5 mg/ml G418 (active) which was replaced every 3 d during the 21 d 'high selection' period. At this time colony number could be assessed by phase

contrast microscopy. Colonies were lifted and dispersed using trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco-BRL), and then replated in 60 mm dishes in DMEM supplemented with 10% FBS and 0.1 mg/ml G418 and grown to mass. G418 was removed from the growth medium during experiments. Stable gene transfer of FGFR-1 hybrid genes into H9c2 cells was done by Dr. P.A. Cattini.

3.4.2 Transient gene transfer in neonatal cardiac myocytes

Transient gene transfer experiments with FGFR-1 hybrid genes were performed using the calcium phosphate/DNA precipitation method, essentially as previously described {Cattini et al., 1988}. Briefly, neonatal cardiac myocytes were plated on 60 and 35 mm dishes as previously described (section ***3.1***) and then transfected for 24 h with 10 μg of test plasmid DNA in 10% FBS-DMEM. After 24 h, cells were refed with growth medium (i.e., 10% FBS-F12 DMEM), and maintained for a further 48 to 72 h before processing. Hybrid genes for transfection included: SVenhMLCp.*luc* (control), SVenhMLCp.*FGFR-1(S)* and SVenhMLCp.*FGFR-1(L)*.

3.4.3 Adenoviral mediated gene transfer in neonatal cardiac myocytes

For ΔkTGF-βRII experiments, neonatal cardiac myocytes were plated onto 35mm plates as described previously (section ***3.1***), and transfected with an adenovirus expressing ΔkTGF-βRII (Ad.Δ*kTGF-βRII*) at a multiplicity of infection (MOI) of 50 for 24 h in 10% FBS F12-DMEM. As a control, neonatal cardiac myocytes were transfected with an adenovirus expressing β-gal (Ad.*β-gal*) at an equivalent MOI. After 24 h, cardiac myocytes were refed with growth medium (i.e., 10% FBS-F12 DMEM) in the absence or

presence of recombinant FGF-2 (Upstate Biotechnology; Lake Placid, NY) at doses of 1, 10 and 1000 ng/ml for a further 24 h before processing.

3.4.4 Transgenic mice

The pronuclear injection protocol to generate transgenic mice was carried out by Ms. Agnes Fresnoza under the direction of Dr. M.L. Duckworth at the University of Manitoba's Transgenic Facility. Two homozygous transgenic (TG) mouse lines (no. 5318 and no. 5323) were generated by pronuclear injection of CD-1 mouse eggs with a modified rat FGF-2 cDNA coding specifically for the 18 kD or LMW FGF-2 (RSVp.*met FGF* linearized with *Sac*I) (section ***3.2***). Age-matched CD-1 mice were used as non-TG controls for all experiments outlined with FGF-2 transgenics. Two TG mouse lines (P300 and P66) expressing the -1058FGFp.*luc* transgene have been previously established and characterized to express the transgene in the adult heart, as well as other tissues {Detillieux, 1999; Detillieux et al., 1999}. Adult mice (8-12 weeks of age, both genders) were euthanized by cervical dislocation and various tissues (heart, lung, skeletal muscle, brain, kidney, spleen and liver) were dissected aseptically for the purposes of the experiments outlined below. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council on Animal care.

3.5 DNA Isolation and Blotting

Genomic DNA from stably transfected H9c2 cells was isolated as described previously {Nickel et al., 1990}. FGFR-1 plasmid DNA (pBSmFGFR(L) and pBSmFGFR(S)) {Jin et al., 1994} was isolated using a Qiagen Maxi Kit (Qiagen Inc., CA, USA) according to manufacturer's instructions. DNA was digested with *Xba*I/*Sal*I,

electrophoresed in 1% (w/v) agarose gels and blotted to nitrocellulose as previously described {Maniatis et al., 1982}. The full-length FGFR-1(S) cDNA {Jin et al., 1994} was radiolabelled to a specific activity of approximately 1 x 10^9 cpm/μg using α^{32}P dATP by random priming method (Promega "Prime-A-Gene" Kit). DNA blots were hybridized to the radiolabelled probe at 42°C in the presence of 50% formamide for 20-24 h, washed three times for 15 min each time at 65 °C in 0.1x SSC (20 x SSC; 3 M sodium chloride, 0.3 M sodium citrate) with 0.1% sodium dodecyl sulphate (SDS) and visualized by autoradiography.

As an initial screen for positive FGF-2 TG mice (RSVp.*metFGF*), tail tips from mice were collected and genomic DNA was isolated as described previously {Nickel et al., 1990}. Both RSVp.*metFGF* plasmid (prepared as described above) and genomic tail DNA were digested with *NcoI*/*XbaI*, electrophoresed in 1% (w/v) agarose gels and blotted to nitrocellulose as described previously {Maniatis et al., 1982}. The 500 bp *XhoI* metFGF (FGF-2) fragment was radiolabelled to a specific activity of approximately 1 x 10^9 cpm/μg using α^{32}P dCTP by random priming method {Promega "Prime-A-Gene" Kit) and hybridized to DNA blots as described on the previous page. With subsequent generations of FGF-2 TG mice, DNA analysis was done by slot blot analysis using the RSV promoter region as a probe. Both DNA blotting and slot blot analysis revealed that mice from the no. 5323 and 5318 lines carried four and two copies of the FGF-2 transgene, respectively (data not shown).

3.6 RNA Isolation and Blotting

Total RNA was isolated from stably transfected H9c2 cells or transiently transfected neonatal cardiac myocytes cultures, which had been maintained for 48 h in 10% FBS F12-DMEM , as well as adult mouse tissues as described by Chomczyski and Sacchi {Chomczyski and Sacchi, 1987} or using a Trizol extraction method (Gibco-BRL), respectively. Total RNA (50-75 μg) was denatured with formaldehyde and resolved by electrophoresis through a 1% agarose gel {Maniatis et al., 1982}. The RNA was blotted to nitrocellulose, hybridized with either radiolabelled (i) mouse FGFR-1 cDNA (*XbaI/SalI*) {Jin et al., 1994}, (ii) rat FGF-2 (*XhoI*) cDNA {Cattini et al., 1998}, and (iii) rat ANF (*PstI*) cDNAs {kindly provided by Dr. M. Nemer, Clinical Research Institute, Montreal, PQ, Canada; Nemer et al., 1988} as well as (iv) α-MHC or β-MHC {Robbins et al., 1990} oligonucleotides (5′-CTGCTGGAGAGGTTATTCCTCG-3′ and 5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′) as described previously {Cattini et al., 1998; Nickel and Cattini, 1991}. Following hybridization, the blots were washed according to previously established protocols {Cattini et al., 1998; Nickel and Cattini, 1991} and exposed to film. Hybridization with GAPDH (*PstI/BglI*) cDNA (kindly provided by Dr. I. Dixon, Institute of Cardiovascular Sciences, University of Manitoba) or detection of 28S ribosomal RNA by ethidium bromide staining was used to assess RNA loading. Autoradiographs from RNA blots were assessed by densitometry.

3.7 Protein Isolation and Blotting

Protein was isolated from mouse hearts essentially as described previously {Kardami and Fandrich, 1989} and quantitated using the Bicinchoninic Acid (BCA)

protein assay (Pierce, Rockford, IL). Immunodetection of FGF-2 in cardiac heparin-binding protein was performed by using mouse monoclonal antibodies (1 μg/ml; Upstate Biotechnology) followed by horseradish peroxidase-conjugated anti-mouse Ig (Bio-rad Laboratories; Hercules, CA). Cytosolic and membrane fractions from mouse hearts were extracted as described previously {Padua et al., 1998}. Immunodetection of of the 54 kD JNK and 38 kD p38 kinase was performed in cardiac cytosolic fractions by using rabbit polyclonal antibodies to: (i) phospho-stress-activated protein kinase (SAPK)/JNK (Thr^{183}/Tyr^{185}), which detects the dually phosphorylated isoforms of all three SAPK/JNKs (1:1000, New England Biolabs; Mississauga, ON, Canada); (ii) SAPK/JNK, which detects total SAPK/JNK levels (1:1000, New England Biolabs); (iii) phospho-p38 MAPK (Thr^{180}/Tyr^{182}), which detects the dually phosphorylated isoform of p38 (1:1000, New England Biolabs); and (iv) p38 MAPK, which detects total p38 MAPK (phosphorylation-state independent) levels (1:1000, New England Biolabs). Immunodetection of the 82 kD PKCα and the 90 kD PKCε in cardiac cytosolic and membrane fractions was performed by using rabbit polyclonal antibodies to the carboxyl terminus of PKCα (1:200, Santa Cruz Biotechnology; Santa Cruz, CA) or the carboxyl terminus PKCε (1:200, Santa Cruz Biotechnology). Protein lysate preparations from mouse hearts and immunoblotting were done by Mr. R.R. Fandrich from Dr. E. Kardami's laboratory at the Institute of Cardiovascular Sciences at the University of Manitoba.

For FGFR-1 experiments, transiently transfected cardiac myocytes were maintained in 10% FBS F12-DMEM for 24 h and then in 0.5% FBS for 46 h. Cardiac

myocytes were subsequently refed with serum free F12-DMEM medium containing 1 x Insulin-Transferrin-Selenium-A supplement (Redu-Ser II, Upstate Biotechnology), 0.02 mg/ml ascorbic acid, and antibiotics for 2 h and then stimulated with 10% FBS for 5 min. Cardiac myocytes were subsequently rinsed with calcium and magnesium free (CMF) PBS [containing 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and complete™, Mini protease inhibitors (Roche Diagnostics; Laval, PQ, Canada)], and total protein was isolated using a protein lysis buffer (200 µl/ 35 mm plate) containing 2% SDS, 50 mM Tris-hydrochloric acid (HCl) pH 6.8, 60 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF and complete™, Mini protease inhibitors (Roche Diagnostics; Laval, PQ, Canada). Lysates were sonicated (3 x 10 seconds (s)), pelleted at 10,000 g for 15 min at 4 °C to clear lysate of insoluble material, and the supernatant (protein) was quantitated using the BCA protein assay (Pierce). Twenty micrograms of protein was resolved by SDS-PAGE on a 10 % gel, transferred onto Immobilon P membrane (Millipore, ON, Canada) and blocked with 10 % milk-TBS-T (10 mM Tris-HCl pH 8.0, 150 mM sodium chloride, and 0.05% Tween 20) overnight at 4 °C. Immunodetection of phosphorylated (active) or non-phosphorylated forms of MAPK was performed as described previously {Padua et al., 1998}, using specific rabbit polyclonal antibodies that either preferentially detect: (i) dually phosphorylated, active form of MAPK enzymes (ERK1 and ERK2) (1:5,000; Promega) or (ii) total ERK1 and ERK2 which is phosphorylation-state independent {1:1,000; New England Biolabs).

For the kinase-deficient TGFβ-RII (ΔkTGF-βRII) experiments, transfected cardiac myocyte cultures were rinsed with calcium and magnesium free (CMF) PBS, and total protein was isolated and quantitated as described on previous page. Twenty micrograms of protein was resolved by SDS-PAGE on a 12.5% gel, transferred onto Immobilon P membrane and blocked with 10 % milk-TBS-T (10 mM Tris-HCl pH 8.0, 150 mM sodium chloride, and 0.05% Tween 20) overnight at 4 °C. Immunodetection of TGF-βRII was performed using specific rabbit polyclonal antibodies raised against the carboxyl terminal residues 550-565 of the human TGF-βRII (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), for 1 hour at room temperature. TGF-βRII antibodies can detect both full-length and kinase-deficient TGF-βRII and have been shown not to cross-react with TGF-βRI (Santa Cruz Biotechnology).

All rabbit polyclonal antibodies were followed by horseradish peroxidase-conjugated anti-rabbit Ig (Bio-Rad Laboratories) for 1 hour at room temperature. Antigen-antibody complexes were visualized by using enhanced chemiluminescence (Pierce; Rockford, IL). Autoradiographs from protein blots were assessed by densitometry. Assessment of protein loading was determined by staining blots with 0.2% Ponceau S (Sigma-Aldrich) in 3% trichloroacetic acid (TCA) prior to immunoblotting and staining with 0.1 % amido black subsequent immunoblotting as described previously {Harlow and Lane, 1988}.

3.8 Immunofluorescence Microscopy

Mouse hearts were excised, blotted dry to remove blood, placed in TissueTek OCT compound (Miles Laboratories; Elkhart, IN), immediately frozen on dry ice, and then cut into 7 μm thin cryosections. Sections were fixed in 1% paraformaldehyde-PBS for 15 min at. To detect FGF-2, sections were incubated overnight at 4 °C in 1% bovine serum albumin (BSA; Sigma-Aldrich)-PBS containing specific and well-characterized FGF-2 antibodies (1:1000) {Kardami and Fandrich, 1989; Kardami et al., 1990} and counterstained with either mouse α-actinin (1:400, Sigma-Aldrich), mouse α-smooth muscle actin (1:200, Sigma-Aldrich), and goat collagen IV (1:40, Southern Biotechnology Associates; Birmingham, AL) antibodies to detect muscle, smooth muscle-containing blood vessels, and extracellular matrix. To detect endothelial cells (capillaries), sections were incubated overnight at 4 °C in 1% BSA-PBS containing rabbit human von Willebrand factor antibodies (1:100, Sigma). Normal rabbit or mouse Ig were substituted for primary antibodies at equivalent dilutions as controls. Sections were then incubated with biotinylated donkey rabbit Ig (1:50, Amersham; Arlington Heights, IL) antibodies in 1% BSA-PBS for 1.5 h at room temperature. Subsequently, sections were incubated overnight at 4 °C with FITC-streptavidin conjugate (1:20, Amersham) and Texas Red conjugated donkey anti-mouse Ig (1:20, Amersham) or Texas Red conjugated donkey anti-mouse Ig (1:20, Jackson Immunoresearch Laboratories; Westgrove, PA) antibodies in 1% BSA-PBS. For counterstaining of nuclei, sections were incubated for 5 min with 0.0125% Hoescht-33342 (Calbiochem-Behring, San Diego, CA) in PBS and then mounted in mounting medium (Crystal/Mount, Biomedia; Foster City, CA) and

examined by epifluorescence and photographed using a Nikon ECLIPSE 800 microscope.

To assess FGFR-1 protein in neonatal cardiac myocytes transiently transfected with FGFR-1 hybrid genes, cardiac myocytes were fixed 72 h after transfection using 1% paraformaldehyde for 15 min at 4 °C. Coverslips were first incubated with affinity purified rabbit FGFR-1 (flg) antibodies (1:200; Santa Cruz Biotechnology) or nonimmune serum at the same dilution in 1% BSA in PBS for 16 h at 4 °C, then with biotinylated anti-rabbit immunoglobulins (Ig, 1:20; Amersham) for 1 h at room temperature, followed by incubation with fluorescein conjugated to streptavidin (Strep-FITC, 1:20, Amersham) for 1 h at room temperature, followed. The rabbit FGFR-1 antibodies were raised against the carboxyl terminal residues 802-822 of the human flg receptor and, thus, detect both the 'long' and 'short' FGFR-1 isoforms. These antibodies are highly specific and do not crossreact with FGFR-2, FGFR-3 and FGFR-4 {Hanneken et al., 1995}. Labeling for myosin, to identify myocytes, was performed using monoclonal antibodies specific for striated muscle myosin (1:50, MF20) in 1% BSA-PBS followed by visualization with Texas Red conjugated anti-mouse Ig (1:20, Amersham). The MF20 hybridoma was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. When identification of nuclei was necessary, cellular DNA was stained with 0.0125% Hoechst dye 33342 (Calbiochem-Behring). Coverslips were mounted using

mounting media for fluorescence (Vector Vectashield; Burlingame, CA), examined and photographed with a Nikon Diaphot microscope equipped with epifluorescence optics.

To assess TGF-βRII protein and subcellular localization in neonatal cardiac myocytes transfected with Ad.ΔkTGF-βRII or Ad.β–gal, cardiac myocytes were fixed 24 h after transfection using 1% paraformaldehyde for 15 min at 4 °C and permeabilized with 0.1% Triton X-100 for 15 min at 4 °C. Coverslips were first incubated with affinity purified rabbit TGF-βRII antibodies (1:200; Santa Cruz Biotechnology) or nonimmune serum at the same dilution in 1% BSA in PBS for 16 h at 4 °C, then with biotinylated anti-rabbit immunoglobulins (Ig, 1:20; Amersham) for 1 h at room temperature, followed by incubation with fluorescein conjugated to streptavidin (Strep-FITC, 1:20, Amersham) for 1 h at room temperature, followed. The rabbit TGF-βRII antibodies used for detection have been described in section ***3.7***. Myocytes were identified by counterstaining with mouse α-actinin (1:400, Sigma) which was visualized with Texas red-conjugated anti-mouse Ig (1:20; Amersham), and nuclei were identified by staining cellular DNA with 0.0125% Hoechst dye 33342 (Calbiochem-Behring). A description of immunofluorescence staining for bromodeoxyuridine (BrdU) in cells is found in section ***3.10***. Coverslips were mounted in mounting medium for fluorescence (Crystal/ Mount, Biomedia; Foster City, CA) and examined by epifluorescence and photographed using a Nikon ECLIPSE 800 microscope.

3.9 FGFR-1 Crosslinking Assay

For FGFR-1 crosslinking studies, neonatal cardiac myocytes were plated on collagen coated dishes, transfected for 24 h with either SVenhMLCp.*FGFR-1(L)* or SVenhMLCp.*FGFR-1(S)* as well as control DNA (section ***3.2***), maintained for 48 h, and incubated with ^{125}I-FGF-2 (2.5 ng/sample; Dupont Canada Inc., Mississauga, ON) for 90 min at 4°C in the absence or presence of 0.1 µg unlabeled FGF-2 (Upstate Biotechnology). The crosslinking reaction was initiated by adding 0.15 mol/L disuccinimidyl suberate (DSS; Pierce) to transfected cultures for 15 min at room temperature. The reaction was terminated by adding 10 µl of 0.5 mol/L Tris-HCl, pH 7.4. Cells were subsequently scraped off, followed by centrifugation (10 min, 10,000 g) and resuspension of the pellet in 30 µl of homogenizing buffer consisting of 0.02 mol/L HEPES pH 7.4, 0.25 mol/L sucrose, 1 µmol/L EDTA, 1 mg/ml leupeptin and 1% Triton X-100. Insoluble residue was removed by centrifugation (10 min, 16,000 g) and the supernatant was analyzed by SDS-PAGE in a 7.5% gel, and visualized by autoradiography. Transfection and crosslinking of iodinated FGF-2 were performed by Dr. B. Doble and Mr. R.R. Fandrich, respectively, who were members of Dr. E. Kardami's laboratory at the Institute of Cardiovascular Sciences at the University of Manitoba. Levels of labeled FGF-2 complexes were assessed from autoradiographs by scanning densitometry.

3.10 Bromodeoxyuridine(BrdU) and Phosphorylated (Phospho)-H1 Labeling Assay

For FGFR-1 experiments, neonatal cardiac myocytes were plated onto collagen-coated dishes (containing three coverslips per 60 mm dish or one coverslip per 35 mm

dish), transfected for 24 h, maintained for a further 24 h and then incubated in the presence of 3 μg/ml (w/v) BrdU (Sigma-Aldrich) for a further 24 h in the presence of 10% FBS F12-DMEM. To assess for plasma-membrane receptor mediated effects of FGF-2, cardiac myocyte cultures were exposed to neutralizing antibodies to FGF-2 using conditions previously described {Pasumarthi et al., 1996}. Neutralizing antibodies have been demonstrated to be highly specific for the non-denatured form of FGF-2 and shown not to cross-react with FGF-1 (Upstate Biotechnology). Briefly, myocytes were maintained for 24 h in either the presence of (i) normal mouse Ig (10 μg/ml; Sigma); or (ii) anti-bovine FGF-2, type I, monoclonal neutralizing antibodies (10 μg/ml; Upstate Biotechnology), followed by incubation in the presence of 3 μg/ml (w/v) BrdU. Myocyte cultures were subsequently fixed with 1% paraformaldehyde for 15 min at 4 °C and then with 70% ethanol for 30 min at room temperature, followed by treatment with 0.07 M sodium hydroxide for 2 min at room temperature. Simultaneous labelling for myosin (to identify myocytes) and/or BrdU in ventricular myocytes was done using monoclonal antibodies against striated myosin (1:50, MF20) and BrdU (1:2, Amersham; or 1:7, Becton Dickinson, Cockeysville, MD) in 1% (w/v) BSA-PBS. Both myosin and BrdU were visualized with Texas red-conjugated anti-mouse Ig (1:20; Amersham). For quantitative analysis, approximately 2000 cardiac myocytes were assessed from cultures transfected with SVenhMLCp.*luc*, SVenhMLCp.*FGFR-1(L)* or SVenhMLCp.*FGFR-1(S)* genes (total approximately 6000), from 15-20 randomly selected fields on three separate coverslips, representing three or four independent transfection experiments.

For ΔkTGF-βRII experiments, neonatal cardiac myocytes were plated onto collagen-coated dishes (containing one coverslip per 35 mm dish), transfected for 24 h, and then incubated in the presence of 1, 10 and 1000 ng/ml FGF-2 (Upstate Biotechnology) for a further 24 h in the presence of 10% FBS F12-DMEM. Cardiac myocytes were pulsed for the final 8 hours of FGF-2 treatment with 15 μg/ml (w/v) BrdU (Sigma-Aldrich). Myocyte cultures were subsequently fixed with 1% paraformaldehyde for 15 min at 4 °C, permeabilized with 0.1% Triton X-100 for 15 min at 4°C followed by treatment with 0.07 M sodium hydroxide for 2 min at room temperature. Simultaneous labelling for α-actinin (to identify myocytes) and/or BrdU in ventricular myocytes was done using monoclonal antibodies against mouse α-actinin (1:200, Sigma-Aldrich) and BrdU (1:1, Amersham) in 1% (w/v) BSA-PBS. In some cases, cardiac myocytes were labeled with phospho-H1 histones , to assess for cardiac myocyte cell cycle entry into the early M phase (i.e. marker associated with mitotic condensation), using specific polyclonal phospho H1 antibodies (1:250; a generous gift from Dr. J. Davie in the Cancer Centre at the University of Manitoba) which have been previously characterized {Chadee et al., 1999; Chadee et al., 1995; Hendzel et al., 1998}. Both α-actinin and BrdU were visualized with Texas red-conjugated anti-mouse Ig (1:20; Amersham), whereas phospho-H1 was visualized with biotinylated anti-rabbit immunoglobulins (Ig, 1:20; Amersham), followed by incubation with fluorescein conjugated to streptavidin (Strep-FITC, 1:20, Amersham). For quantitative analysis of DNA synthesis (BrdU labeling index) in Ad.β-gal versus Ad.ΔkTGF-βRII groups, 10-15 randomly selected fields on three separate coverslips, representing two independent transfection experiments was assessed. For quantitative analysis of DNA synthesis in FGF-2 treatment groups in Ad.β-

gal versus Ad.ΔkTGF-βRII groups, six separate coverslips in 15 randomly selected fields, representing one independent transfection experiment was assessed. For quantitative analysis of phospho-H1 labelling index in Ad.β-gal versus Ad.ΔkTGF-βRII groups, 15 randomly selected fields on three separate coverslips was assessed. In all cases, approximately 550-650 cells was assessed per coverslip.

For DNA synthesis, a BrdU labelling index (LI) was obtained by expressing the number of cardiac myocyte nuclei staining positively for BrdU as a percentage of the total number of cardiac myocyte nuclei assessed. Phospho-H1 LI was obtained by expressing the number of cardiac myocyte nuclei staining positively for phospho-H1 as a percentage of the total number of cardiac myocyte nuclei assessed.

3.11 Cell Number Assay

To assess cell division in stably transfected or control H9c2 cells, cells were plated at a density of 1-2 x 10^4 per 100 mm dish in the presence of 10% FBS and then attached cells were counted at 24 h intervals for 5 days using a hemacytometer. During this period the medium was changed every 48 h. For FGF-2 treatment, FGF-2 (Upstate Biotechnology, Lake Placid, NY; 10 ng/ml) was added to cells 6-7 days after plating and cells were harvested and counted 24 h later.

To assess cell number in transiently transfected neonatal cardiac myocytes, cardiac myocytes were plated and transfected as described for 'Bromodeoxyuridine Labeling' experiments. After 48 h, myocytes were rinsed with PBS, and fixed with 1%

paraformaldehyde for 15 min at 4 °C. Cardiac myocytes were labeled for myosin using monoclonal antibodies against striated myosin (1:50, MF20) and visualized with Texas-red conjugated anti-mouse Ig (1:20; Amersham). Cell number was assessed by counting the number of myocytes from 15 randomly selected fields on at least three coverslips representing two independent experiments. Cell number was expressed as the fold difference relative to control, which was arbitrarily set to 1.0.

3.12 Langendörff Perfusion Apparatus

Adult mice (8-12 weeks old) were euthanized by cervical dislocation, and their hearts were excised and perfused by using a retrograde Langendörff method {Ng et al., 1991}. The ascending aorta was cannulated by using a 21-gauge needle tied with a 6-0 silk suture and perfused within 5 min of excision. The perfusate, consisting of a Krebs-Henseleit (KH) solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 glucose, 24 $NaHCO_3$, and 3% BSA (Roche Molecular Biochemicals), was bubbled with 95% O_2-5% CO_2 (pH 7.4, 37 °C) under nonrecirculating conditions at a constant pressure of 60 mmHg. The atria were removed and a KH-filled latex balloon was inserted into the left ventricle through the mitral valve. This allows monitoring of systolic left ventricular pressure, defined as developed pressure, and left ventricular end-diastolic pressure (EDP) using a Digimed Heart Performance Analyzer (Micro-Med; Louisville, KY). In addition, a thermocouple was inserted into the right ventricle to monitor the temperature of the KH in the heart, which was kept at 37 °C. All hearts were electrically paced by using a platinum electrodes placed on the top of the right ventricle with 1-ms pulses at 6 Hz and 3 volts throughout

the experiment. Preload in all hearts was adjusted to achieve maximal developed pressure while maintaining a positive EDP (2-5 mmHg) to monitor for balloon integrity. Only hearts demonstrating minimal developed pressure of 70 mmHg and stable EDP were utilized for experimentation. Establishment of this retrograde perfusion apparatus for isolated mouse hearts was done in collaboration with Mr. David Sontag from Dr. P.A. Cattini's laboratory.

The experimental protocol to assess injury in the mouse model was adopted from a previously established rat heart Langendörff preparation {Padua et al., 1998}. After an equilibration period of 30 min, hearts were subjected to 30 min of global ischemia by turning off flow of perfusion medium to the heart. Perfusion was restored after 30 min of global ischemia, and continued for 60 min. The volume of perfusate during 1-min periods was collected from TG and non-TG adult mouse hearts at various time points during the period of preischemia and ischemia-reperfusion. Time points include preischemia (30-min equilibration time) and reperfusion time points of 1, 5, 10 , 15, 30, 45 and 60 min. The coronary flow rate was determined by measuring the volume of perfusate collected during 1-min periods before ischemia (30-min equilibration time) and during reperfusion (1, 5, 10, 15, 30, 45 and 60 min) and by normalizing these values to heart weight (in $ml \cdot min^{-1} \cdot g^{-1}$). For exogenous FGF-2 studies, hearts were equilibrated for 25 min with KH and then supplemented with either vehicle (20 mM Tris·HCl pH7.9, 0.5 M NaCl, 10% glycerol, 260 mM imidazol, 5 mM β-mercaptoethanol, and 1 mM EDTA) or 10 μg of recombinant rat FGF-2 {Padua et al., 1995b; Padua et al., 1998} dissolved in 1 ml KH for

2 min followed by KH solution for 3 min, before 30-min global ischemia and 60 min reperfusion.

To extract FGF-2 from extracellular matrix using the Langendörff preparation, hearts were equilibrated for 20 min with KH and then perfused for 5 min with a high salt buffer (1.6 M NaCl, 10 mM Tris pH 7.0) followed by KH solution for 5 min. Hearts were then sectioned and processed for immunofluorescence microscopy as described above.

3.13 LactateDehydrogenase Assay

Perfusates from mouse hearts were collected on ice for 1 min at various time points before and during ischemia-reperfusion. The time points include: before ischemia (30-min equilibration) and postischemic times of 1, 5, 10, 15, 30, 45, and 60 min. Quantitative kinetic determination of lactate dehydrogenase (LDH) activity in perfusates was assessed according to the manufacturer's instructions (LDH Optimized, Sigma-Aldrich). LDH activity was normalized for coronary flow rate and heart weight (in $U \cdot min^{-1} \cdot g$ heart wt^{-1}). As an additional control, LDH activity was assessed in non-TG mouse hearts during a 2-h period without ischemia-reperfusion injury.

3.14 FGF-2 ELISA Assay

Perfusates from mouse hearts were collected for 1 min at various times during the equilibration period (before ischemia). Blood was collected from euthanized from euthanized adult mice and was allowed to clot for 30 min before centrifugation at 10,000 g for 30 min to collect serum. Conditioned medium from neonatal cardiac myocytes

transiently transfected with control or FGFR-1 hybrid genes, was collected 48 h subsequent transfection. Quantitative determination of FGF-2 in perfusates, serum and conditioned medium was assessed using a Quantikine HS human FGF basic immunoassay (R&D systems; Minneapolis, MN) as described by the manufacturer's instructions. FGF-2 in perfusates was normalized for coronary flow rate and heart weight (in $pg \cdot min^{-1} \cdot g$ heart wt^{-1}).

3.15 Reporter Gene Assays

Transfection efficiency for transient gene transfer or adenoviral transfection experiments was assessed by using the β-gal assay {Xu et al., 1992}. Cardiac myocytes, in the transient FGFR-1 experiments were co-transfected with 10 μg RSVp.β-gal, whereas controls cells for adenoviral ΔkTGF-βRII experiments were transfected with Ad.βgal (MOI 50) and processed after 48 h and 24 h following transfection, respectively. Cardiac myocytes were rinsed with PBS-CMF, fixed (1.8% formaldehyde, 0.2% glutaraldehyde, 2 μmol/L magnesium chloride, 50 mM sodium phosphate, pH 7.4) and incubated with X-gal solution containing 1 mM magnesium chloride, 3.3 μmol/L potassium ferrocyanide, 3.3 μmol/L potassium ferricyanide, 0.15 mol/L sodium chloride, 0.01 mol/L sodium phosphate buffer pH 7.4 and 0.2% (w/v) X-gal for 18 h at 37 °C. For transient transfection experiments, quantitative analysis of about 12,000 cardiac myocytes were assessed for β-gal staining by counting from 15 randomly selected fields on three separate coverslips, representing four independent transfections experiments. The percentage of β-gal stained cells was determined.

Studies involving -1058FGFp.*luc* mice are described in Chapter 5. At the end of the norepinephrine (Research Biochemicals International; 0.01 M) stimulation period, –1058FGFp.*luc* adult cardiac myocytes were rinsed with PBS-CMF, harvested with PBS-CMF, pelleted, and lysed on ice in 50 µl of 1x Promega Lysis buffer. After 15 min on ice, insoluble material was removed by centrifugation (30 min, 10,000 g), and 20 µl of supernatant was assayed for luciferase activity using the Promega "Luciferase Assay System" and a luminometer (LUMAT LB9507 Luminometer, BERTHOLD GmBH & Co. KG) according to the manufacturer's instructions. Luciferase activity was normalized against lysate protein content as determined by the BCA protein assay (Pierce).

3.16 StatisticalAnalysis

Data presented in the text and figures represent the means ± standard error mean (SEM) from at least two independent experiments each done in triplicate, unless stated otherwise. Statistical analysis of the results was done in most cases, using the parametric student's t-test, however, non-parametric Mann-Whitney was used when the standard deviations were significantly different. Alternate Welch and ANOVA with Dunn's multiple-comparison post hoc tests was used for multiple column comparisons. In all cases, a value was considered statistically significant if p was determined to be < 0.05.

CHAPTER 4

RESULTS: The Effects of Endogenous Overexpression of FGF-2 in Adult Mouse Hearts *In Vivo* and after Ischemia-Reperfusion Injury

4.1 Basic Characterization of the Effects of Endogenous FGF-2 Overexpression in Adult Mouse Hearts In Vivo.

4.1.1 The FGF-2 transgene is expressed in striated muscle.

Two independent transgenic mouse lines (#5323 and #5318) expressing the low mwt. form of FGF-2 were established for *in vivo* and *ex vivo* studies on the heart. To assess the level and range of transgene expression, RNA (50 μg) was isolated from various tissues of FGF-2 TG mice and examined by RNA blot analysis. A 1.3 kb transcript, consistent with expression of the FGF-2 transgene, was detected in cardiac and skeletal muscle, but not in lung, brain, kidney, spleen or liver (closed arrowhead, Fig. 6A). Transcripts from the FGF-2 transgene were observed in both cardiac atria and ventricles. Although endogenous FGF-2 mRNA was too low to be detectable in cardiac tissue, it was detected in the lung, brain and liver as indicated by a 6.1 kb transcript (open arrowhead, Fig. 6A). Overexpression of FGF-2 in the cardiac (ventricle) muscle of both FGF-2 TG lines was confirmed by protein blot analysis using specific monoclonal antibodies. Based on densitometry, levels of 18 kD FGF-2 protein were increased about 22 and 34 fold (n=6-9) in the #5318 and #5323 lines respectively (Fig. 6B). The protein band above the 18 kD FGF-2 present in both FGF-2 TG and non-TG hearts, likely represents 18 kD FGF-2 protein that has undergone post-translational modifications *in vivo* or that has been translated or transcribed from an alternative start site in the RSV promoter. An alternative transcription site in the RSV promoter could be a possibility since a larger sized FGF-2 transcript, above the FGF-2 transgene transcript was also

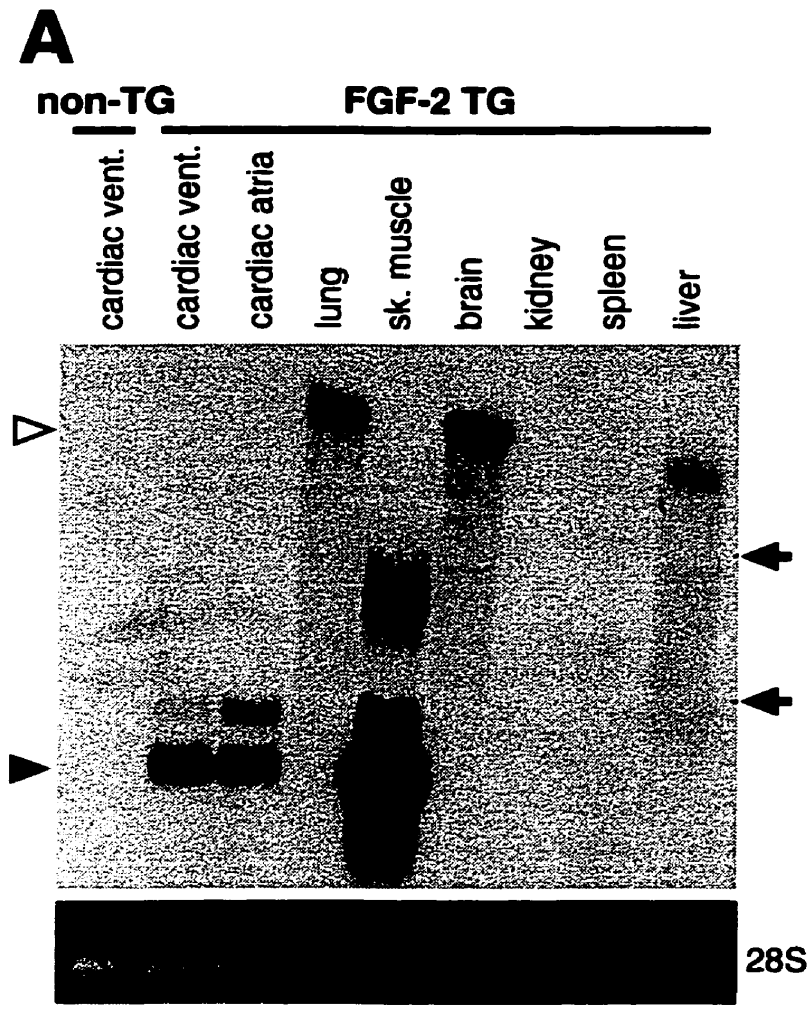
Figure 6.

Expression of FGF-2 in adult mouse hearts *in vivo*.

(A) Detection of transgene (1.3 kb, closed arrowhead) or endogenous (6.1 kb, open arrowhead) FGF-2 transcripts in tissues (as indicated) from non-TG and FGF-2 TG (line #5323) mice by RNA blotting and autoradiography. Arrows indicate mobilities of 28S and 18S RNAs. The 28S RNA band for each sample stained with ethidium bromide and photographed before blotting is also shown (lower panel).

(B) Detection of 18 kD FGF-2 in non-TG and FGF-2 TG (lines #5323 and #5318) mouse ventricles by protein blotting, immunodetection using specific FGF-2 antibodies and chemiluminescence. A sample of recombinant 18 kD FGF-2 was used as a positive control.

This figure is reproduced from Sheikh et al., 2001.


A
non-TG
FGF-2 TG
cardiac vent.
cardiac vent.
cardiac atria
lung
sk. muscle
brain
kidney
spleen
liver
28S
B
18 kD FGF-2
2 ng
1 ng
FGF-2 TG #5323
FGF-2 TG #5318
non-TG
31.0
21.5
14.5
6.0

observed in RNA blots (Fig. 6A). Alternate possibilities could include induction of expression of endogenous CUG-initiated FGF-2 or aggregated species of FGF-2. Serum FGF-2 levels in the #5318 (1.75 ± 0.8 ng/ml, n=4) and #5323 (2.23 ± 0.52 ng/ml, n=5) lines were not significantly different from non-TG mouse values (1.35 ± 0.32 ng/ml, n=5) in 3 month old adults.

Immunofluorescence microscopy was used to visualize FGF-2 protein in ventricular tissue sections from non-TG and TG mouse hearts using specific polyclonal antibodies to FGF-2 (Fig. 7) {Kardami and Fandrich, 1989; Kardami et al., 1990}. Ventricular tissue was triple-labeled for FGF-2, DNA and either α-actinin or α-smooth muscle actin, to specifically identify myocytes or smooth muscle cells (blood vessels) staining for FGF-2. In the case of non-TG mice, nuclei and cytoplasm of cardiac myocytes stained specifically for FGF-2 at levels clearly above the background observed with control Ig (Fig. 7, A and B). No FGF-2 staining of smooth muscle cells/blood vessels was observed (Fig. 7B). In contrast, cardiac myocytes from TG mice were stained uniformly and more intensely for FGF-2. We also observed the accumulation of specific FGF-2 staining surrounding the cardiac myocytes (Fig. 7, A and B). Again, no FGF-2 staining of smooth muscle cells/blood vessels was observed (Fig. 7B).

4.1.2 Heart weight-to-body weight ratio is unchanged in the FGF-2 TG mouse.

Adult mice (9-12 weeks) and their excised hearts, removed of atria, were weighed to determine heart weight-to-body weight ratios (μg/g). There was no significant difference between the heart weight-to-body weight ratios for FGF-2 TG lines #5323

(5.75 ± 0.51 μg/g, n=4) and #5318 (4.97 ± 0.27 μg/g, n=5) and non-TG (5.14 ± 0.18 μg/g, n=10) mice. In addition, we used RNA blotting (assessed 50 μg RNA) to compare the expression of the cardiac differentiation markers ANF, α-MHC and β-MHC in FGF-2 TG (#5323 – highest FGF-2 expressing line) and non-TG mouse ventricles. Expected transcript sizes of 0.9 kb and 6.0 kb for ANF and α-MHC, respectively, were observed (Fig. 8). Based on densitometry (n=4), there were no significant differences in ANF and α-MHC RNA levels in TG versus non-TG mouse ventricles. Although β-MHC transcripts (6.0 kb) were detected in embryonic mouse heart RNA {Sheikh and Cattini, unpublished results}, no expression, and thus, difference was detected in either FGF-2 TG or non-TG adult mouse ventricles due, presumably, to low abundance. FGF-2 transgene expression was also confirmed in these RNA samples by detection of the 1.3 kb FGF-2 (transgene) transcript (Fig. 8). An assessment of the 4.3/4.1 kb FGFR-1 RNA levels showed no difference in FGF-2 TG versus non-TG mouse hearts (Fig. 8).

4.1.3 Capillary density is increased in the FGF-2 TG mouse heart.

The density of blood vessels was estimated in cardiac ventricular sections from FGF-2 TG hearts (n=4) by staining for α-smooth muscle actin or, for capillaries with von Willebrand factor. For smooth muscle-containing blood vessels, four fields (1.1 mm^2 using x20 objective) from three sections from each of four FGF-2 TG and four non-TG mice were counted. Similar values of 19.5 ± 0.4 blood vessels/mm^2 and 20.7 ± 1.4 blood vessels/mm^2 were obtained for FGF-2 TG and non-TG mice, respectively. To assess capillary density, forty fields (0.02 mm^2 using x40 objective) from four sections from four FGF-2 TG and four non-TG mice were counted. The value for capillary density was

increased significantly (about 1.2 fold) from 1866 ± 169 capillaries/mm^2 in non-TG to 2297 ± 52 capillaries/mm^2 in TG mouse hearts ($p<0.05$).

4.1.4 Relative levels of JNK, p38 kinase and PKC, are increased in FGF-2 TG mouse hearts.

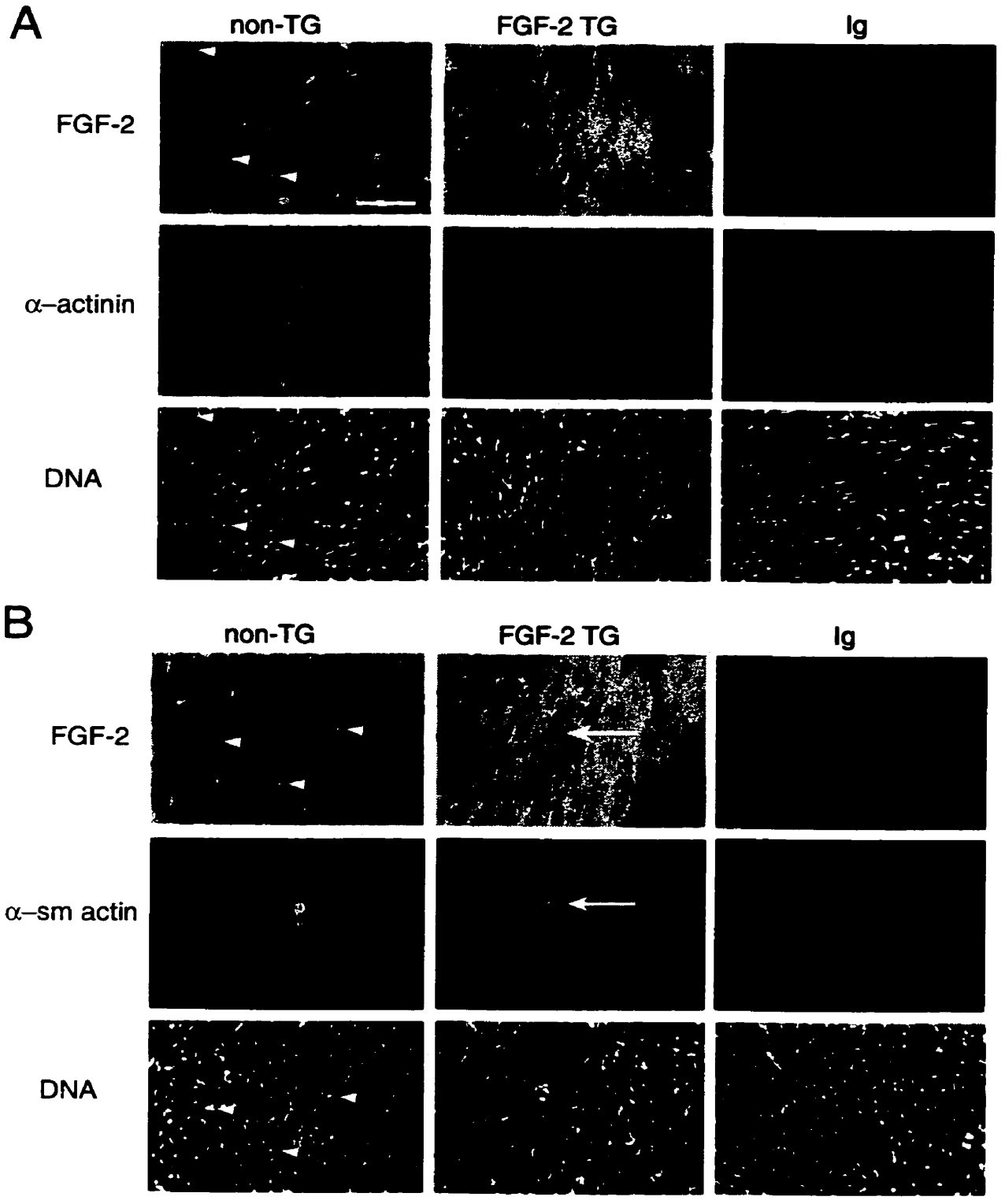
Stress-activated MAP kinases (JNK and p38) and PKC isoforms are known downstream targets of FGF-2 signaling{Hrzenjak and Shain, 1997; Le and Corry, 1999; Liu et al., 1999; Maher, 1999; Padua et al., 1998}. The relative levels of these kinases in FGF-2 TG (#5323 line) versus non-TG mouse hearts (n=3) were assessed in membrane and/or cytosolic fractions by protein blotting. For JNK and p38 MAPK, antibodies to both phosphorylated (active) and phosphorylation-state independent (active + inactive) forms were used to probe cytosolic protein (Fig. 9). Based on densitometry, levels of phosphorylated JNK and p38 were increased about 14 and 42 fold, respectively, in TG mouse hearts ($p<0.05$, n=3). There was no significant difference, however, in the "total" levels of JNK and p38 kinase in TG versus non-TG mouse hearts. For PKCs, relative levels of membrane-associated αPKC were significantly increased about 15 fold in TG mouse hearts ($p<0.05$, n=3), but cytosolic levels were unchanged (Fig. 9). In contrast, cytosolic levels of εPKC were increased significantly about 2 fold in TG mouse hearts ($p<0.05$, n=3), however, membrane-associated levels were not significantly different from controls (Fig. 9).

Figure 7.

Subcellular distribution of FGF-2 in adult non-TG and FGF-2 TG mouse ventricles.

Non-TG and FGF-2 TG (line #5323) mouse ventricles were triple-immunostained for **(A)** FGF-2, α-actinin and DNA or **(B)** FGF-2, α-smooth muscle actin and DNA as indicated. α-Actinin or α-smooth muscle actin antibodies were used to confirm the identity of cardiac myocytes or smooth muscle/blood vessels, respectively. DNA (Hoeschst 33342) stain was used to identify nuclei. Endogenous FGF-2 visible in cardiac myocyte nuclei of non-TG ventricles is indicated with white arrowheads **(A and B)**. FGF-2 TG ventricles show strong staining of FGF-2 within the **(A)** cytoplasm of cardiac myocytes and extracellular spaces but not **(B)** smooth muscle/blood vessels (indicated by white arrows). **(C)** FGF-2 staining of adult non TG and FGF-2 TG (line #5323) mouse ventricles (see next page). The pattern observed with non-TG ventricles stained with control Ig is shown for comparison. Bar is equivalent to 75 μm.

This figure is reproduced from Sheikh et al., 2001.


A
non-TG
FGF-2 TG
Ig
FGF-2
α–actinin
DNA
B
non-TG
FGF-2 TG
Ig
FGF-2
α–sm actin
DNA

C

non-TG

FGF-2 TG

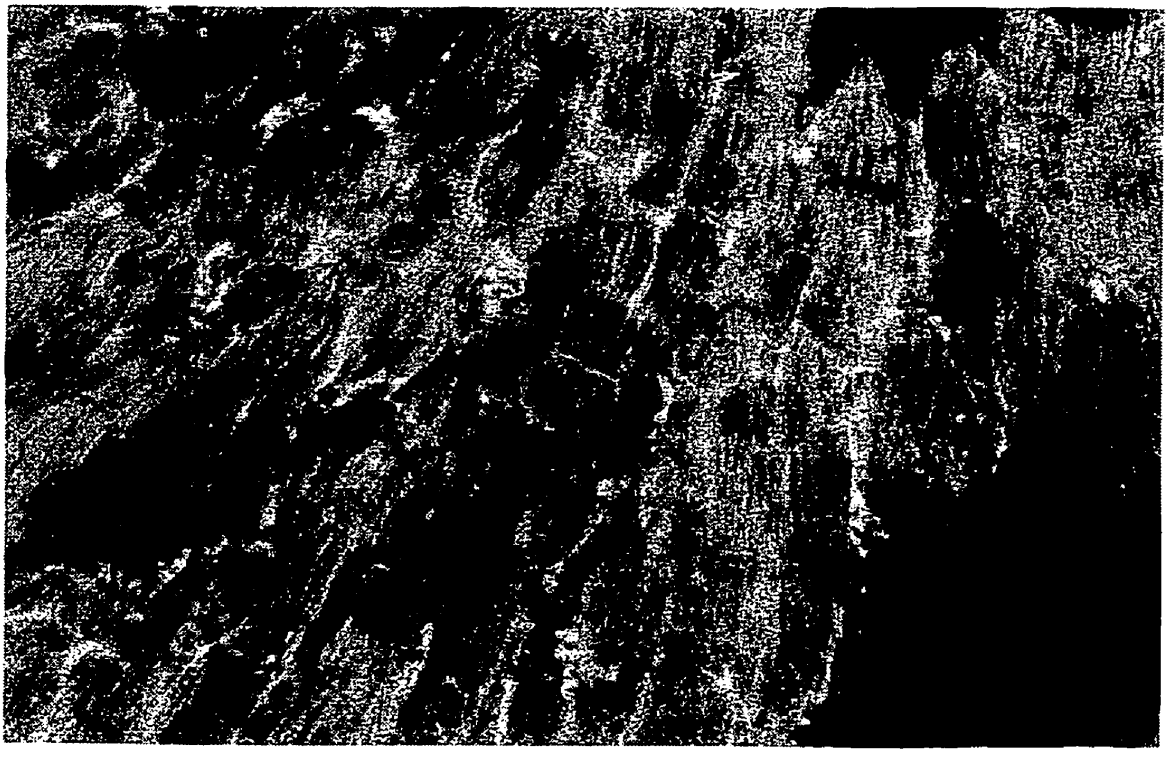


Figure 8.

Autoradiograph showing expression of cardiac differentiation markers and FGFR-1 in FGF-2 TG mouse hearts after RNA blotting.

RNA was isolated from non-TG and FGF-2 TG (line #5323) mouse ventricles, blotted and probed for **(A)** FGF-2 (1.3 kb), α-MHC (6.0 kb), β-MHC (6.0 kb) and ANF (0.9 kb) and **(B)** FGFR-1 (4.1 and 4.3 kb) transcripts as indicated. No change in α-MHC, β-MHC, ANF or FGFR-1 mRNA levels were observed in FGF-2 TG ventricles. Ethidium bromide staining of 28S RNA in each sample is also shown.

This figure is reproduced from Sheikh et al., 2001.

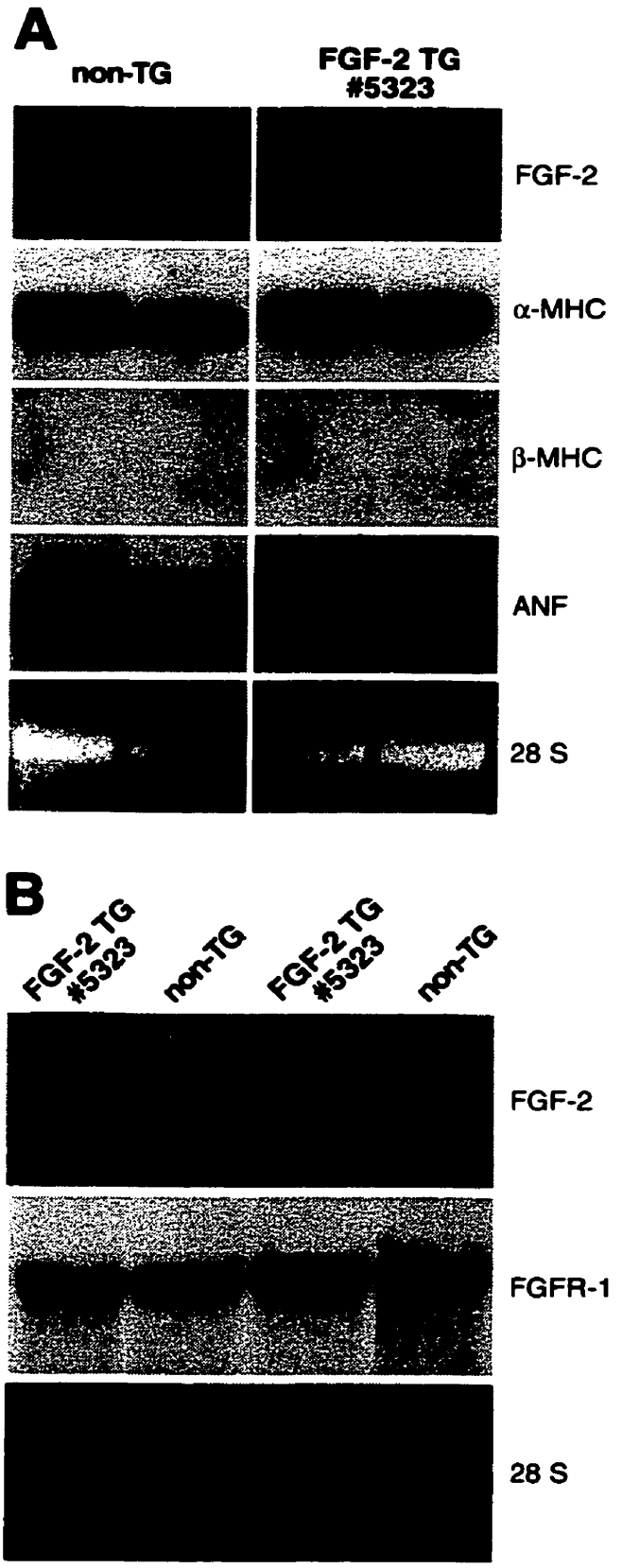
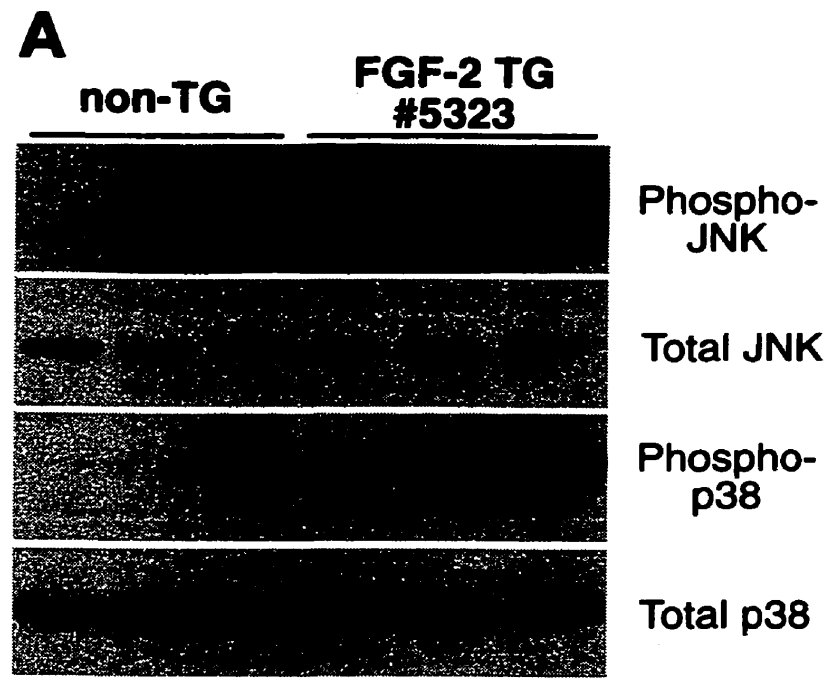

A
non-TG
FGF-2 TG
#5323
FGF-2
α-MHC
β-MHC
ANF
28 S
B
FGF-2 TG
#5323
non-TG
FGF-2 TG
#5323
non-TG
FGF-2
FGFR-1
28 S

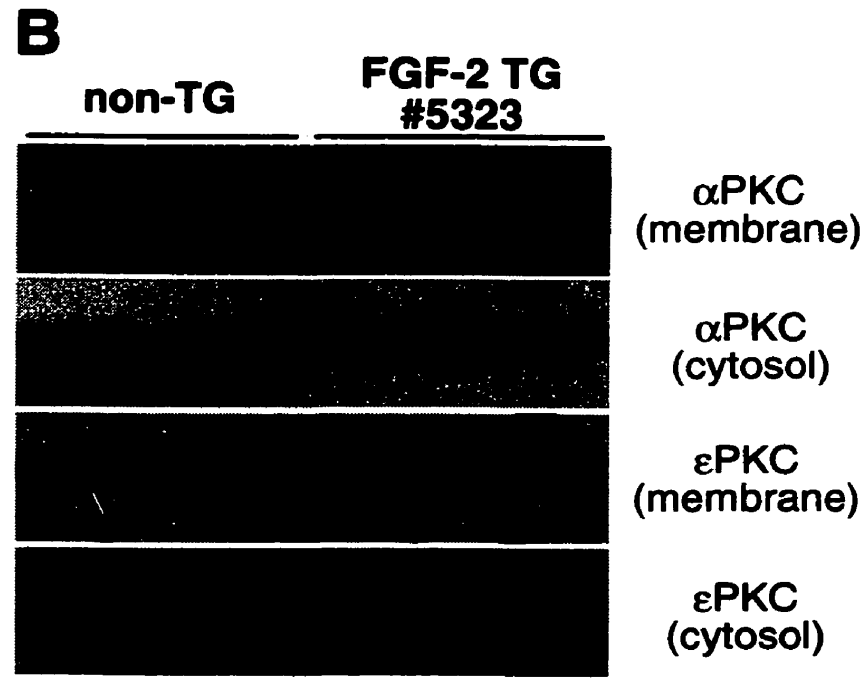
Figure 9.

Autoradiograph showing expression of JNK, p38 kinase as well as αPKC and εPKC in FGF-2 TG mouse hearts after immunoblotting.

Cytosolic and membrane protein fractions were isolated from non-TG and FGF-2 TG (line #5323) mouse ventricles. **(A)** Equal amounts of cytosolic protein were analyzed by SDS-PAGE and immunoblotted with antibodies specific for the phosphorylated forms of JNK and p38 or 'total' JNK and p38 as indicated. **(B)** Equal amounts of cytosolic and membrane-associated protein were analyzed by SDS-PAGE and immunoblotted with antibodies specific for the carboxyl terminus of αPKC and εPKC.

This figure is reproduced from Sheikh et al., 2001.


A
non-TG
FGF-2 TG
#5323
Phospho-
JNK
Total JNK
Phospho-
p38
Total p38


B
non-TG
FGF-2 TG
#5323
αPKC
(membrane)
αPKC
(cytosol)
εPKC
(membrane)
εPKC
(cytosol)

4.2 Establishment of a Retrograde Perfusion Apparatus for Isolated Mouse Hearts to Assess FGF-2 Release and to Induce Ischemia-Reperfusion Injury in FGF-2 TG Mouse Hearts.

4.2.1 Characterization of the isolated mouse heart (Langendörff) preparation.

The stability of our mouse Langendörff preparation was determined in isolated non-TG mouse hearts throughout a two-hour period. Both contractile force and percent cell damage as measured by developed pressure and LDH activity, respectively, were assessed. Representative profiles are shown for two non-TG mice and reveal stable developed pressures that are above 70 mmHg (Fig. 10A). Perfusates taken from these same non-TG mice showed no significant changes in LDH release from baseline at various time points throughout the two-hour period (Fig. 10B). Based on the stability of our preparation during a two hour period as well as existing protocols for the rat Langendörff preparation, an experimental protocol for myocardial injury was devised (Fig. 10C).

4.3 FGF-2 Release Studies

4.3.1 Distribution and release of FGF-2 in isolated and perfused TG versus non-TG mouse hearts.

Immunofluorescence microscopy was used to visualize FGF-2 protein in ventricular tissue sections from non-TG and TG perfused mouse hearts after 30-min equilibration (Fig. 11). Cardiac myocytes from "equilibrated" non-TG mouse hearts displayed the same predominantly nuclear and weaker cytoplasmic FGF-2 staining (Fig. 11) as seen in "freshly" isolated non-TG mouse hearts (Fig. 11A). In the case of FGF-2 TG mouse hearts, FGF-2 was localized predominantly around cardiac myocytes, and intracellular staining for FGF-2 was more intense than seen in non-TG cardiac myocytes

(Fig. 11A). No FGF-2 staining of smooth muscle cells/blood vessels was observed in either non-TG or TG mouse hearts (Fig. 11B).

Ventricular tissue sections from "equilibrated" FGF-2 TG mouse hearts were triple labeled for FGF-2, DNA and collagen IV as a marker for the basal lamina and extracellular matrix. In addition to nuclear staining (arrows), we observed the accumulation of specific FGF-2 staining surrounding the cardiac myocytes, which co-localized with collagen IV staining (Fig. 12). Immunofluorescence microscopy of ventricular sections from "equilibrated" FGF-2 TG mouse hearts perfused with a high salt buffer resulted in the loss of FGF-2 staining from the extracellular matrix (Fig. 12). In contrast, nuclear FGF-2 staining was still evident (arrows, Fig. 12).

Given the accumulation of FGF-2 in the extracellular matrix, the effect of FGF-2 overexpression on release of FGF-2 from isolated TG mouse hearts was assessed. The level of FGF-2 release from non-TG and FGF-2 TG mouse line #5323 was measured by ELISA of perfusates collected at various time points. Levels of FGF-2 were significantly higher in FGF-2 TG versus versus non-TG mouse hearts at 3 min equilibration. A decrease in FGF-2 release over the 30 min equilibration time was detected for both non-TG and FGF-2 TG mouse hearts (Fig. 13). The level of FGF-2 release, however, was consistently (about 2.5 fold) higher from FGF-2 TG hearts throughout the equilibration period, as seen clearly using a logarithmic scale (Fig. 13).

Figure 10.

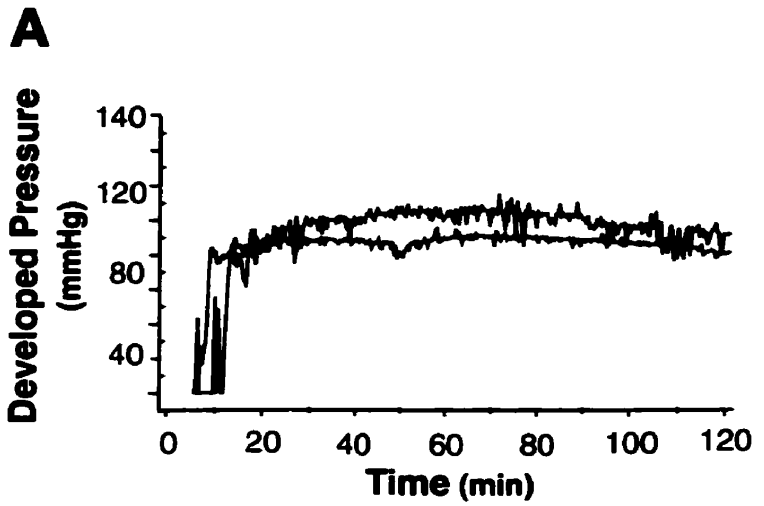
Assessment of developed pressure and LDH release in isolated mouse hearts.

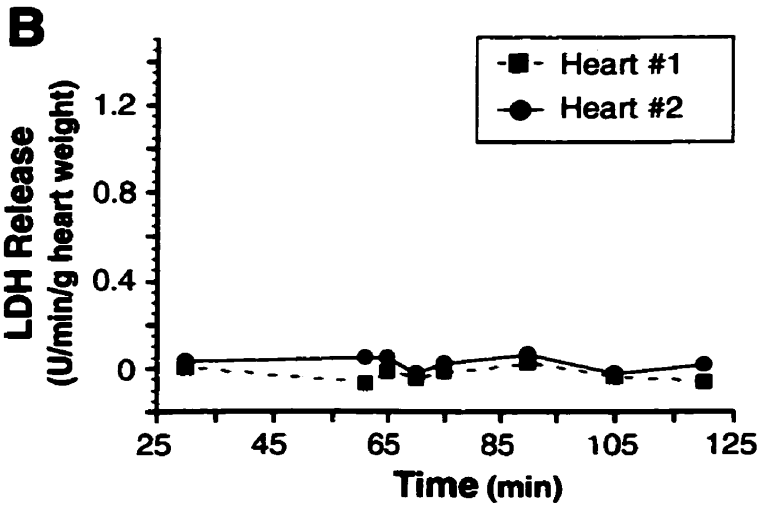
(A) Developed pressures were monitored and recorded for 120 min in isolated non-TG mouse hearts (n=2) perfused on a Langendörff preparation. This section of the figure was plotted by Mr. D. Sontag from Dr. P.A. Cattini's laboratory.

(B) LDH release was measured in perfusates from isolated non-TG mouse hearts (n=2) for 1 min at the times indicated throughout the 120 min period. Values for LDH release were normalized to coronary flow rate.

(C) Experimental protocol for ischemia-reperfusion in isolated perfused mouse hearts. All hearts were equilibrated over a 30-min period and then subjected to 30-min global ischemia and 60 min reperfusion. For studies involving exogenous addition of FGF-2, hearts were equilibrated for 25 min and then supplemented with either vehicle or 10 μg FGF-2 for 2 min followed by KH solution for 3 min, prior to global ischemia-reperfusion. Cardiac function and cellular damage (LDH) were determined at the indicated sample time points.

This figure is reproduced from Sheikh et al., 2001.


A
Developed Pressure
(mmHg)
140
120
80
40
0
20
40
60
80
100
120
Time (min)


B
LDH Release
(U/min/g heart weight)
1.2
0.8
0.4
0
Heart #1
Heart #2
25
45
65
85
105
125
Time (min)

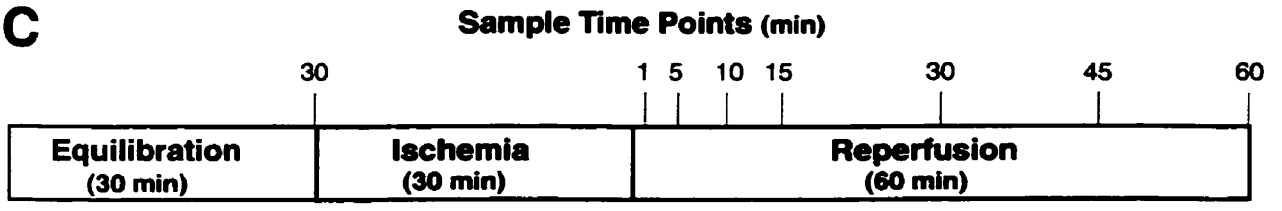
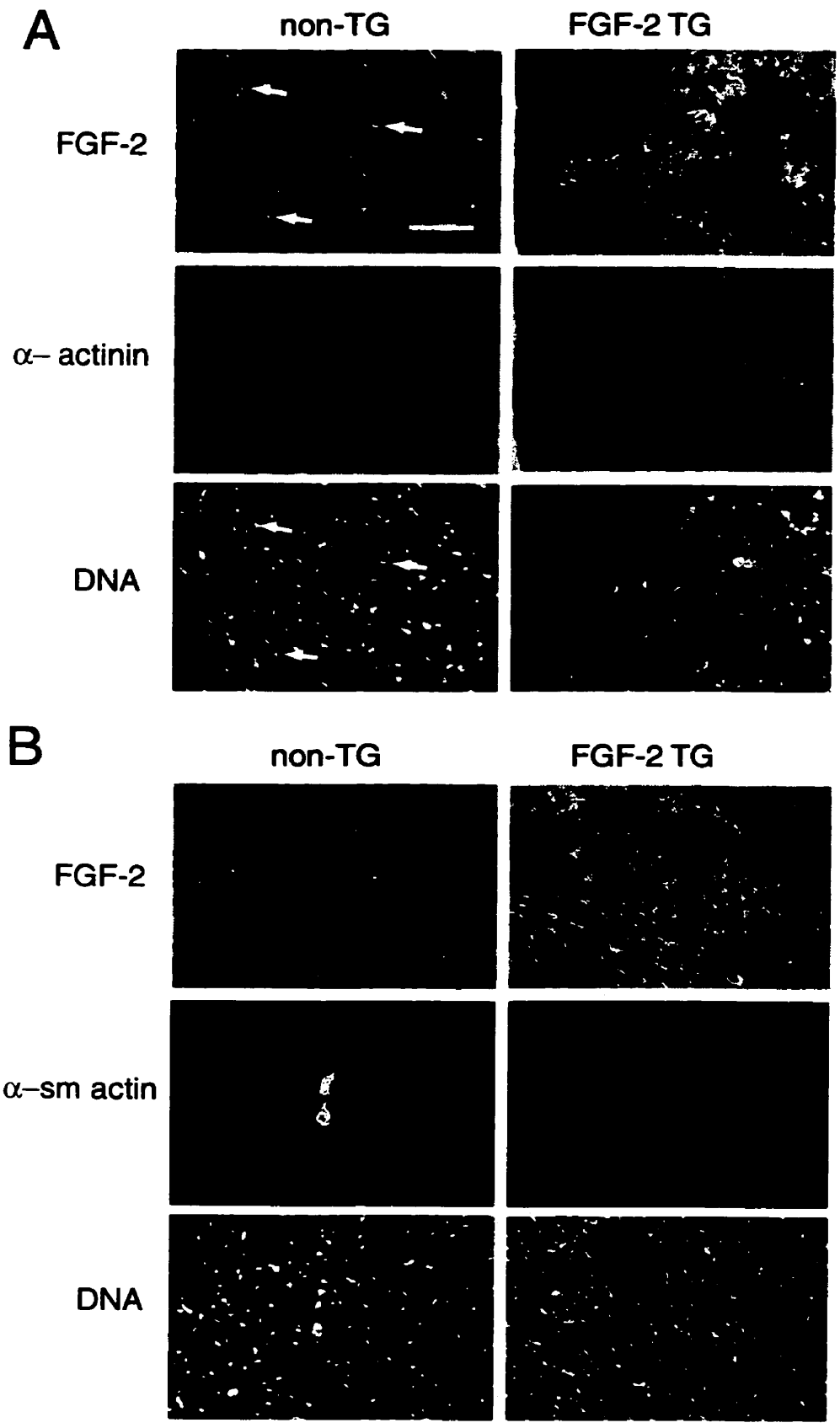

C
Sample Time Points (min)
30
1
5
10
15
30
45
60
Equilibration
(30 min)
Ischemia
(30 min)
Reperfusion
(60 min)

Figure 11.

Subcellular distribution of FGF-2 in isolated non-TG and FGF-2 TG mouse hearts after 30 min equilibration.

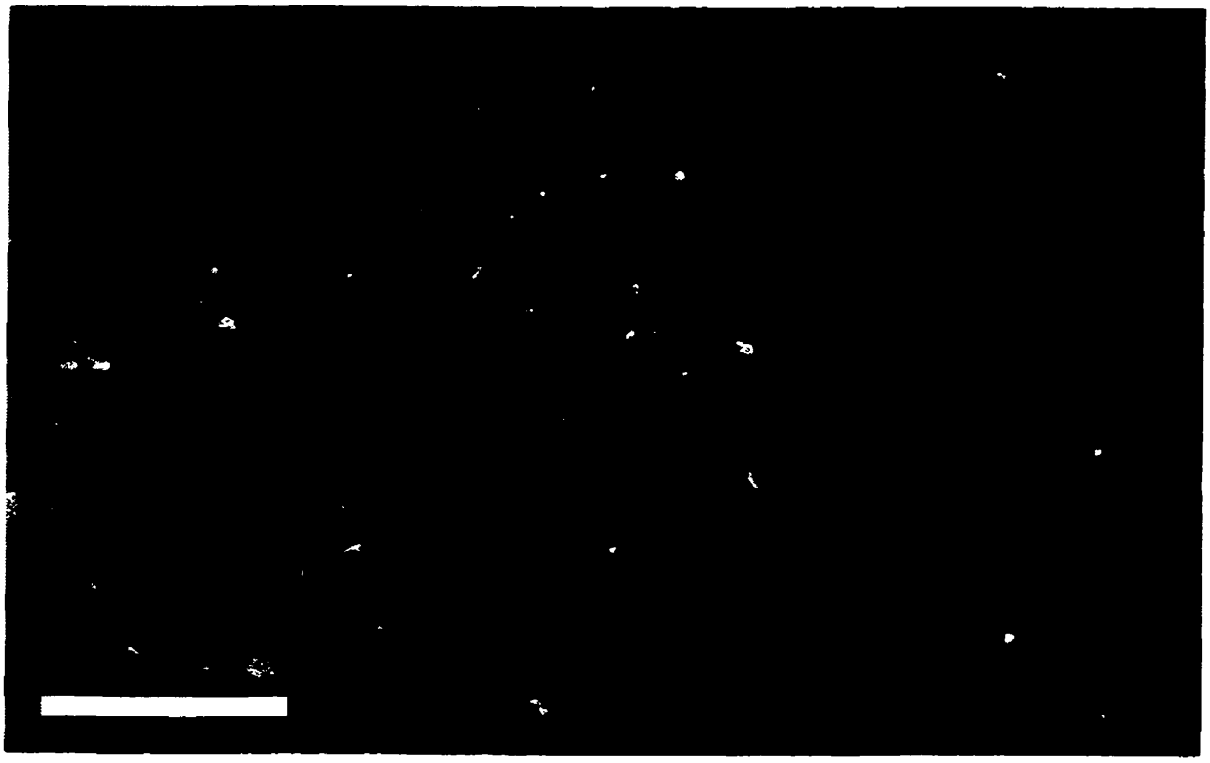
Ventricle sections were triple-stained for **(A)** FGF-2, α-actinin, and DNA or **(B)** FGF-2, α-smooth muscle actin and DNA. Low levels of endogenous FGF-2 staining was observed in nuclei of cardiac myocytes (white arrows, **A**). FGF-2 TG mouse hearts showed strong FGF-2 staining in the cytoplasm and extracellular spaces of cardiac myocytes **(A and B)**. FGF-2 staining of smooth muscle cells was not observed in FGF-2 TG mouse hearts. **(C)** FGF-2 staining of non-TG and FGF-2 TG mouse heart subsequent 30 min equilibration. Bar is equivalent to 75 μm.

This figure is reproduced from Sheikh et al., 2001.

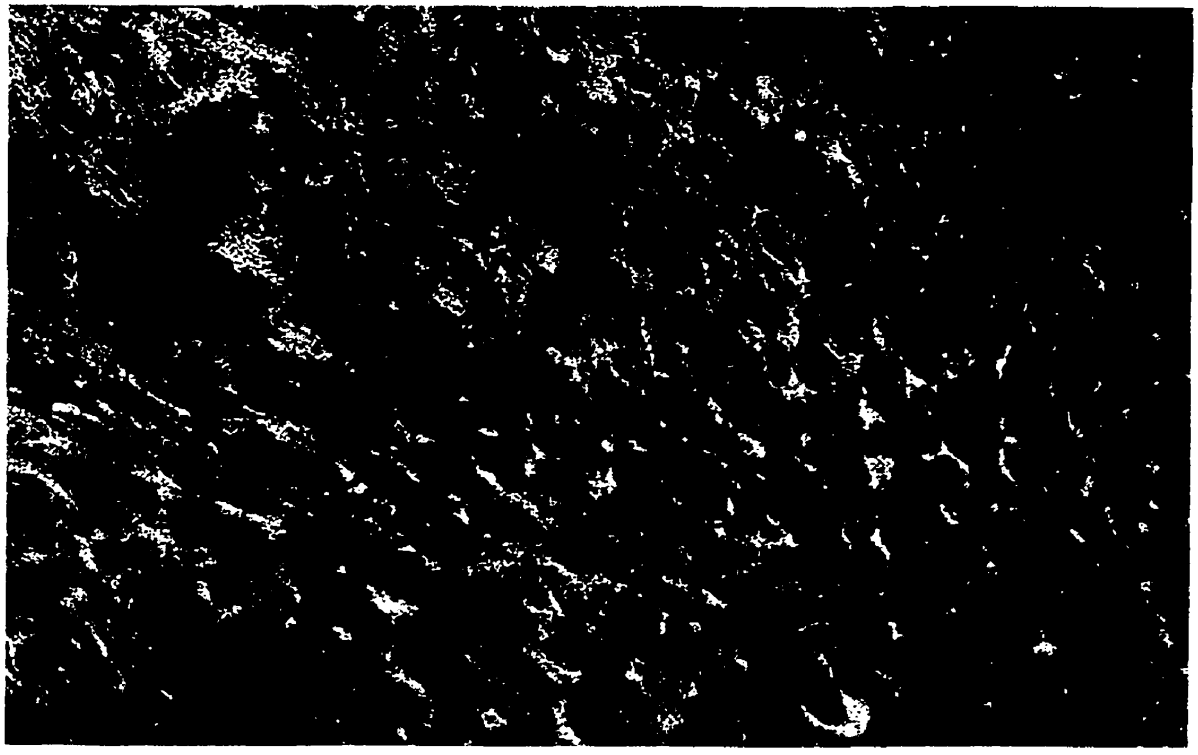

A
non-TG
FGF-2 TG
FGF-2
α– actinin
DNA
B
non-TG
FGF-2 TG
FGF-2
α–sm actin
DNA

C

non-TG



FGF-2 -TG



157B

Figure 12.

Subcellular distribution of FGF-2 in the extracellular matrix in isolated FGF-2 TG mouse hearts after 30 min equilibration and high salt wash.

Ventricle sections were triple stained for FGF-2, collagen IV, and DNA in the absence or presence of 5-min high salt perfusion. Intense FGF-2 staining in the extracelullar spaces of cardiac myocytes was observed. FGF-2 TG hearts perfused with high salt resulted in a loss of FGF-2 staining in areas surrounding cardiac myocytes or extracellular matrix. Examples of nuclei stained for FGF-2 are indicated with arrows. The bar is equivalent to 75 μm.

This figure is reproduced from Sheikh et al., 2001.

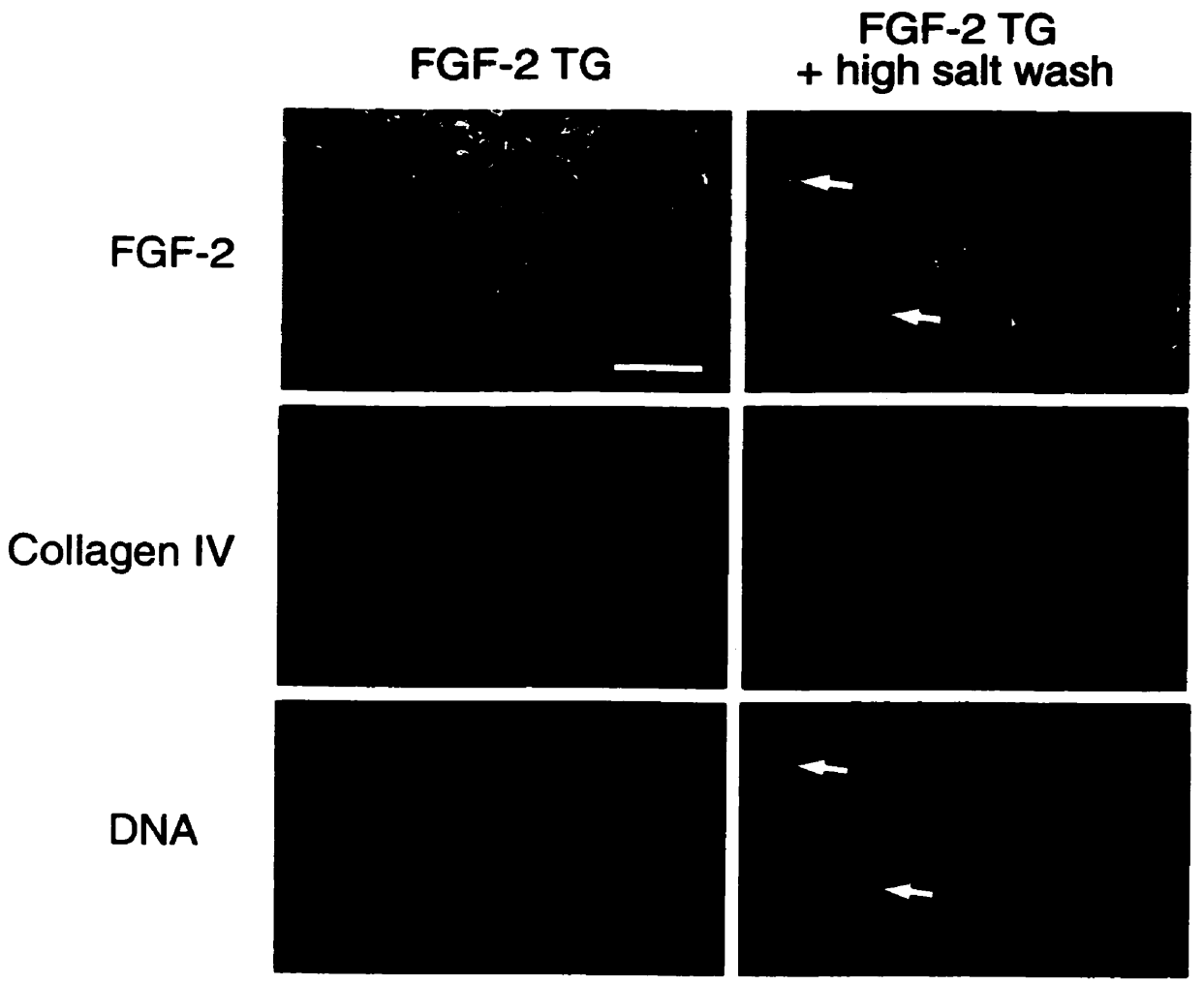
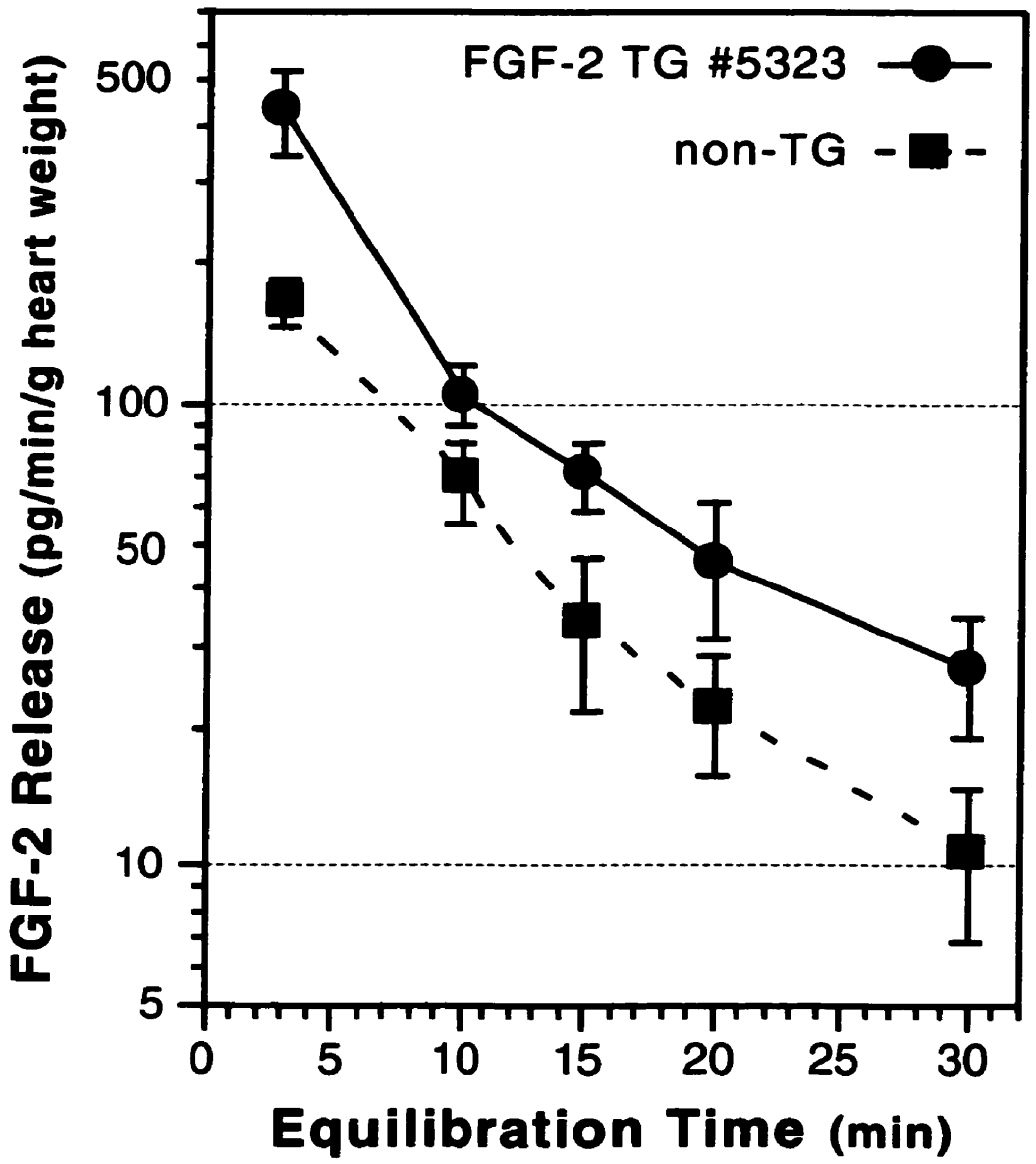

FGF-2 TG
FGF-2 TG
+ high salt wash
FGF-2
Collagen IV
DNA

Figure 13.

Measurement of FGF-2 levels in perfusates from FGF-2 TG mouse hearts during 30 min equilibration.

FGF-2 levels were determined in perfusates from isolated non-TG and FGF-2 TG (line #5323) mouse hearts during equilibration using an ELISA. FGF-2 in perfusates was normalized for coronary flow rate and heart weight (pg/min/g heart wt). The absolute levels were expressed as the mean plus or minus standard error of the mean (n=3).

This figure is reproduced from Sheikh et al., 2001.


FGF-2 TG #5323
non-TG
FGF-2 Release (pg/min/g heart weight)
500
100
50
10
5
0
5
10
15
20
25
30
Equilibration Time (min)

4.4 Ischemia-Reperfusion Injury Studies in Isolated Mouse Hearts

4.4.1 Decreased myocyte damage is observed in FGF-2 TG mouse hearts after injury.

To examine resistance to injury, isolated non-TG and FGF-2 TG mouse hearts from both lines were subjected to global ischemia-reperfusion injury (Fig. 10C). Myocardial performance of both FGF-2 TG and non-TG mouse hearts were measured as percent left ventricular contractile recovery in developed pressure after reperfusion. Absolute values obtained for developed pressures just prior to ischemia were used to represent maximal recovery and arbitrarily set to 100% (see legend of Fig. 14). FGF-2 TG mouse hearts displayed no significant difference in contractile recovery after 30, 45 and 60 min of reperfusion when compared to non-TG hearts (Fig. 14, A and B). In contrast, a significant decrease in perfusate LDH activity and thus, increase in cardiac myocyte viability, was observed at the 30, 45 and 60 min reperfusion time points for the #5318 FGF-2 TG line as well as 1 and 60 min time points for the #5323 line (Fig. 14, C and D). When the total LDH release/activity was assessed throughout 60 min of reperfusion, the decreases (and thus increases in cell viability) were highly significant for both the #5318 (38%, $p<0.0001$, n=28-41) and #5323 (45%,$p<0.0001$, n=28) FGF-2 TG mouse lines.

4.4.2 Exogenous addition of FGF-2 increases contractile function and myocyte viability in the mouse heart.

Exogenous FGF-2 addition increases both contractile recovery and myocyte viability in the isolated rat heart after injury {Padua et al., 1998; Padua et al., 1995b}. Thus, it was possible that the lack of improved contractile recovery in the isolated FGF-2

TG mouse hearts after injury may reflect differences based on: (i) chronic (transgenic) versus acute FGF-2 expression, which could reflect the structural differences observed in FGF-2 TG hearts (ii) endogenous (transgenic) versus exogenous delivery of FGF-2, or (iii) species (mouse versus rat) –related effect. To address these three questions, we determined the effect of exogenous FGF-2 (10 μg) or vehicle on isolated non-TG mouse hearts subjected to global ischemia-reperfusion injury (Fig. 10C). Myocardial performance of both FGF-2 and vehicle-treated hearts was measured as percent left ventricular contractile recovery in developed pressure at 30, 45 and 60 min reperfusion. The absolute values obtained for developed pressure in FGF-2 and vehicle-treated hearts prior to ischemia were 88.6 ± 5.3 (n=4) and 90.4 ± 2.1 mmHg (n=4), respectively. These absolute values were used to represent maximal recovery and arbitrarily set to 100%. The contractile recovery increased from 34.3 ± 3.9, 43.5 ± 4.3, and 38.9 ± 3.3 % with vehicle, to 61.0 ± 1.0, 64.9 ± 2.7, and 62.6 ± 4.6 % (n=4) with FGF-2 treatment, after 30, 45 and 60 min reperfusion, respectively (Fig. 15A).

Cardiac cellular damage in FGF-2 and vehicle-treated hearts was assessed by measuring LDH release in perfusates at various time points during ischemia-reperfusion (Fig. 10C). Mouse hearts treated with FGF-2 showed a significant 34% reduction in LDH release at 15, 30, 45 and 60 min of reperfusion when compared to vehicle-treated hearts (Fig. 15B).

Immunofluorescence microscopy was used to visualize FGF-2 protein in ventricular tissue sections from non-TG mouse hearts treated with exogenous FGF-2

(Fig. 16). FGF-2 was localized to the nuclei and cytoplasm of cardiac myocytes as well as extracellular spaces (Fig. 16). Unlike FGF-2 TG hearts (Fig. 11), intense FGF-2 staining of smooth muscle cells/blood vessels was observed (Fig. 16).

Figure 14.

Effect of FGF-2 transgene expression on myocardial performance and cellular damage after ischemia-reperfusion injury.

(A and B) Developed pressure for non-TG and FGF-2 TG hearts during reperfusion were expressed as percentage of the corresponding values of the same heart obtained before ischemia. The absolute values obtained for developed pressure after 30 min (prior to ischemia) in FGF-2 TG (line #5323) and age-matched non-TG hearts were 77.1 ● 4.9 (n=4), and 78.6 ● 6.7 (n=4), respectively **(A)**, and for FGF-2 TG (line #5318) and age-matched non TG hearts were 75.4 ● 2.3 (n=4) and 82.8 ● 3.5 mmHg (n=6), respectively **(B)**. These absolute values were used to represent maximal recovery and arbitrarily set to 100%.

(C and D) LDH levels in perfusates from non-TG and FGF-2 TG mouse hearts were measured before ischemia (equil.) and during reperfusion. All values were presented as mean plus or minus standard error mean (n=4-6). Asterisks were used to indicate points at which a statistically significant reduction in LDH levels was observed with FGF-2 TG versus non-TG hearts (* $p<0.05$).

This figure is reproduced from Sheikh et al., 2001.

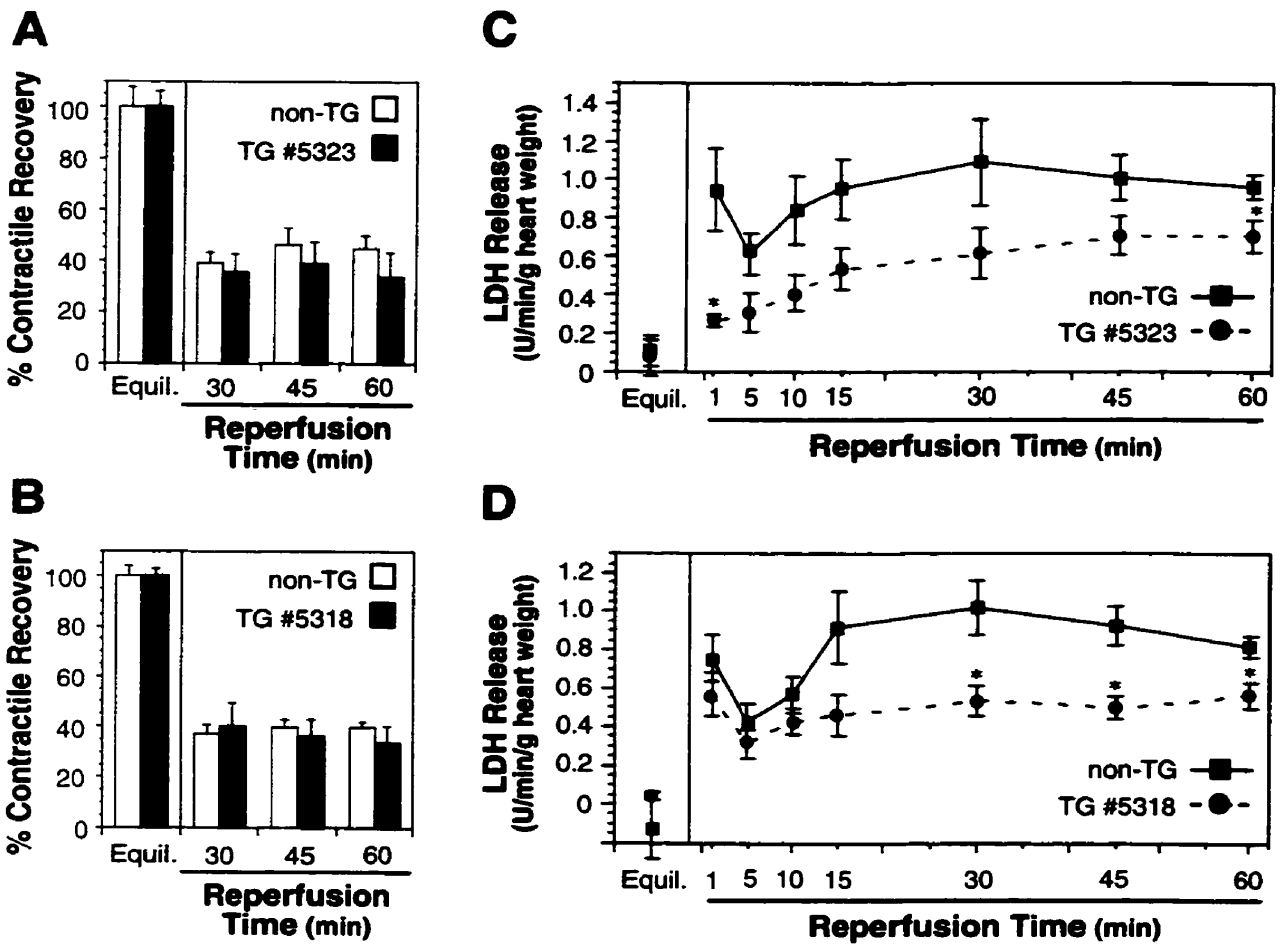

A
% Contractile Recovery
100
80
60
40
20
0
non-TG
TG #5323
Equil.
30
45
60
Reperfusion Time (min)
B
% Contractile Recovery
100
80
60
40
20
0
non-TG
TG #5318
Equil.
30
45
60
Reperfusion Time (min)
C
LDH Release (U/min/g heart weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
non-TG
TG #5323
Equil.
1
5
10
15
30
45
60
Reperfusion Time (min)
D
LDH Release (U/min/g heart weight)
1.2
1.0
0.8
0.6
0.4
0.2
0
non-TG
TG #5318
Equil.
1
5
10
15
30
45
60
Reperfusion Time (min)

Figure 15.

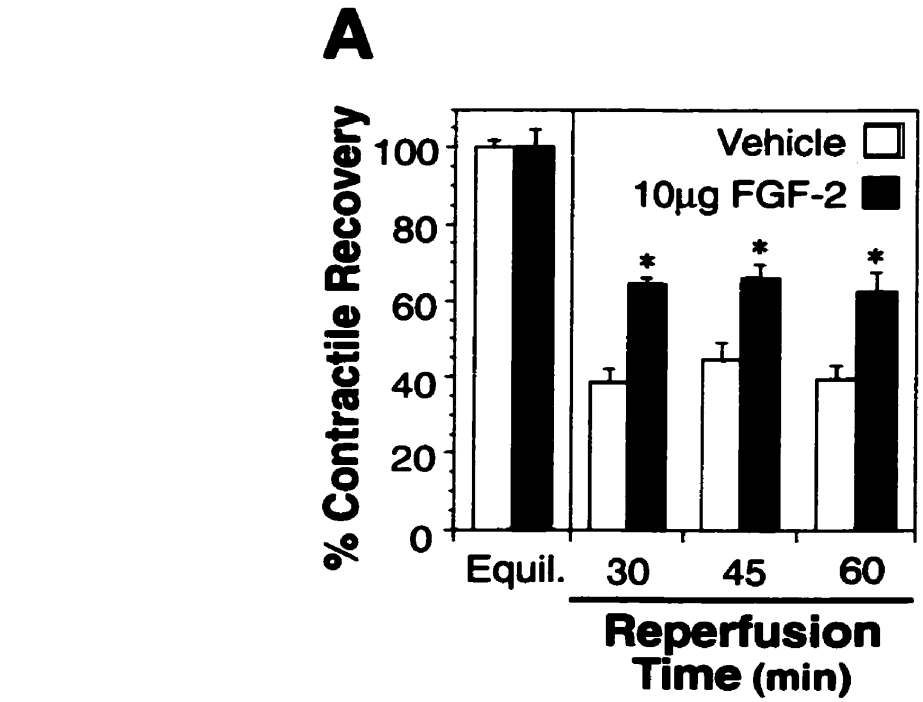
Effect of exogenous addition of FGF-2 in mouse hearts on contractile recovery and cellular damage after ischemia-reperfusion injury.

(A) Functional measurement as defined as developed pressure for each heart after either vehicle or FGF-2 treatment at time points during reperfusion. Values were expressed as percentages of the corresponding values of the same heart obtained before ischemia (equil.). The absolute values measured for developed pressure after 30 min equilibration (prior to ischemia) in vehicle and FGF-2 treated hearts were 90.4 ± 2.1 (n=4) and 88.6 ● 5.3 mmHg (n=4), respectively.

(B) LDH levels in perfusates from vehicle and FGF-2 treated mouse hearts before ischemia and during reperfusion.

All values were presented as mean plus or minus standard error of the mean (n=4). Asterisks in panels indicate statistically significant differences between FGF-2-treated hearts compared to vehicle-treated values (*$p<0.05$, **$p<0.01$).

This figure is reproduced from Sheikh et al., 2001.


A
% Contractile Recovery
100
80
60
40
20
0
Vehicle
10μg FGF-2
*
*
*
Equil.
30
45
60
Reperfusion
Time (min)

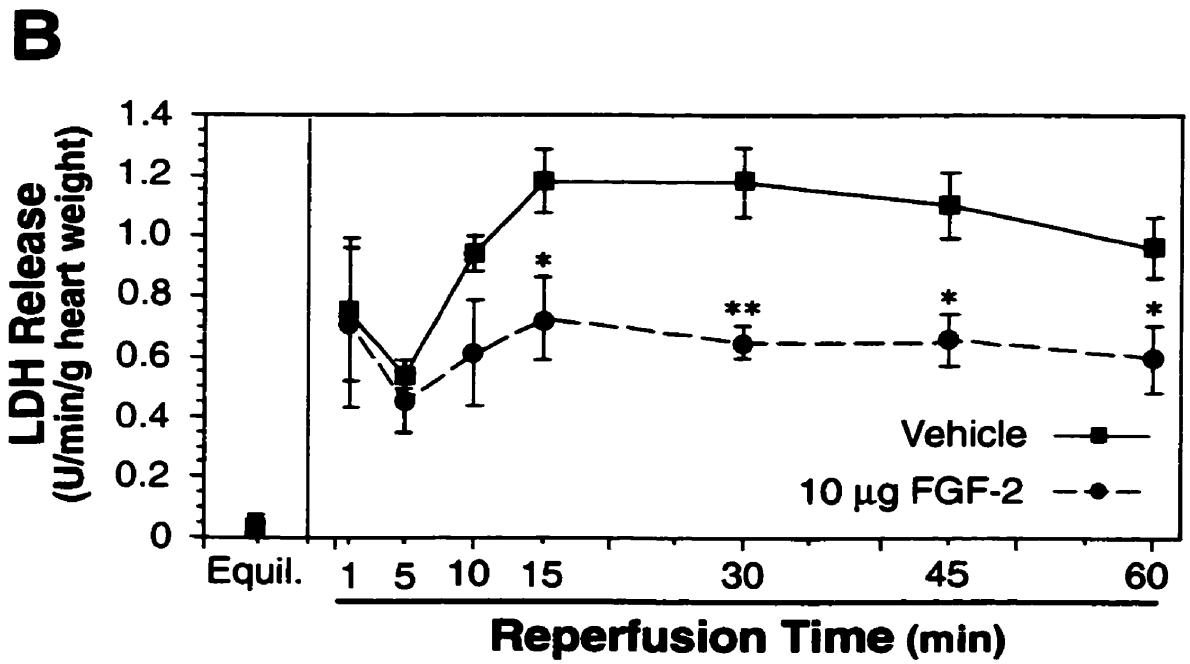

B
LDH Release
(U/min/g heart weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
*
*
Vehicle
10 μg FGF-2
Equil.
1
5
10
15
30
45
60
Reperfusion Time (min)

Figure 16.

Subcellular distribution of FGF-2 in isolated FGF-2 treated mouse hearts after 30 min equilibration.

Ventricle sections were triple-stained for **(A)** FGF-2, α-actinin and DNA or **(B)** FGF-2, α-smooth muscle actin and DNA. Intense FGF-2 staining in the cytoplasm and extracellular spaces of cardiac myocytes **(A and B)**, as well as smooth muscle cells was observed (white arrowheads, **B**). Bar is equivalent to 75 μm.

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A

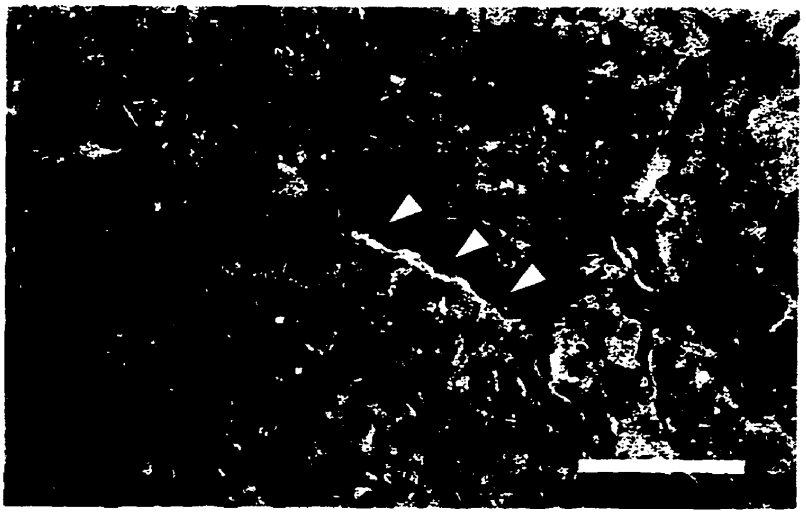
FGF-2

α– actinin

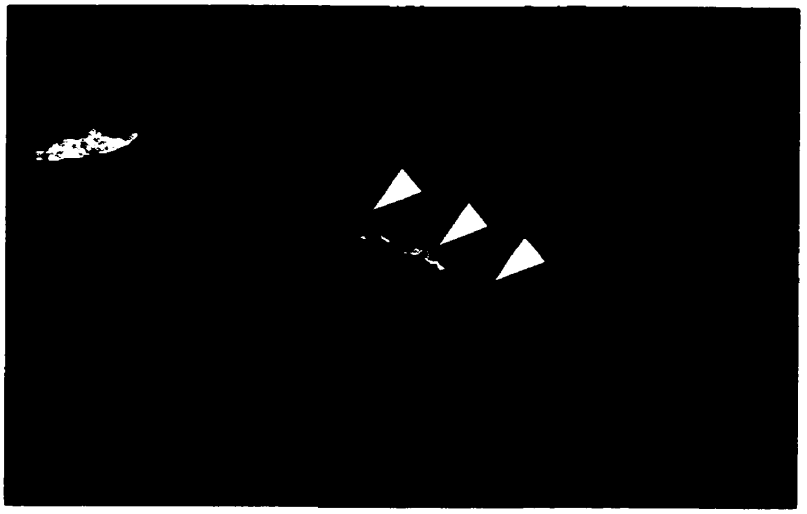
DNA

B

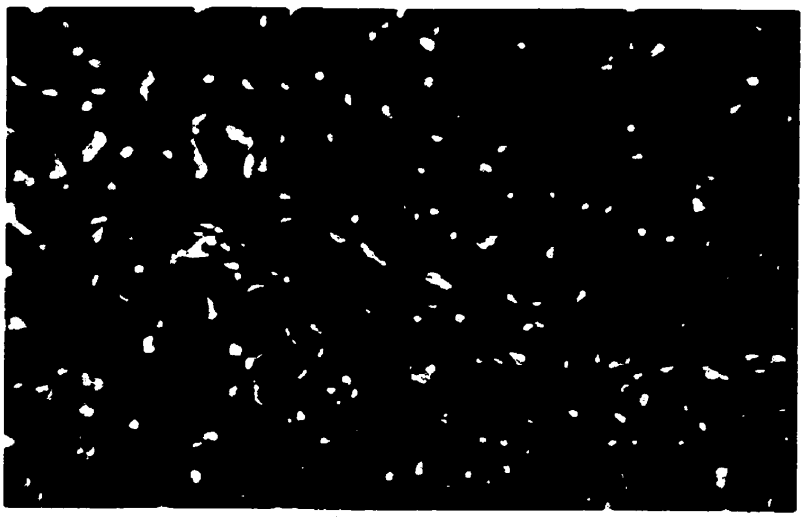
FGF-2



α–sm actin



DNA



CHAPTER 5

RESULTS: Isolation and Culturing of Adult Mouse Cardiac Myocytes for Studies in the -1058FGFp.*luc* Transgenic Mouse Model.

5.1 Viable Adult Cardiac Myocytes can be Isolated and Cultured From Mouse Hearts.

To date, several methods have been used to isolate adult cardiac myocytes from mice, however, there are few, which involve either short or long term culture of these cells. As a result, the method described in this chapter was assembled using modifications of two protocols which relate to methods reported for isolation of adult cardiac myocytes from mouse hearts {Maxwell et al., 1999} and culturing techniques reported for adult cardiac myocytes in the rat heart {Padua et al., 1998}. This method was assembled largely under the guidance of Dr. John Scott (laboratory of Dr. L. Hryshko) and Dr. Pierre Merle (laboratory of Dr. E. Kardami) of the Institute of Cardiovascular Sciences at the University of Manitoba, as well as Dr. Bill Lester (laboratory of Dr. E. Kranias) of the University of Cinncinatti, with additional information from the existing literature.

Adult mice (8-15 weeks of age, 25-35 g in weight) were euthanized by cervical dislocation and their hearts were quickly excised and submerged into ice-cold nominally calcium-free Tyrode solution (137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 5 mM HEPES, 10 mM glucose, filtered, oxygenated using 100% O_2 and buffered to pH 7.4 at 37 °C using NaOH). Injectable heparin was not used as an anticoagulant prior to dissection of the heart, since future isolations would involve the use of mice expressing the heparin-binding growth factor, FGF-2. While in this Tyrode solution, the aorta was trimmed and freed of surrounding tissue under a magnifying lens.

Subsequently, under a magnifying lens, the aorta was tied with 6-0 suture thread to a 21 gauge stainless steel cannula which was mounted on a Langendörff perfusion apparatus. Time for excision and mounting of the heart onto Langendörff perfusion apparatus is critical to the success of the isolation procedure and should take less than five minutes for viable adult cardiac myocytes. Prior to mounting, care should have been taken to make sure that there were no air bubbles trapped in the cannula or perfusion solution chambers. Once mounted, the heart is then retrogradely perfused under constant flow rate (2 ml/min) using nominally calcium-free Tyrode solution for five minutes for blood wash out and equilibration. Perfusion with this Tyrode solution resulted in cessation of the heartbeat. During this perfusion period, (i) the heart was gently massaged to remove blood as the heart was no longer beating, (ii) a thermocouple (IT-1E), connected to a Physitemp Thermalert TH-5, was inserted into the right ventricle to monitor temperature and (iii) a water-jacketed glass chamber was raised to enclose the heart, allowing the temperature of the heart to be maintained at 37 °C by means of a recirculating water bath. All perfusion solutions were made using double distilled water (specific resistance was $\geq$ 18 ΩM•cm (25 °C)) from Mega-pure water system, filtered, buffered to pH 7.4, maintained at 37 °C and oxygenated with 100% O_2. Following this initial five minute perfusion, the heart was perfused under constant flow (2 ml/min) with the same nominally calcium-free Tyrode solution, supplemented with 1.25 mg/ml (w/v) collagenase with an enzymatic activity of 226 U/ml (Sigma, type I), 0.07 mg/ml (w/v) protease (Sigma-Aldrich, type XIV) and 0.94 mg/ml (w/v) fatty acid-free BSA (Sigma Aldrich). A total of 32 ml of this solution is sufficient to perfuse the heart in our system for approximately 16 min (i.e., 2 ml/min.). Since enzyme digestion times can vary from

heart to heart, the perfusate falling from the heart was collected from the sealed bottom of the water-jacketed chamber enclosing the heart, to be recirculated in the event that a longer perfusion time was needed.

The enzymatic digestion period was critical in terms of attaining viable adult mouse cardiac myocytes and is mainly dependent on heart perfusion (i.e., cannulation procedure and clots) and enzyme activity. Perfusion of mouse hearts with enzyme solutions varied between 18-22 min. The enzyme activity can be followed based on heart appearance, solution drip rate and shape of drops falling from the heart. In terms of heart appearance, enzymatic digestion caused the right ventricle to swell or balloon within 5 minutes of perfusion, turned heart tissue from a dark red to a light red/tan colour and finally caused the heart to become flaccid (can be touched with fingers to assess this parameter) and acquire a drop-like shape. In terms of solution drip rate, enzymatic digestion caused the drip rate to slightly decrease at the beginning of digestion and then readily increase as digestion continued. In terms of shape of drops, at the end of digestion, the drops became more oval-shaped. The end of digestion was usually determined based on the flaccid and drop-like appearance of the heart as well as the colour (light red/tan) of the heart.

Once the heart was digested, it was removed from the Langendörff perfusion apparatus and submerged into a solution containing the same nominally calcium-free Tyrode solution but supplemented with 50 μM calcium and 1% (w/v) albuMAX (Gibco-BRL), which was in a sterile glass petri dish and kept at 37 °C. Under sterile conditions

(i.e., laminar flow hood), the heart was removed of atria and the aorta, and subsequently teased apart with forceps. Complete heart tissue digestion can be determined at this stage, by (i) the colour of tissue inside the heart (i.e., dark red signified "underperfused" areas and white areas signified "overdigested" areas) and (ii) what was known as the "pillow effect". The "pillow effect" referred to an analogy, which could be used to help visualize the effect of tearing the heart open, right after digestion. In this analogy, the dissociated cardiac myocytes would be the equivalent of the "feathers" in a pillow and the outer part of the heart or epicardium would be the equivalent of the "pillow-case". Since complete tissue perfusion would result in complete dissociation of cardiac myocytes, tearing open the heart tissue with forceps, would resemble ripping a feathered pillow open. This effect was only visualized when there was complete heart tissue digestion. The adult cardiac myocytes were then completely dissociated by further trituration using a sterile transfer pipette, filtered using a 250 μM polypropylene mesh (Small Parts, Florida, USA), pelleted using low speed centrifugation (400 g) for 4 min at room temperature, and the supernatant was then disgarded. At this point, the pellet should be large (at least between 2-5 ml volume in a 50 ml orange-capped tube) and dark red in colour. Next, cardiac myocytes were brought to calcium tolerance by resuspending these cells in three solutions, which had incremental increases in levels of calcium. The three solutions were made using nominally calcium-free Tyrode solution and supplemented with either: (i) 200 μM calcium with 1% albumax, (ii) 500 μM calcium with 1% albumax or (iii) 1 mM calcium with 4% albumax. These solutions were kept at 37 °C. It was critical that time (ranged from 5-10 minutes) be taken between the additions of calcium since this allowed adult cardiac myocytes time to adapt to the increase in calcium levels. This was essential

since calcium overload could play a major role in limiting the success of this procedure. Also, since adult cardiac myocytes, at this stage, are prone to damage, the cells were pelleted between each calcium increment under gravity, as opposed to low speed centrifugation, as this allowed for less mechanical manipulation of the cells. Again, pelleting of cells occurred at 37 °C. Once cardiac myocytes were brought to calcium tolerance, they were resuspended in 10 ml of culture medium M 199 (Gibco-BRL), supplemented with 10% (v/v) FBS, 2 mM (w/v) DL-carnitine (Sigma), 5 mM (w/v) creatinine (Sigma-Aldrich), 5 mM (w/v) taurine (Sigma-Aldrich), and antibiotics (1000 units/ml penicillin, 1 mg/ml streptomycin).

To determine the viability of adult cardiac myocytes using this isolation procedure, both cardiac myocyte shape (i.e, microscopic assessment) and cardiac myocyte membrane integrity (i.e., trypan blue exclusion assay) were assessed. As shown in Fig. 17, this procedure resulted in the isolation of rectangular rod-shaped adult cardiac myocytes. To assess adult cardiac myocyte membrane integrity, a 20 µl aliquot of the adult cardiac myocyte resuspension was added to 20 µl of 0.4% trypan blue (Gibco-BRL), mixed and 10 µl of this mixture was assessed for trypan exclusion using a hemacytometer. Approximately 70 ± 7 % (n=6) of rod-shaped adult cardiac myocytes were shown to exclude trypan blue. Based on this assessement, 4.1 ± 0.4) x 10^5 (n=6) adult cardiac myocytes could be isolated per mouse heart.

Once adult cardiac mouse myocytes were counted, approximately 5 x 10^4 cells were plated onto 35 mm dishes (Corning) which had been pre-coated with laminin

(Becton-Dickinson). Laminin was diluted in 10% FBS-M 199 medium and incubated on plates for 24 h at 37 °C prior to adding cells. Adult cardiac mouse myocytes were plated for 4-6 hours to allow for cell attachment and then washed twice with and maintained in M 199 medium supplemented with 2% FBS, 2 mM (w/v) carnitine, 5 mM (w/v) creatinine, 5 mM (w/v) taurine, and antibiotics (1000 units/ml penicillin, 1 mg/ml streptomycin) in culture at 37 °C in the presence of 5% CO_2. For maintenance, the M199 maintenance medium was changed daily. Observation by light microscopy after one day in culture revealed that striated adult cardiac myocytes retained their rectangular rod-like shape (Fig. 17). Only rod-shaped striated adult cardiac myocytes could be observed on plates through assessment by light microscopy, suggesting that this preparation was pure for myocytes. Adult mouse cardiac myocytes were maintained in culture for up to three days. After three days, however, adult mouse cardiac myocytes began to lose their rod-like shape appearance, detach from the plate and undergo hypercontracture, which resulted in cardiac myocyte death.

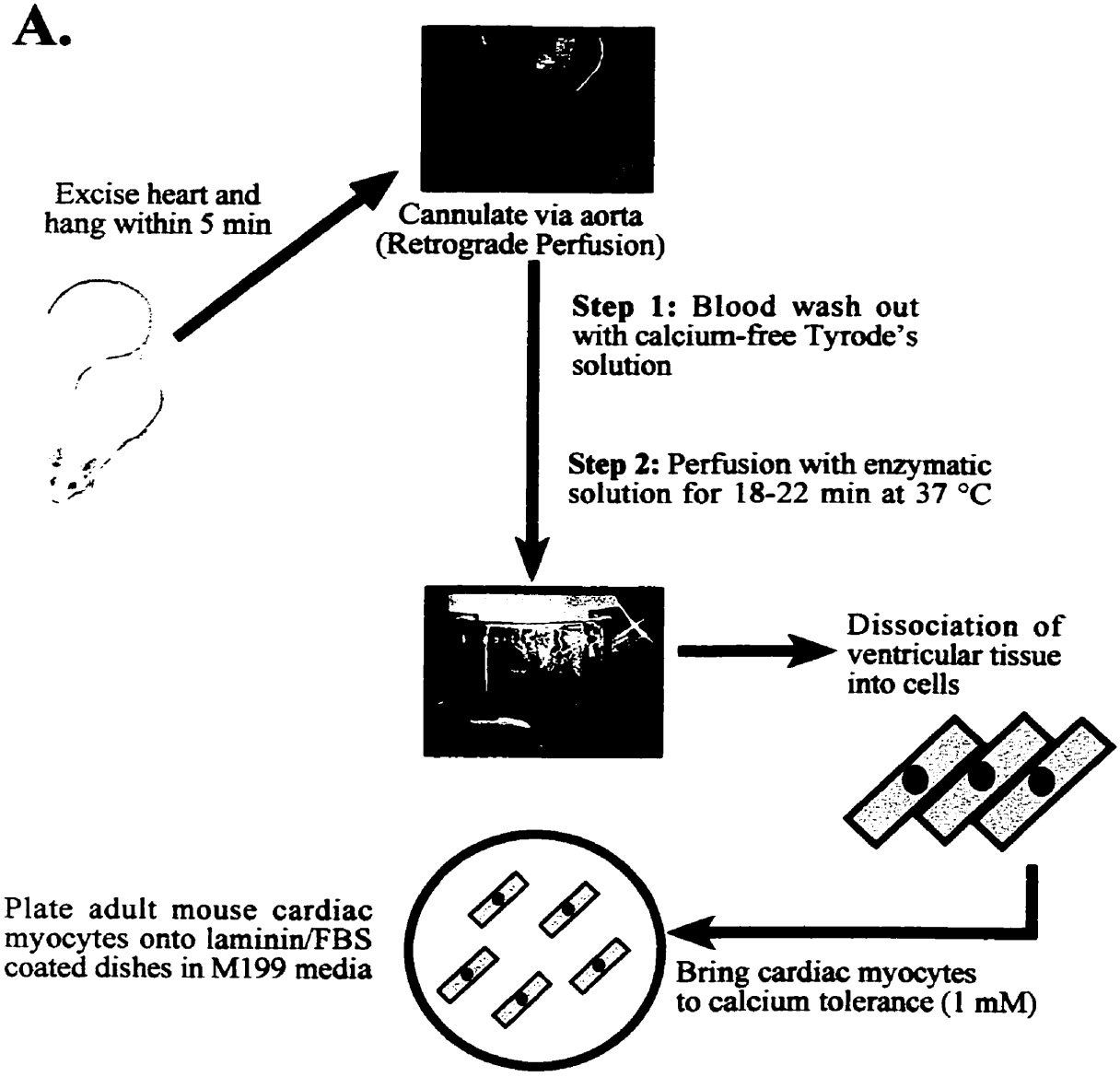
Figure 17.

Cell morphology of adult cardiac myocytes isolated and cultured from the mouse heart.

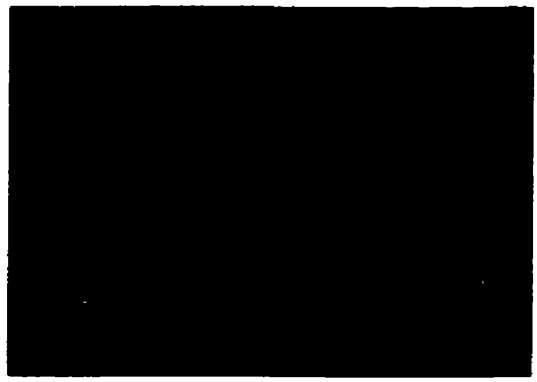
(A) Synopsis of the procedure involved in the isolation and short-term culture of adult mouse cardiac myocytes, which briefly include: **(a)** photograph of mounted heart onto Langendörff apparatus, **(b)** initial heart perfusion for blood wash out, **(c)** perfusion of heart with enzymatic solution, **(d)** heart tissue dissociation to cardiac myocytes, **(e)** bringing adult cardiac myocytes to calcium tolerance, and **(f)** culturing and maintaining cells on laminin-coated dishes.

(B) Light micrograph of an isolated adult cardiac myocyte after one day in culture. Bar is equivalent to 25 μM.

A.


Excise heart and hang within 5 min
Cannulate via aorta (Retrograde Perfusion)
Step 1: Blood wash out with calcium-free Tyrode's solution
Step 2: Perfusion with enzymatic solution for 18-22 min at 37 °C
Dissociation of ventricular tissue into cells
Bring cardiac myocytes to calcium tolerance (1 mM)
Plate adult mouse cardiac myocytes onto laminin/FBS coated dishes in M199 media

B.



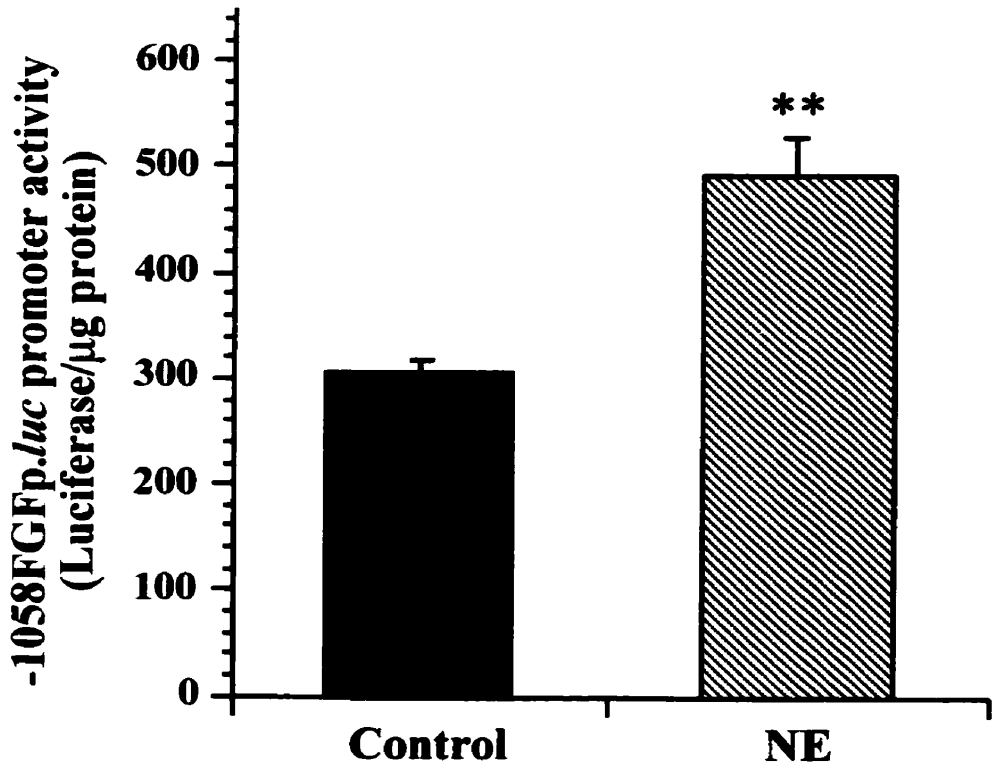
5.2 *Norepinephrine Stimulates FGF-2 Promoter Activity in Adult MouseCardiac Myocytes Isolated From -1058FGFp.luc Transgenic Mice.*

One of the major reasons for the generation of adult mouse cardiac myocyte cultures was to make use of the increasing number of transgenic mouse models which are being developed to address specific questions in the area of heart research. As an example, we used our procedure to obtain adult cardiac mouse myocyte cultures from the −1058FGFp.*luc* transgenic model which was developed in our laboratory to address specific questions relating to the role of FGF-2 transcription in the heart {Detillieux et al., 1999}. Viable adult cardiac myocytes were isolated from -1058FGFp.*luc* mice (P300 line), plated for 4 h, maintained in culture for 12 h and then treated without (control) or with the natural catecholamine, norepinephrine for 2 h to assess its effect on FGF-2 promoter (-1058FGFp.*luc*) activity. The results are shown in Fig. 18. The −1058FGFp.luc activity (expressed per μg protein) was detected in control adult mouse cardiac myocyte cultures and norepinephrine evoked a significant 1.6-fold increase in −1058FGFp.*luc* activity after 2 h of stimulation, when compared to control cultures ($n=5$; $p<0.05$). No differences in viability were observed between adult cardiac myocytes isolated from −1058FGFp.*luc* versus normal mice.

Figure 18.

Effect of norepinephrine on FGF-2 promoter activity in adult cardiac myocytes cultured from –1058FGFp.*luc* transgenic mice.

Cultured adult cardiac myocytes from –1058FGFp.*luc* transgenic mice (P300 line) were treated without or with 0.01 mM NE as previously described {Iwaki et al., 1990; Lockhart et al., 1997}, harvested and assayed for luciferase activity and protein concentration. The promoter activities (luciferase/μg protein) for the –1058FGFp.*luc* gene are shown as the mean from five determinations. Bars represent standard error of the mean. * *= significant at $p<0.01$.


-1058FGFp.luc promoter activity
(Luciferase/µg protein)
600
500
400
300
200
100
0
**
Control
NE

CHAPTER 6

RESULTS: The Effects of Fibroblast Growth Factor Receptor (FGFR)-1 Overexpression on Cardiac Cell Proliferation *In Vitro.*

6.1 FGFR-1 Isoform Expression Studies in Rat Heart H9c2 Myoblasts.

6.1.1 H9c2 cells are deficient in FGFR-1 mRNA.

Previous studies from our laboratory have shown that no RNA corresponding to either long or short species of FGFR-1 could be detected by RT-PCR in H9c2 cells {Sheikh et al., 1997}. Based on this result, the H9c2 cell line was determined to be an excellent candidate cell system to attempt expression of FGFR-1 and assess effects on cell division. The results of these experiments are described in the following sections.

6.1.2 Characterization of H9c2 cells stably transfected with FGFR-1 cDNAs.

Both cDNAs coding for long and short isoforms of FGFR-1, under the control of MLC-2 promoter and SV40 enhancer sequences, were used to stably transfect H9c2 cells {Sheikh et al., 1997}. The SV40enh/MLC-2 promoter was used to express FGFR-1 isoforms since studies in our laboratory have shown that this promoter is active in H9c2 cells {Jin et al., 1995}. Stable integration was accomplished by co-transfection with a neomycin resistance-containing vector and G418 selection. Genomic DNA was isolated from transfected and non-transfected H9c2 cells, and digested with the restriction endonucleases *Xba*1/*Sal*1 to release the diagnostic fragments of 2,526 bp and 2,259 bp for 'long' and 'short' FGFR-1 RNA, respectively. Electrophoresis and DNA blotting using the full-length FGFR-1 (S) cDNA as a radiolabeled probe and subsequent autoradiography was used to detect the diagnostic fragments (Fig. 19A). Control

fragments of the correct size were generated by *Xba*1/*Sal*1 digestion of plasmids containing the cDNAs coding for the long and short FGFR-1 isoforms (Fig. 19, lanes a and b). Bands of the expected size were detected in the DNA from stably transfected cells (Fig. 19A, lane d and e), but were absent from the non-transfected cells (Fig. 19A, lane c). However, bands greater than 2.5 kb were detected in all H9c2 DNA samples and, presumably, represent fragments containing endogenous FGFR-1, or FGFR-1-like genes (Fig. 19A, lanes c-e).

RNA blotting was used to assess whether the stably transfected FGFR-1 cDNAs were expressed and the MLC-2 promoter was active (Fig. 19B). A transcript of the expected size of about 4.3 kb was detected with the FGFR-1 cDNA probe in RNA from H9c2 cells stably transfected with the 'long' FGFR-1 cDNA. A band with slightly faster mobility was detected in cells stably transfected with the cDNA coding for the short FGFR-1 isoform. As expected, no transcript was detected in non-transfected cell RNA.

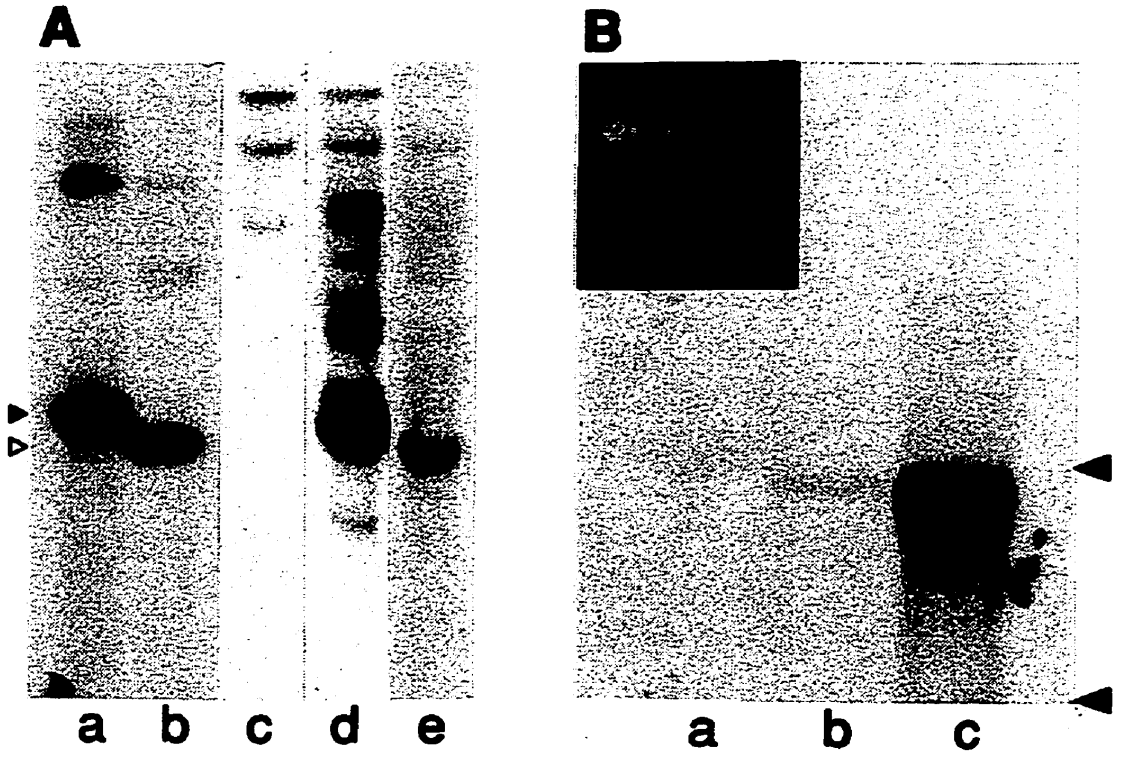
Figure 19.

Detection of 'long' and 'short' FGFR-1 isoforms in stably transfected H9c2 cells by DNA and RNA blotting.

(A) DNA was isolated from plasmids (pBSmFGFR(L) and pBSmFGFR(S)) containing the **(a)** FGFR-1 (L) and **(b)** FGFR-1 (S) cDNAs, as well as **(c)** H9c2, **(d)** H9c2[Long] and **(e)** H9c2[Short] cells. The DNA was cut with *Xba*1/*Sal*1 to release the diagnostic fragments of 2,526 bp and 2,259 bp for the 'long' (closed arrowhead) and 'short' (open arrowhead), respectively. The DNA fragments were resolved in a 1.5% agarose gel, transferred to nitrocellulose, probed with radiolabelled FGFR-1(S) cDNA and visualized by autoradiography.

(B) Expression of FGFR-1 RNA in **(a)** H9c2, **(b)** H9c2[Long] and **(c)** H9c2[Short] cells. Total RNA (70 μg) was resolved in a 1.5% denaturing agarose-formaldehyde gel, blotted to nitrocellulose, probed with radiolabelled FGFR-1(S) cDNA and visualized by autoradiography. The arrowheads are used to indicate the mobilities of 28S and 18S RNAs. The inset shows the 28S and 18S RNA bands for each of the three lanes **(a-c)** as visualized by ethidium bromide staining before blotting, as an indication of RNA loading.

This figure is reproduced from Sheikh et al., 1997.


A
B
a b c d e
a b c

*6.1.3 **FGFR-1 cDNA overexpression increases H9c2 cell proliferation.***

H9c2 cells stably transfected with the cDNA coding for the long (H9c2[Long]) or short FGFR-1 isoforms (H9c2[Short]) as well as non-transfected H9c2 cells were plated at equal densities in the presence of 10% FBS. A growth curve was determined by counting cells at 24 h interval for 5 d, and the results from two separate experiments, each done in triplicate, are shown (Fig. 20). The rate of cell division for H9c2[Long] and H9c2[Short] cells was increased 1.6 and 3.1 fold, respectively, compared to non-transfected H9c2 cells. Furthermore, a significant increase in cell number was observed in the first 24 h of assessment in the cells expressing FGFR-1 but not in control H9c2 cells (Fig. 20).

After 5 d in culture, transfected and non-transfected H9c2 cells were treated with or without 10 ng/ml FGF-2 and cell number was determined 24 h later (Fig. 21). There was no effect on the cell number of non-transfected H9c2 cells. There was a slight but significant increase in H9c2 [Long] (1.3 fold, $p<0.02$, $n=6$) cell number in response to FGF-2 treatment. A more potent effect on proliferation was observed in H9c2[Short] cells (1.5 fold, $p<0.0005$, $n=6$).

Figure 20.

Comparison of H9c2, H9c2[Long] and H9c2[Short] growth characteristics.

Cells were plated at equal density in the presence of 10% FBS and the cell number was counted using a hemacytometer at 24 h intervals for 5 days. Each point represents the mean of six determinations from two independent experiments. The bars are standard error of the mean.

This figure is reproduced from Sheikh et al., 1997.

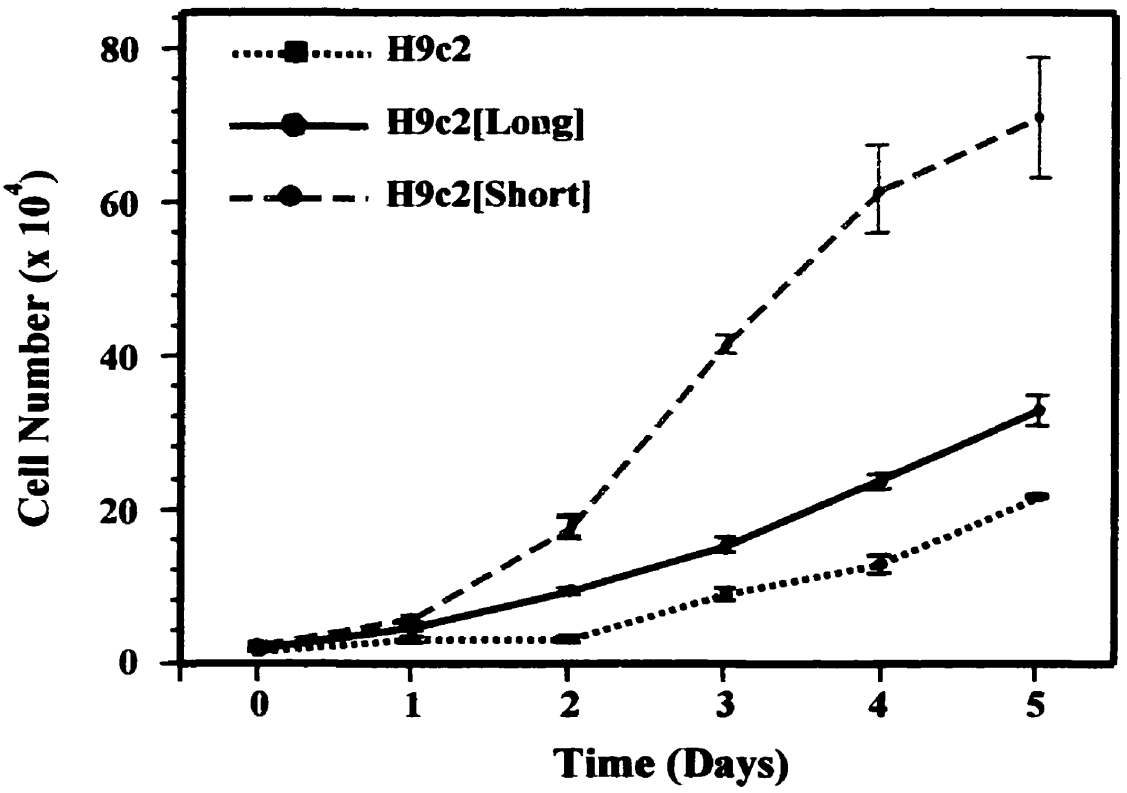
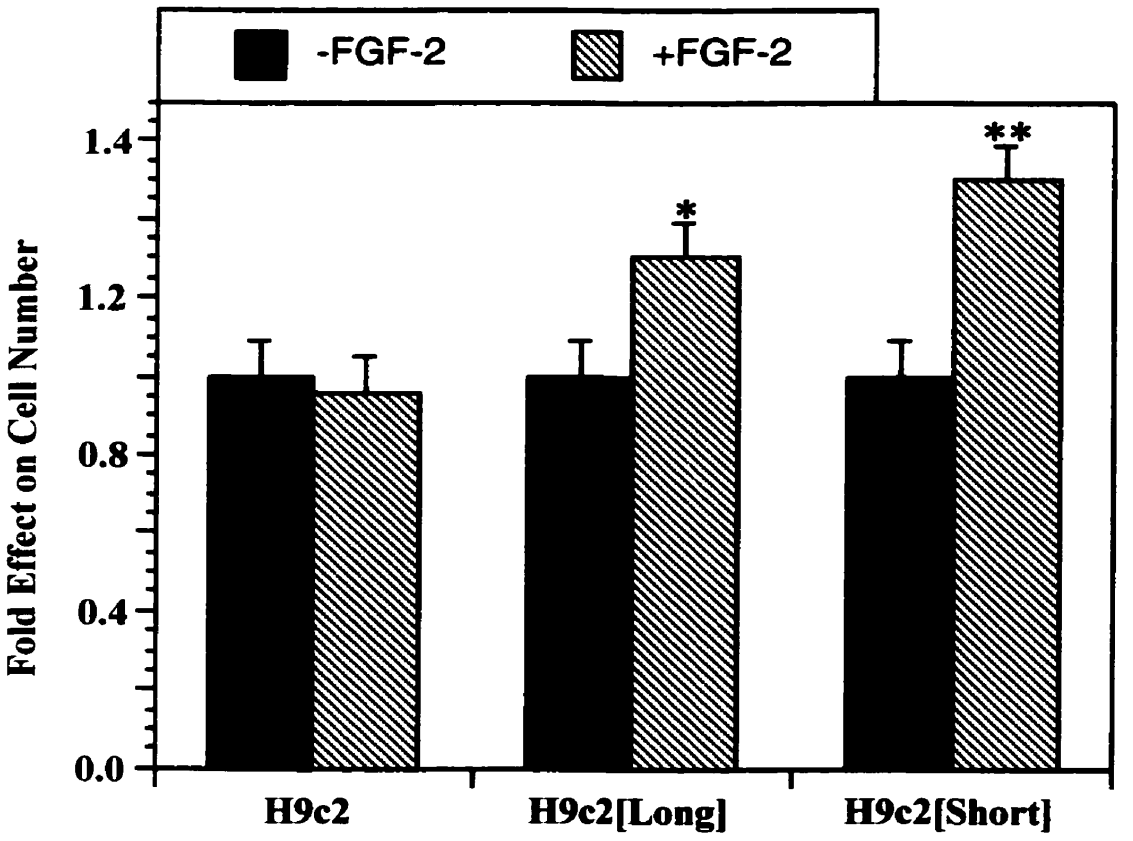

H9c2
H9c2[Long]
H9c2[Short]
Cell Number (x 10^4)
80
60
40
20
0
0
1
2
3
4
5
Time (Days)

Figure 21.

Effect of FGF-2 treatment on H9c2, H9c2[Long] and H9c2[Short] proliferation.

Cells were cultured in the presence of 10% FBS and then treated with or without 10 ng/ml FGF-2 for 24 h. The cell number was counted and the results represent the mean (plus or minus standard error of the mean) from six determinations and are expressed as the fold difference between treated and the untreated cells, where the values for untreated cells were arbitrarily set to 1.0. The number of untreated H9c2, H9c2[Long] and H9c2[Short] cells was 3.0×10^5, 4.0×10^5 and 6.4×10^5, respectively. * $p < 0.05$, * $p < 0.001$

This figure is reproduced from Sheikh et al., 1997.


-FGF-2
+FGF-2
Fold Effect on Cell Number
1.4
1.2
0.8
0.4
0.0
*
**
H9c2
H9c2[Long]
H9c2[Short]

6.2 *FGFR-1 Isoform Overexpression Studies in Neonatal Rat Ventricular Cardiac Myocyte Cultures.*

6.2.1 *FGFR-1 mRNA levels are increased in neonatal cardiac myocytes transiently transfected with FGFR-1(L) and (S) cDNAs.*

Neonatal (1-2 days) rat ventricular myocyte cultures were used to test the expression of the same FGFR-1 cDNA constructs coding for either 'long' (L) or 'short' (S) isoforms used in section ***6.1.2***, after gene transfer and RNA blotting. Neonatal rat ventricular cardiac myocytes were used since they represented "postnatal" cells with a reduced capacity to divide. In addition, the same constructs were used to express FGFR-1 isoforms as the SVenhMLC2 promoter has been shown to be active in neonatal rat cardiac myocytes {Jin et al., 1995}. Transfection efficiency for the myocyte cultures was determined initially by expression and detection of β-galactosidase following co-transfection with RSVp.β-gal (Fig. 26C). Staining was detected in 9.2 ± 0.3 % (n=4) cells of transfected cultures. As a negative control, ventricular cardiac myocytes were transfected with a hybrid gene using the same promoter used to direct the FGFR-1 cDNAs, but fused to the firefly luciferase gene. Total RNA was isolated from myocytes 48 h after infection with SVenhMLCp.*FGFR-1(L)* and SVenhMLCp.*FGFR-1(S)*, as well as SVenhMLCp.*luc*, as a negative control. Samples were transferred to nitrocellulose and probed for FGFR-1 using a fragment of the cDNA capable of detecting both FGFR-1(L) and FGFR-1(S) transcripts (Fig. 22A). Subsequently, the blot was reprobed with GAPDH to allow a standardization of RNA levels after densitometry of autoradiographs (Fig. 22B). A transcript of about 4.3 kb, reflecting endogenous FGFR-1 mRNA and consistent with the expected size for FGFR-1(L) {Jin et al., 1994; Pasumarthi et al., 1995}, was detected in the control lane using the FGFR-1 probe (Fig. 22A, lane a). Transfection with

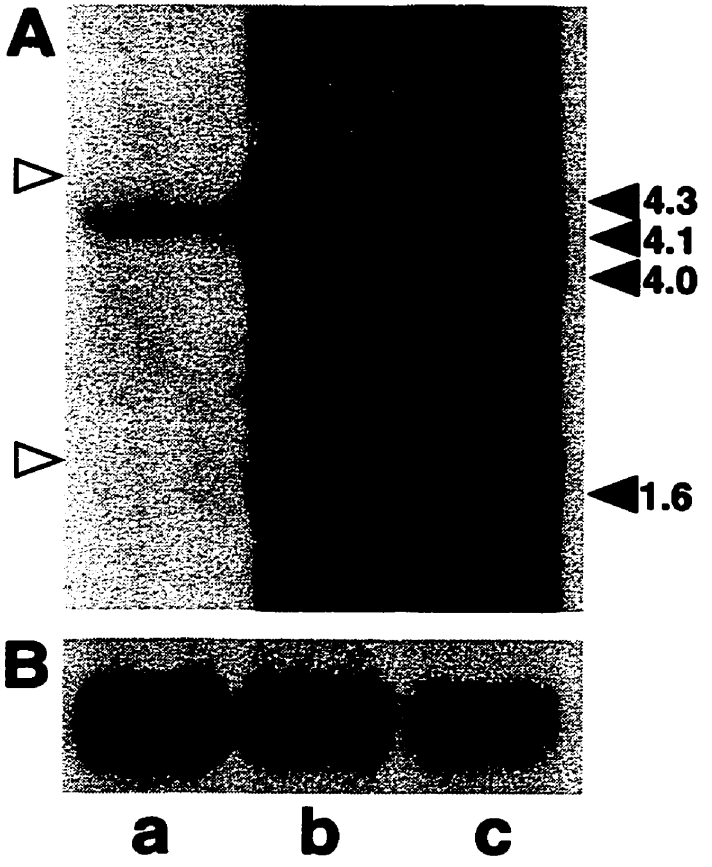
the FGFR-1 cDNA resulted in about a ten-fold increase in the intensity of the 4.3 kb transcript (Fig. 22A, lane b). In contrast to the low level of endogenous FGFR-1(L) RNA observed, no endogenous FGFR-1(S) RNA was detected. As a result, transfection with the FGFR-1(S) cDNA resulted in the induction of a 4.1 kb transcript consistent with the predicted size of FGFR-1(S) RNA (Fig. 22A, lane c). This estimate was based on the size determined for FGFR-1(L) and subtraction of the 267 bp associated with the first Ig loop-like domain {Jin et al., 1994; Pasumarthi et al., 1995}. Minor bands were detected at 4.3 and 4.0 kb, which likely reflect endogenous FGFR-1(L) RNA and an alternative FGFR-1(S) transcript, respectively. A further minor transcript of 1.6 kb was also detected that was common to cardiac myocytes transfected with either FGFR-1(L) or (S) cDNAs (Fig. 22A, lanes b and c). This transcript initiates, presumably, from an internal start site downstream of the first Ig loop-like domain, since this loop sequence would be absent from the FGFR-1(S) cDNA {Jin et al., 1994}. It is possible that this transcript is also generated from the endogenous FGFR-1 gene, but is not detected because of the relatively lower levels of expression.

Figure 22.

Detection of FGFR-1 mRNA levels in neonatal rat cardiac myocyte cultures transfected with FGFR-1 cDNAs.

Total RNA (75 μg) from cardiac myocyte cultures transfected with **(a)** SVenhMLCp.*luc*, **(b)** SVenhMLCp.*FGFR-1(S)* and **(c)** SVenhMLCp.*FGFR-1(L)* was resolved in a 1.0% denaturing agarose gel containing formaldehyde, blotted to nitrocellulose and assessed for **(A)** FGFR-1 isoform transcripts using the FGFR-1(S) cDNA. **(B)** RNA loading was normalized by subsequently reprobing with a radiolabelled cDNA for GAPDH and detection of the corresponding 1.4 kb transcript. All RNAs were visualized by autoradiography. The mobility of 28S and 18S RNAs as well as the transcripts detected with the FGFR-1 cDNA probe are indicated by open and closed arrowheads, respectively.

This figure is reproduced from Sheikh et al., 1999.


A
4.3
4.1
4.0
1.6
B
a
b
c

6.2.2 Subcellular localization of FGFR-1 isoforms in neonatal cardiac myocytes.

Immunofluorescence microscopy was used to visualize FGFR-1 protein in neonatal rat cardiac myocytes transfected with control plasmid (SVenhMLCp.*luc*) as well as FGFR-1(L) and (S) cDNAs (Fig. 23). Cells were double-labeled for myosin and FGFR-1 to verify that cells expressing FGFR-1 were myocytes. The endogenous pattern of FGFR-1 localization was assessed in cultures transfected with control plasmid. The overall FGFR-1 specific staining was relatively weak. FGFR-1 was localized to the perinuclear region and a speckled pattern of staining was present in some nuclei. In cultures transfected with FGFR-1(L) or (S) cDNAs (but not control), intense and specific staining for FGFR-1 was observed. This represented localization of FGFR-1 in overexpressing cardiac myocytes. The majority of these overexpressing cells displayed strong perinuclear staining and/or 'particles' staining intensely for FGFR-1 extended out along the length of the myocyte (Fig. 23).

6.2.3 Overexpression of FGFR-1 isoforms increases specific binding of ^{125}I-FGF-2 on neonatal cardiac myocyte membranes.

Crosslinking and SDS-PAGE were used to determine whether transfection of neonatal rat cardiac myocytes with FGFR-1 cDNAs increased specific FGF-2 binding on the cell membrane, and, thus, the presence of receptors capable of binding FGF-2. Cardiac myocytes were transfected with expression plasmids containing FGFR-1 cDNAs or control DNA. Following transfection, cardiac myocytes were maintained for 48 h and incubated for 90 min with iodinated FGF-2 in the absence or presence of greater than 40-fold molar excess of non-radiolabelled FGF-2. Membranes were isolated after crosslinking with DSS and analyzed by SDS-PAGE and autoradiography (Fig. 24). In the

absence of 'cold' FGF-2, samples from cardiac myocytes transfected with control DNA (Fig. 24, lane a) or FGFR-1(S) (Fig. 24, lanes c-e) and FGFR-1(L) cDNAs (Fig. 24, lanes g-i) revealed crosslinked products of about 35, 50 and 155 kD forming on the membrane. A further band of 135 kD was only present in the sample resulting from FGFR-1(S) cDNA expression, and gave rise to a four-fold (n=2, six determinations) increase in overall receptor (135 + 155 kD) levels (Fig. 24, lane c). Overexpression of the FGFR-1(L) cDNA resulted in a seven-fold (n=2, six determinations) increase in the intensity of the 155-kD sized band compared to control. Both the 155-kD sized band in the control and FGFR-1(L) related samples as well as the 135-kD band in the FGFR-1(S) related samples were competed effectively in the presence of nonradiolabeled FGF-2 (Fig. 24, lanes b, f and j). The 155 and 135 kD band values for the FGF-2/FGFR-1(L) and FGF-2/FGFR-1(S) complexes would be consistent with receptor sizes of about 137 and 117 kD, respectively, after subtracting 18 kD for FGF-2. The bands with mobilities corresponding to proteins of about 35 and 50 kD likely reflect crosslinked multimers of radiolabelled FGF-2 as described previously {Liu et al., 1995}.

Figure 23.

Distribution of endogenous FGFR-1 in neonatal rat cardiac myocytes.

Cardiac myocyte cultures transfected with control SVenhMLCp.luc plasmid, SVenhMLCp.FGFR-1(S) or SVenhMLCp.FGFR-1(L) cDNA, were stained with FGFR-1 antibodies **(a, c, e, i)**, and co-stained for striated muscle myosin to confirm the identity of myocytes **(b, d, f, j)**. Low levels of endogenous FGFR-1 staining, including the perinuclear region, was seen **(a)**. Cardiac myocytes overexpressing either 'short' **(c, i)** or 'long' **(e)** FGFR-1 show strong perinuclear staining with FGFR-1 antibodies and/or an accumulation of particles staining intensely for FGFR-1 around the nucleus and throughout the cytoplasm. The pattern observed with transfected cultures stained with non-immune serum is shown for comparison **(g)**. Identification of myocytes was again confirmed by co-staining with an antibody to striated muscle myosin **(h)**. Bar is equivalent to 25 μm in **a-h**, and to 10 μm in **i** and **j**.

This figure is reproduced from Sheikh et al., 1999.

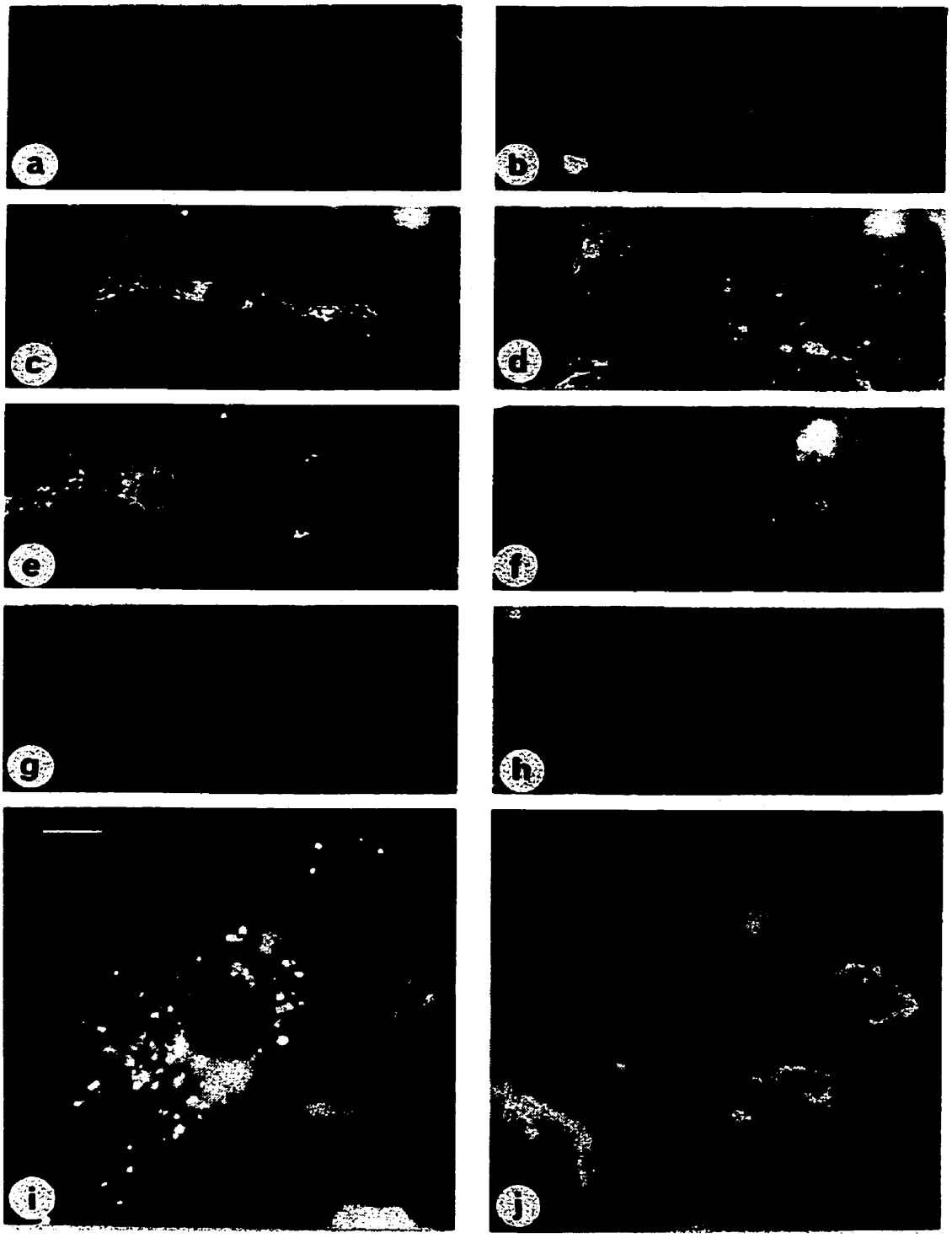
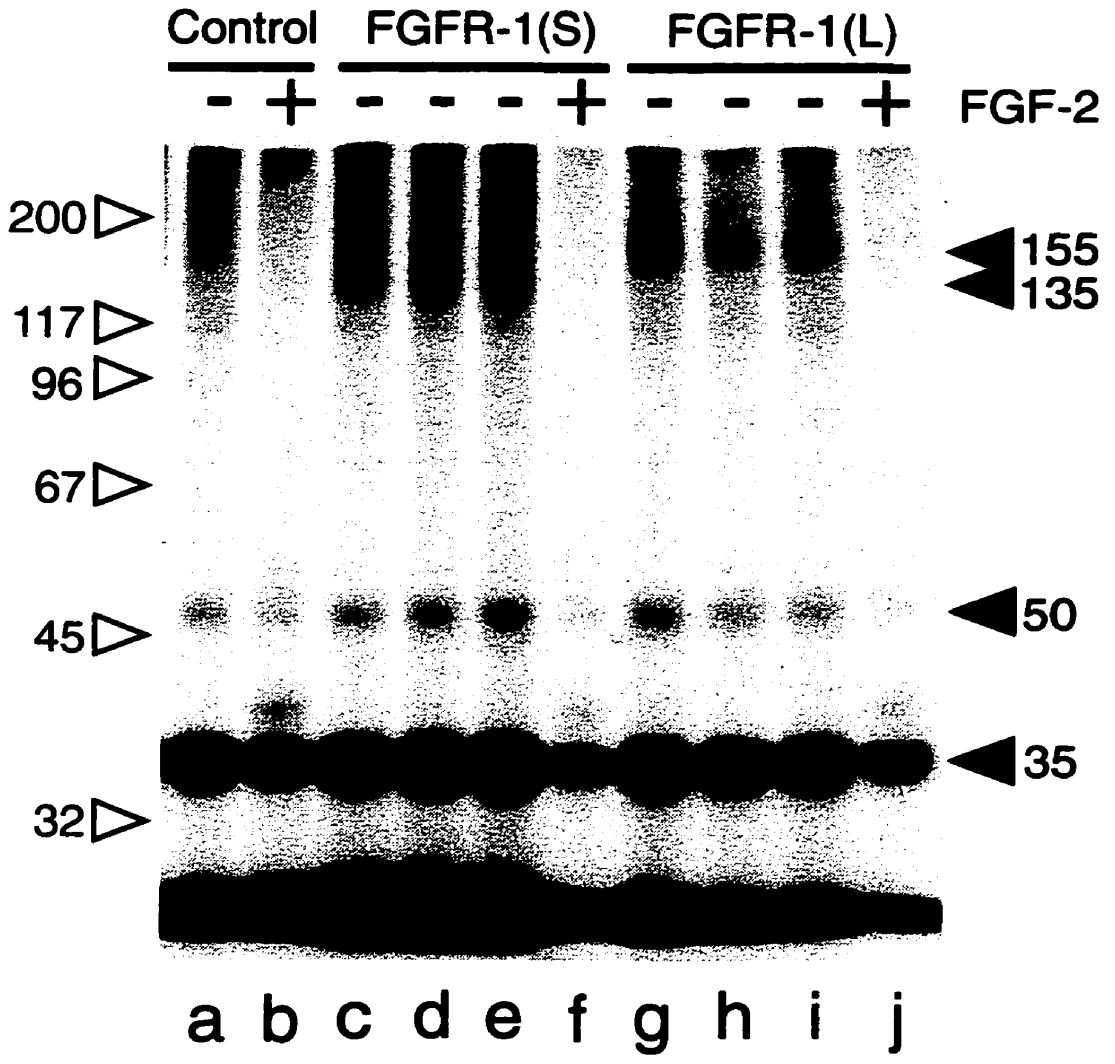

a
b
c
d
e
f
g
h
i
j

Figure 24.

Effect of FGFR-1 isoform overexpression on specific FGF-2 binding on neonatal cardiac myocyte membranes.

Cardiac myocyte cultures transfected with control **(a,b)**, SVenhMLCp.FGFR-1(S) **(c-f)**, or SVenhMLCp.FGFR-1(L) DNA **(g-j)**, were incubated with ^{125}I-FGF-2 in the absence **(a, c-e, g-i)** or presence **(b, f, j)** of 0.1μg unlabelled FGF-2, and then crosslinked with DSS. Cells were harvested and a cell membrane preparation was assessed by SDS-PAGE in a 7.5% gel, and protein visualized by autoradiography. The size (kD)/mobility of molecular mass standards and FGF-2 crosslinked bands detected are indicated by open and closed arrowheads, respectively.

This figure is reproduced from Sheikh et al., 1999.


Control
FGFR-1(S)
FGFR-1(L)
- + - - - + - - - +
FGF-2
200
117
96
67
45
32
155
135
50
35
a b c d e f g h i j

187A

6.2.4 Overexpression of FGFR-1 isoforms increases levels of phosphorylated MAPK in neonatal rat cardiac myocytes.

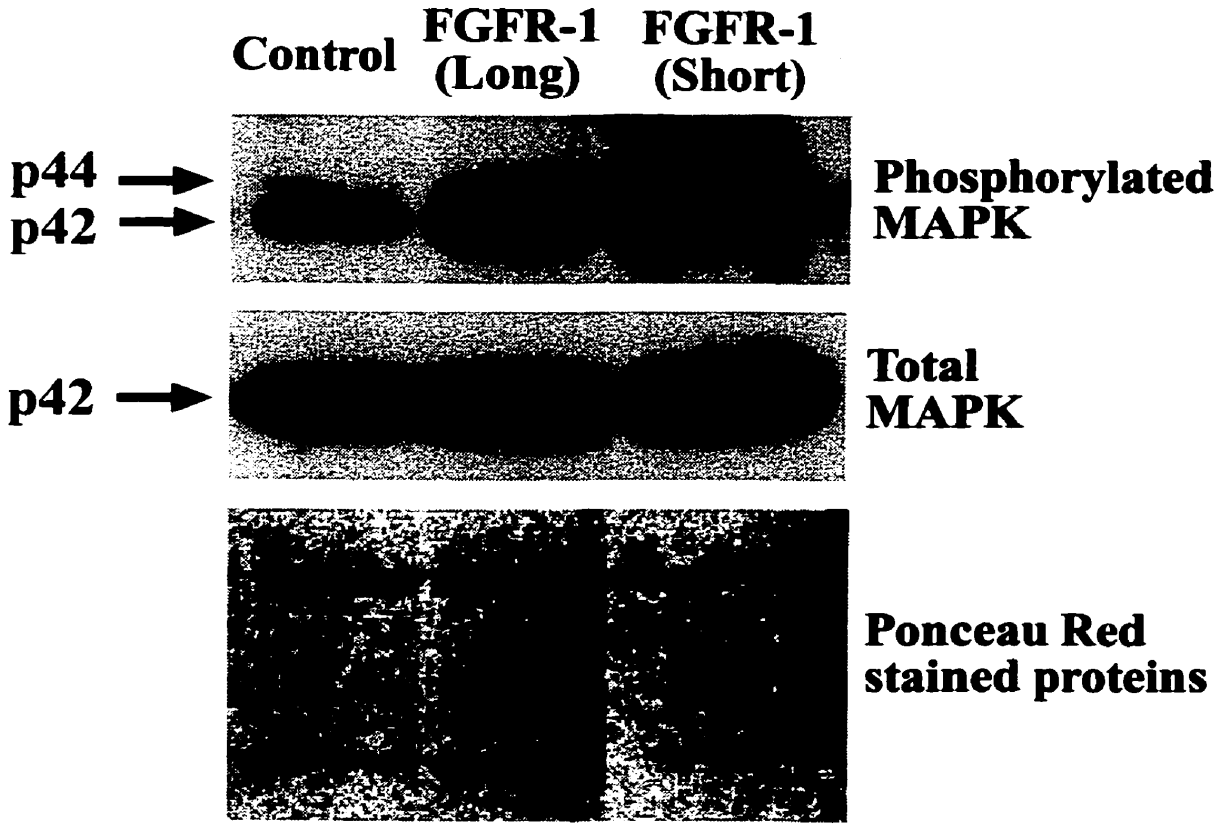
FGF-2 has been previously shown to activate the MAPK (ERK1/ERK2) pathway both in adult cardiomyocytes and whole hearts {Liu et al., 1995; Padua et al., 1998}. To determine whether the FGFR-1 cDNAs can signal a downstream target of FGF-2, we assessed the effects of FGFR-1 overexpression on 'active' ERK1 and ERK2 levels in neonatal cardiac myocytes. The relative levels of ERK1 and ERK2 were assessed in total protein isolated from cardiac myocyte cultures transfected with either control or FGFR-1 cDNAs (long or short isoforms), which were stimulated with 10% FBS for 5 min. As shown in Fig. 25, increased levels of dually phosphorylated and therefore activated ERK1 and ERK2 (42 and 44 kD proteins) were observed in myocytes overexpressing either FGFR-1 isoform, when compared to myocytes transfected with control DNA. Both FGFR-1 isoforms, also, appeared to be equally potent in increasing relative levels of active ERK1 and ERK2. In contrast, no change in either 'total' ERK1 and ERK2 levels or protein loading, as assessed by Ponceau Red stained proteins, was observed between control, SVenhMLC2p.*FGFR-1(L)* or SVenhMLC2p.*FGFR-1(S)* transfected cultures (Fig. 25, lower two panels).

Figure 25.

Effect of FGFR-1 isoform overexpression on phosphorylated MAPK levels.

Cardiac myocyte cultures transfected with control, SVenhMLCp.*FGFR-1(L)*, or SVenhMLCp.*FGFR-1(S)* hybrid genes, were maintained for 24 h in 10% FBS, 46 h in 0.5% FBS, 2 h in serum-free medium, and then stimulated with 10% FBS (containing FGF-2) for five minutes. Total protein was isolated from cultures and equal amounts of protein (20 μg) were assessed by SDS-PAGE and protein blotting, using antibodies recognizing either phosphorylated (active) or both phosphorylated and non-phosphorylated (total) forms of ERK1 (p44) and ERK2 (p42), as indicated. The size (kD)/mobility of bands detected are indicated by closed arrows. Ponceau S stained proteins, present on the nitrocellulose prior to antibody incubation, were also shown as an indication of protein loading (lower panel).

This figure is modified from Kardami et al., 2001.


Control
FGFR-1 (Long)
FGFR-1 (Short)
p44
p42
Phosphorylated MAPK
p42
Total MAPK
Ponceau Red stained proteins

6.2.5 DNA synthesis and cell number are increased significantly in neonatal cardiac myocytes cultures transfected with FGFR-1 cDNAs.

The effects of FGFR-1 overexpression on DNA synthesis and cell number were assessed as markers of cardiac myocyte proliferation in cultures maintained in the presence of 10% FBS-DMEM. Immunofluorescence staining with monoclonal antibodies to myosin and BrdU were used to determine a BrdU LI, or the proportion of myocytes undergoing DNA synthesis (S-phase nuclei) in cultures transfected with FGFR-1 cDNAs or a control gene. Anti-BrdU staining was confined to the nucleus, whereas anti-myosin staining was exclusively cytoplasmic in the rat cardiac myocytes. The results are shown in Fig. 26A and are presented as the proportion of myocytes staining for BrdU and, thus, showing evidence of DNA synthesis. There was a significant approximately three-fold increase in the number of myocyte nuclei staining for BrdU in cultures transfected with SVenhMLCp.*FGFR-1(L)* (2.8 fold) compared with cells transfected with control plasmid ($p<0.001$, n=4). There was no significant difference between the results obtained following overexpression of the 'short' versus 'long' FGFR-1 isoforms.

Myosin positive cells were also scored in 15 random field per coverslip (n=3) to determine whether the increase in BrdU staining (Fig. 26A) also reflected an increase in cardiac myocyte cell number (Fig. 26B). Myocyte number was significantly increased (approximately 1.7 fold) in cultures transfected with SVenhMLCp.*FGFR-1(S)* or SVenhMLCp.*FGFR-1(L)* compared with cells transfected with control SVenhMLCp.*luc* plasmid ($p<0.001$, n=3) (Fig. 26B). Myocyte number was not significantly different in cultures overexpressing FGFR-1(S) versus FGFR-1 (L) (Fig. 26B).

Figure 26.

Effect of FGFR-1 isoform overexpression on cardiac myocyte DNA synthesis and cell number.

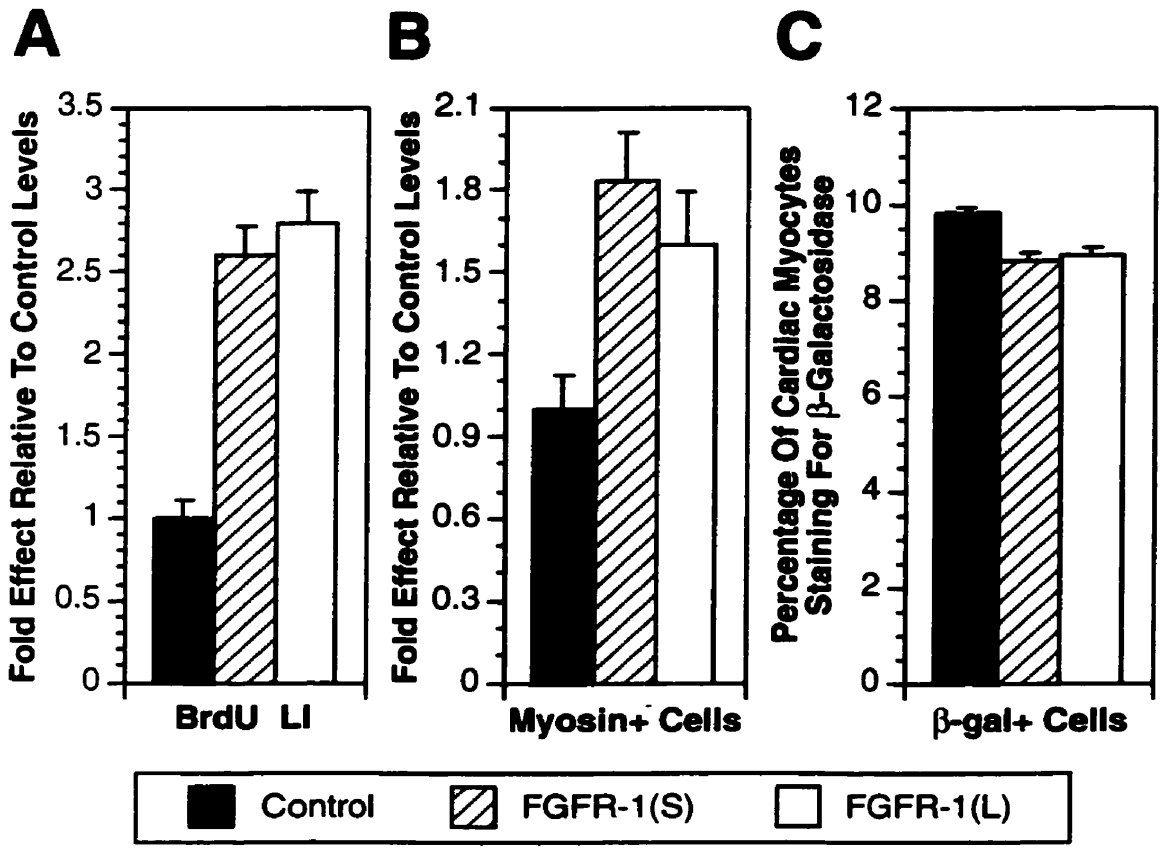
(A) DNA synthesis in transfected cardiac myocytes was assessed by BrdU incorporation. A BrdU labelling index (BrdU LI= BrDU positive cells staining for myosin/total number of cells staining for myosin) was determined (n=4) using immunofluorescence staining with monoclonal antibodies to BrdU and myosin.

(B) Cardiac myocyte proliferation was determined by scoring myosin positive cell number in random fields (n=3) in cultures transfected with FGFR-1 cDNAs and SVenhMLCp.*luc*.

In both cases, the results are presented as fold differences relative to the control (SVenhMLCp.*luc*) value, which was arbitrarily set to 1.0. Bars represented standard error of the mean.

(C) Transfection efficiency was determined by scoring β-galactosidase positive cells in random fields (n=4) in cultures transfected with FGFR-1 cDNAs or SVenhMLCp.*luc*/total number of cells and expressed as a percentage. Bars represented standard error of the mean.

This figure is modified from Sheikh et al., 1999.


A
B
C
Fold Effect Relative To Control Levels
3.5
3
2.5
2
1.5
1
0.5
0
BrdU LI
Fold Effect Relative To Control Levels
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
Myosin+ Cells
Percentage Of Cardiac Myocytes Staining For β-Galactosidase
12
10
8
6
4
2
0
β-gal+ Cells
Control
FGFR-1(S)
FGFR-1(L)

6.2.6 FGF-2 levels are higher in conditioned media from cardiac myocytes overexpressing FGFR-1 cDNAs versus control cDNA.

A highly sensitive immunoassay specific for quantitating FGF-2 levels (Quantikine HS Human FGF basic Immunoassay) was used to detect FGF-2 in samples of conditioned media from myocyte cultures transfected with FGFR-1(S), FGFR-1(L) cDNAs or control DNA after 48 h. A standard curve for FGF-2 levels in the conditioned medium of cultures transfected with SVenhMLCp.*luc*, SVenhMLCp.*FGFR-1(S)* or SVenhMLCp.*FGFR-1(L)* were determined and the results are expressed as mean values (pg/ml) from at least nine determinations (Fig. 27A). A level of 0.12 pg/ml was observed in cultures transfected with control (SVenhMLCp.*luc*) plasmid DNA. However, the levels of FGF-2 in the conditioned medium of cultures overexpressing FGFR-1 (S) or FGFR-1(L) were 3.2 and 2.9 fold higher, respectively ($p<0.05$, n=9) (Fig. 27A). Subsequently, RNA was isolated from these cultures, and assessed for FGF-2 expression by RNA blotting (Fig. 27B). The blot was also probed for GAPDH to allow standardization of FGF-2 mRNA levels following densitometry of resulting autoradiographs. A 6.0 kb FGF-2 transcript was detected in all samples. After correction for RNA loading, the levels of FGF-2 mRNA were not found to be different between cultures transfected with control, FGFR-1(L) or (S) DNA.

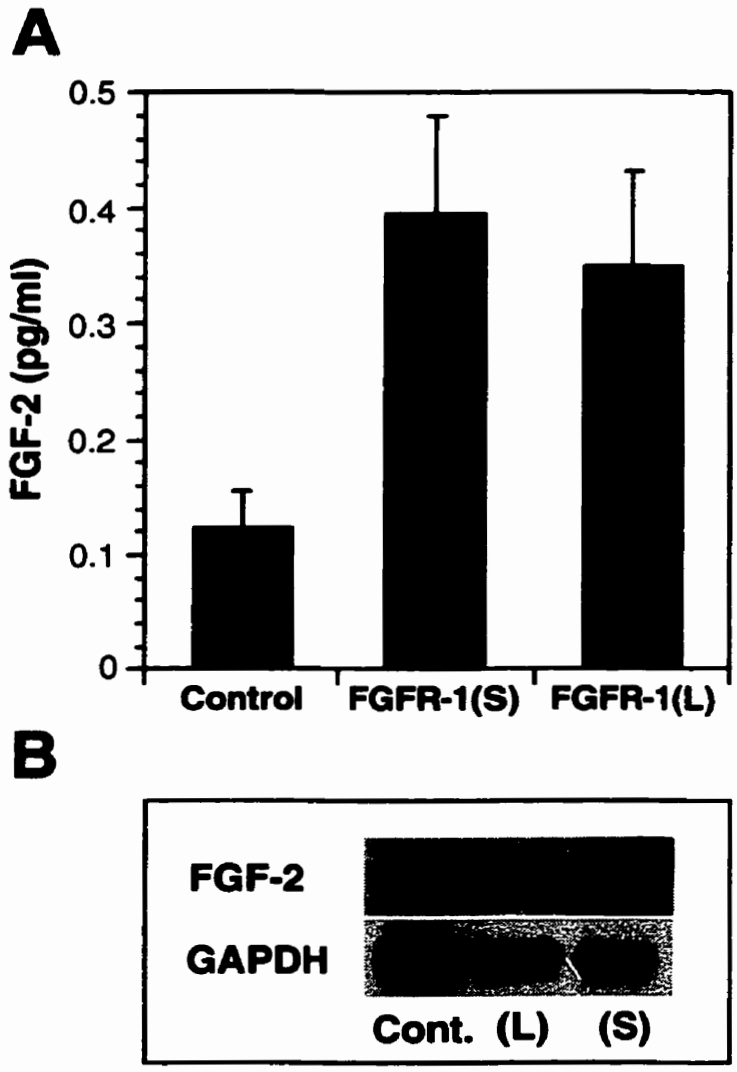
Figure 27.

Effect of FGFR-1 isoform overexpression on FGF-2 levels in the conditioned medium of neonatal cardiac myocyte cultures and FGF-2 mRNA levels in neonatal cardiac myocyte cultures.

(A) Cardiac myocyte cultures transfected with SVenhMLCp.*luc*, SVenhMLCp.*FGFR-1(S)* or SVenhMLCp.*FGFR-1(L)* were maintained for 48 h before FGF-2 levels in the culture medium were determined. The results are expressed as the mean FGF-2 level plus or minus standard error of the mean (n=5).

(B) RNA was isolated from each transfected cardiac myocyte culture, electrophoresed, transferred to nitrocellulose and probed subsequently with radiolabelled FGF-2 and GAPDH. Hybridized bands were visualized by autoradiography.

This figure is reproduced from Sheikh et al., 1999.


A
0.5
0.4
0.3
0.2
0.1
0
FGF-2 (pg/ml)
Control
FGFR-1(S)
FGFR-1(L)
B
FGF-2
GAPDH
Cont.
(L)
(S)

6.2.7 The increase in DNA synthesis with FGFR-1 overexpression is blocked with neutralizing antibodies to FGF-2.

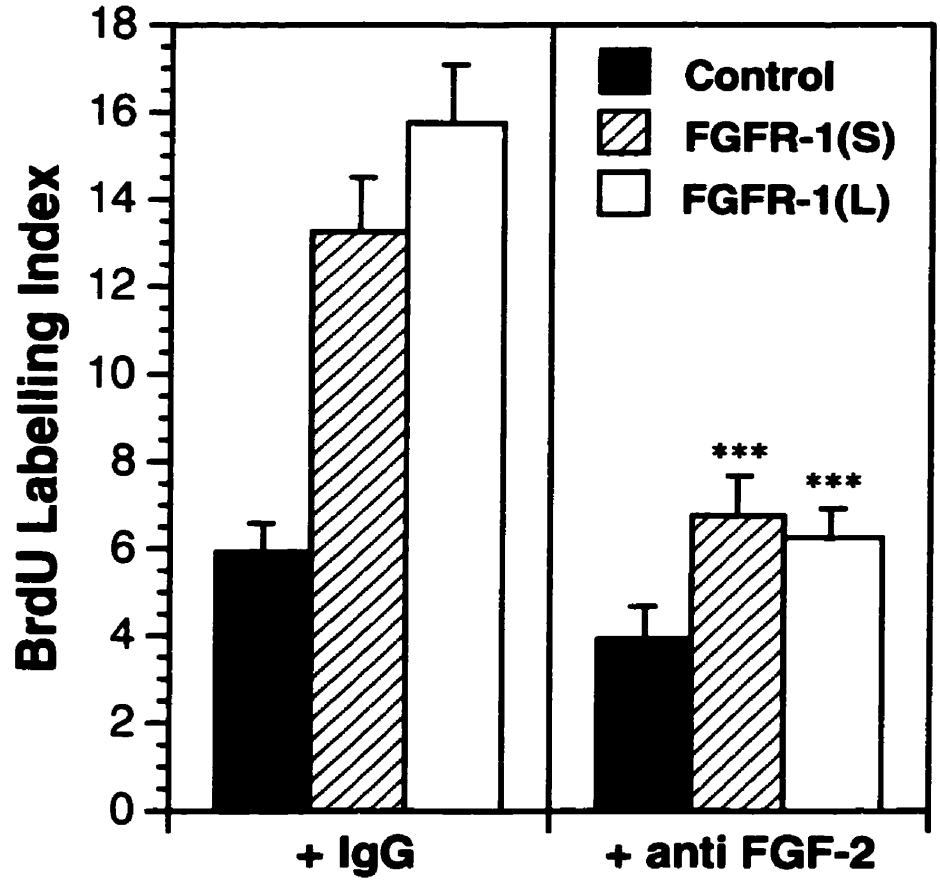
We examined the BrdU LI in the presence of neutralizing antibodies to FGF-2 to determine whether the increased proliferation seen in FGFR-1 overexpressing cultures was FGF-2 dependent (Fig. 28). Cultures were incubated with normal mouse serum (NMS) as a control for the mouse anti-FGF-2 serum. A significant two to three fold increase in BrdU LI was seen with both FGFR-1(S) and (L) transfected versus control cells in the presence of mouse Ig ($p<0.001$, $n=3$). The presence of neutralizing antibodies against FGF-2 (10 μg/ml) blocked this increase so that there was no significant difference between the BrdU LI for cardiac myocytes transfected with FGFR-1 cDNAs versus control DNA ($p>0.05$, $n=3$). Regardless of the presence or absence of antibodies, no significant difference was detected between the effects observed following FGFR-1(S) versus FGFR-1(L) overexpression (Fig. 28).

Figure 28.

Effect of FGF-2 neutralizing antibodies on the increased cardiac myocyte DNA synthesis observed with FGFR-1 isoform overexpression.

The BrdU LI for cardiac myocytes was determined for cultures transfected with SVenhMLCp.luc, SVenhMLCp.FGFR-1(S) or SVenhMLCp.FGFR-1(L), and maintained in the presence of normal mouse Ig (IgG) or FGF-2 neutralizing antibodies (n=3). Bars represent standard error of the mean. *** $p<0.001$ (when compared to FGFR-1(S) and FGFR-1(L) in the presence of Ig)

This figure is reproduced from Sheikh et al., 1999.


BrdU Labelling Index
18
16
14
12
10
8
6
4
2
0
Control
FGFR-1(S)
FGFR-1(L)
***
***
+ IgG
+ anti FGF-2

CHAPTER 7

RESULTS: The Effects of the Kinase-Deficient TGF-β Receptor Type II (TGF-βRII) on Neonatal Cardiac Myocyte DNA Synthesis

7.1 Characterization of Expression of the Kinase-Deficient TGF-βRII in Neonatal Cardiac Myocytes.

Protein blotting and immunofluorescence microscopy were used to assess and visualize TGF-βRII protein expression and localization in neonatal cardiac myocyte cultures transfected with either a "control" virus encoding the β-galactosidase gene (Ad.β-gal) or a virus encoding the kinase-deficient TGF-βRII cDNA (Ad.ΔkTGF-βRII) for 24 h. Specific polyclonal antibodies to the carboxyl terminus of the TGF-βRII were used to recognize both the "endogenous" and "truncated" forms of TGF-βRII. A protein band of approximately 70 kD was detected in cardiac myocytes transfected with the "control" virus, which represented "endogenous" TGF-βRII. The size of "endogenous" TGF-βRII observed in cardiac myocyte cultures is consistent with the reported size of TGF-βRII (Fig. 29) {Cheifetz et al., 1990; Lin et al., 1992; Wrana et al., 1992}. In contrast, transfection of cardiac myocyte cultures with Ad.ΔkTGFβRII (MOI 50) resulted in a major protein band of 50 kD (Fig. 29), which was not only consistent with the predicted amino acid sequence and but also consistent with the only ΔkTGFβRII protein product observed when overexpressed in C6 cells (data not shown). The 50 kD kinase-deficient or "truncated" form of TGF-βRII was approximately 50 fold (n=2) in excess of the "endogenous" 70 kD TGF-βRII form (Fig. 29). The larger, less abundant protein likely represents post-translational modifications of ΔkTGF-βRII, including glycosylation

and/or phosphorylation, and the smaller less abundant proteins likely represent degraded products of the 50 kD ΔkTGF-βRII protein.

Immunofluorescence microscopy was used to visualize the expression of TGF-βR II in transfected neonatal rat cardiac myocyte cultures (Fig. 30). Cardiac myocytes were triple-labeled for α-actinin, BrdU and TGF-βRII to specifically identify myocytes expressing TGF-βRII that were in DNA synthesis. The endogenous pattern of TGF-βRII was assessed in cultures infected with the "control" Ad.β-gal. The overall TGF-βRII staining was weak and localized to the perinuclear region of myocytes (Fig. 30A). In cultures transfected with the Ad.ΔkTGFβRII (but not control), intense and specific staining for TGF-βRII was observed (Fig. 30A). This represented localization of the kinase-deficient TGF-βRII in overexpressing cardiac myocytes. The majority of these overexpressing cells displayed strong perinuclear, cytoplasmic as well as membrane staining (Fig. 30A). ΔkTGFβRII transfected cultures incubated with non-immune serum showed no detectable TGF-βRII staining (Fig. 30B), confirming the specificity of the TGF-βRII antibodies. As an additional control, light microscopy was used to visualize β-galactosidase expression in transfected neonatal cardiac myocyte cultures. β-galactosidase expression was detected in cardiac myocytes transfected with the "control" Ad.β-gal but not in cultures transfected with Ad.ΔkTGFβRII (data not shown).

Figure 29.

Detection of "endogenous" and "kinase-deficient" TGF-βRII in neonatal cardiac myocytes by protein blotting.

Total protein was isolated from neonatal cardiac myocyte cultures transfected with either "control" Ad.β-gal (MOI 50) or Ad.ΔkTGFβRII (MOI 50), analyzed by SDS-PAGE and immunoblotted using antibodies specific against the carboxyl terminus of human TGF-βRII. Open and closed arrowheads denote bands corresponding to either "endogenous" or major "kinase-deficient" TGF-βRII proteins, respectively. The mobility of molecular mass standards is indicated on the right.

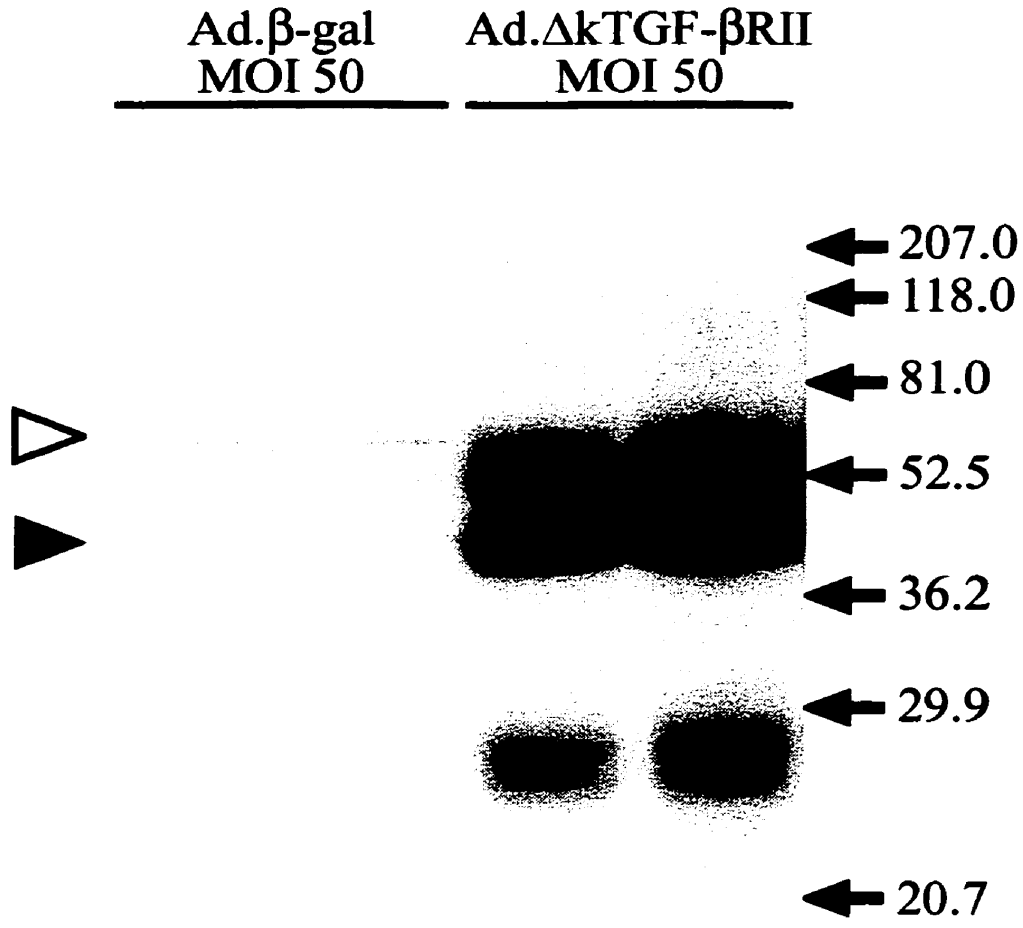

Ad.β-gal
MOI 50
Ad.ΔkTGF-βRII
MOI 50
207.0
118.0
81.0
52.5
36.2
29.9
20.7

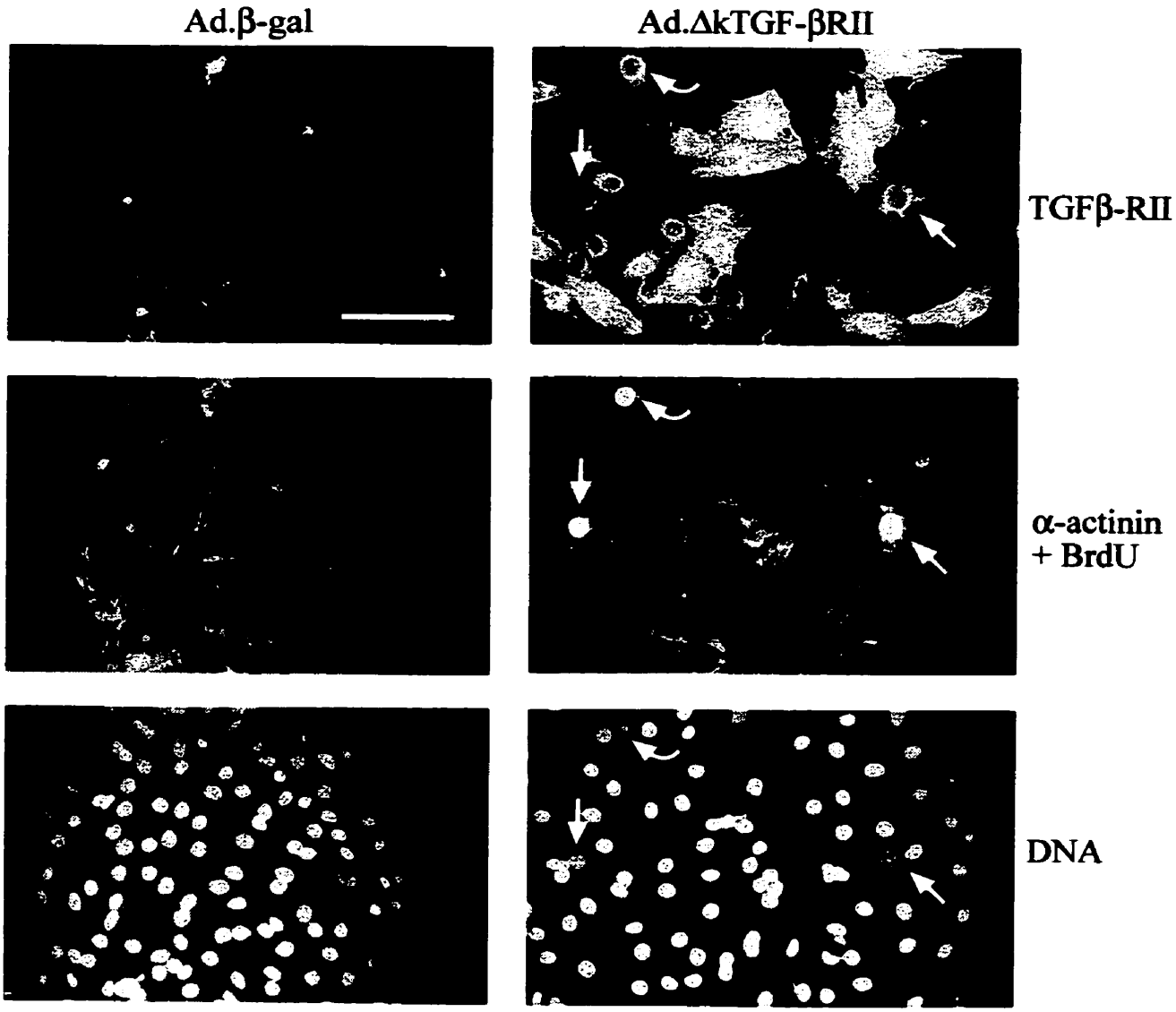
Figure 30.

Detection of "endogenous" and "kinase-deficient" TGF-βRII in neonatal cardiac myocytes by immunofluorescence microscopy.

(A) Neonatal cardiac myocytes cultures transfected with either "control" Ad.β-gal (MOI 50) or Ad.ΔkTGFβRII (MOI 50) were triple stained for TGF-βRII, α-actinin and DNA, as indicated. Low levels of endogenous TGF-βRII staining were observed in perinuclear areas of cardiac myocytes, however, specific TGF-βRII staining was observed in cultures transfected with the kinase-deficient TGF-βRII. White arrows denote ΔkTGFβRII expressing cardiac myocytes that incorporate BrdU. Bar is equivalent to 75 μm.

(B) The pattern observed with Ad.ΔkTGFβRII transfected cultures stained with non-immune serum is shown for comparison. Identification of myocytes was again confirmed by co-staining with an antibody to α-actinin. Bar is equivalent to 75 μm.

A.



B.

7.2 *DNA Synthesis is Significantly Increased in Neonatal Rat Ventricular Cardiac Myocyte Cultures Expressing the Kinase-Deficient TGF-β RII.*

Studies have demonstrated that the kinase-deficient TGF-βRII can inhibit TGF-β signaling in neonatal cardiac myocytes, resulting in a "dominant-negative" phenotype {Brand et al., 1993}. As a result, the effects of the kinase deficient TGF-βRII were assessed on BrdU incorporation and phosphorylation of histone-H1 (phospho-H1) as markers of cardiac myocyte cell cycle entry (DNA synthesis and early mitosis), in transfected cultures maintained in the presence of 10% FBS. Immunofluorescence staining with monoclonal antibodies to α-actinin and either BrdU, or polyclonal antibodies to phospho-H1 were used to determine BrdU or phosph-H1 labelling indexes (LI). As a result, the proportion of myocytes undergoing DNA synthesis (S-phase nuclei) and early mitosis (late G2 phase, early M phase nuclei) in cultures infected with Ad.ΔkTGFβRII versus "control" Ad.β-gal were assessed. Both anti-BrdU and anti-phospho-H1 staining were confined to the nucleus, whereas anti-α-actinin staining was exclusively cytoplasmic in cardiac myocytes. The results are shown in Fig. 31 and are presented as the proportion of myocytes staining for BrdU and phospho-H1. There was a significant 2.4 fold increase in the number of myocyte nuclei staining for BrdU in cultures transfected with Ad.ΔkTGFBRII when compared with cells transfected with "control" Ad.β-gal ($p<0.05$, $n=6$). The effects of the ΔkTGFβRII expression versus control (Ad.β-gal) on increasing DNA synthesis can also be observed by immunofluorescence microscopy in Fig. 30. The proportion of myocytes staining for phospho-H1 mirrored the effect observed on BrdU. As a result, there was also a significant 2.8 fold increase in the number of myocyte nuclei staining for phospho-H1 in cultures transfected with Ad.ΔkTGFBRII when compared with cells infected with

"control" Ad.β-gal ($p<0.05$, n=3). Nuclear co-localization of BrdU and phospho-H1 staining in myocytes is shown in Fig. 32, demonstrating that cardiac myocytes undergoing DNA synthesis can proceed to the late G2/M phase.

7.3 Overexpression of the "Kinase-Deficient" TGF-βRII Amplifies the Effects of FGF-2 on Cardiac Myocyte DNA Synthesis.

TGF-β has been shown to inhibit the increase in cardiac myocyte proliferation observed with FGF-2 {Engelmann et al., 1992; Kardami et al., 1990; Kardami et al., 1993}. To determine whether "neutralizing" the effects of TGF-β signaling can influence the effects of FGF-2 on cardiac myocyte cell cycle entry (S phase or DNA synthesis), we examined the BrdU LI in cardiac myocytes transfected with the kinase-deficient TGFβ-RII (Ad.ΔkTGF-βRII) in the presence of increased levels of added FGF-2. Varying concentrations of FGF-2, 1, 10 and 1000 ng/ml, were used to ensure that FGF-2 was maximized and thus, not a limiting factor to cardiac myocyte cultures. As a control, cultures were incubated with a "control" (Ad.β-gal) virus in the absence or presence of increasing concentrations of FGF-2, as previously established. As expected, exogenous addition of FGF-2 significantly increased cardiac myocyte DNA synthesis between 3 and 5 fold in a dose-dependent manner when compared to cells without FGF-2 treatment ($p< 0.05$, n=6; Fig. 33). In addition, the effects observed with 10 ng/ml FGF-2 were not significantly different from 1000 ng/ml FGF-2, suggesting that FGF-2 was indeed maximized in the cardiac myocyte cultures (n=6; Fig 33). Cardiac myocytes transfected with Ad.ΔkTGF-βRII when treated with 1, 10 or 1000 ng/ml FGF-2 resulted in a significant 14 , 12 and 13 fold increase in DNA synthesis, respectively, when compared to control cells ($p<0.01$, n=6; Fig. 33). The average 13 fold increase in cardiac myocyte

DNA synthesis (based on averaging fold increases with 1, 10 and 1000 ng/ml FGF-2 in cardiac myocytes transfected with Ad.ΔkTGFβ-RII) was significantly greater than the increase in DNA synthesis observed with either the kinase-deficient TGF-βRII (2.4 fold) or FGF-2 (3.5 fold) alone, suggesting an amplified effect ($p<0.05$, n=6; Fig. 33).

Figure 31.

Effect of "kinase-deficient" TGF-βRII overexpression on cardiac myocyte entry into S phase.

DNA synthesis and early mitosis in Ad.β-gal and Ad.ΔkTGF-βRII transfected cardiac myocytes was assessed by bromodeoxyuridine (BrdU) and phosphorylated H1 (phospho-H1) staining. Both BrdU and phospho-H1 labeling indices (BrdU LI = BrdU positive cells staining for α-actinin/total number of cells staining for α-actinin and phospho-H1 LI = phoshorylated H1 positive cells staining for α-actinin/total number of cells staining for α-actinin) were determined using immunofluorescence staining with monoclonal antibodies to BrdU and α-actinin as well as polyclonal antibodies to phosphorylated H1. In both cases, the BrdU LI (n=6, two independent experiments) and phospho-H1 LI (n=3) are expressed as fold differences relative to a control (Ad.β-gal) value, which was arbitrarily set to 1.0. Bars represent standard error of the mean and * = $p<0.05$.

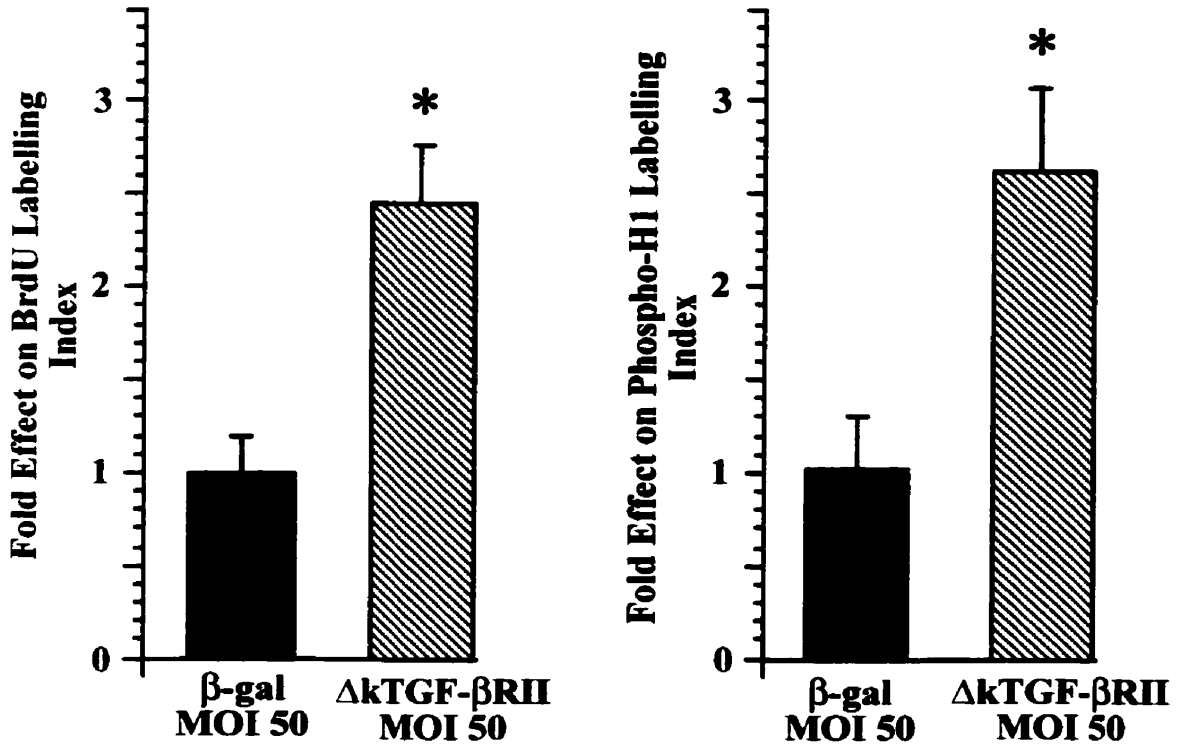

Fold Effect on BrdU Labelling Index
0
1
2
3
*
β-gal MOI 50
ΔkTGF-βRII MOI 50
Fold Effect on Phospho-H1 Labelling Index
0
1
2
3
*
β-gal MOI 50
ΔkTGF-βRII MOI 50

Figure 32.

Co-localization of BrdU and Phospho-H1 Labeling in ΔkTGF-βRII transfected cardiac myocytes.

Ad.ΔkTGF-βRII transfected cardiac myocyte cultures were quadruple-stained for (A) phospho-H1, (B) α-actinin and BrdU as well as (C) DNA. White arrow denotes co-localization of phospho-H1 and BrdU labeling in cardiac myocyte cultures transfected with ΔkTGF-βRII. Bar is equivalent to 38 μm.

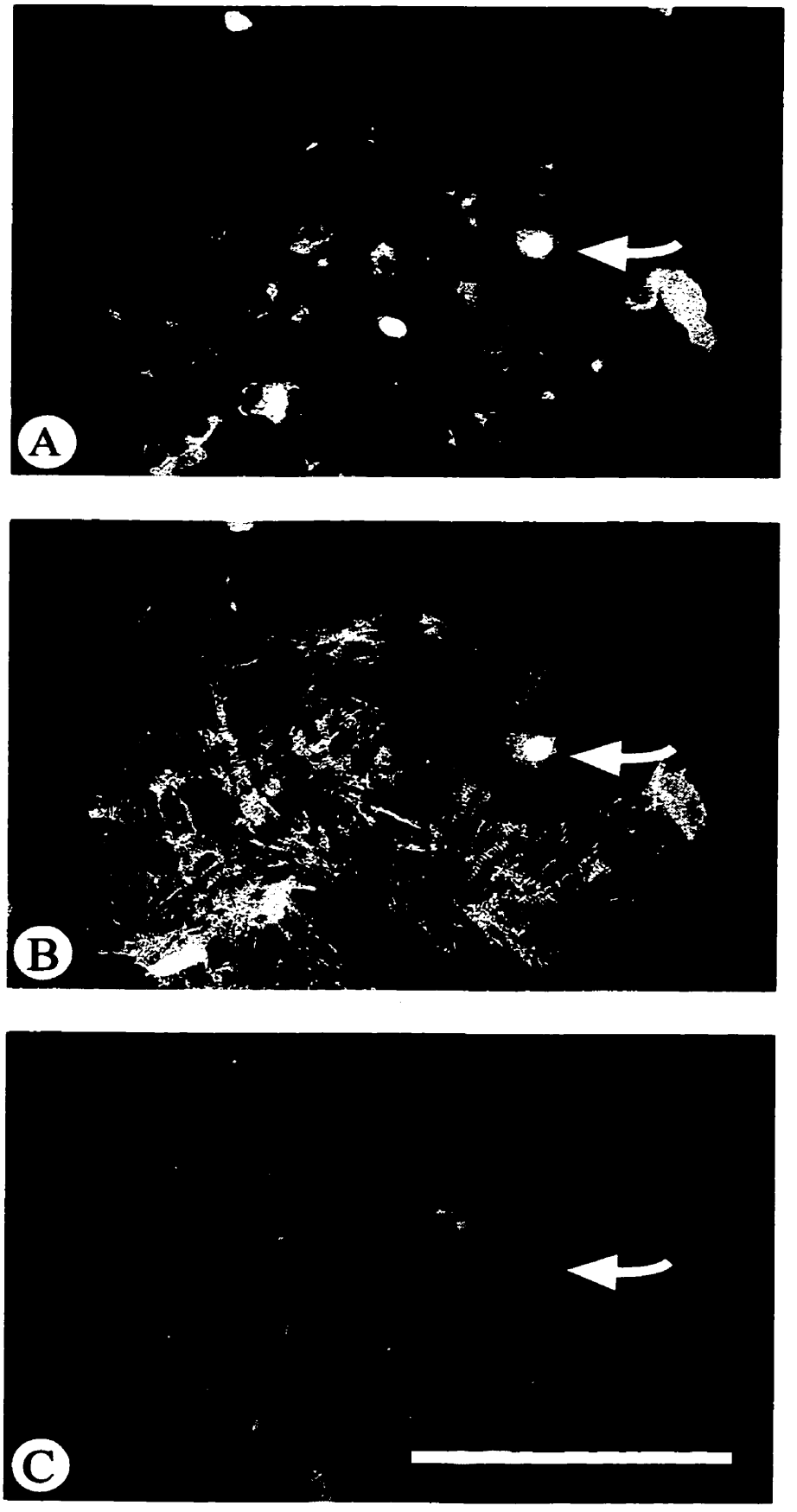
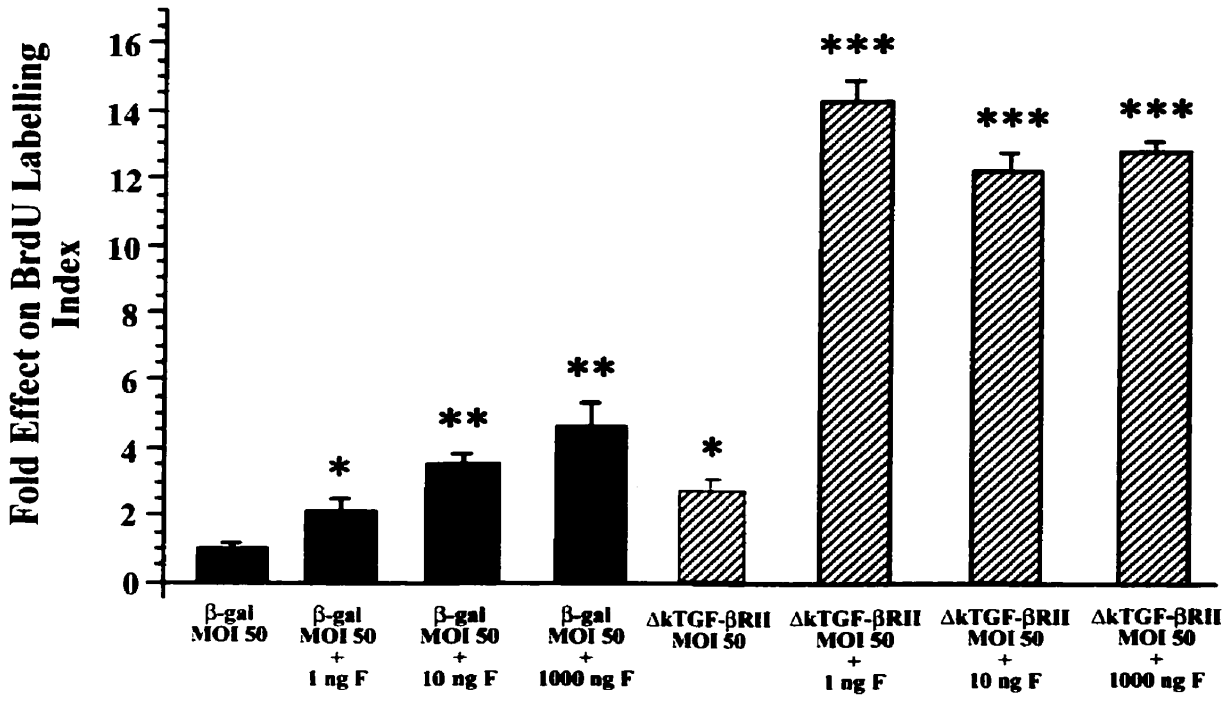

A
B
C

Figure 33.

Effect of exogenous addition of FGF-2 on the increased cardiac DNA synthesis observed with the "kinase-deficient" TGF-βRII.

The BrdU LI for cardiac myocytes was determined for cultures transfected with Ad.ΔkTGF-βRII and Ad.β-gal, and maintained in the absence or presence of 1, 10 and 1000 ng/ml FGF-2 (n=6). The BrdU LI is expressed as fold differences relative to a control (Ad.β-gal in the absence of FGF-2) value, which was arbitrarily set to 1.0. Bars represent standard error of the mean, * = $p<0.05$, ** = $p<0.01$, and ***=$p<0.001$.


Fold Effect on BrdU Labelling Index
0
2
4
6
8
10
12
14
16
*
**
**
*
***
***
***
β-gal MOI 50
β-gal MOI 50 + 1 ng F
β-gal MOI 50 + 10 ng F
β-gal MOI 50 + 1000 ng F
ΔkTGF-βRII MOI 50
ΔkTGF-βRII MOI 50 + 1 ng F
ΔkTGF-βRII MOI 50 + 10 ng F
ΔkTGF-βRII MOI 50 + 1000 ng F

CHAPTER 8

DISCUSSION

8.1 The Role of Endogenous FGF-2 Overexpression in Mouse Hearts In Vivo and in the Context of FGF-2 Release and Cardioprotection

Considerable attention has focussed on the use of polypeptide growth factors and in particular FGF-2 in the treatment of cardiovascular disease {Waltenberger, 1997}. Although efforts have targeted the protective effects of exogenously supplied FGF-2 and the development of delivery systems, there has been little effort to exploit these effects by controlling endogenous production of FGF-2. Targeting endogenous FGF-2 production may serve as an additional strategy to improve cardiac resistance to injury as this approach would clearly take advantage of the proposed natural mode of FGF-2 release from the myocardium. As a result, increased FGF-2 synthesis would allow for more prolonged and increased FGF-2 release from intracellular pools upon contraction, which could then lead to targeted effects on adult cardiac myocytes to mediate protection of the heart. This may have benefits in both the short term, such as is in the event of injury, as well as the long term, which may be important in maintaining a healthy myocardium.

We generated TG mice overexpressing 18 kD FGF-2 and established an isolated mouse heart Langendörff preparation to assess the release and the potential cardioprotective effect of increased "endogenous" FGF-2. The RSV promoter was used to generate FGF-2 TG mice, because previous reports have demonstrated that the RSV promoter can direct preferential overexpression of genes in striated muscles *in vivo* {Conti et al., 1995; Jackson et al., 1990}. Our results are consistent with these findings and demonstrate that the RSV promoter directs preferential overexpression of FGF-2 in

striated muscles, including adult cardiac myocytes *in vivo* (Figs. 6 and 7). An isolated mouse heart model system was established as it allows us to: (i) directly measure "local" release of factors in a functioning heart without the influence of systemic factors, (ii) easily deliver factors (via additions to the perfusate) directly to the heart to assess their response to cardiac injury and (iii) directly measure functional and cellular responses to cardiac injury without the interference of the immune system. The immune system, in particular, was of concern in the FGF-2 TG mouse model since recent evidence from our laboratory using this model implicated a role for FGF-2 in the inflammatory response observed in cardiac tissue after injury *in vivo* {Meij et al., 1998}.

Novel findings presented in this section of the thesis are that stimulation of "endogenous" production of FGF-2 as achieved in the FGF-2 TG mouse hearts resulted in increased: (i) FGF-2 release, (ii) FGF-2 in apparent association with the extracellular matrix (basement membrane), (iii) capillary density, (iv) activity of downstream kinases (JNK, p38, αPKC) associated with ischemic preconditioning and/or angiogenesis as well as (v) resistance of the myocardium to ischemia-reperfusion injury. These results suggest that myocardial protection in FGF-2 TG mice may reflect a direct effect of FGF-2 on cardiomyocytes, including the activation of stress MAP kinases and αPKC. These data support the notion that stimulation of endogenous FGF-2 expression might provide a strategy for improving cardiac resistance to injury.

8.1.1 FGF-2 release in the context of the FGF-2 TG mouse model

Since it is widely accepted that FGF-2 released from cells is retained by the extracellular matrix and its specialized component the basement membrane, the effects of FGF-2 overexpression would be expected to manifest locally at the tissue level and not systemically. Our results confirm this notion since there were no significant changes in serum FGF-2 levels between FGF-2 TG and non-TG mice. These results also provide supporting, albeit indirect, evidence for a role of the extracellular matrix in tightly regulating FGF-2 bioavailability (reviewed in section ***2.4.6.2***). Although the basement membrane acts as a dynamic reservoir for FGF-2, binding and release of FGF-2 is rapid and dependent on several factors which include FGF-2 concentration and/or basement membrane integrity {Dabin and Courtois, 1991, Dowd et al., 1999}. However, matrix degradation is not an absolute requirement to 'free' FGF-2 {Nugent and Edelman, 1992}. In the heart, endogenous FGF-2 appears to be released from cardiac myocytes on a beat-to-beat basis through contraction-induced transient remodelling of the myocyte plasma membrane under normal physiological conditions {Clarke et al., 1995; Kaye et al., 1996}. Additional FGF-2 is released with increased heart rate and force of contraction {Clarke et al., 1995}, as well as upon damage to the cell membrane resulting in the liberation of intracellular stores {Kaye et al., 1996}. Two lines of evidence presented in this thesis, show "proof of concept" that FGF-2 overexpression was accompanied by increased "local" FGF-2 release (and thus increased FGF-2 potentially available to cell FGF receptors) in FGF-2 TG mouse hearts. This includes the increased anti-FGF-2 immunostaining in apparent association with the basement membrane (Fig. 12) and the increased levels of FGF-2 in the effluent of FGF-2 TG mouse hearts during the 30 min of

equilibration prior to ischemia (Fig. 13). Furthermore, the increased intracellular FGF-2 levels, indicated by immunostaining (Fig. 7) and inferred by the 22-34 fold increase in total 18 kD FGF-2 extracted from FGF-2 TG hearts, would also be expected to result in increased levels of FGF-2 release both chronically as well as acutely. Thus, our data indicate that "loading" cardiac myocytes with FGF-2 through endogenous overexpression does translate into increased levels of FGF-2 release. This increase in endogenous FGF-2 release would be expected to contribute to the increase in myocyte viability observed in FGF-2 TG mouse hearts subsequent to cardiac injury.

8.1.2 Cardioprotection in the context of the FGF-2 TG mouse model

Specific parameters were used to determine whether endogenous FGF-2 overexpression in mice *in vivo* resulted in any gross morphological changes in FGF-2 TG hearts. This was clearly important to assess since FGF-2 is not only implicated in cardioprotection but also in various aspects of cardiac growth including proliferation, hypertrophy and angiogenesis (reviewed in section ***2.4.8***). FGF-2 TG mice are viable and hearts from adult FGF-2 TG and non-TG mice displayed no gross differences as reflected by similar heart weight-to-body weight ratio and density of smooth muscle-containing blood vessels, as well as expression of cardiac differentiation markers (Fig. 8). Thus, these data do not provide evidence for a hypertrophic effect on the heart. We did not rigorously assess specific parameters related to cardiac myocyte proliferation in adult FGF-2 TG mouse hearts, however, a preliminary assessment of DNA synthesis in FGF-2 TG and non-TG adult cardiac mouse myocytes cultured for 48 h and pulsed for 18 h with BrdU revealed no evidence or change in the level of DNA synthesis (Sheikh and Cattini,

unpublished observations). There was, however, a significant 20% increase in capillary density in FGF-2 overexpressing hearts. In view of the established angiogenic properties of FGF-2, it is likely that FGF-2 overexpression resulted in chronically elevated basal levels of "local" FGF-2 release that would then affect endothelial cells which would result in increased capillary density {Tomanek et al., 1998; Yanagisawa-Miwa et al., 1992}. It is also possible that FGF-2 overexpression may have caused increased capillary density indirectly, perhaps by inducing expression/release of other angiogenic factors. Regardless of the mechanism, an increase in capillary density might be expected to contribute to the increase in myocyte viability during ischemia-reperfusion injury by increasing tissue perfusion to myocytes in areas of damage.

To determine whether endogenous overexpression of FGF-2 in mouse hearts *in vivo* had any effects on FGF-2 signaling, both levels of the high affinity receptor for FGF-2, FGFR-1, and downstream targets of FGF-2 were assessed in FGF-2 TG mouse hearts. Although mRNA expression of FGFR-1 was unchanged in FGF-2 TG mouse hearts (Fig. 8), baseline activity levels of downstream targets of FGF-2 signaling such as stress-activated MAP kinases (JNK and p38) as well as membrane-associated (presumably active) αPKC were augmented (Fig. 9). The upregulation of active JNK, p38 and αPKC have all been implicated in ischemic preconditioning, thus suggesting that FGF-2 TG hearts may be in a "preconditioned" and thus "protected" state prior to injury {Kitakaze et al., 1997; Nakano et al., 2000; Ping et al., 1999; Yoshida et al., 1997}, irrespective, of "freshly" released FGF-2. This is also consistent with the observed increase in εPKC which has also been implicated in FGF-2 induced cardioprotection and

ischemic preconditioning and thus, may reflect a potential mediator of cardioprotection {Padua et al.,1998; Yoshida et al.,1997}. In addition, we cannot exclude the possibility that the upregulation of certain kinases could also be related to the increased capillary density observed, as activation of p38 and αPKC but not JNK are reported to be essential in early and/or late events of angiogenesis {Harrington et al., 1997; Erdreich-Epstein et al., 2000; Mudgett et al., 2000; Rousseau et al., 1997; Yamamura et al., 1996; Yang et al., 2000}. Also, the upregulation of active p38 has been implicated in FGF-2 induced angiogenesis {Tanaka et al., 1999}. The increase in εPKC in FGF-2 TG hearts may also signal angiogenesis, thus increasing tissue perfusion in the event of injury {Yamamura et al., 1996}. Regardless, the expression profiles of the kinases and their potential roles in ischemic preconditioning and angiogenesis suggest that FGF-2 TG hearts may be "primed" for protection in the event of injury. Although the mechanism of protection remains to be defined, the activation of these signaling pathways would be expected to contribute towards the increase in myocyte viability observed in FGF-2 TG hearts during ischemia-reperfusion injury.

To determine whether endogenous FGF-2 overexpression in mouse hearts had any effects on protection, FGF-TG mouse hearts were subject to global ischemia-reperfusion injury in an established mouse Langendörff preparation (Fig. 10) and hearts were assessed for cell viability (as measured by LDH release) and contractile recovery (left ventricular developed pressure). A significant 35-40% increase in myocardial viability, as reflected by a decrease in LDH release in perfusates was observed in FGF-2 TG versus non-TG mouse hearts subsequent to ischemia-reperfusion injury in both FGF-

2 TG lines (Fig. 14 , C and D), which was consistent with the increase in capillary density, levels of kinases (JNK, p38, αPKC) associated with ischemic preconditioning and/or angiogenesis and "local" FGF-2 release. However, in spite of the increase in FGF-2 release and myocyte viability, there was no significant difference between the contractile recovery seen with FGF-2 TG and non-TG mouse hearts after myocardial injury (Fig. 14, A and B). The lack of an apparent improvement in cardiac function was not expected given the positive effect of FGF-2 overexpression on mouse myocyte viability (Fig. 14, C and D) as well as enhanced contractile recovery and cell integrity reported for exogenous FGF-2 treatment of isolated rat heart preparations {Padua et al., 1998; Padua et al., 1995b}. Several factors may have contributed to the differences in cardioprotection observed between exogenously administered and overexpressed endogenous FGF-2. These include: (i) species-related effects (i.e., mouse versus rat), (ii) "chronic" (transgenic) versus "acute" exposure of hearts to FGF-2, (iii) the amount of FGF-2 available to the receptors of cardiomyocytes and other cardiac cells, (iv) endogenous (transgenic) versus exogenous mode of FGF-2 delivery and (v) mechanism of cardioprotection by "endogenous" versus "exogenous" FGF-2.

In terms of species-related effects, we showed that this was not the case by perfusing mouse hearts with exogenous FGF-2. This resulted in an increase in contractile recovery (Fig. 15A) and significantly less damage to the myocardium as reflected in decreased LDH levels (Fig. 15B). The level of contractile recovery in the mouse heart (average improvement from 40.9 ± 1.9% to 64.2 ± 1.1%) was less than reported previously (improvement from 34.1 ± 5.1% to 76.4 ± 4.1%) for the effect of FGF-2 in a

similar isolated rat heart preparation {Padua et al., 1998}. This may reflect differences in the extent of the damage seen with these species as the mouse myocardium is reported to be more sensitive to changes in calcium concentration than the rat myocardium {Brooks et al., 1999}.

In terms of "chronic" versus "acute" exposure of FGF-2, the structural differences in FGF-2 TG hearts (related presumably to chronic exposure) raise the possibility that increased capillary density may contribute to the differences in cardioprotection observed in FGF-2 TG hearts. However, an increase in capillary density is not required for a positive effect on myocyte viability as similar results were obtained through "acute" exposure to FGF-2 via exogenous addition (Fig. 15). Studies in the rat heart indicated that the "acute" cardioprotective effects of exogenous FGF-2 were not dependent on effects on vasculature leading to flow modulation {Padua et al., 1998}. Rather, a direct effect of FGF-2 on adult cardiac myocytes was implied {Padua et al., 1998}. Thus, the increase in myocardial viability caused by the increase in endogenous FGF-2 reflects a direct protective effect on the adult myocytes.

In terms of amount of FGF-2 available to receptors, total FGF-2 released from FGF-2 TG and non-TG mouse hearts during the 30 min period prior to ischemia was about 3 and 1 ng (based on data presented in Fig. 13), respectively. Although FGF-2 TG cardiomyocytes could be considered to have been exposed to at least three times as much FGF-2 as non-TG cardiomyocytes, the absolute levels may have been insufficient for increased contractile recovery. Infusion of 10 μg FGF-2 in the non-TG perfused hearts on

the other hand may have resulted in higher overall levels of exposure to FGF-2, at least for the duration of the experiment. Certainly there is evidence that cardioprotective and angiogenic properties of FGF-2 are dose-dependent {Harada et al., 1994; Laham et al., 1999; Lopez et al., 1997; Padua et al., 1995b}.

In terms of mode of delivery, FGF-2 added exogenously was distributed via the blood vessels to the cardiac myocytes, while FGF-2 released as a consequence of endogenous overexpression was released by cardiac myocytes into the vessels. Blood vessels were intensely stained for FGF-2 (indicating local retention of this factor) in exogenously treated but not FGF-2 TG or non-TG mouse hearts (Fig. 11B versus 15B), a finding consistent with the mode of delivery. Thus, it is possible that exogenous administration of FGF-2 resulted in higher exposure and therefore protection from injury of a wider range of cells, particularly smooth muscle and endothelial cells of blood vessels, compared to local FGF-2 release from cardiac myocytes.

In terms of the mechanism of cardioprotection, it is possible that "endogenous" and "exogenous" FGF-2 may mediate divergent signaling pathways, which may exert and target their effects on protection at different sites within the cell. This concept is not unheard of as divergent signaling pathways have been reported for endogenous and exogenous molecules such as nitric oxide in evoking a toxic response by tert-butyl-hydroperoxide in CHP100 cells {Guidarelli et al., 1999} as well as ceramides to mediate protection of cells against viral infection {Allan-Yorke et al., 1998}. The two measures used to assess the response to cardiac injury include: LDH release and contractile

recovery. LDH release is a measure of cell membrane integrity, while contractile recovery is a measure of myofibrillar integrity and function. Although, we cannot exclude the possibility that exogenous FGF-2 may act through a pathway similar to endogenous FGF-2 to increase cell membrane integrity and that exogenous FGF-2 signaling may mediate effects on myofibrillar function, which are divergent from the effects of endogenous FGF-2 in the event of injury, our data also raise the possibility that endogenous FGF-2 may play a unique role in preserving cell membrane integrity to mediate cardiac resistance to injury. Since LDH is an enzyme originating from mitochondria, our data also raise the intriguing possibility that endogenous FGF-2 may play a role in preserving mitochondrial membrane integrity, and possibly function. Thus, investigating a possible role for endogenous FGF-2 in mediating mitochondrial membrane integrity and cardioprotection offers an exciting new area of research. This is especially intriguing, given that recently (i) mitochondrial K^+ ATP-sensitive channels have been proposed to be the end-effector of signaling pathways implicated in the protective effects of ischemic preconditioning {Baines et al., 1999a}, (ii) both PKC and stress MAP kinases are implicated in the opening of mitochondrial K+ ATP channels {Baines et al., 1999a}and an increase in levels of active p38, JNK and α-PKC as well as increased myocyte viability is observed in FGF-2 TG hearts (Chapter 4), and (iii) a recent report has implicated a direct role for K+ channels in FGF-1 mediated cardioprotection in ischemia-reperfusion injury {Cuevas et al., 2000}. Since (mitochondrial) membrane integrity is also increased in exogenously FGF-2 treated hearts, it is possible, in this scenario, that this effect may due to a response resulting from an amplification of "endogenous" FGF-2 production in FGF-2 treated hearts. Since endogenous FGF-2

synthesis can increase with stimuli related to injury (i.e., ischemic/reperfusion injury) (reviewed in section ***2.4.1***) and autoregulation of FGF-2 gene expression has been described in cardiac cells including endothelial cells, vascular smooth muscle cells and adult rat cardiac myocytes {Alberts et al., 1994; Fischer et al., 1997; Wang et al., 1997; Weich et al., 1991}, we also cannot exclude the possibility that the effects of FGF-2 on cell (including mitochondrial) membrane integrity are mediated by an amplified response of endogenous FGF-2 signaling. Future directions for assessing a role for endogenous FGF-2 on mitochondria during cardiac injury are outlined in Chapter 9.

8.1.3 Conclusions

In conclusion, these results demonstrate that overexpression of FGF-2 *in vivo* has significant phenotypic effects on the adult heart that might influence its response to injury. Chronic FGF-2 overexpression (associated with increased angiogenesis/capillary density and augmentation of kinases linked with ischemic preconditioning, angiogenesis and cardioprotection) which leads to increased "local" FGF-2 release are likely contributing to the increased cardiac myocyte viability observed after ischemia-reperfusion injury. These results also demonstrate, for the first time, that exogenous FGF-2 is cardioprotective in the mouse. The differences in cardioprotection observed between exogenous and endogenous FGF-2 in the mouse heart may reflect differences in FGF-2 concentration, mode of delivery or mechanism of cardioprotection. Taking together these data in combination with previous reports on the cardioprotective effect of exogenous FGF-2 suggest that FGF-2 expression and release from cardiomyocytes could be viewed as part of the normal process for maintaining a healthy myocardium as well as part of the

response to injury. Therefore, stimulation of endogenous expression of FGF-2, and thus targeting endogenous FGF-2 production, may provide a credible method for improving cardiac health and resistance to injury.

8.2 Generation of Adult Cardiac Mouse Myocyte Cultures for FGF-2 Transcriptional Studies in the Heart

Adult cardiac myocyte cultures are an essential tool to answer specific questions at the cellular level, which can be used to either complement or address questions which are impeded by whole animal studies. For example, adult cardiac myocytes have been shown to be a useful model system in assessing various preservation solutions affecting cardiac myocyte metabolism after preservation for successful transplantation and long-term survival of the heart {Schmid et al., 1991}. This was stated to have been impossible to assess systemically in whole hearts {Schmid et al., 1991}. Although there is focus on the development of procedures to isolate and culture adult cardiac myocytes from various species including the rat, guinea pig, dog and rabbit, there is a paucity of information in the literature with regards to similar methods for the mouse. Given the growing use of transgenic and gene "knockout" mouse models for the study of gene expression and regulation in the heart, the ability to isolate and culture viable adult cardiac myocytes from mice would prove to be an invaluable tool to complement studies in the whole animal (transgenic or otherwise).

We have devised a method to isolate and culture viable adult cardiac myocytes from mouse hearts in order to make use of the -1058FGFp.*luc* transgenic mouse model to address specific questions which relate to the role of FGF-2 transcription in the heart.

Transcription is one important level at which gene expression is regulated. However, in the period of 1995 to early 2001 less than 5% of publications in the area of FGF-2 in the heart report on transcriptional regulation of FGF-2. Results from a previous chapter (Chapter 5) have already demonstrated that increasing endogenous FGF-2 production is associated with an increase in cardiac resistance to injury. Therefore, targeting endogenous FGF-2 production through regulation of FGF-2 synthesis/transcription in adult cardiac myocytes could serve as an additional means to exploit the beneficial effects of FGF-2 in the heart. Studies in our laboratory have revealed that FGF-2 transcription/promoter activity can be regulated in -1058FGFp.*luc* mice *in vivo* through adrenergic stimulation (via catecholamines) {Detillieux et al., 1999}. However, since activity was assessed in whole hearts, it remained to be determined whether the FGF-2 promoter is active and/or could be regulated by adrenergic stimulation in adult cardiac myocytes. Addressing the first premise would be essential in exploiting the use of this transgenic model for future studies in the area of transcriptional regulation of FGF-2 in the heart.

Novel findings presented in this section of the thesis are that the procedure developed to isolate and culture adult mouse cardiac myocytes: (i) was successful in generating 70% viable rod-shaped striated adult cardiac myocytes which could be maintained in culture for 3 days and (ii) could be applied to the –1058FGFp.*luc* transgenic model to demonstrate that the FGF-2 promoter is active and can be increased by the catecholamine, norepinephrine (10 μM), in adult cardiac myocytes. These results present a procedure to generate and culture viable adult mouse cardiac myocytes, which

can be used to exploit transgenic mouse models. These data also strengthen the notion that adrenergic regulation of FGF-2 in the heart could be viewed as an additional strategy to exploit the "therapeutic" effects of FGF-2 in the heart, for both normal maintenance of the heart and in the event of injury.

8.2.1 Technical considerations for isolating and culturing adult mouse cardiac myocytes

Isolation of adult cardiac myocytes, in general, is a complicated procedure. Specific modifications of methods for isolation are not only required for each species but also within the same species, dependent on their experimental use {Wolska and Solaro, 1996}. In terms of the mouse, there have been several reports on the isolation of mouse cardiac myocytes, however, these cells were mainly used for electrophysiological studies and not maintained in culture {Albitz et al., 1990; Dorn et al., 1994; Li et al., 1998; Maxwell et al., 1999}. Although there is one report for short-term culture and one report for long-term culture of adult cardiac myocytes, these cells clearly do not retain their striations or rod-shaped features {Kruppenbacher et al., 1993, Zhou et al., 2000}. Since the purpose of our studies required adult cardiac myocytes to be maintained in culture, we found that the existing isolation procedure for mouse myocytes for electrophysiological purposes had to be modified {Maxwell et al., 1999}. Certain variables were crucial to this procedure and they include composition of disaggregation medium, EGTA, calcium, time of enzymatic digestion, pre-treatment of culture dishes with laminin/serum and use of M199 medium as well as low serum to maintain myocytes in culture.

The isolation method used for mouse myocytes involved the use of constant flow with an enzyme solution containing collagenase, protease and BSA at a temperature maintained at or just below 37°C {Maxwell et al., 1999}. Constant flow was used since it was shown to permit efficient and continuous perfusion of tissue during enzymatic digestion in the mouse heart {Maxwell et al., 1999}. A combination of two proteolytic enzymes, which include collagenase (breakdown of connective tissue) and protease (breakdown of polypeptide chains) were used since together they were found, as reported by others, to permit complete dissociation of mouse cardiac myocytes {Dorn et al., 1994; Maxwell et al., 1999}. Quality of collagenase was also an issue, and batches were tested for isolation procedures. Also, consistent with other reports, use of BSA was crucial to the isolation method for mouse myocytes since it was found to minimize myocyte damage and increase viability, presumably due to preservation of membrane integrity {Gambassi et al., 1992; Li et al., 1998; Maxwell et al., 1999}. We have also found that BSA addition to perfusates was essential in maintaining normal heart function in the mouse Langendörff preparation, as it was found to prevent edema (Sontag, Sheikh and Cattini, unpublished observations). BSA may be a necessity, in particular for mouse myocyte isolations, since it has been shown that the mouse myocardium has an increased sensitivity to calcium, and thus possibly damage, when compared to the rat myocardium {Brooks and Conrad, 1999}.

The main modification from the existing mouse myocyte isolation procedure used was removal of a wash perfusion step which involved using a buffer containing EGTA {Maxwell et al., 1999}. Although EGTA (or calcium chelation) can facilitate release of calcium-tolerant rod-shaped adult cardiac myocytes for electrophysiological

studies {Maxwell et al., 1999; Isenberg and Klockner, 1982}, we found that this step generated viable cardiac myocytes but caused the cells to become irreversibly rounded when maintained in culture. Removal of this step in the isolation procedure greatly improved yield of viable adult cardiac myocytes and resulted in survival in culture. Although the reasons for these differences are largely unknown, it has been shown that chelator-supplemented medium tend to yield calcium-insensitive cells, however, this was dependent on time of exposure to the medium {Dow et al., 1981}. Thus, it is possible that membrane integrity changes with time (i.e., when maintained in culture) and the sensitivity of isolated adult cardiac myocytes towards calcium increases, leading to cell death. In addition, temperature and oxygenation may also play a role in the increased sensitivity of cardiac myocytes to calcium when maintained in culture.

Extracellular calcium is also a crucial parameter in attaining viable adult cardiac myocytes. Calcium must be present at concentrations sufficiently high to preserve the functional and structural integrity of the cardiac myocyte sarcolemma without causing "calcium overload", but low enough to ensure separation of cells at their intercalated discs {Dow et al., 1981}. As a result, we have used a nominally calcium-free solution, as others have reported for mouse myocyte isolation procedures and used incremental increases in levels of calcium after isolation to obtain viable cells {Li et al., 1998; Maxwell et al., 1999}. Supplementation of BSA in these graded calcium concentrations was also found to increase both yield and viability of adult cardiac myocytes, presumably by preserving membrane integrity. Although, the calcium concentration was not assessed during enzymatic digestion using the "nominally" calcium-free solution, others have reported calcium concentrations of up to 30 to 40 μmol/L at the end of organ perfusion in

hearts {Powell and Twist; 1981}. This is based on endogenous calcium, calcium contaminants in collagenase, calcium present in albumin and salts and calcium leached from the tissue during perfusion {Powell and Twist, 1981}. The use of a nominally calcium-free solution and increasing calcium with graded concentrations has been shown previously to greatly reduce adult cardiac myocyte sensitivity to calcium, and thus increase viability {Dow et al., 1981}.

Duration of enzymatic digestion was the most crucial parameter in the "success" of the isolation procedure and the ability to culture adult cardiac myocytes from normal mouse hearts. Three key observations were assessed which include colour, texture and shape of heart tissue to indicate the termination of enzymatic digestion. The most meaningful test was "the pinch method" which involved directly palpitating the heart to determine flaccidity or sponginess of heart tissue. This method has been described previously by others to indicate termination of digestion {Wolska and Solaro, 1996}. From at least ten trials, it was found that enzymatic digestion took between 18-22 min to isolate viable adult mouse cardiac myocytes that could be maintained in culture. Perfusion with enzymatic solutions for shorter or longer periods resulted in underdigestion or overdigestion of mouse heart tissue, reducing cell viability and yield. Timings could, however, vary depending on different properties observed in both normal and/or transgenic mouse hearts. We have observed that a reduced enzymatic digestion period (i.e., 10 min) was required to isolate adult cardiac myocytes from FGF-2 TG mice (Sheikh and Cattini, unpublished observations). Since FGF-2 TG mouse hearts have been characterized with a 20% increase in capillary density {Sheikh et al., 2001}, a reduced enzymatic digestion period is presumably required because of better delivery of

proteolytic enzymes for tissue disaggregation. In addition, we also found that enzymatic digestion of hearts from mice that were greater than eight months old was not as effective, yielding reduced numbers of viable cardiac myocytes. This is presumably due to increased extracellular matrix and collagen deposition which is reported in aged animals {Eghbali and Weber, 1990; Weisfeldt et al., 1971}.

The ability to culture adult cardiac myocytes relies on three key parameters, (i) viability of adult cardiac myocytes from isolation procedure, (ii) pre-treatment of culture dishes with laminin/10% FBS-M199 and (iii) the use of M199 and low serum conditions for maintenance in culture. Since adult cardiac myocytes do not divide in culture, replication rates and mitotic indices cannot be used as methods to measure viability of isolated cells {Dow et al., 1981}. The simplest test for cell viability, albeit crude, is the dye exclusion assay {Dow et al., 1981}. Using this assay, rod-shaped cardiac myocytes tend to exclude trypan blue while contracted cells are permeable to this dye {Dow et al., 1981}. Using our isolation method, approximately 70% of the striated rod-shaped cardiac myocytes excluded trypan blue. These percentages are greater than reported by others which are in the range of 30-58% {Kruppenbacher et al., 1993; Li et al., 1998; Redaelli et al., 1998; Wolska and Solaro, 1996}. Recently another group reported mouse myocyte viability of approximately 70%, however, these cells did not retain their rod-shaped features after one day in culture {Zhou et al., 2000}. Dye exclusion has also been shown to parallel respiration features (i.e., oxygen consumption) of adult cardiac myocytes, used as another indicator of viability {Farmer et al., 1977; Rajs et al., 1978}. Other tests described to measure viability include measurement of LDH release in medium and

calcium transients in cells exposed to increased extracellular calcium {Dow et al., 1981}. In terms of cell number, we reported isolating approximately 4.1×10^5 viable adult cardiac myocytes per mouse heart, however, this may be an underestimate, since counting of adult cardiac myocyte number using a hemacytometer may be inaccurate due to their large size {Horackova et al., 1997}. We have also found that the the survival of myocytes in culture is proportional to the viability/yield of isolated myocytes with 70% viability allowing adult cardiac myocytes to survive for up to 3 days, without undergoing spontaneous hypercontracture. In terms of culturing conditions, although laminin is commonly used as the substrate to facilitate attachment of adult cardiac myocytes to culture dishes {Maxwell et al., 1999; Padua et al., 1998}, we have found that dishes pre-treated with a combination of laminin and 10% FBS-M199 can increase the survivability of adult cardiac myocytes in culture. Pre-treatment of culture dishes with serum has previously been shown to be a crucial prerequisite towards attachment and maintenance of adult mouse cardiac myocytes, presumably due to factors present in serum which may promote attachment {Kruppenbacher et al., 1993}. We have also found like others that M199 medium, as opposed to other medium, and using low serum conditions can be used to sustain adult cardiac myocytes in a differentiated state in short-term culture {Kruppenbacher et al., 1993; Padua et al., 1998}.

After three days in culture adult cardiac myocytes undergo hypercontracture or increased sensitivity to calcium and die. Although the reason for the hypercontracture at three days has not been determined it could be related to two possibilities. The first possibility includes the isolation procedure used for mouse myocytes, which may not be

as efficient as protocols used for other species to maintain these cells in long-term culture. We have reported a 70% viability of rod-shaped adult cardiac myocytes, however, isolation methods to maintain adult rat and guinea pig cardiac myocyte cultures in long-term culture have reported >80% viability of rod-shaped adult cardiac myocytes {Horackova et al., 1997; Horackova and Byczko, 1997}. A second possibility could relate to plating densities of adult mouse cardiac myocytes in culture, which may relate to theformation of cell contacts, as this was reported to be a crucial parameter in maintaining long-term cultures of adult guinea pig cardiac myocytes {Horackova et al., 1997}. In addition, the presence of survival factors not present within low serum could also account for differences.

8.2.2 FGF-2 gene regulation in –1058FGFp.luc adult cardiac myocytes

There is limited information on the transcriptional regulation of FGF-2 in the adult heart. This largely relates to the fact that (i) transcriptional regulation of FGF-2 is an understudied area (5% publications on FGF-2 in the heart since 1995), (ii) difficulties in detecting endogenous FGF-2 mRNA due to instability and low levels of message in cardiac cells as well as (iii) difficulties in using conventional methods to introduce genes into adult cardiac myocytes for FGF-2 gene regulation studies. To increase the sensitivity of detecting FGF-2 transcription in the heart, our laboratory has generated and characterized a transgenic mouse model containing the rat FGF-2 promoter region driving the luciferase reporter gene. The use of luciferase as a reporter gene has greatly increased the sensitivity of detection of FGF-2 transcription in a variety of tissues, including the heart *in vivo* {Detillieux, 1999; Detillieux et al., 1999}. However, to date,

studies on the transcriptional regulation of FGF-2 in the heart, using this model, have been limited to whole hearts because of difficulties in detecting luciferase at the cellular level. Attempts at detecting luciferase using conventional methods like *in situ* hybridization and immunlocalization have been unsuccessful in our laboratory as well as others, due to lack of specific methods and reliable commercially available luciferase antibodies {Grothe, Detillieux and Cattini, unpublished observations}. Clearly, detection of luciferase/FGF-2 transcription in adult cardiac myocytes would be crucial in terms of exploiting this model and determining its role in the context of adrenergic regulation, to complement studies in the whole heart. Therefore, to circumvent the difficulties of luciferase detection, adult cardiac myocytes were isolated and cultured from −1058FGFp.*luc* mice to determine whether the FGF-2 promoter was active and/or responsive to adrenergic stimulation in adult cardiac myocytes.

We were thus able to confirm that the FGF-2 promoter is active and is responsive to adrenergic stimulation using norepinephrine (10 μM) in adult cardiac myocytes derived from -1058FGFp.luc mice (Fig. 18). According to the dose reponse curve for NE in studies in isolated right ventricular papillary muscles, 10 μM NE was shown to be within the pharmacological dose response range {Chidsey and Braunwald, 1966}. These results also suggest that the genetic information contained within 1 kb of the rat FGF-2 promoter region is sufficient to drive FGF-2 gene expression in adult cardiac myocytes. The use of adult cardiac myocyte cultures, in this case, has extended and complemented *in vivo* observations in the -1058FGFp.luc model, related to the adrenergic regulation of FGF-2 transcription in the heart. Previous studies from our

laboratory have shown that FGF-2 transcription in -1058FGFp.*luc* mouse hearts *in vivo* can be increased by both alpha-adrenergic regulation, using phenylephrine {Detillieux et al., 1999} as well as beta-adrenergic regulation, using isoproterenol from our laboratory. The use of norepinephrine (10 μM), which mediates its effects through both alpha and beta adrenergic receptors, has demonstrated that FGF-2 synthesis can potentially be regulated through both pathways in adult cardiac myocytes. Further studies would be required to determine the specificity of this response using various adrenergic blockers and/or specific agonists (i.e., phenylephrine or isoproterenol). In addition, these results have provided a credible means to exploit the -1058FGFp.*luc* transgenic mouse model for future studies on cardiac myocyte growth, development and/or injury. Furthermore, since adult cardiac myocytes release FGF-2 upon contraction {Clarke et al., 1995; Kaye et al., 1996} and release can be increased by adrenergic regulation {Clarke et al., 1995}, these observations strengthen the notion that FGF-2 transcription may be a natural part of this process in order to maintain a healthy myocardium (via β-adrenergic regulation) as well as a mechanism to exploit the protective effects of endogenous FGF-2 in the heart in the event of injury or congestive heart failure (via α-adrenergic regulation).

8.2.3 Conclusions

In conclusion, we were successful in establishing a procedure for the isolation of adult mouse cardiac myocytes and theri maintenance in short term culture. As a consequence, we were able to confirm that endogenous FGF-2 synthesis can be regulated by adrenergic stimulation, using 10 μM norepinephrine, at the transcriptional level in adult cardiac myocytes using -1058FGFp.luc transgenic mouse model. These results also reveal avenues to exploit the use of the –1058FGFp.luc transgenic mouse model for

future studies in the area of FGF-2 transcription in the heart. Together these results strengthen the notion that adult cardiac myocytes are a useful model system and that studies on the transcriptional regulation of FGF-2 in this system can be used as a credible means to target and exploit the protective effects of FGF-2.

8.3 The role of FGFR-1 isoforms in cardiac cell proliferation

The FGF-2 axis and thus, signaling, clearly plays an important role in cardiac myocyte growth, and in particular cardiac myocyte proliferation {Kardami et al, 2001}. However, despite efforts, the lack of a measurable proliferative response by FGF-2 in adult cardiac myocytes *in vitro* and *in vivo* raise the possibility that FGF-2 signaling/axis may be limited or antagonized in the postnatal heart.

With a view towards increasing proliferative potential of cardiac cells, we generated expression vectors containing the myosin light chain-2 promoter and SV40 enhancer sequences to overexpress the 'long' and 'short' FGFR-1 isoforms to assess their effects on cell division in two cell systems, rat cardiac H9c2 cells and neonatal rat ventricular cardiac myocytes. The myosin light chain-2 promoter and SV40 enhancer sequences were used because previous characterization of this promoter revealed that it is active in both H9c2 cells and neonatal rat ventricular cardiac myocytes {Jin et al., 1995}. H9c2 cells were selected for this task since no RNA corresponding to either long or short species of FGFR-1 was detected in these cells using a highly sensitive RT-PCR assay {Sheikh et al., 1997}. These results suggested that FGFR-1 RNA in H9c2 cells was either (i) absent, (ii) present at extremely low levels or (iii) unstable, and as a result, these cells

presented as an excellent model system to attempt overexpression of FGFR-1 to assess any effect on cell division. Neonatal rat ventricular cardiac myocytes (1-2 days) were used as a second cell system, as they were primary cardiac myocytes derived directly from postnatal heart tissue which had a limited ability to divide, and thus, represented a more relevant cardiac model system to attempt FGFR-1 overexpression to assess effects on cell division.

Novel findings presented in this section of the thesis are that stimulation of FGFR-1 isoform expression, as achieved by stable gene transfer in H9c2 cells and transient gene transfer in neonatal rat ventricular cardiac myocytes, resulted in a significant increase in cardiac cell proliferation (as assessed by DNA synthesis and cell number), which was clearly FGF-2 dependent. However, these results suggested that there were no preferential effects by either FGFR-1 isoforms in this response. These results also suggested that the FGFR-1 mediated proliferation, may at least in part be mediated by the increase in FGF-2 release as well as ERK/MAPK pathway, as FGFR-1 overexpression was associated with increased levels of activated ERK1/2 MAPK. These data support the notion that increasing FGFR-1 levels could provide a strategy towards stimulating FGF-2 mediated postnatal cardiac myocyte proliferation/regeneration in the event of injury *in vivo*.

8.3.1 The role of FGFR-1 isoforms in cardiac H9c2 cell proliferation

Stable gene transfer with the hybrid MLC-2/FGFR-1 genes, was used to assess cell division given the length of H9c2 doubling time (~36 hours, Fig. 20). DNA and RNA

blotting (Fig. 19) confirmed the presence of the transfected genes. The detection of FGFR-1 transcript supported previous observations that the modified cardiac-specific MLC-2 is active in H9c2 cells {Jin et al., 1995}. The H9c2 cells stably expressing 'short' or 'long' FGFR-1 cDNA required less time to recover their proliferative activity after plating in 10% FBS-DMEM compared to control H9c2 cells (Fig. 20; compare results at 1 versus 3 d). Furthermore, the cells overexpressing FGFR-1 displayed significant increases in their rates of cell division over a period of 5 d which, presumably, reflected the effect of FGF in 10% FBS or endogenous FGF (Fig. 20). It was likely that the larger response seen with the short isoform reflected higher levels of receptor as suggested by the amount of FGFR-1 transcript detected in samples from H9c2 cells stably expressing the 'short' versus the 'long' FGFR-1 cDNAs (Fig. 19B). A mitogenic response signaled via long or short FGFR-1 was also indicated by the modest but significant increase (approximately 1.4 fold) in cell number observed following treatment of H9c2[Long] or H9c2[Short] with FGF-2 for 24 h (Fig. 21). There was no effect of FGF-2 on 'control' H9c2 proliferation during the same period (Fig. 21). The effects on proliferation observed with stable gene transfer experiments were also supported by transient gene transfer experiments in H9c2 cells {Sheikh et al., 1997}. In these studies, a significant two fold increase in tyrosine phosphorylation and tritiated thymidine incorporation was observed following transient overexpression of FGFR-1 isoforms in H9c2 cells in the presence of 0.5% FBS {Sheikh et al., 1997}.

8.3.2 The role of FGFR-1 isoforms in neonatal rat ventricular cardiac myocyte proliferation

Expression of FGFR-1 (L) and (S) cDNAs in neonatal rat cardiac myocyte cultures was demonstrated by RNA blotting, through an increase in the levels of the 4.3 and 4.1 kb FGFR-1 transcripts, respectively (Fig. 22). FGFR-1 overexpression in cardiac myocytes was confirmed by immunohistochemistry and co-staining for FGFR-1 and striated muscle myosin (Fig. 23). The FGFR-1 antibodies were raised to the intracellular kinase domain and, thus, recognize an epitope common to both FGFR-1(L) and FGFR-1(S). Finally, as evidence of an increase in FGFR-1 levels, the presence of increased levels of specific plasma membrane FGF-2 binding sites was detected in cultures overexpressing either FGFR-1 isoforms (Fig. 24). The ability to overexpress FGFR-1 isoforms in neonatal cardiac myocytes supported previous observations that the modified cardiac-specific MLC-2 is active in these cells {Jin et al., 1995}.

A significant 2.6-2.8 fold increase in DNA synthesis and 1.6-1.8 fold increase in overall cell number (Fig. 26, A and B) confirmed stimulation of postnatal cardiac myocyte proliferation in cultures overexpressing FGFR-1(L) and (S). These increases, however, are quite high and disproportionate to the fraction of myocytes (~10%, based on β-galactosidase), expected to be stimulated to divide due to FGFR-1 overexpression, and suggest that all myocytes (not only the overexpressing fraction) may have been subjected to increased mitogenic stimulation. Indeed, overexpression of either FGFR-1 (L) or (S) resulted in a three-fold increase in FGF-2 present in the culture medium (Fig. 27A), thus raising the possibility that increased levels of FGF-2 contribute to the overall cardiac myocyte proliferation observed. Exogenous addition of FGF-2 to the culture medium has

been previously shown to increase neonatal cardiac myocyte proliferation, to a degree similar to the stimulation observed in this study {Kardami, 1990}. Furthermore, the stimulatory effect of FGFR-1 overexpression was fully blocked by neutralizing antibodies to FGF-2 (Fig. 28), indicating that the increase in overall myocyte proliferation is FGF-2 dependent and similar for the 'long' and 'short' FGFR-1 isoforms. These results are also consistent with unpublished observations from Dr. E. Kardami's laboratory which suggest that a kinase-deficient FGFR-1(S) can inhibit FGF-2 induced DNA synthesis in neonatal cardiac myocytes {Augustin et al., 2001}. The mechanism resulting in increased FGF-2 levels in the medium of FGFR-1 overexpressing myocytes is not as yet known but it does not appear to include stimulation of FGF-2 mRNA expression (Fig. 27B). FGFR-1 overexpression may regulate the expression of proteases or heparin sulfate degrading molecules directly or indirectly in cardiac myocytes to release entrapped FGF-2 from the extracellular matrix. A similar mechanism was implied in prostate cancer cells which did not express FGF-2 mRNA, but where serum FGF-2 which was trapped in the extracellular matrix of cells could be released by heparinase activity in the culture to increase cell proliferation {Kassen et al., 2000}. There is limited information on the role of heparan sulfate proteoglycan degrading molecules in cardiac myocytes, however, there are reports that nitric oxide, plasmin, thrombin, collagenase, low affinity receptors such as syndecan-1, and factors that increase cell density, possess properties that could degrade heparan sulfate proteoglycans, which would presumably release FGF-2 into the medium {Kato et al., 1998; Richardson et al., 1999; Vilar et al., 1997; Whitelock et al., 1996}.

8.3.3 FGFR-1 signaling in the context of cardiac cell proliferation

There is scant information about the specific signaling mechanisms associated with FGFR-1 mediated proliferation in cardiac cells and specifically, whether long and short isoforms are associated with similar signaling mechanisms. However, what is clear is that signaling via FGF-2 and FGFR-1 has two major components. The first mode is dependent on ligand-plasma membrane receptor interaction in the extracellular space. Ligand binding causes FGFR dimerization, autophosphorylation, and activation of downstream signaling cascades, including increased phosphorylation of raf-1 and activation MAPK which has been associated with stimulation of proliferation by FGF-2 {Kouhara et al., 1997; Morrison et al., 1988}. Although not established in cardiac cells, it was suggested the FGF-induced mitogenesis requires phosphorylation of FGFR at either Tyr 653 alone or both Tyr 653 and Tyr 766 residues {Mason, 1994}. The MAPK pathway has been implicated in FGF-2 mediated cardiac myocyte growth effects {Kardami et al., 2001}. To this end, we have also demonstrated that overexpression of either FGFR-1 isoform in cardiac myocytes resulted in downstream stimulation of the ERK1/2 MAPK pathway, which may be involved in proliferation (Fig. 25). This would be consistent with studies which suggest that MAPK activation is required for FGF-2 mediated cardiac myocyte proliferation, as MAP kinase kinase (MEK-1) inhibitors was able to block 70% of the increase in DNA synthesis observed with FGF-2 {Kardami et al., 2001}. The inhibition of the stimulatory effect of 'long' or 'short' receptor overexpression on cardiac myocyte proliferation by neutralizing antibodies to FGF-2 (Fig. 28) and stimulation of proliferation by FGF-2 in stable H9c2 cells expressing FGFR-1 isoforms (Fig. 20 and 21), indicate that a ligand-dependent triggering

mechanism is largely operating in both of these systems. Consistent with this observation, no differences have been reported between the intracellular signaling elicited by FGF-2 binding to the 'long' versus 'short' FGFR-1 isoform. The affinity of FGF-2 for the 'long' (50-150 pm) or 'short' (100 pm) FGFR-1 is comparable {Johnson and Williams, 1993}. Although more controversial, this also appears to be the case for FGF-1 and the 'long' (20-80 pm) or 'short' (50-200 pm) receptor isoform {Johnson and Williams, 1993; Shi et al., 1993}.

The second mode of signaling appears to involve direct intracellular action of FGF-2 and/or FGFR-1. Previous reports from our laboratory have shown that nuclear localization of the CUG, but not the AUG-initiated form of FGF-2 exerts specific, and apparently receptor- and/or proliferation-independent, effects on cardiac myocytes {Pasumarthi et al., 1996}. Using antibodies to FGFR-1, staining of the perinuclear region and cytoplasmic 'particles' was observed in overexpressing cardiac myocytes (Fig. 23). This distribution of FGFR-1 was similar for the two receptor isoforms, and was consistent with overexpression of FGFR-1 and its presence in the cytoplasm at presumably, different stages of synthesis and processing. However, mobilization and accumulation of FGFR-1 to a region surrounding the nucleus has been described as nuclear trafficking in several systems, although not cardiac myocytes {Feng et al., 1996; Prudovsky et al., 1996; Prudovsky et al., 1994; Stachowiak et al., 1996; Stachowiak et al., 1997}. A translocation of FGFR-1, with its ligand, to the nucleus during the G1 phase of the cell cycle {Prudovsky et al., 1994} has been associated with a transition from a quiescent to a proliferative cellular state {Stachowiak et al., 1997}. Thus, the presence of

FGFR-1 and its ligand at the nucleus could indicate an increase in proliferative potential, and play a role in signaling this process. Although the mechanisms involved in intracellular signaling effects of FGF-2 and FGFR-1 are largely unknown, it was suggested that this may be independent of the MAPK pathway {Kardami et al., 2001}.

8.3.4 The role of the long isoform of FGFR-1 in cardiac cell proliferation

It has been suggested that expression of 'long' FGFR-1 isoforms correlates with restriction of cell growth, malignancy and enhanced differentiated function {Feng et al., 1996}. However, this is not supported by the pattern of FGFR-1 mRNA expression in the developing heart. Previously, we used reverse transcriptase-polymerase chain reaction to assess the relative levels of FGFR-1(S) versus FGFR-1(L) in embryonic (dividing) and adult (non dividing) mouse cardiac myocytes {Jin et al., 1994}. There appeared to be a switch in the pattern of FGFR-1 expression during development as the 'long' and 'short' RNAs represented the major transcript detected in embryonic and adult cells, respectively. Thus, there was a correlation between predominantly FGFR-1 (L) expression and a proliferative cardiac myocyte phenotype. The continued presence of endogenous FGFR-1(L) transcript in neonatal rat cardiac myocytes (Fig. 22) would be consistent with this idea, reflecting a limited proliferative capacity at this stage. Although transfection with the FGFR-1(L) cDNA increased cardiac myocyte growth, no significant difference was detected between the levels of stimulation of activated ERK1/2 MAPK and proliferation that was associated with FGFR-1(L) versus FGFR-1(S) overexpression (Figs. 25 and 26). Although proliferation of H9c2 cells stably transfected with FGFR-1 (L) was not stimulated to the same extent as in those transfected with FGFR-1 (S), which

may be possibly due to the difference in expression level between both FGFR-1 isoforms (Fig. 19B). Thus, while we have shown that an increase in either 'long' or 'short' FGFR-1 levels can stimulate postnatal cardiac myocyte proliferation in an FGF-2 dependent manner, there is no evidence that either of the two receptor isoforms stimulated these effects preferentially.

8.3.4 Conclusions

In conclusion, FGFR-1 levels play a significant role in cardiac cell proliferation. 'Long' and 'short' forms of FGFR-1 are capable of stimulating a mitogenic response in both cardiac H9c2 tumor cells and postnatal primary neonatal cardiac myocytes. No differences in proliferation were observed between isoforms, suggesting that the 'long' form of FGFR-1 does not preferentially direct cardiac cell proliferation. In addition, we demonstrated that the proliferative effects of FGFR-1 were FGF-2 mediated and in neonatal cardiac myocytes may involve activation of ERK1/2 MAPK pathway. A novel finding presented was that FGFR-1 overexpression promoted FGF-2 release in cardiac myocytes, and although the mechanism remains unexamined, it could be used to exploit the mitogenic effects of FGF-2 in cardiac cells. Our results strengthen the notion that a targeting strategy to increase FGFR-1 levels would be expected to contribute towards stimulation of regeneration/proliferation in response to FGF-2 in adult cardiac myocytes in the event of injury *in vivo*.

8.4 The Role of TGF-β in FGF-2 Mediated Cardiac Myocyte DNA Synthesis

The absence of positive signals, such as FGFR-1, in the postnatal heart may not be the only variable which could limit the effects of FGF-2 on adult cardiac myocyte proliferation *in vitro* and *in vivo*. The proliferative effects of FGF-2 can also be antagonized by the presence of negative factors (present in serum and/or cardiac cells). TGF-β, in particular, is proposed to be an endogenous inhibitor of growth *in vivo* (reviewed in section ***2.5.2***). In addition, TGF-β has been shown to cancel the mitogenic effects observed with FGF-2 in embryonic and neonatal cardiac myocytes *in vitro* {Engelmann et al., 1992; Kardami, 1990; Kardami et al., 1993}. Also, both TGF-β and FGF-2 can accumulate in cardiac myocytes at the margins of infarcted regions of the adult heart in the event of injury *in vivo* {Padua and Kardami, 1993; Thompson et al., 1988; Wunsch et al., 1991}. These data suggest that TGF-β signaling may limit cardiac myocyte proliferation as well as the effects of FGF-2 on proliferation in the postnatal heart *in vivo*.

With a view towards increasing postnatal cardiac myocyte proliferation we generated an adenovirus encoding a kinase-deficient TGF-βRII, which would prevent autophosphorylation of the TGF-βRI and subsequent downstream events involved with TGF-β signaling, to assess the effects of "knocking out" TGF-β function on cell cycle entry in neonatal rat ventricular cardiac myocytes. A "dominant-negative" approach was used as a strategy because it could inhibit TGF-β signaling via all mammalian forms of TGF-β, thus surmounting the possibility of redundancy. In fact, transfection studies have already demonstrated that the kinase-deficient TGF-βRII can block gene expression and

signaling by all mammalian forms of TGF-β in neonatal ventricular cardiac myocytes {Brand et al., 1993; Brand and Schneider, 1996}. The dominant negative approach may also offer advantages *in vivo* as it has the capacity to block theoretical consequences of maternally derived growth factors, which may act to compensate for loss of function phenotypes {Schneider and Brand, 1995}. Studies have suggested that the normal program of development in some of the TGF-β1 null mice may be attributed to transplacental transmission of maternally derived TGF-β1 to embryos {Letterio et al., 1994}. In addition, the use of a dominant negative approach *in vitro* and *in vivo* can test more mechanistically the function assigned to TGF-β in cardiac myocytes. An adenovirus was generated for gene delivery as it allowed for easy control of expression of the kinase-deficient TGF-βRII in cardiac myocytes. This was a key requirement since sufficient expression of the dominant inhibitor must be assured to overcome the effects of the wild type protein {Schneider and Brand, 1995}. Neonatal rat ventricular myocytes were used as a model system, as they represent cardiac myocytes directly derived from postnatal heart tissue, which have a limited ability to divide, thus representing a relevant cardiac model system to assess the effects of overexpressing the kinase-deficient TGF-βRII on cardiac myocyte cell cycle entry. DNA synthesis was used as the main parameter to assess the effects of overexpressing the kinase-deficient TGF-βRII, as it represents entry into the S phase of the cell cycle {Field and Soonpaa, 1998}.

Novel findings presented in this section of the thesis are that the inhibition of TGF-β signaling, as achieved by overexpression of the kinase-deficient TGF-βRII, resulted in a (i) significant increase in cardiac myocyte DNA synthesis and early mitosis

in 10% serum and (ii) an amplification of FGF-2 induced DNA synthesis in neonatal cardiac myocytes. Inhibition of TGF-β signaling in cardiac myocytes, using the kinase-deficient TGF-βRII, as we have shown provides an additional strategy towards stimulating FGF-2 mediated postnatal cardiac myocyte proliferation/regeneration in the event of injury *in vivo*.

8.4.1 The role of the kinase-deficient TGF-βRII in inhibiting TGF-β signaling in neonatal ventricular cardiac myocytes

Adenoviral expression of the kinase-deficient TGF-βRII cDNA in neonatal rat cardiac myocyte cultures was demonstrated by protein blotting, and was observed by the induction and expression of the major protein of approximately 50 kD in size (Fig. 29). This protein band was not apparent in control transfected cardiac myocytes, which contained the approximately 70 kD sized endogenous TGF-βRII protein (Fig. 29). Assessment of the ratio of endogenous versus kinase-deficient TGF-βRII protein (using MOI 50) suggested that the kinase-deficient TGF-βRII was in excess of approximately 50 fold (n=2) compared to endogenous TGF-βRII. This level of excess would be expected to overcome the function of the endogenous TGF-βRII proteins, as previous reports suggested that at least a five fold excess of the dominant inhibitor is needed {Chang et al., 1994; Chen et al., 1993}. Expression of the kinase-deficient TGF-βRII in cardiac myocytes was also confirmed by immunohistochemistry and co-staining for TGF-βRII and α-actinin (Fig. 30). TGF-βRII expression was localized to the perinuclear, cytoplasmic and membrane regions of cardiac myocytes, consistent with different stages of synthesis and processing as well as its presence on the cardiac myocyte cell surface (Fig. 30). The TGFβR-II antibodies used for protein blotting and immunohistochemistry

were raised to the intracellular kinase domain and recognize an epitope that is common to both the endogenous and kinase-deficient TGF-βRII.

A significant 2.4 fold increase in DNA synthesis (as assessed by BrdU incorporation) and 2.6 fold increase in cardiac myocytes in early mitosis (as assessed by phospho-H1 staining) confirmed stimulation of postnatal cardiac myocyte cell cycle entry in cultures overexpressing the kinase-deficient TGF-βRII in the presence of 10% serum (Fig. 31). The increase in cardiac myocyte DNA synthesis in ΔkTGF-βRII transfected cultures is also shown through immunofluorescence microscopy (Fig. 30). Although it remains to be assessed, the increase in cardiac myocyte DNA synthesis would be expected to result in an increase in proliferation, as it was demonstrated by immunofluorescence staining that ΔkTGF-βRII transfected cardiac myocytes in DNA synthesis can proceed to late G2/M phase (Fig. 32). These results are also consistent with a previous report, which suggested that inhibition of endogenous and/or serum derived TGF-β in cardiac myocytes, using neutralizing antibodies to TGF-β1, can increase cardiac myocyte DNA synthesis {Kardami et al., 1995}. In addition, these results also support studies which demonstrate that TGF-β can cancel the mitogenic effects of serum in cardiac myocytes {Kardami, 1990}. As a result, deletion of the kinase domain of TGF-βRII is capable of generating a dominant inhibitor of TGF-β signal transduction, which can "neutralize" the "inhibitory" effects of TGF-β on cardiac myocyte cell cycle entry.

8.4.2 TGF-β and FGF-2 signaling pathways involved in cardiac myocyte DNA synthesis

TGF-β requires the presence of mitogens to exert its effects on cardiac myocyte growth as no acute effects on growth have been reported in the absence of mitogens {Parker et al., 1990}. Since serum contains mitogenic factors that promote cardiac myocyte growth, this raises the possibility that the increased DNA synthesis observed with the kinase-deficient TGF-βRII in 10% serum may be due to the ability of mitogens present in serum, such as FGF-2, to signal a proliferative response. This is supported by studies which demonstrate that TGF-β can cancel the mitogenic effects of FGF-2 in cardiac myocytes {Engelmann et al., 1992; Kardami, 1990; Kardami et al., 1993}. Indeed, stimulation of cardiac myocyte cultures overexpressing the kinase-deficient TGF-βRII with FGF-2 resulted in an approximately 13 fold increase in cardiac myocyte DNA synthesis when compared to control transfected cultures in the absence of FGF-2 (Fig. 33). The level of cardiac myocyte DNA synthesis observed was significantly greater than the sum of the fold effects observed with either FGF-2 stimulation (3.5 fold) or overexpression of the kinase-deficient TGF-βRII (2.4 fold) alone, suggestive of a synergistic effect (Fig. 33). The increase in cardiac myocyte DNA synthesis observed with FGF-2 alone was consistent with previous reports {Kardami, 1990}. Therefore, these results suggested that expression of the kinase-deficient TGF-βRII can amplify the fold effect on FGF-2 mediated cardiac myocyte DNA synthesis by approximately 10 fold.

There is limited information on the cross-talk between downstream signaling events involved in TGF-β and FGF-2 signaling in cardiac myocytes. Preliminary studies

from our laboratory have demonstrated that the increase in cardiac myocyte DNA synthesis observed with the kinase-deficient TGF-βRII could be blocked using the kinase-deficient or "dominant-negative" FGFR-1 {Sheikh and Cattini, unpublished observations}. These results suggest that the intracellular signaling pathways which mediate TGF-βR and FGFR-1 signaling pathways cross-talk in cardiac myocytes and could be involved in mediate proliferation. There is evidence to suggest that the PKC pathway may be a likely target in neonatal cardiac myocytes {Kardami et al., 2001}. This is largely based on studies which demonstrate that TGF-β could block FGF-2 induced PKC translocation to the membrane(and presumably its activation) but not the activation of ERK1/2 MAPK in cardiac myocytes {Kardami et al., 2001}. TGF-β can also block FGF-2 induced connexin43 phosphorylation, which was also shown to be mediated by PKC {Doble et al., 2000; Kardami et al., 2001}. The role of PKC in the amplification of FGF-2 mediated cardiac myocyte cell cycle entry by the kinase-deficient TGF-βRII remains to be determined.

8.4.3 Conclusions

In conclusion, TGF-β signaling clearly plays an important role in limiting cardiac myocyte growth. Using a dominant negative approach, our results demonstrated that overexpression of the kinase-deficient TGF-βRII could stimulate serum-induced cardiac myocyte cell cycle entry and amplify FGF-2 induced DNA synthesis in neonatal cardiac myocytes. These results also suggest that dominant-negative genes, like the kinase-deficient TGF-βRII, could serve as generic approach complementary to gene ablation to create loss-of-function mutations *in vivo*. These results strengthen the notion that

inhibition of TGF-β function could serve as an additional strategy to increase/amplify FGF-2 mediated regeneration/ proliferation in postnatal cardiac myocytes in the event of injury *in vivo*.

CHAPTER 9

FUTURE DIRECTIONS AND FINAL REMARKS

9.1 Preamble

The four studies described in this thesis were designed to regulate the FGF-2 axis in order to exploit its effects on cardiac myocyte protection and growth, to provide the basis for novel treatments and/or prevention of cardiovascular diseases. The following section will discuss future directions for the study of FGF-2 in the areas of: (i) cardioprotection, (ii) cardiac angiogenesis, (iii) cardiac hypertrophy, and (iv) cardiac regeneration, which have stemmed from the work described in this thesis. In addition, the therapeutic utilities of FGF-2 in acute myocardial infarction and the pathogenesis of cardiac disease will also be discussed.

9.2 Endogenous FGF-2: Part of the Endogenous Cardioprotective Response ?

Parallels have been drawn between the signaling pathways which mediate FGF-2 induced cardioprotection (via exogenous addition) and ischemic preconditioning (section ***2.4.8.7***). Ischemic preconditioning is described as the endogenous cardiac myocyte protection that occurs following brief periods of ischemia {Murry et al., 1986}. Considerable attention is focussed in this area as the factors and signaling pathways which mediate ischemic preconditioning are currently being exploited in treatment strategies for patients undergoing cardiac surgery and/or suffering from cardiac disease. To date there is no information on the role of endogenous FGF-2 in ischemic preconditioning. Future studies in the area of FGF-2 and cardioprotection could be aimed

at elucidating the role of endogenous FGF-2 in ischemic preconditioning through the use of both *in vitro* and *in vivo* model systems.

There is limited information on the mechanisms involved in the increased cardiac myocyte resistance to injury afforded by FGF-2, however, our results suggest that both PKC and stress activated MAP kinases may play a role (Chapter 4), since these pathways are upregulated in FGF-2 TG mice and have both been implicated in ischemic preconditioning and cardioprotection {Kitakaze et al., 1997; Nakano et al., 2000; Ping et al., 1999; Yoshida et al., 1997}. In fact, evidence from isolated rat heart studies using exogenous FGF-2 demonstrated that the cardioprotective effects of FGF-2 are mediated by PKC {Padua et al., 1998}. Other major players in ischemic preconditioning include, mitochondrial K^+ATP channels, which are implicated as the end effectors of acute ischemic preconditioning {Baines et al., 1999a}, and NO which is the prime mediator of delayed acute preconditioning {Baines et al., 1999a}. Thus, future studies could be aimed at elucidating the role of PKC, stress activated MAP kinases, as well as mitochondrial K^+ATP channels and NO (i.e., pathways associated with ischemic preconditioning and known to be triggered by FGF-2) in the mechanisms involved in endogenous FGF-2 mediated cardioprotection. Recent evidence has implicated a role for NO in mediating the cardioprotective effects of FGF-2 (exogenous administration) during stunning {Hampton et al., 2000}. Our results also do not exclude the possibility that the protective effects of exogenous FGF-2 are due to an amplification of endogenous FGF-2 production, future studies aimed at blocking endogenous FGF-2 production in FGF-2 treated hearts (i.e., antisense strategies or inducible systems with cardiac specific promoters) could be used

to assess the contribution of the protective of effects of exogenous versus endogenous FGF-2 in the FGF-2 TG mouse model.

Our results also suggest that release of FGF-2 contributes to the increased cardiac myocyte resistance to injury observed in FGF-2 TG mice. To confirm this link, future directions could be aimed at determining a direct role for FGF-2 release in FGF-2 mediated cardioprotection. Employing a strategy to inhibit or increase FGF-2 release (i.e., β-adrenergic agonists and antagonists) in the FGF-2 TG hearts prior to cardiac injury and then assessing myocyte viability could be used. FGF-2 released from cardiac myocytes would be expected to act on cardiac myocyte cell surface FGF-2 receptors to mediate protection. Although FGFR-1 was demonstrated to be essential in mediating the biological effects of FGF-2 on cardiac myocyte proliferation (Chapter 6), its role in cardioprotection is unknown. Thus, future directions could also be aimed at determining the role of FGFR-1 in FGF-2 mediated cardioprotection. This would be an important avenue of research, since this could lead to novel drug designs aimed at modulating FGFR activity in an effort to mediate cardioprotection, which would presumably take advantage of the natural mechanism of FGF-2 release (i.e., availability) from the heart. There is some evidence to suggest that FGFR-1 may be important in FGF-2 mediated cardioprotection as exogenous administration of a mutated FGF-2 protein with reduced affinity to FGFR-1 could not afford cardioprotection, unlike the wild type FGF-2 protein subsequent to cardiac ischemia-reperfusion injury {Jiang et al., 2001}. Alternatively, blocking activation of FGFR-1 signaling in postnatal myocytes could be used as an approach. Thus, generating a transgenic mouse model that can induce expression of a

dominant-negative (kinase-deficient) form of FGFR-1 in postnatal cardiac myocytes (use of α-MHC cardiac specific promoter) at different times during injury could be used to address the role of FGFR-1 in FGF-2 mediated cardioprotection.

9.3 Endogenous FGF-2 Gene Regulation and Cardioprotection

The FGF-2 TG mouse model has also contributed significantly towards the notion that targeting endogenous FGF-2 production, may be a credible method for improving cardiac health and resistance to injury. Although a genetic approach can be used to increase endogenous FGF-2 production (i.e., gene therapy), clearly, alternate methods, which are perhaps less invasive, can be used to increase endogenous FGF-2 production. Although our results demonstrated that the natural catecholamine, NE, which acts via α and β-adrenergic receptors can increase FGF-2 synthesis at the level of transcription in adult cardiac myocytes from –1058FGFp.*luc* mice, future studies could, however, be aimed at determining the specificity of the adrenergic response in adult cardiac myocytes. Determining the underlying mechanisms which regulate these pathways would be important in not only exploiting strategies to increase endogenous FGF-2 production and thus, protection, in cases of cardiac injury or in maintaining a healthy myocardium but also in "tailoring" therapies for individuals dependent on their physical health and/or use of medications.

β-Adrenergic receptor signaling is perhaps the most prominent pathway to modulate cardiac function under normal physiological conditions. Since FGF-2 release can be increased by β-adrenergic agonists from the myocardium through contractions

{Clarke et al., 1995} and endogenous FGF-2 is implicated in cardiac myocyte resistance to injury (Chapter 5), it is thought that β-adrenergic regulation of FGF-2 synthesis may be important in maintaining a healthy myocardium. This strategy could conceivably be used as a "preventative" approach towards decreasing the risk of cardiac injury. β-Adrenergic signaling has been proposed to increase FGF-2 synthesis largely through a mechanism involving autoregulation of FGF-2 (see section ***2.4.1***), however to date there is no direct evidence of this relationship. Thus, future studies could be aimed at determining the role of autoregulation of FGF-2 (i.e., FGF-2 release) on FGF-2 transcription in adult cardiac myocytes as well as its' role in β-adrenergic stimulation of FGF-2 synthesis. There is already evidence in the literature, albeit indirect, to suggest that β-adrenergic regulation of FGF-2 synthesis may reduce the risk of cardiac injury. Physical exercise could conceivably result in an increase in endogenous FGF-2 release from the heart (via increase in heart rate), which could lead to increased FGF-2 synthesis (via autoregulation). The benefits of physical exercise are undisputed in the prevention of cardiovascular diseases, and given FGF-2's role in cardioprotection it is conceivable that FGF-2 would be a component of this scenario. Future studies could also be aimed at determining whether physical exercise plays a role in regulating FGF-2 synthesis (-1058FGFp.*luc* mice) as well as cardioprotection in cardiac injury models in the FGF-2 TG mouse models. Clearly, future studies aimed at elucidating the role of β-adrenergic stimulation (via other regulators) of FGF-2 synthesis in *in vitro* and *in vivo* models of cardiac injury would be of great value in determining the benefits of regulating FGF-2 transcription as an approach towards mediating cardioprotection.

α-Adrenergic receptor signaling, on the other hand, is thought to play a more prominent role in the heart in a "disease-state", once β-adrenergic receptors are downregulated and responsiveness is lost. In this case, it has been proposed that α-adrenergic receptors serve as a reserve mechanism in the heart to maintain its responsiveness to catecholamines {Will-Shahab and Schubert, 1991}. Thus, stimulating FGF-2 synthesis via α-adrenergic stimulation could play an important role in increasing cardiac resistance to injury in patients with various pathological conditions (i.e., lost β-adrenergic receptor responsiveness). Future directions in the area of α_1-adrenergic receptor regulation of FGF-2 synthesis could be to assess its role in chronic models of injury or pathological conditions. Egr-1 is proposed to be one of the central mediators of FGF-2 synthesis in cardiac myocytes as well as other cell types (section ***2.4.1***). Although a role for transcriptional regulation of FGF-2 was not established in these studies, Egr-1 knockout mice were demonstrated to be prone to increased sensitivity to cardiac stress, according to the pattern of gene expression following catecholamine-treatment {Saadane et al., 2000}. Future studies could be aimed at identifying other regulators of Egr-1 as a means to modulate FGF-2 synthesis in cardiac myocytes and thus, possibly cardioprotection in various cardiac injury/stress models. PKC is implicated as the cytoplasmic intermediate involved in mediating the effects of Egr-1 on FGF-2 transcription in cardiac myocytes (section ***2.4.1***) as well as FGF-2 mediated cardioprotection {Padua et al., 1998}. Therefore, another future avenue of research could involve delineating the role of the PKC pathway in mediating FGF-2 synthesis in adult cardiac myocytes in both *in vitro* and *in vivo* model of injury.

9.4 Endogenous FGF-2 and Cardiac Angiogenesis

Consistent with a role for FGF-2 in the vasculature, chronic overexpression of FGF-2 during development resulted in an increase in capillary density in the adult heart *in vivo*. Future studies could be aimed at more fully characterizing the effects of FGF-2 on other vascular parameters including, vessel branching and vascular tone. Vascular tone would be an important parameter to assess, since ablation of the endogenous FGF-2 gene in mice was associated with reduced vascular tone resulting in a hypotensive phenotype {Dono et al., 1998; Zhou et al., 1998}. Although the results presented in Chapter 4 suggested that the protective effects of FGF-2 were independent of effects on the vasculature, the ex-vivo cardiac injury model used for these experiments may have precluded a role for the increased angiogenesis in FGF-2 TG hearts during injury. Several studies have demonstrated that the cardioprotective effects of FGF-2 in various *in vivo* models of cardiac injury are attributed to the angiogenic effects of FGF-2 {Harada et al., 1994; Yanagisawa-Miwa et al., 1992}. Future studies aimed at using *in vivo* models of cardiac injury (e.g., coronary ligation) could be used to fully establish the role of the increased angiogenesis observed in FGF-2 TG hearts during injury.

Although, FGF-2 is one of the most extensively studied angiogenic growth factors to date {Simons et al., 2000}, the mechanisms which mediate angiogenesis are not fully established. FGF-2 TG mouse hearts were demonstrated to contain increased levels of activated p38 and α-PKC (Fig. 9) and both αPKC and p38 are implicated in angiogenesis and/or FGF-2 mediated angiogenesis, respectively {Harrington et al., 1997; Erdreich-Epstein et al., 2000; Mudgett et al., 2000; Rousseau et al., 1997; Tanaka et al.,

1999; Yamamura et al., 1996; Yang et al., 2000}. Thus, future studies could be aimed at determining the roles of these pathways in FGF-2 mediated angiogenesis. VEGF has also been implicated to play an important role in FGF-2 mediated angiogenesis {Seghezzi et al., 1998}. Thus, future studies could also be aimed at determining the role of VEGF in FGF-2 mediated angiogenesis in FGF-2 TG mice. Crossing FGF-2 TG mice with transgenic mice which can conditionally turn off VEGF expression specifically in vascular cell types at different times during development can be used as a strategy to determine the role of VEGF in FGF-2 mediated angiogenesis.

9.5 Endogenous FGF-2 and Cardiac Hypertrophy

To reconcile whether FGF-2 has a role in cardiac hypertrophy, future studies could be aimed at inducing cardiac hypertrophy using a variety of methods (e.g., aortic banding, catecholamines) in FGF-2 TG mice *in vivo* and then assessing the hypertrophic response compared to non-TG mice. Determining a role for FGF-2 in cardiac hypertrophy would be essential in gaining an understanding of the role that FGF-2 may play in the "big picture" of the pathogenesis of cardiac disease.

9.6 Regulation of the FGF-2 Axis by Overexpression of FGFR-1 and Kinase-Deficient TGF-βRII: Potential Role in Cardiac Myocyte Regeneration

The results presented in Chapters 6 and 7, have demonstrated at least two strategies to regulate the FGF-2 axis (FGFR-1 and TGF-β), which can significantly influence the effects of FGF-2 on postnatal cardiac myocyte proliferation and/or cardiac myocyte cell cycle entry *in vitro*. In terms of the kinase-deficient TGF-βRII experiments, future studies could be aimed at determining whether this response translates into an

increase in cell number *in vitro*, which would be indicative of M phase entry. Ultimately, future directions in the area of FGF-2 and cardiac regeneration could be aimed at testing both the effects of overexpression of FGFR-1 as well as kinase-deficient TGF-βRII in adult cardiac myocyte proliferation *in vitro* and/or *in vivo*. Generating transgenic mice which inducibly overexpress FGFR-1 or kinase-deficient TGF-βRII in cardiac myocytes (use of the cardiac specific α-MHC promoter) could be used as a means to assess the effects of both strategies on cardiac myocyte proliferation/cell cycle entry in adult hearts during development and after cardiac injury *in vivo*. In addition, we could use a combinatory approach to inhibit "anti" FGF-2 signaling pathways and stimulate "pro" FGF-2 signaling pathways by crossing transgenic mice overexpressing TGF-βRII with mice overexpressing FGFR-1 as well as FGF-2 to determine whether this strategy could be infinitely more potent in stimulating a proliferative/regenerative response by FGF-2 during development and/or after injury. The could in essence maximize the chances for FGF-2 to exert a mitogenic effect in adult cardiac myocytes *in vivo*. Future directions could also be aimed at identifying the underlying intracellular signaling pathways (i.e., PKC, MAPK, Smad) which mediate the proliferative/regenerative response by FGF-2 via overexpression of FGFR-1, kinase-deficient TGF-βRII and FGF-2. This may uncover novel intracellular targets which could be used to stimulate a proliferative/regenerative response in the postnatal heart *in vivo*. Recently a study by Orlic and colleagues, provided the first account of an approach to induce cardiac myocyte regeneration in the postnatal heart *in vivo* following cardiac injury {Orlic et al., 2001}. This study demonstrated that local delivery of bone marrow cells have the capability of regenerating acutely significant amounts of contracting myocardium following injury *in vivo* thereby ameliorating the

outcome of cardiac disease {Orlic et al., 2001}. It is conceivable, given FGF-2's role in cardiac myocyte proliferation that it may be one of the key players, which may be part of the regenerative response. Future directions could also be aimed at assessing a role for the FGF-2 axis in this model of cardiac regeneration.

9.7 Therapeutic Potential of FGF-2 forCardiac Disease

There are several key events following acute cardiac injury *in vivo* which define the pathogenesis of cardiac disease and these include: (i) ischemia-reperfusion injury, (ii) inflammatory response, (iii) cardiac hypertrophy, (iv) cardiac fibrosis and (v) cardiac arrhythmias. Although the role of FGF-2 has not been defined in all of these events, it is conceivable that given the pleiotropic actions of FGF-2 in the heart, that FGF-2 could have effects at all of these stages. The following section will seek to define a role for FGF-2 in these events and postulate on the possible beneficial or adverse effects of using FGF-2 as a therapeutic agent in the heart during these various stages.

In terms of ischemia-reperfusion injury, several reports support a role for FGF-2 (endogenous and exogenous) in stimulating cardiac myocyte protection in various animal models of acute ischemia-reperfusion injury, when supplied to the heart prior to ischemia (Chapter 4; section ***2.4.8.7***}. However, there is limited information on whether the protective effects of FGF-2 would prevail if it were supplied to the heart <u>after</u> ischemia. In other words, would the heart receive the same type of protection if FGF-2 were increased during reperfusion injury? In the setting of acute myocardial ischemia using isolated hearts the data is controversial. Preliminary observations using isolated rat

hearts, demonstrated that if FGF-2 was administered after 20 min of ischemia it was almost as effective in increasing contractile recovery as when it was administered before ischemia {Padua, 1998}. On the other hand, administration of FGF-2 at the time of reperfusion, in isolated mouse hearts after stunning (causing severe ventricular dysfunction) did not improve myocardial recovery when compared to administration prior to stunning {Hampton et al., 2000}. This difference may, however, reflect the type and severity of injury incurred in isolated hearts thus, raising the possibility that the amount of viable myocardium remaining after injury may dictate whether FGF-2 can protect the heart, if administered subsequent to ischemic injury. Clearly, a role for FGF-2 during cardiac reperfusion injury *in vivo* remains to be defined.

A key event following myocardial reperfusion injury is its association with a dramatic inflammatory response, which could lead to myocyte injury {Entman et al., 1994}. Although a role for FGF-2 in the cardiac inflammatory response following injury remains to be defined, several pieces of evidence suggest a possible link between FGF-2 signaling and infiltrating T cells. With regard to recruitment of T cells, FGF-2 is known to be a chemoattractant affecting cell attachment and migration (see section ***2.4.8***) {Nicosia and Villaschi, 1999}, accumulating at sites of myocardial injury *in vivo* {Padua and Kardami, 1993}. With regard to proliferation of T cells, the (co)-stimulatory effects of FGF-1 and FGF-2 on proliferation of FGFR-1 expressing T cells have been demonstrated *in vitro* {Miller et al., 1998; Woodley et al., 1991}. In addition, preliminary studies from our laboratory using FGF-2 TG mice, have demonstrated that endogenous overexpression of FGF-2 can exacerbate the cardiac inflammatory response following

cardiac injury *in vivo* in a T cell dependent manner {Meij et al., 1998}. The inflammatory response can be viewed as either a beneficial or detrimental component of injury (i.e., increase or decrease myocardial healing) as it has been shown to be clearly dependent on the cytokines expressed {O'Garra and Murphy, 1996}. Future directions could, as a result, be aimed at assessing the cytokines involved in mediating the inflammatory response by FGF-2 to clearly determine the long-term effects on myocardial repair.

There is currently limited information on the role of FGF-2 in ventricular remodelling. In terms of cardiac hypertrophy, however, there is evidence, albeit indirect, via FGF-2 knockout mice to suggest that FGF-2 promotes cardiac hypertrophy *in vivo* {Schultz et al., 1998}. Since hypertrophy is viewed as an adaptive response, this raises the possibility that increasing FGF-2 levels after cardiac injury could promote increased adaptation to injury by increasing hypertrophy. It remains to be determined if FGF-2's proposed role in cardiac hypertrophy would be beneficial to the heart subsequent to injury *in vivo*. Clearly, a direct evidence for a role for increased FGF-2 on stimulation of cardiac hypertrophy *in vivo* remains to be defined. In terms of cardiac fibrosis, FGF-2 has been shown to increase both fibroblast/myofibroblast proliferation {Galzie et al., 1997; Hoerstrup et al., 2000}, which could conceivably have an adverse effect in the heart leading to increased scar formation or a "stiffer" heart in the event of cardiac injury *in vivo*. On the other hand, studies have demonstrated that FGF-2 potently inhibits collagen fiber production by human smooth muscle cells {Pickering et al., 1997}. It is thought that this represents a mechanism for thinning the local collagen environment during vascular remodeling, which could in turn be important in intimal accumulation of smooth

muscle cells or destabilization of an atherosclerotic plaque {Pickering et al., 1997}. Clearly, FGF-2's role as a therapeutic agent during cardiac fibrosis remains to be defined. Arrhythmias are another complication following cardiac injury which is dependent on the degree of fibrosis in the heart {Poole-Wilson et al., 1997}. Although responses to increased load or injury are generally adaptive, remodeling of intercellular junctions under conditions of severe stress creates anatomic substrates conducive to the development of lethal ventricular arrhythmias {Saffitz, 2000}. Potential mechanisms controlling the level of intercellular communication in the heart include regulation of connexin turnover and dynamics {Saffitz, 2000}. FGF-2 has been shown decrease cardiac myocyte communication through a mechanism involving phosphorylation of connexin43 which is mediated by PKC {Doble et al., 1996; Doble et al., 2000}. Since there is a strong correlation between decreased communication and increased proliferation, it has been advocated that in the event of injury FGF-2's role in decreasing cardiac myocyte communication could potentially stimulate a proliferative/regenerative response in the postnatal heart {Kardami et al., 2001}. However, increasing FGF-2 levels in the adaptive phase of cardiac disease, could also possibly play a role in exacerbating cardiac arrhythmias. Preliminary observations in FGF-TG mice, revealed that increasing endogenous FGF-2 levels in the heart led to a significant decrease in total levels of connexin 43 {Kardami, Fandrich, Sheikh and Cattini, unpublished observations}. The physiological effects of this decrease and its role in cardiac myocyte proliferation and/or risk towards cardiac arrhythmias following injury *in vivo* remains to be determined.

However, if we assume that physical exercise can increase FGF-2 synthesis (via increased heart rate and autoregulation), then there is evidence to support the hypothesis that increasing endogenous FGF-2 levels after injury may be beneficial. In fact, in randomized trials it was shown that patients that exercise after suffering from a myocardial infarction can reduce their overall mortality rate by 20%, when compared to patients that don't exercise {O'Connor et al., 1989}. In addition, if we are advocating a role for FGF-2 as a form of therapy for patients following myocardial infarction, we must also assess its role in the scheme of other drug therapies (e.g., β-blockers and angiotensin converting enzyme inhibitors). This would be important since many patients suffering from myocardial infarction or congestive heart failure are on a variety of medications, which have a various effects including reducing heart rate and decreasing fluid retention {Poole-Wilson et al., 1997}. Given their diverse role, it is not clear how these drugs would impact endogenous FGF-2 release and synthesis in the ischemic heart. However, tailoring drug therapies or using drugs, which act to increase endogenous FGF-2 production may be an alternate strategy at increasing cardiac myocyte protection. In addition, we cannot ignore the outcome of phase I clinical trials which have indeed, demonstrated that exogenous administration of FGF-2 can result in functionally significant benefits in the human ischemic myocardium (see section ***2.4.8.9***). Although current studies in patients primarily exploit the angiogenic effects of FGF-2 on the vasculature to promote collateral formation in ischemic hearts {Laham et al., 2000; Udelson et al., 2000}, future studies could also be aimed to exploit FGF-2's direct protective effects on adult cardiac myocytes as well as the repair response after injury.

9.8 Final Remarks

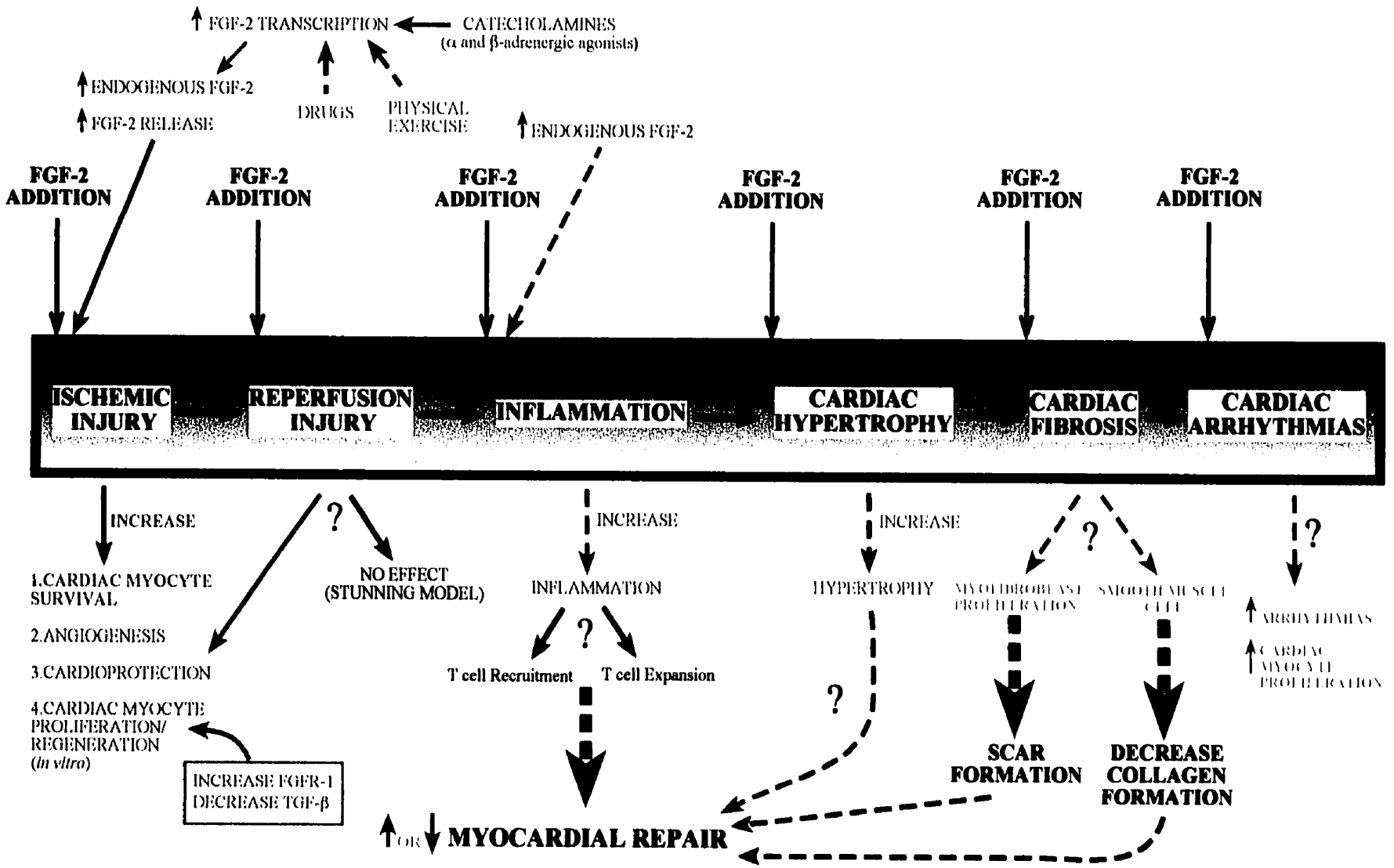
Figure 34 gives a schematic overview of the ideas presented in the discussion and future directions. Using the FGF-2 TG model, we have demonstrated that increasing endogenous FGF-2 production can be used as a strategy to increase "local" FGF-2 release from the heart and increase cardiac myocyte resistance to injury. A direct effect of FGF-2 on cardiac myocyte protection was implicated. A role for the activation of stress MAP kinases and αPKC was also discussed. By developing a method to isolate and culture adult mouse cardiac myocytes, we were able to exploit the -1058FGFp.*luc* mouse model to identify regulators of FGF-2 synthesis, at the transcriptional level, in adult cardiac myocytes. This strategy could potentially be used to exploit the protective effects of endogenous FGF-2 in maintaining cardiac health and increasing resistance to injury. Specifically, a role for adrenergic receptor signaling pathways was implicated. A role for endogenous FGF-2 in angiogenesis and cardiac hypertrophy was also discussed. In terms of stimulation of cardiac myocyte growth, we have demonstrated that FGFR-1 availability and levels clearly play a significant role in entry of cardiac cells into the cell cycle. However, the significance of the FGFR-1 isoform switch during cardiac development remains to be determined. We also demonstrated using a dominant negative approach, that inhibiting TGF-β signaling can amplify FGF-2 induced entry of postnatal cardiac myocytes into the cell cycle. Future studies could be directed at determining the mechanisms involved in FGF-2 mediated cardiac myocyte protection and proliferation, as well as exploiting the possibilities of using combinatory approaches to maximize the beneficial effects of FGF-2 in the response following injury.

Figure 34.

Therapeutic Potential of FGF-2 in Cardiac Disease.

Schematic representation of the events following acute cardiac injury *in vivo* which describe the pathogenesis of cardiac disease as well as the postulated (dashed arrows) and known (solid arrows) biological consequences of increasing FGF-2 at various stages during this process. The stages include: (i) acute ischemic injury, (ii) reperfusion injury, (iii) inflammatory response, (iv) cardiac hypertrophy, (v) cardiac fibrosis and (vi) cardiac arrhythmias. Text in red directly relate to work presented in this thesis. This includes, the role of endogenous FGF-2 in cardioprotection (Chapter 4), adrenergic regulation of endogenous FGF-2 gene expression in adult cardiac myocytes (Chapter 5), as well as the role of FGFR-1 (Chapter 6) and TGF-β (Chapter 7) in regulating FGF-2 mediated cardiac myocyte cell cycle entry. The postulated effects of FGF-2 on an inflammatory response in the heart, in FGF-2 TG mice, are also presented in this schemata {Meij et al., 1998}. The effects of FGF-2 on reperfusion injury remain controversial as the cardioprotective effects of FGF-2 have been observed in isolated rat hearts in a global ischemia-reperfusion injury model {Padua, 1998}, but not in a stunning model of injury {Hampton et al., 2000}. Additionally, the postulated effects of FGF-2 on increasing cardiac hypertrophy, regulating scar formation and collagen deposition during cardiac fibrosis as well as modulating cardiac arrhythmias are also outlined.

THERAPEUTIC POTENTIAL OF FGF-2 IN CARDIAC DISEASE



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