CHARACTERIZATION OF FIBROBLAST GROWTH FACTOR-16 EXPRESSION AND BIOLOGICAL FUNCTION IN THE HEART

BY

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Abstract

Every year millions of people suffer myocardial infarction. Although many survive if given proper medical treatment in a timely fashion, the damage which occurs is often irreversible and results in cardiovascular disease and eventually death. While some animal species possess the ability to regenerate damaged adult myocardium (i.e. amphibians), mammals have a limited regenerative ability. Thus, it is prudent to investigate the mechanisms and factors that regulate mammalian cardiac development, injury-repair response and remodeling in order to gain insight as to potential targets for future therapies.

The fibroblast growth factor (FGF) family is of particular interest with regards to cardiac development and remodeling. Several of these growth factors have not only been shown to be required for proper cardiac development but also show promise as therapeutic agents for the prevention and treatment of cardiac injury. The sixteenth member of this family, FGF-16, has been cloned from the RNA of adult rat and human tissues, and is the only FGF preferentially expressed in the heart. Despite a close association with the heartits basic properties and function remain unknown. A series of studies was thus undertaken to generate the tools necessary to examine FGF-16 and gain insight into: 1) the primary structure of mouse FGF-16; 2) the expression pattern of mouse FGF-16 RNA and protein; 3) glycosylation and secretory properties of FGF-16 protein; 4) the cardiac cell types expressing FGF-16; and 5) possible signaling pathways and targets of FGF-16.

The cDNA for FGF-16 was cloned from adult mouse heart by RT-PCR and sequenced. The nucleotide sequence revealed 95% and 97% identity when compared to that of human and rat, respectively. The amino acid sequence translated to a protein with an even higher degree of similarity (99%) between species, differing by only two amino acids.

In order to determine the tissue distribution of FGF-16 in the mouse, FGF-16 cDNA was used as a probe for RNA blotting using RNA isolated from various mouse tissues. Of those examined (including brain, spleen, lung, liver, skeletal muscle, kidney and testes), a signal corresponding to the expected 1.8 kb transcript was detected only in heart. The timing of FGF-16 expression was also examined using RNA isolated from the hearts of mice at various ages. Levels of FGF-16 mRNA were found to increase dramatically from the time of birth and reached levels that were similar to that of adult heart within 4 days after birth. FGF-16 expression was detected in primary cultures of neonatal rat cardiac myocytes, suggesting that myocytes are a major source of FGF-16 within the heart.

Expression systems for FGF-16 were developed for the purpose of testing of antibodies as well as biological activity of FGF-16. Transfection of human embryonic kidney (HEK-293) cells with FGF-16 expression vectors resulted in the secretion of FGF-16 protein, which was detectable by protein blotting. However, FGF-16 detected in the conditioned media from HEK-293 cells with an apparent molecular weight (25.4 kDa) larger than that of the predicted protein or recombinant protein (23.7 kDa). This difference was determined to be due to glycosylation of FGF-16. A glycosylated form of FGF-16 was detected in the conditioned media from neonatal cardiac myocytes but not

non-myocytes. This is consistent with data obtained by RNA blotting in these same cell types.

Recombinant as well as FGF-16 from conditioned media was tested for biological activity through the use of a cell line (BAF-3) expressing an FGF receptor (FGFR-2c) known to be activated by recombinant FGF-16. Cells treated with either source of FGF-16 resulted in a decreased rate of apoptosis when cells were starved of a specific factor necessary for survival of this cell type (interleukin-3). Examination of adult mouse heart, as well as primary cultures of cardiac myocytes and non myocytes revealed the presence of transcript for FGFR-2c. However, a similar concentration of FGF-16 did not protect cardiac myocytes from cell death in an in vitro model of oxidative stress (H_2O_2).

Primary cultures of neonatal cardiac myocytes and non myocytes were also examined for evidence of MAPK activation, as an indicator of responsiveness to this factor. FGF-16 activated ERK1/2, p38 and p54 JNK MAPKs in cardiac myocytes, and a combination of FGF-2 and FGF-16 resulted in a greater stimulation of p38 and p54 JNK MAPKs as compared to FGF-.. Non-myocyte cultures demonstrated no such MAPK responses toward FGF-16.

In conclusion, we have shown that the FGF-16 transcript appears shortly after birth and results in the production of a glycosylated and secreted protein, which suggests the capacity for autocrine, paracrine and/or endocrine function of this protein. The ability to affect MAPK signaling in myocytes but not non-myocytes is unique and indicates myocyte-specific roles for FGF-16. Its developmental pattern of expression furthermore implies that FGF-16 may be directly involved in myocyte hypertrophy, binucleation, and differentiation that occur shortly after birth.

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Abbreviations

Ab60	antibody from rabbit #61660
Ab61	antibody from rabbit #61661
AbPeprotech	commercial antibody from Peprotech
AbSantacruz	commercial antibody from Santacruz
Ask	apoptosis signal regulating kinase
ATF	activated transcription factor
BAF-3	bovine progenitor cell line
Bp	base pair
BSA	bovine serum albumen
CDNA	complementary DNA
c-terminal	carboxy terminal
ECM	extracellular matrix
EDTA	ethylenedinitrilotetraacetic acid
EGFP	enhanced green fluorescent protein
EGFP-FGF-16	enhanced green fluorescent protein FGF-16 fusion protein
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ERK	extracellular regulated kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGF-1	fibroblast growth factor-1 or acidic FGF
FGF-2	fibroblast growth factor-2 or basic FGF

FGFR	fibroblast growth factor receptor
FGFRL-1	fibroblast growth factor receptor like
FRS2	FGFR substrate 2
Gab-1	GRb2 associated binder
H_2O_2	hydrogen peroxide
HEK 293	human embryonic kidney
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hFGF-16	human fibroblast growth factor 16
HRP	horse radish peroxidase
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
IL-3	interleukin-3
IPTG	isopropyl-beta-D-thiogalactopyranoside
ITS	insulin transferrin selenium
JNK	c-Jun NH_2-terminal kinase
kb	kilobase
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic
MAPK	mitogen activated protein kinase
min	minutes
MKK	mitogen activated protein kinase kinase
MKK4	mitogen activated protein kinase kinase 4
MKK7	mitogen activated protein kinase kinase 7

M-MLV	(Moloney Murine Leukemia Virus) Reverse Transcriptase
$NaC_6H_5O_7$	sodium citrate
NaCl	sodium chloride
NaH_2PO_4	sodium phophate monobasic
Na_3O_4V	sodium orthovanadate
NRCM	neonatal rat cardiac myocytes
n-terminal	amino-terminal
OCT	optimal cutting temperature
p38	p38 mitogen activated protein kinase
p42	ERK2
p44	ERK1
p46	c-Jun NH2-terminal kinase
p54	c-Jun NH2-terminal kinase
PBDS	phosphate buffered saline digestion solution
PBS	phoshate buffered salt solution
PBS-CMF	phosphate buffered salt solution calcium and magnesium free
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase C-gamma
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidine fluoride
QCB	Quality Controlled Biochemicals Inc.

RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcriptase polymerase chain reaction
SAPK	stress activated protein kinase
SDS-PAGE	sodium dodecylsufate polyacrylamide gel electrophoresis
Shp2	a protein tyrosine phosphatase
SNT	Suc1-associated neurotrophic factor target
TAK1	transforming beta activated kinase
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween-20
TNF-α	tumor necrosis factor alpha
UV	ultraviolet
WEHI	IL-3 expressing leukemia blood cell line

Chapter 1

1.0 Introduction

The following will introduce the family of fibroblast growth factor (FGF) proteins of which FGF-16 is the sixteenth member. The basic characteristics of the family as well as some of the differences between members will be discussed. This includes what is known about the structure, posttranslational modifications, different FGF variants and cellular trafficking. Particular attention will focus on the many different FGF receptor(s) (FGFR) and how the combination of both FGF and FGFR provide for an enormous plasticity in terms of signaling capability. Attention will then focus on what is known about the different FGFs as they appear during cardiac development and how FGF-16 is different from that of other previously reported members. Finally, some of the fundamental questions that are raised by the lack of basic characterization of FGF-16 will be presented. This will be followed by a summary of the methods used to address these questions.

1.1 Fibroblast growth factor family

FGF molecules or (fibroblast growth factor) proteins were initially cloned and identified based on their mitogenic properties. The first FGFs to be cloned were acidic fibroblast growth (Jaye et al., 1986) and basic fibroblast growth factor (Abraham et al., 1986). Since then a plethora of growth factors have been identified and named in a numerical fashion based on the order of discovery. However, FGF-1 (acidic) and FGF-2 (basic) still remain the prototypic growth factors for the family and are perhaps the best

characterized of all the family members. Among those discovered is FGF-16, which is most closely related to FGF-9 and FGF-20 in terms of sequence identity (see Figure 1.1 for relationships between the various FGF family members).

There are currently 23 members of the FGF family, but only 22 members are found in humans and are believed to have arisen as a result of gene duplication and translocation (Baird and Klagsbrun 1991). The isoform missing member in humans, FGF-15, has been described for mouse but is believed to be a possible ortholog to human FGF-19 (Ornitz and Itoh 2001). Although there has been a great deal of research characterizing the function of some of the first family members, their functions and relative importance is still not fully understood. While the term fibroblast growth factor implies the stimulation of fibroblasts, the family has been found to affect numerous cell types with specificity and not necessarily fibroblasts. For example, FGF-7 has been demonstrated not to target fibroblasts but rather endothelial cells (Rubin et al., 1989).

1.1.1 FGF structure

The FGF structure consists of a core region comprised of 140 amino acids whose homology is highly conserved. Within this core region are six identical amino acid residues with 28 conserved residues, ten of which interact with the FGF receptor (Ornitz et al., 2001). The core region has a secondary structure comprised of twelve antiparallel β-strands that form a cylindrical barrel. At either end, the carboxy and amino-terminal regions are more variable between family members (Ago et al., 1991, and Zhang et al., 1991). Some of the FGFs contain cysteine residues which result in the formation of disulfide bridges within the protein, while others do not (Powers et al., 2000). Although

these bridges may affect the structure of the protein, they do not seem to affect the function in the case of FGF-2 as substitution of the cysteine residues through mutation still results in a biologically active protein (Arakawa et al., 1989). An investigation into the secondary structure of FGF-16 reveals two cysteine residues corresponding to positions C^{67} and C^{133}, however they do not appear to form a disulfide bridge (Danilenko et al., 1999).

1.1.2 Different FGF isoforms

Some of the genes for FGFs have been found to code not only for one but several different sized transcripts. For example, FGF-3 is known to have at least six different size transcripts that all code for variations of the same size protein (Acland et al., 1990). Alternate splicing of the first exon in FGF-13 has been found to result in an alternate amino-terminus; however, the biological consequences if any have not yet been determined (Munoz-Sanjuan et al., 1999). Variation in protein size has also been found to result from alternative translation start sites, as is the case with FGF-2 (Florkiewicz and Sommer 1989, Prats et al.,1989). These result in 18 kDa and 34 kDa forms of FGF-2 which Kardami and colleagues propose to have different functions on cardiac physiology. While the 18 kDa form is said to be part of an adaptive response to cardiac injury, the 34 kDa form results in hypertrophy and can thus result in a maladaptive response (Kardami et al., 2004). There is currently no evidence of different transcript sizes for FGF-16. There is evidence that the recombinant form of FGF-16 produced in an insect cell line is subject to proteolysis, however the consequence does not appear to affect biological activity (Danilenko et al., 1999).

1.1.3 Posttranslational modification of FGFs

There are many different forms of posttranslational modification that can occur to FGFs. For instance, FGF-1 is acetylated in mammalian cells (Crabb et al.,1986), however this does not appear to be required for biological activity as recombinant protein lacking the acetylation is also active. In terms of phosphorylation, there is little information regarding the involvement of either kinase A (PKA) or protein kinase C (PKC) in the regulation of FGFs. FGF-2, for example, contains motifs for recognition by both PKC and PKA (Feige and Baird 1989). Phosphorylation by PKA has been shown to result in a higher affinity of FGF-2 for heparin (Feige and Baird 1989). Although one could speculate as to the consequence of such a modification, it has not yet been linked to any particular physiological role. However, there is currently no evidence of altered function or biological activity as a consequence of phosphorylation by PKC. Similarly, an analysis of FGF-16 sequence also reveals target motifs for both PKC and PKA. However, evidence of phosphorylation or altered function remains to be investigated.

Several FGFs have also been shown to be glycosylated (Figure 1.1), for example FGF-4, FGF-5, FGF-9 and FGF-16 (Miyagawa et al., 1991, Bates et al., 1991, Revest et al., 2000, Miyakowa 1999, 2003). Although there has been no evidence as to the importance of this modification for normal physiological function, some differences exist between glycosylated and non glycosylated forms. Such is the case of FGF-4 for example, whereby glycosylation does not appear to interfere with its ability to stimulate angiogenesis (Yoshida et al., 1994). However, removal of the glycosylation site by mutation results in cleavage of the protein, increased heparin affinity and increased

biological activity for at least one of the fragments (Bellosta et al., 1993). There has been no indication of altered activity with FGF-5 with respect to glycosylation (Bates et al., 1991, Clements et al.,1993). Other glycosylated members (FGF-9 and 16) have not been investigated for differences in activity.

1.1.4 FGF secretion

Many FGFs are known to exert their effects through high-affinity extracellular receptors (Safran et al., 1990). However, in order for this interaction to take place, this requires that the FGF molecule be released from the cell (see Figure 1.1 for summary). This mechanism of secretion is often, but not always achieved through the presence of a signal sequence. Some members of the family such as FGF-4 for example, contain a signal sequence that is required for secretion. Removal of the sequence results in loss of function in a mouse embryonic fibroblast cell line (NIH 3T3) which suggests that the protein must first get to the cell surface in order to exert its effects (Talarico and Basilico 1991).

Although some FGFs contain a classic signal recognition sequence for secretion, others do not. Nonetheless, FGF-1 (Christofori and Luef, 1997), FGF-2 (Taverna et al., 2003) and FGF-9 (Miyamoto et al., 1993) for example, are still secreted through atypical mechanisms. With regard to FGF-16, recently Miyakawa and Imamura (2003) demonstrated through mutation and deletion that both a hydrophobic core sequence and a signal in the N-terminal region are necessary for secretion. Although Revest et al. (2000) claimed that only the N-terminal region of FGF-9 was necessary for secretion, they did not take into account that the green fluorescent protein used as a tag also has minor

secretory properties (Tanudji et al., 2003). Various atypical methods of secretion or release of growth factors have been proposed for FGFs lacking the classical leader sequences. McNeil et al. (1989) for example, proposed that FGF-2 release occurs through the damaged membrane under conditions of mechanical stress. Other conditions of stress resulting in release include the use of heat shock, which results in the release of FGF-1 (Jackson et al., 1992). However, the protein released from NIH 3T3 cells did not possess mitogenic or heparin binding properties (Jackson et al., 1992). This mechanism of release was also found to be dependent on *de novo* protein synthesis as it was prevented by the inhibition of Golgi function by brefeldin A (Jackson et al., 1995). Other novel mechanisms of release include the dependence on the $Na^+ K^+$ ATPase for FGF-2 release from COS-1 cells (Florkiewicz et al., 1998), and shedding through membrane vesicles (Taverna et al., 2003). As previously mentioned there are several reports of FGF-16 being secreted following transfection of various eukaryotic cells (Danilenko et al., 1999, Revest et al., 2000, Miyakawa and Imamura 2003). However, there has been no investigation as to the properties of endogenous cardiac FGF-16.

1.2 Fibroblast growth factors and heparan sulfate proteoglycans

One of the hallmark features of FGFs is their ability to bind heparin and heparan sulphate proteoglycan(s) (HSPG) (Gould et al. 1995, Folkman et al. 1988, Allen et al. 2001). HSPGs are a group of molecules which are characterized by the presence of one or more heparan sulphate chains attached to a core protein. They are often associated with the extracellular matrix of many cells or basement membrane and are known to play important roles in the regulation of cell migration and development. The concept that the

extracellular matrix serves as a reservoir for FGFs was first proposed by Folkman and colleagues (Folkman et al., 1988). In addition, the matrix also protects FGFs against degradation by acid and heat (Gospodarowics and Cheng 1986) as well as various enzymes such as trypsin, plasmin and thrombin (Lobb et al., 1988, Saksela et al., 1988, Sommer and Rifkin 1989).

It was proposed by Gould and colleagues (Gould et al., 1995), that the expression of specific cell surface heparin-like glycosoaminoglycans provide a mechanism to regulate the localization of FGFs. More recently however, studies have revealed that HSPGs serve a much more complex and integral role in FGF function. Not only does heparin interact with FGF upon binding to the membrane receptor, in some cases it is necessary for receptor activation. For example Allen and colleagues (Allen et al., 2001) demonstrated that the type of heparan sulphate present can dictate which FGF binds the extracellular matrix. In addition, they also suggest that the activation of FGF receptor is dependent not only on the appropriate FGF but also the right heparan sulphate. Their evidence stems from a model in which cultured endothelial cells respond to FGF-2 by drastic morphological changes but do not respond to FGF-4. Upon addition of heparin with FGF-4 however, the cells now respond like those treated with FGF-2. This suggests that the receptor interaction is dependent on heparin or the appropriate HSPG to facilitate the interaction.

Heparan sulphates have also been proposed to function in an FGF receptor (FGFR) independent fashion and function as receptors themselves. Chua and colleagues (Chua et al., 2004) base this theory on evidence whereby the activation of extracellular signal regulated kinases 1 and 2 (ERK1/2) are activated by FGF-2 independent of FGFR.

Despite inhibition of FGFR pathway through a combination of pharmacological and dominant negative receptor expression, vascular smooth muscle cells demonstrate activation of ERK1/2 by FGF-2 stimulation. However, activation is sensitive to treatment with heparatinase III, suggesting a dependence on HSPG(s). This dependence, is not present when normal cells are treated with heparatinase III, but rather, they demonstrate a different time course of ERK1/2 activation, suggesting alternate pathways of activation independent of FGFR (Chua et al., 2004).

Although all FGF members bind heparin, they do not all possess the same heparin binding affinity. For example, FGF-7 has a lower affinity for heparin than FGF-10. It is this difference in affinity, which may also be responsible for the reduced activity of FGF-7 and the potentiation of FGF-10 by the addition of heparin (Igarashi et al., 1998). Not only is there an innate heparin binding affinity for FGFs but there is evidence that the affinity can be modified or regulated. With FGF-2 for example, phosphorylation by PKA can increase the heparin affinity of the protein (Feige and Baird 1989). Thus, not only variations in heparin affinity exist between members but the potential for regulation of the members also exists.

Although some studies have used heparin to purify or concentrate FGF-16, thus confirming an affinity for heparin (Danilenko et al., 1999, Miyakawa and Imamura 2003), there is currently no information regarding the interaction of FGF-16 with HSPGs.

1.3 Fibroblast growth factor receptors

The first FGF receptor (FGFR) to be identified (Olwin et al., 1986) and sequenced was isolated through the use of affinity purification using FGF-2 (Lee et al.,1989). Since

then, there have been four FGFR genes identified in mammals. Each of the receptors represents an individual gene, which codes for many different splice isoforms. As with the FGF molecules, the receptors have also been named based on the chronological order of their discovery. In addition to the four receptor types there has recently been a new FGFR like receptor (FGFRL-1) which seems to have a different function than that of the other receptors (Wiedemann and Trueb 2000, Trueb et al., 2005).

1.3.1 FGF receptor structure

Starting from the amino terminus, each of the first four FGFRs consists of an extracellular region comprised of up to three immunoglobuin-like domains, a hydrophobic region, transmembrane segment and a cytoplasmic tyrosine kinase domain at the carboxy terminus. While FGFs are known to demonstrate preferential activation of certain FGFRs, the mechanism becomes much more complex once all the different splice variants of each receptor are factored in. Alternative splicing for each of the different FGFRs provides for close to a hundred different receptor proteins. Differences between variants are sometimes as subtle as a single amino acid, however the biological significance of these variants is not fully understood.

1.3.1a The extracellular domain

The amino terminus, or extracellular domain of FGFR's is the region which interacts with FGFs and thus can lead to activation of the receptor. This region contains up to three immunoglobulin (Ig) like sequences or domains which have been shown to be involved in determining FGF binding specificity (Ornitz et al., 1996). Most receptor

splice isoforms are divided into one of three subtypes based on which Ig sequences are present. As seen in Figure 1.2, starting with the outermost Ig domain furthest from the membrane, they are numbered I, II, and III. Alternative splicing of the third (III) Ig domain results in one of three main subtypes of receptor labeled IIIa, IIIb, and IIIc. Within each of these subtypes there is the possibility for many other splice variants which lack Ig like domains through alternative splicing (Johnson et al., 1990) or premature truncation (Johnson and Williams 1993).

The genes for FGFR-1 and FGFR-2 each result in three main variants IIIa, IIIb and IIIc (Johnson et al., 1991) while FGFR-3 and FGFR-4 are more limited. FGFR-3 only results in IIIb and IIIc subtypes (Chellaiah et al., 1994) while FGFR-4 is restricted to only the IIIc subtype (Vainikka et al., 1992). Each of the different Ig domains share a high degree of homology between the different receptor genes. In fact, the similarity of each Ig like domain is even higher between the genes than within a particular FGFR gene. However, contrary to what one might expect, analogous FGFRs sharing the same Ig arrangement do not necessarily show the same specificity toward a given FGF. For example, while FGF-7 is capable of activating FGFR-2IIIb, it does not activate FGFR-1IIIb or FGFR-3IIIb (Ornitz et al., 1996). In addition, between domains I and II is an "acidic region" which is not necessarily present in all receptor subtypes. Alternate splicing events also take place within the intracellular and transmembrane regions of FGFR.

1.3.1b The transmembrane domain

Most FGFR variants described to date contain a hydrophobic region after the Ig segments which serves to anchor the receptor in the plasma membrane. This region

consists of 21 amino acids and may be absent for certain receptor forms which are secreted (Terada et al., 2001) The secreted form has been shown to bind both FGF-1 and FGF-2 and is thought to perhaps act as an extracellular reservoir for FGFs and/or serve to block the action of FGFs by preventing their interaction with membrane bound receptors (Johnson et al., 1991). There is currently no information regarding FGF-16 and possible interactions with secreted FGFR variants.

1.3.1c The cytoplasmic region

Although some FGFRs do not contain a transmembrane region and can be secreted, this is not the case for most FGFRs. The majority of FGFR variants contain intracellular motifs that serve to relay the message from extracellular signals such as the presence of FGF ligand. These include but are not necessarily limited to the phosphorylation of tyrosine residues following receptor dimerization (Lemmon and Schlessinger 1994). However, the receptor dimerization is not restricted to one specific subtype (homodimer) of receptor but may involve different receptors (heterodimer) (Bellot et al., 1991). The downstream effects of receptor dimerization or activation will be covered in the following section.

1.3.2 FGFR isoform specificity

Perhaps the best evidence for FGFR isoform selectivity towards certain FGFs has come from work done by Ornitz and colleagues (Ornitz et al., 1996). They've developed a system which uses a hematopoetic cell line, BAF-3, which is devoid of FGF receptor. Through stable transfection of the cells with constructs which code for specific receptor

variants they are able to examine responses to various FGFs. Responses are then evaluated by tritiated thymidine incorporation that is indicative of DNA synthesis.

Results from the study indicated that FGF-1 was very effective at activating all of the FGFR splice isoforms examined. A comparison in terms of relative activity to that of FGF-1 (100%) revealed that FGF-2 was less effective at activating FGFR-2b (9%) and FGFR-3b (1.2%). An examination of FGF-9 (the closest member of the FGF family to FGF-16), revealed that unlike FGF-2 it was effective at activating FGFR-3b (41.5%) (Ornitz et al., 1996). A recent examination of FGF-16 reveals a preference for the same receptors as FGF-9. However, unlike FGF-9, FGF-16 is less effective at activating FGFR-4 (5 versus 75% that of FGF-1) (Lavine et al., 2005). However, there is a conflicting report in the literature in regards to which FGFRs FGF-16 is thought to interact. Konishi and collegues (Konishi et al., 2000) examined interactions between FGF-16 and brown adipocytes and report that activation occurs through binding with FGFR-4 and not FGFR-2c in primary cultures of brown adipocytes. However, lack of receptor activation may have been due to the absence of endogenous HSPG or exogenous heparin in the assay used.

1.3.3 FGFR signal transduction

Activation of FGFR within the plasma membrane through the binding of appropriate FGF results in receptor dimerization. As mentioned, receptors often contain a cytoplasmic tyrosine kinase domain which results in the autophosphorylation of the receptor and thus activation (Pawson, 1995). This then allows for the recruitment of a variety of different intracellular second messengers, which relay the signal (refer to

Figure 1.3). Pathways that have been identified in this process include proteins with Src-homology 2, or SH2 domains (Mohammadi et al., 1991). This domain serves as a recognition site for many target and adapter proteins, which can then result in a cascade of different second messengers within the cell. Although there are many different tyrosine amino acid residues within the C-terminus of the receptor which are candidates for autophosphorylation, Mohammadi and colleagues (Mohammadi et al., 1992) demonstrated that mutation of all but one tyrosine kinase residue (Tyr^{776}) resulted in no loss of mitogen activated protein kinase (MAPK) activity or mitogenic signaling. Mutation of residue Tyr^{776} to a phenylalanine residue prevented the activation of phospholipase C-gamma (PLCγ), which normally binds through interaction with the SH2 domain (Mohammadi et al., 1992, Peters et al., 1992). The activation of PLCγ results in the formation of many second messengers such as diacylglycerol and inositol trisphosphate formation, which in turn, results in protein kinase C activation and release of calcium from intracellular stores. The mutation of the Tyr^{766} residue, while inhibiting PLCγ, does not however affect mitogenesis, neuronal differentiation (Spivac-Kroizman et al., 1994) or mesoderm-induction in a Xenopus model (Muslin et al., 1994). This suggests that while PLCγ is activated by FGFR activation, other pathways independent of Tyr phosphorylation exist.

1.3.3a The Src signaling pathway

Alternate pathways that do not involve tyrosine kinase activation may instead signal through Src, a non-receptor tyrosine kinase, which may indirectly interact with FGFR. One candidate for such an interaction is cortactin, a focal adhesion-associated

protein (Wu et al., 1991). Evidence of an interaction between Src and FGFR comes from the immunoprecipitation of Src with FGFR (Zhan et al., 1994). Although, others have proposed that Src activation is a consequence of PLCγ inhibition, Langgren et al., (1995) suggest that mutation of the FGFR Tyr^{766}, as discussed in the previous section, inhibits a negative feedback system that normally keeps Src from being phsophorylated. Evidence for this comes from elevated levels of phosphorylated Src as a consequence of the Tyr^{766}/Phe^{766} mutation.

1.3.3b Crk Signaling

Crk is an adapter protein, which interacts via an SH2/SH3 domain that is thought to allow FGFR to activate other downstream pathways. These include several Src homology adapter proteins such as Shc, C3G and Cas, which may be responsible for the mitogenic signals resulting from FGFR activation. Evidence from this stems from an interaction observed between Crk and the Sh2 domain to the Tyr^{463} residue of the activated FGFR (Larsson et al., 1999). Endothelial cells expressing FGFR-1 do not demonstrate extracellular receptor kinase (ERK)2 and c-Jun NH_2-terminal kinase (JNK) activation or proliferation following mutation of the Tyr^{463} residue. These results are contradictory to those discussed in section 1.3.3 where Mohammadi et al., (1996) reported no affect as a result of the same mutation. However, the differences observed may be due to the cell types used (i.e., fibroblasts versus endothelial cells).

1.3.3c SNT-1/FRS2 pathway

Even when FGF receptors are mutated such that they lack all the non-catalytic tyrosine residues, a 90-kDa protein is still found to be phosphorylated. This protein has been identified by two independent groups (Wang et al., 1996, Kouhara et al., 1997) and has thus been named Suc1-associated neurotrophic factor target (SNT) or FGFR substrate 2 (FRS2). They found that FGFR activation is linked to the RAS/MAPK signaling pathway which is important for FGF induced cell proliferation. This mechanism involves the activation of SNT-1/FRS2 which, through the use of an adapter protein, Grb-2/Sos, results in interaction of the small molecular weight G-protein Ras with the FGFR complex (Kouhara et al., 1997). Through immunoprecipitation studies, a protein tyrosine phosphatase (Shp2) has also been found to bind not only FRS2 but a docker protein Grb2 associated binder (Gab-1) (Ong et al., 2000). Furthermore, SNT-1/FRS2 has been linked to activation of atypical PKC isoforms (Lim et al., 1999). The association between SNT-1/FRS2 and FGFR-1 is constitutive and independent of receptor activation (Ong et al., 2000).

1.3.3d Mitogen activated protein kinase pathways

The family of mitogen activated protein kinases (MAPK) are a group of proteins which function as messengers for intracellular signaling (Widmann et al., 1999, Ravingerová et al., 2003). They have been shown to be activated by a wide array of cell signaling molecules (such as FGFs) as well as in response to physical changes in cellular environment including heat, acidity, oxidative stress etc. some of which are associated with conditions that arise as a consequence of cardiac ischemia for example (Padua et al.,

1995, Sheng et al., 1997, Stephanou et al., 1998, Buerke et al., 2001). Consequently, MAPKs have been implicated in cell proliferation, apoptosis, cytoprotection and development (Widmann et al., 1999, Molkentin 2004). These cellular processes and conditions have direct implications in cardiovascular physiology and pathophysiology. The following sections will focus on what is known about the involvement of the MAPKs investigated in the present study, namely extracellular regulated MAPK (ERK1 and ERK2), p38, p46 and p54 or stress activated MAPK (SAPK). Particular emphasis will placed on what is known about the effects or roles that MAPKs have in relation to cardiovascular injury/repair and disease. For a general synopsis of the different pathways that regulate the various MAPK covered please refer to Figure 1.3.

1.3.4.dI Extracellular regulated MAPK (ERK)

There are five different isoforms of ERK identified to date, but of these, the ERK1/2 (p44 and p42 respectively) are considered a separate subfamily. They are activated by phosphorylation of threonine and tyrosine residues, which is often the result of cell stimulation from growth or trophic factors (Htun et al., 1988, Bogoyevitch et al., 1994 Padua et al., 1995, Kuwahara et al., 2000,).

The relation between ERK activation and cardioprotection has been of great interest to cardiovascular science for the past decade. Several reports of ERK1/2 activation coinciding with increased cell survivival (Parrizas et al., 1997, Stephanou et al., 1998) and reduced myocardial damage as a result of ischemic insult have been described (Padua et al., 1995, Sheng et al., 1997, Stephanou et al., 1998, Buerke et al., 2001). The benefits of ERK activation, however, are not limited to ischemia alone as β-

receptors, which are intimately involved in the pathology of cardiovascular hypertrophy, have also been linked to ERK activation. For example, activation of β_1-adrenergic receptor results in the suppression of the ERK1/2 phosphorylation and increased apoptosis, while stimulation of the β_2-adrenergic receptor results in activation of ERK1/2 and myocyte protection (Shizukuda et al., 2002). The protective effects of ERK activation are believed to be due to the prevention or inhibition of apoptosis. Similarly, the protective or anti-apoptotic effects of FGF-2 on myocytes observed following hypoxic injury through the administration of H_2O_2 (a model of hypoxia) is also mediated by ERK1/2 activation (Iwai-Kanai et al., 2002). Many inhibitors such as PD98059 and UO126 have been used in hopes of elucidating the roles of ERK1/2 (Alessi et al., 1995, Favata et al., 1998, Strohm et al., 2000). Consequently, inhibition of ERK1/2 results in increased injury following ischemia/reperfusion in isolated rat hearts (Alessi et al., 1995, Favata et al., 1998, Strohm et al., 2000) and *in vitro* cultures of neonatal rat cardiac myocytes are more prone to apoptosis (Yue et al., 2000).

The use of gene deletion has recently been used to further characterize the roles of each ERK in myocardial protection. For instance, a model of ischemia-reperfusion injury using transgenic mice lacking ERK1 was reported not to have any differences in the degree of damage following injury (Lips et al., 2004). However, heterozygotes for the ERK2 deletion were found to have significant increases in myocardial injury (Lips et al., 2004). As predicted, transgenic mice expressing a constitutively active mutant form of MEK1, which is an upstream activator of ERK results in hearts that are more resistant to ischemia reperfusion injury (Lips et al., 2004).

There are several mechanisms by which ERKs are believed to prevent apoptosis. These include possible interactions with cyclooxygenase-2 (Adderley et al., 1999), PKC_{ε} in the mitochondria and p90 ribosomal S6 kinases which can serve to inhibit pro-apoptotic proteins such as Bad (Smith et al., 1999, Baines et al.,2002, Valks et al., 2002). Other protective mechanisms by which ERK functions include promoting the enhanced degradation of pro-apoptotic factors such as BimEL (Ley et al., 2003) as well as the inhibition of caspase-8 cleavage which is one of the first steps in the apoptosis cascade (Holmstrom et al., 2000, Park et al.,2003).

1.3.3.dII P38 MAPK

The p38 subfamily of MAPK are osmoregulatory protein kinases that are activated by various forms of cellular stress. They are also referred to as the cytokine-suppressive or anti-inflamatory drug-binding proteins (Lee et al., 1994). There are four isoforms of p38 to date (α, β, γ, δ), but only p38α and p38β are known to exist in the heart (Sugden and Clerk, 1998) with p38α being the most dominant (Liao et al., 2001, Lemke et al., 2001, Braz et al., 2003). Several transcription factors have also been proposed as substrates for p38, such as activated transcription factor (ATF), Elk-1 and serum response factor (Han et al., 1994, Whitmarsh et al., 1995, Raingeaud et al., 1995). Other targets which are thought to be cytoprotective include MAPK-activated protein kinase 2 (Freshney et al., 1994) and the activation of the small heat shock protein HSP27 which can prevent oxygen radicals from inducing cell damage (Guay et al., 1997). Activation of p38 by transfection of active MAPK kinase (MKK) six, or MKK6, has also been found to prevent apoptosis in myocytes induced by anisomysin and is said to be

NFκB mediated (Zechner et al., 1998, Craig et al., 2000, Hoover et al., 2000). Thus, based on these data it would appear that p38 serves a protective role, however, there is also a large body of work which suggests the contrary.

Most model systems of ischemia have found that p38 is activated during ischemia and throughout reperfusion. However, inhibition of p38 reduces the extent of injury observed following ischemic insult (Meldrum et al., 1998, Ma et al., 1999, Barancik et al., 2000, Yue et al., 2000, Schneider et al., 2001, Gysembergh et al., 2001, Marais et al., 2001). The degree of protection from the inhibition of p38 is limited however as more severe ischemic insults reveal no benefit (Armstrong et al., 1999, Nakano et al., 2000, Mocanu et al., 2000, Sanada et al., 2001). Extended p38 inhibition was recently found to improve cardiac performance and inhibit cardiac remodeling, whereby inhibition resulted in reduced myocyte hypertrophy and interstitial fibrosis (Liu et al., 2005). This is consistent with the known involvement of p38 in myocyte growth (Hefti et al., 1997, Wang et al., 1998, Ono and Han, 2000). For example, it is involved in the initiation of collagen expression by fibroblasts (Obata et al., 2000) as well as playing a role in apoptosis with both *in vitro* and *in vivo* models (Ichijo et al., 1997, Adams et al., 1998, Wang et al., 1998, Galán et al., 2000).

Various transgenic models have been used to clarify the role of p38 in cardioprotection/injury. Activation of p38 by the expression of an active mutant of transforming beta activated kinase (TAK1), which is an upstream activator of p38, was found to result in increased cell death (Zhang et al., 2000). A similar approach using a mutant and constitutively active form of an upstream activator of p38 (MKK3b) did not result in differences in apoptosis despite p38 activation (Liao et al., 2001). The use of a

heterozygous p38α knockout mouse was found to result in hearts that were less susceptible to ischemia reperfusion injury when examined using a reversible coronary occlusion model (Ostu et al., 2003). This is similar in finding to the study of a dominant negative p38α expressing transgenic mouse which resulted in decreased infarct size following ischemic insult (Kaiser et al., 2004).

Taken together, the more elegant experiments using gene targeting techniques would appear to paint a clearer picture of p38 activation as being a negative factor for recovery following cardiac injury. Possible reasons for the discrepancy may include non specific and unintended effects of the p38 inhibitors used in the first series of experiments, in addition to differences in the assays themselves in terms of the method of injury.

1.3.3.dIII c-Jun NH_2-terminal kinases (JNK)

The JNK family of kinases represent three genes that encode over ten isoforms of JNK as a result of alternate splicing (Gupta et al., 1996). The activation or phosphorylation of JNK results from the initiation of GTP-binding proteins. Exposure of cells to stress such as UV irradiation, osmotic shock, toxicity and oncogenes as well as mitotic stimuli such as growth factors often lead to JNK activation (Johnson et al., 1996). Both JNK1 (p46) and JNK2 (p54) are expressed in the heart but not JNK3 (Itoh et al., 1999, Liang et al., 2003).

The investigation of JNK in relation to cardiac injury is currently ongoing. Although JNK has been shown to be activated by ischemia reperfusion injury as well as pressure overload (Ramirez et al., 1997, He et al., 1999, Choukroun et al., 1999), there is

confusion as to whether JNKs play a role in the regulation of cardiac hypertrophy following injury. The use of adenovirus for expressing active MKK seven (MKK7) which serves to activate JNKs, was found to result in hypertrophy of neonatal rat ventricular myocyte cultures (Wang et al., 1998). Conversely, the expression of a dominant negative form of MKK4 which would otherwise activate JNKs, resulted in the prevention of hypertrophy in the same system when cells were exposed to endothelin-1 as a hypertrophic stimulus (Choukroun et al., 1998). Similar results were also obtained by expression of a dominant-negative MKK4 through adenoviral transfection, whereby the mutant was able reduce pressure overload induced cardiac hypertrophy (Choukroun et al., 1999). However, a transgenic MKK knockout mouse model that does not demonstrate normal JNK activation as a result of pressure overload (aortic banding), still resulted in the same degree of hypertrophy as was observed in wild type mice (Sadoshima et al., 2002). Furthermore, a lack of JNK through gene deletion has been found not to prevent hypertrophy in transgenic animals subjected to aortic banding, although animals undergo spontaneous cardiac hypertrophy with age (Liang et al., 2003).

Several studies have implicated JNK signaling in the regulation of apoptotic processes. Deletion of either p54 JNK or a double deletion of p46/p54 JNKs have been found to result in altered apoptotic events associated with development in other tissues (Sabapathy et al., 1999, Kuan et al., 1999). In addition, the deletion of apoptosis signal regulating kinase (Ask), which is an upstream regulator of JNKs, results in altered responses to hydrogen peroxide (H_2O_2, a model of hypoxic injury) and tumor necrosis factor alpha (TNF-α) induced cell death (Tobiume et al., 2001). However, deletion of the MKK1 gene which was previously described as having no effect on hypertrophy as a

result of aortic banding in mice (Sadoshima et al., 2002), results in the differentiation of embryonic stem cells to myocytes with an increased sensitivity toward oxydative stress (Minamino et al., 1999). It should be noted that the effects of MKK1 are not limited to JNK activation but can also affect ERK. The role of JNKs however, may not all be pro-apototic in nature as dominant negative p46 JNK in cultured cardiac myocytes results in increased apoptosis after nitric oxide treatment (Andreka et al., 2001). Similarly, expression of dominant negative JNK mutants were found to result in increased apoptosis as a result of hypoxia in neonatal rat myocyte cultures (Dougherty et al., 2002). Conversely, anti-apoptotic effects were reported by the inhibition of p46 JNK but not p54 JNK for cardiac myocytes subjected to ischemic conditions (Hreniuk et al., 2001). Similar results were also reported for adult myocytes expressing dominant negative JNK, whereby the incidence of apoptosis was less following H_2O_2 insult (Kwon et al., 2003). Therefore, there appear to be discrepancies as to whether JNK inhibition is beneficial or deleterious to myocyte survival depending on the system or experimental design.

1.4 FGFs and cardiac physiology

Several members of the FGF family have been identified as being present in the heart during embryonic development and/or in the postnatal heart. However, the knowledge regarding the involvement of different FGFs in cardiac physiology varies greatly. A number of FGFs have been detected in the heart and the following will provide some information regarding their role in cardiac development.

1.4.1 Embryonic cardiac development

The early stages of cardiac development (cardiogenesis) require interactions between endoderm and mesoderm. Signals between the two types of cells initiate a sequence of events that promote the differentiation of the mesoderm into precardiac tissue. This tissue later forms the heart tube, which loops and divides into a four chambered organ. This complex process requires signals that regulate cell proliferation, differentiation, migration and apoptosis. For comprehensive reviews on the subject and the factors involved please refer to Lough and Sugi (2000), Zaffran and Frasch (2002) and Brand (2003).

Certain FGFs are associated with specific regions of the early embryo or cells which ultimately differentiate to form the heart. For example, FGF-8 is found in precardiac mesoderm (Crossley and Martin, 1995), FGF-10 is associated with cells of the anterior heart forming field in the mouse (Kelly et al., 2002) and FGF-4 corresponds to sites occupied by precardiac progenitor cells (Garcia-Martinez and Schoenwolf, 1993). Not only is there a close association between these growth factors and heart development but evidence of function has also been established. For example, FGF-1 and FGF-4 are capable of inducing cardiogenesis of non-precardiac mesoderm explants. In addition, these effects can be blocked by inhibition of FGF/FGFR interactions using sodium cholate (Zhu et al., 1996) or reduced by blocking with an antibody that binds and prevents interaction with the extracellular domain of FGFR-1 (Zhu et al., 1999).

There is also evidence that while there is some overlap between receptors, not all FGFs are interchangeable. For example, FGF-7 has a lack of cardiogenic properties unlike that of more distantly related FGF-2 and FGF-4 (Barron et al., 2000). Even FGF-1

and FGF-4, which are both present in the developing heart differ in their ability to stimulate proliferation (Zhu et al., 1996).

The timing and level of FGF expression also varies during cardiogenesis. For example, while FGF-2 appears at Carnegie stage 9 (day 1) of the developing chick heart (equivalent to mouse embryonic day 9), FGF-1 and FGF-4 are delayed in their appearance until stages 11 and 15 respectively (day 10-11 for mouse), with levels peaking between stages 18-24 (day 12-13 for mouse) (Zhu et al., 1996, Madiai and Hackshaw 2002). These time points coincide with important events in cardiogenesis such as the breakdown of the dorsal mesocardium, division of the truncus anteriosus, formation of the cardiac cushions and septation. Staining for FGF-1 and FGF-2 was said to be positive, uniform and superimposable when the cardiac cushions first became recognizable. (day 13-14 in the rat), while later stages (day 15-17) had a non uniform staining of the cardiac cushions with some areas being intense and others faint (Spirito et al., 1991). These regions are considered to be very active in terms of cellular division (Jeter et al., 1971, Franciosi et al., 2000). Given the mitotic effects caused by the addition of FGFs with *in vitro* (Kardami et al., 1989, 1990, Sugi eta l 1993, Sugi and Lough 1995) as well as *in vivo* assays (Franciosi et al., 2000), one would postulate detrimental effects when removed. However, knockout models of either FGF-1, FGF-2, or a combination of both results in perfectly viable offspring with functional hearts (Ortega et al., 1996, Miller et al., 2000). Similarly, the deletion of either FGFR-1 or FGFR-2 do not appear to result in lethality or abnormal heart size. However, deletion of both receptors results in a lethal hypoplatic cardiac phenotype, thus illustrating the need for FGF signaling for proper heart development (Lavine et al., 2005). Unlike FGF-1 and FGF-2, deletion of

FGF-9 alone results in a hypoplastic phenotype but not as severe as that observed by the double receptor deletion (Lavine et al., 2005). Deletions of other FGFs such as FGF-8 and FGF-4 also result in lethal conditions, however, animals die during the very early stages of mesoderm induction or formation (Feldman et al., 1995, Sun et al., 1999). The relative importance in terms of lethality between the FGFs is probably related to several factors: 1) the specificity of the FGFs toward given receptors, 2) the receptor subtypes present and 3) the presence of another FGF(s) which might substitute for the deleted one.

Recently Ornitz and colleagues (Lavine et al., 2005) reported the presence of transcript in embryonic heart for FGF-9 as well as closely related family members FGF-16 and FGF-20 (see Figure 1.1 for relationship between members) through the use of *in situ* hybridization. FGF-16 transcript had also been detected in embryonic heart using RT-PCR and is said to increase during embryonic development (Miyake et al., 1998). However, the reported distribution for the FGF-16 transcript is different between the two studies. While Lavine et al., (2005) reported localization to the endocardium and epicardium in the mouse, Miyake et al.,(1998) detected a weak and diffuse signal in the embryonic heart. These apparent differences in distribution may be explained by the different time points used (embryonic day 10.5 versus 19.5), sensitivity of the label used for detection (fluorescence versus autoradiography) or differences between the species examined (rat versus mouse).

1.4.2 The role of Adult heart FGFs

There are several important distinctions to be made between the adult heart and embryonic heart. During development, heart formation requires the specification,

differentiation and division of many cell types for proper heart formation. The post natal heart demonstrates a gradual loss of mitotic activity within the first few days after birth (Soonpa and Field, 1996). This terminal differentiation of the cells has been the subject of intense research for many years as cardiac cells are unable or reluctant to divide. However, as previously mentioned, many of the growth factors described, such as FGF-1 and FGF-2, decrease in the later stages of embryonic development and post partum (Cummins et al., 1991). Although several FGFs have been detected in the adult heart, for example FGF-1 (Weiner and Swain, 1989), FGF-2 (Kardami et al., 1991), FGF-12, FGF-13, (Hartung et al., 1997), and FGF-16 (Miyake et al., 1998), very few have been defined as to their role in cardiac physiology, or to injury and pathology of heart disease. There have been links drawn between injury and increases in FGF (Padua and Kardami, 1993, Iwakura et al., 2000) as well circulating levels (Abramov et al., 2000) suggesting that perhaps the heart already has an endogenous repair/protection systems in place that involve FGFs. Evidence in support of this comes from transgenic animals lacking FGF-1 or FGF-2. Although the animals appear normal, for the most part, their abilities to recover from cardiac injury (Pellieux et al., 2001, House et al., 2003) and wounds in general is compromised (Miller et al. 2000). Several FGFs such as FGF-2 and FGF-4 have proven beneficial in terms of therapy (Iwakura et al., 2003, Grines et al., 2003). Their benefits are believed to come from a combination of myocyte preservation through anti apoptotic mechanisms (Horrigan et al., 1996, 1999, Cuevas et al., 1997a, 1997b) as well as positive effects in terms of vascular remodeling (Buehler et al., 2002, Caron et al., 2004, Jiang et al., 2004). Despite the therapeutic effects of those examined, for example FGF-1, FGF-2, FGF-4, and FGF-5 (Grines et al., 2003, Palmen et al., 2004, Detillieux et

al., 2004, Suzuki et al., 2005) several others remain to be characterized. While transcripts for FGF-12, FGF-13 and FGF-16 have been reported to be present in the adult heart (Hartung et al., 1997, Miyake et al., 1998), their relative protein expressions, distribution or function remain undefined.

1.4.3 Ischemic injury

A reduction or complete loss of blood flow through the coronary arteries is one of the major causes of cardiac injury. However the paradox being that restoration of blood flow or reperfusion causes further injury. This mechanism of injury has many components beginning with the stress associated with increased metabolites, decreased pH and oxygen deprivation caused by the initial decrease in blood flow followed by other compontents upon reperfusion such as oxidative stress and the generation of reactive oxygen species (Aikawa et al 1997). The combination of these factors result in the loss of myocytes either by apoptosis (von Harsdorf et al. 1999) or necrosis (Buerke et al 1995, Zhao et al 2000), which are instead replaced by other cell types which do not posess the same properties (Itescu et al 2003). This often results in compromised myocardial function as is the case with contractile, coronary perfusion and electrical properties (Aronson 1980, Kohya et al. 1988, Botchway et al 2003). Although the heart does compensate to some degree through hypertrophy, on a cellular level, myocytes increase in size rather than divide (Kellerman et al. 1992,Soonpaa and Field 1997). The benefits of cardiac hypertrophy as a means of compensation however, are limited and can eventually become maladaptive.

1.5 Summary of what is known about FGF-16

In summary of what is known, the levels of embryonic heart FGF-16 can be detected by RT-PCR and are said to increase during development (Miyake et al, 1998). Although FGF-16 is reported to be most abundant in heart, transcript has also been detected in other tissues but to a much lesser degree when examined by RT-PCR. These include small intestine, muscle, thymus, stomach pancreas spleen and testis (Miyake et al 1998) as well as retina (Wilson et al. 2003) and the inner ear during development (Wright et al 2003). FGF-16 is capable of promoting mitosis in some systems as is evidenced by BrdU incorporation (an indicator of DNA synthesis) in rat brown adipose tissue where it is highly expressed in the embryo (Konishi et al 2000), as well as explants from embryonic mouse heart (Lavine et al 2005) while also promoting cell motility in teratocarcinoma cells (Granerus and Engström 2000). Expression of the protein in other eukaryotic cell lines including Sf9 insect cells (Miyake et al 1998) and COS-1 cells (Miyakawa and Imamura 2003) results in a secreted protein which is capable of binding heparin (Danilenko et al 1999, Miyakawa and Imamura 2003). Of greatest interest however is that RNA blotting of adult rat tissues has revealed that the heart contains the largest concentration of FGF-16 by far with only minor detection in brown adipose tissue (Miyake et al., 1998). To date, no other FGF has been described which demonstrates such a restricted and heart specific pattern of expression as that reported for FGF-16. This suggests that FGF-16 plays a role in normal heart physiology. Although FGF-16 transcript levels are said to increase during development, there is currently no information regarding which cell type(s) produce or respond to the protein or what the relative levels are between that of the embryonic, newborn and adult heart. With the

limited information available it is difficult to hypothesize the precise function of FGF-16 ***Furthermore, the potential for FGFs as therapeutic molecules makes basic characterization of FGF-16 essential, especially given its cardiac specificity, pattern of expression and the differentiated state of the adult heart.***

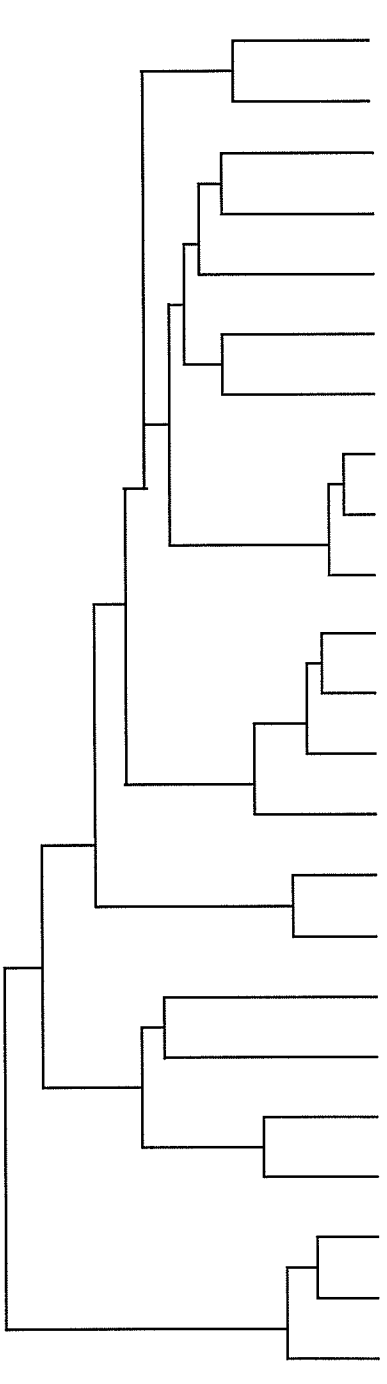
1.6 Research Objectives

The 16th member of the FGF family, FGF-16, expression appears to be relatively specific to the heart. The main objective of this thesis, therefore, is ***to gain a better understanding of FGF-16 expression, location and function in relation to the heart. With particular attention paid to the mouse given the future possibility of generating transgenic models.***

Fulfillment of this objective will be accomplished through the following specific aims:

1. Clone and sequence the murine FGF-16 cDNA.
2. Design and develop antibodies against FGF-16.
3. Using RNA and protein detection techniques, determine when and where FGF-16 transcript and protein are most abundant in relation to cardiac development.
4. Assess the biological activity of FGF-16 in various cell types, including cardiac myocytes.

Fig. 1.1



	Glycosylated	Secretion	Heart
hFGF-1		at_{15}	e_{21},a_{19}
hFGF-2		at_{15}	e_{21},a_{8}
hFGF-10	g_{1}	s_{15}	e_{9}
hFGF-7	g_{16}	s_{15}	e_{11}
hFGF-22		s_{15}	
hFGF-3	g_{4}	s_{15}	
hFGF-5	g_{16}	s_{15}	
hFGF-16	g_{16}	b_{15}	$e_{10,12},a_{12}$
hFGF-9		b_{15}	
hFGF-20		b_{15}	$e_{10,12}$
hFGF-12			a_{6}
hFGF-14	g_{16}	b_{7}	$e_{10,12}$
hFGF-13			a_{14}
hFGF-11			
hFGF-4	g_{16}	s_{15}	e_{5}
hFGF-6		s_{15}	a_{3}
hFGF-21		s_{15}	a_{13}
hFGF-23		s_{15}	a_{20}
mFGF-19		s_{15}	
hFGF-15		s_{15}	
hFGF-17		s_{15}	
hFGF-8	g_{18}	s_{15}	e_{2},a_{17}
hFGF-18	$g_{7,14}$	s_{15}	a_{7}

Figure 1.1 Relationship based on sequence similarity for the 23 members of the FGF family. Known properties of the members identified are indicated as glycosylation (g) and method of secretion through a secretory sequence (s), atypical mechanism (at) or bipartite secretory sequence (b). FGF members previously identified in either the embryonic (e) or adult (a) heart are indicated. Modified from Powers et al., (2000) and Ornitz and Itoh (2001). References for specific FGF properties are provided in subscript in a numerical fashion.

References for Figure 1.1

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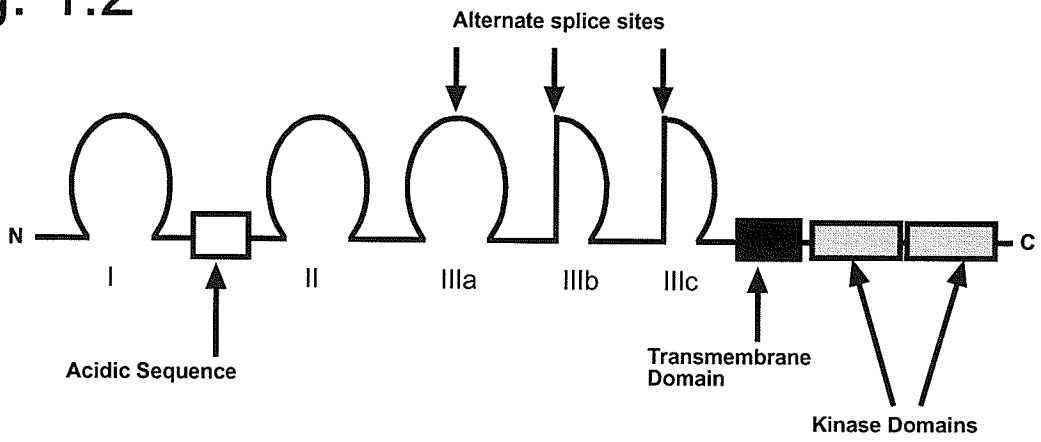
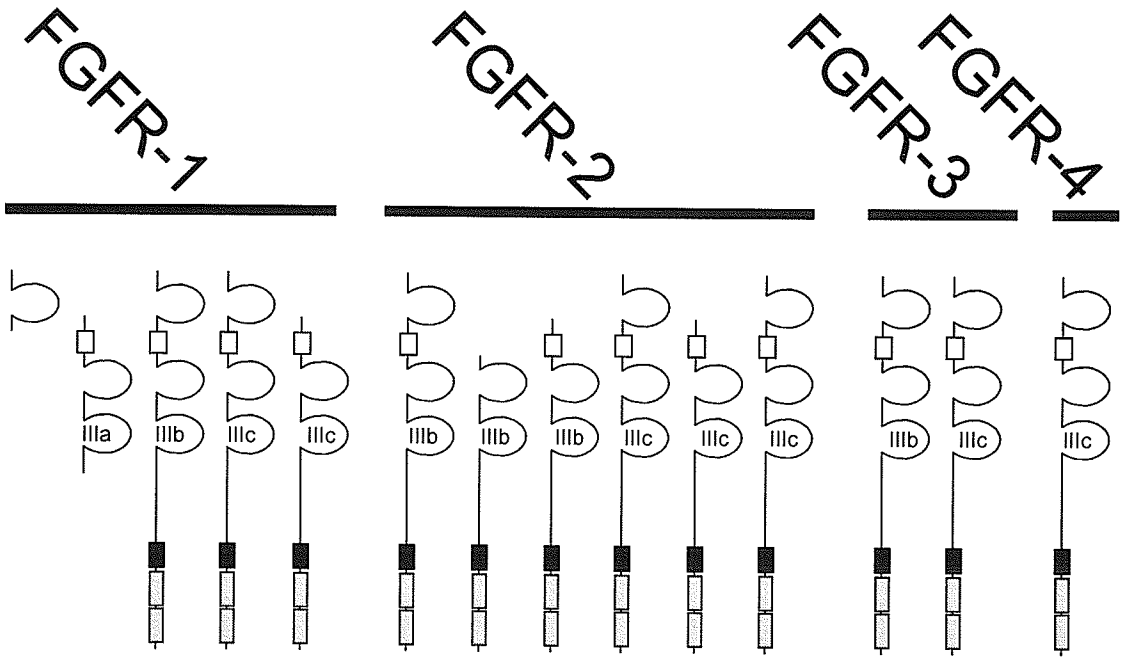
Fig. 1.2


Alternate splice sites
N
C
I
II
IIIa
IIIb
IIIc
Acidic Sequence
Transmembrane Domain
Kinase Domains


FGFR-1
FGFR-2
FGFR-3
FGFR-4
IIIa
IIIb
IIIc
IIIc
IIIb
IIIb
IIIc
IIIc
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IIIb
IIIc
IIIc

Figure 1.2 Model of the FGF receptor showing the key alternate splice sites which determine FGF specificity. There are up to three main alternate splice exons which code for three immunoglobulin (Ig)-like domains designated IIIa, IIIb and IIIc. These are used to identify the different variants (as indicated in the top panel). Alternate usage of the exons leads to several major receptor forms as indicated (bottom panel). Note that not all of the genes for FGFR (for example FGFR-3 and FGFR-4) lead to all three variant types. The combination of Ig-like domains used dictate the specificity between FGF and FGFR interactions. There also exist the possibility for other alternative splice events within each of the FGFR variant types. Modified from Johnson et al., (1991), Johnson and Williams (1993) Powers et al., (2000).

Fig. 1.3

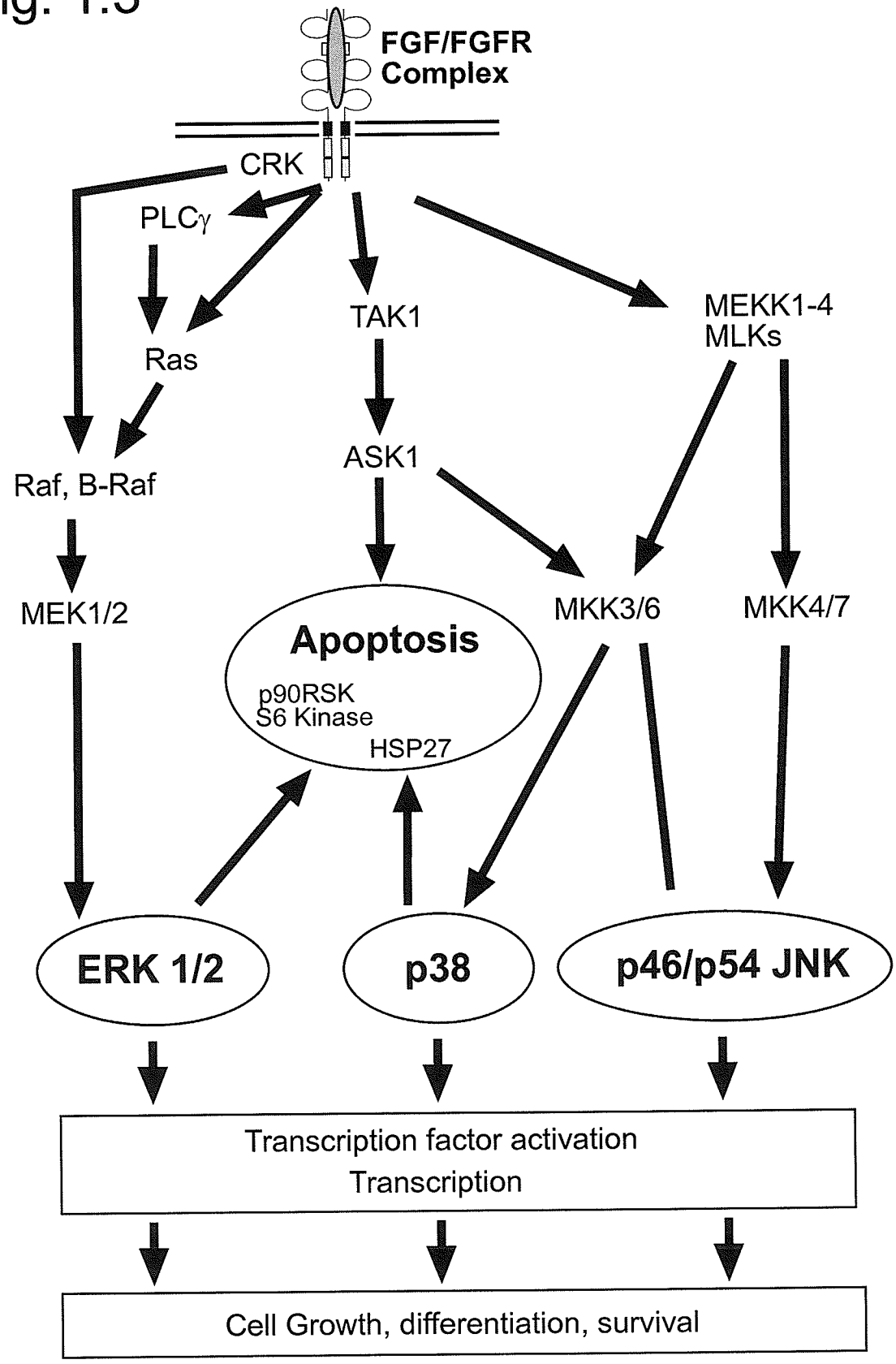

FGF/FGFR
Complex
CRK
PLCγ
Ras
TAK1
MEKK1-4
MLKs
Raf, B-Raf
ASK1
MEK1/2
Apoptosis
p90RSK
S6 Kinase
HSP27
MKK3/6
MKK4/7
ERK 1/2
p38
p46/p54 JNK
Transcription factor activation
Transcription
Cell Growth, differentiation, survival

Figure 1.3 Schematic illustrating some of the basic pathways by which FGFR activates MAPK. Arrows indicate direction of the cascades and can involve one or more intermediate signaling molecules. Modified from Wildman et al., (1999) and Powers et al., (2000).

Chapter 2

2.0 Materials and Methods

2.1 Development of tools

2.1.1 Cloning of mouse FGF-16 cDNA

Mouse FGF-16 was cloned by reverse transcription polymerase chain reaction (RT-PCR). Primers were designed based on the reported sequence for rat FGF-16 cDNA as previously described (Sontag and Cattini 2003). Additional terminal sequences coding for *Hin*dIII, *Xho*I, and *Eco*RV restriction endonuclease sites were also added for future subcloning purposes. The sequences used were as follows: Forward 5′-TTTAAGCTTCTCGAGATGGCGGAGGTCGGGGGCGTCTTTGCC-3′ and Reverse 5′-TTTGATATCTTACCTATAGCGGAAGAGGTCTCTGG-3′. Reverse transcriptase reactions were performed using RNA isolated from mouse heart (see section 2.3.4 for details). Briefly, total RNA (1 μg/10 μl) was incubated with the buffer cocktail provided by the manufacturer (Invitrogen, 28057-018). Samples treated with water instead of enzyme, M-MLV (Invitrogen, 28025-013), served as negative controls for the assay. PCR reactions were performed using RT reaction as a template. The following sequence of temperatures and times were used to amplify the product; 1) 95°C for 4 min to denature, 2) 60°C for 1 minutes (min) to anneal, 3) 72°C for 1.5 min for elongation, 5) 95°C for 1 min to denature, repeating steps 2-5 for 39 cycles before ending with 60°C for 1 min, 72°C for 10 min and storing at –20 °C until use. Components from the reactions were

obtained from Gibco Invitrogen (10342-053) A standard 1.5 mM $MgCl_2$ concentration was used for all PCR reactions.

2.1.2 Generation of recombinant FGF-16

FGF-16 cDNA was inserted in frame within the pET-19b bacterial expression vector (Novagen) and sequenced to verify for the correct reading frame. BL21(DE3)pLyS (Novagen) bacteria were then transformed with the vector from which a positive clones was identified. A culture of the clone was then stimulated with IPTG to produce the protein of interest. Bacteria were allowed to express the protein for a period of 6 hours before isolating by centrifugation for 15 min at 6500 x g. The pellet was then lysed and processed as per the manufactures directions (Novagen). Briefly, the pellet was lysed using 4 ml/pellet (from 250 ml culture) of buffer containing 5mM imadazol, 0.5 M NaCl, 10% (by volume) glycerol, 0.1% IGEPAL CA630 (Sigma, I-3021) and 20 mM Tris HCl pH 7.9 and 1 protease inhibitor tablet/10ml (Roche Diagnostics, 1-836-170). The mixture was sonicated briefly (10-20 seconds) until the viscosity was consistent throughout the sample, after which the sample was centrifuged at 16,000 x g. The supernatant was then passed through a column prepared with His-Bind Resin (Novagen, Cat# 69670-3) and washed with a similar solution containing 60 μM imidazol (Sigma, I-2399). The protein was eluted by the addition of a solution containing 20 mM Tris HCl pH 7.9, 0.5 M NaCl and gradually increasing the concentration of imidazol until the protein came off the column (at ~260 μM imidazol). Samples of the elutants were tested using SDS-PAGE to determine purity and protein, by staining with Coomasie G250 (Fein Biochemica, 35050)

and concentration by comparing against a set of known bovine serum albumen (BSA) standards.

For biological assays and protein blotting using recombinant FGF-16, human FGF-16 (hFGF-16) and FGF-9 (hFGF-9) were purchased (Peprotech, 100-29 and 100-23 respectively). The short form of recombinant rat FGF-2 (18 kDa) was obtained as a generous gift (Dr. Kardami, University of Manitoba, Canada).

2.1.3 Generation of antibodies

Antibody generation and purification was performed by Quality Controlled Biochemicals Inc. (QCB). Antibodies were generated by injecting 2 adult rabbits with a synthesized peptide (ALNKDGSPREGYRTKRHQK) conjugated to keyhole limpet hemocyanin. The peptide corresponded to a portion of the protein predicted to be one of the more antigenic regions. Animals were subject to 1 immunization and 4 boosts before blood was taken for the final antibody purification. The antibody from each rabbit (61660 and 61661) was purified by QCB using an affinity column created with the peptide used for the immunizations and subsequently referred to as Ab60 and Ab61.

Two sources of commercial antibodies were obtained for use against FGF-16 (Peprotech, 500-p160G and Santa Cruz SC-16820) as well as FGFR-1, FGFR-2, FGFR-3 or FGFR-4 (Santa Cruz, SC-121, SC-122, SC-123 and SC-124, respectively).

2.2 Cell culture

2.2.3 WEHI cells

A leukemia blood cell line (WEHI) was purchased from American Type Culture Collection (TIB-68) for the purpose of conditioning media with interleukin 3 (IL-3). Briefly, cells were grown to confluence in Iscove's modified Dulbecco's medium (Sigma, I-7633) with 4 mM glutamine (Gibco Invitrogen Corp., 25030-149) adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 50 μM β-mercaptoethanol (Sigma M-3148), 10% fetal bovine serum (FBS) (Gibco Invitrogen Corp., 261040-079) and 1 x Penstrep (Gibco Invitrogen Corp., 15140-122). After cells had reached confluency, cells were washed and fed RPMI (Gibco Invitrogen Corp., 23400-062) supplemented with 50 μM β-mercaptoethanol, 10% FBS and 1 x Penstrep. Media was conditioned for a period of 48 hours before filtering (0.2 μm) and being stored at -20°C until needed.

2.2.4 BAF-3 cells

A bone marrow progenitor cell line (BAF-3) expressing FGFR-2c was obtained as a generous gift from Dr. David Ornitz (Washington University Medical School, St. Louis, MO). Cells were grown in a Feeding Media consisting of RPMI (Invitrogen) containing 0.6 mg/ml Gentamycin (Gibco Invitrogen Corp., 11811-031), 10% Conditioned Media from WEHI cells, 10% FBS, 1% glutamine 4mM, 1 x Penstrep and 50 μM β-mercaptoethanol. Cells were split 1:6 when they reached ~70% confluency.

2.2.5 HEK 293 cells

Human embryonic kidney cells (obtained as a gift from Dr. Kardami, University of Manitoba, Canada) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen Corp., 12100-061) supplemented with 1 x Penstrep, 5% FBS and 8.3 mM glutamine. Cells were grown until 60-80% confluency before splitting by washing with a phosphate buffered salt solution free of calcium and magnesium (PBS-CMF) and released from the plate using Trypsin-ethylenedinitrilotetraacetic acid (EDTA) (Gibco Invitrogen Corp., 25300-054), counted using a hemocytometer and diluted for plating at the desired concentration. Cells were plated at a density of 0.1 million per 100 mm culture dish (Corning, 430167) and allowed to grow overnight before transfecting. Cells were transfected using a calcium phosphate method previously described (Pasumarthi et al., 1997). After 24 hours cells were washed with PBS-CMF and fed the same media used to culture the cells. Cells were left to express and condition media for 48 hours before harvesting media. Media was then passed through a 0.2 μm filter and frozen at –20°C for later use.

The PBS-CMF solution consisted of 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.5 mM NaH_2PO_4 which was filtered (0.2 μM) and stored in autoclaved bottles at 4°C until used. The solution was warmed to 37 °C prior to use for washing cells.

2.2.6 Primary cultures of myocytes and non myocytes

2.2.6a Myocytes

Neonatal rat cardiac myocytes (NRCM) were isolated as previously described (Detillieux et al., 1999). Briefly, neonatal rats less than 24 hours of age were decapitated and their hearts removed. Hearts were placed in a phosphate buffered saline digestion solution (PBDS) comprised of 136.9 mM NaCl, 2.7 mM KCl, 1.5 mM Na_2HPO_4, 4.3 mM KH_2PO_4 and 55.5 mM dextrose at a pH of 7.3. Hearts were then minced into small pieces ~$2mm^2$ and placed in a water jacketed spinner flask at 37°C. A PBDS enzyme cocktail containing 740 units of Collagenase, 340 units Trypsin and 2280 units DNAse with a total volume of 10 ml was added to the flask and stirred for 10 min at 37°C. The supernatant was removed and stored in a bottle containing 10 ml of FBS. The remaining pieces were subjected to another 6 enzyme incubations each time pooling the supernatants. The supernatant was then strained through a nytex membrane and centrifuged at 300 x g for a period of 2 min. The cells were resuspended in a N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffered solution consisting of 116.4 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.6 mM dextrose, 5.4 mM KCl and 0.8 mM $MgSO_4$ at a pH of 7.3. The suspension was placed on a HEPES buffered Percoll (Amersham Biosciences, 17-0891-01) with the same components mentioned but with the upper and lower portions containing 40.5% and 58.5% Percoll by volume. After a 30 minute centrifugation at 2000 x g the cells separated into 2 distinct layers. The top layer consisted of predominantly non myocytes, while the bottom contained mostly myocytes. Each population of cells was washed by diluting the Percoll with HEPES buffer, followed by centrifugation for 2 min at 300 x g and removal of the

supernatant. The pellet was resuspended a second time and the process repeated only this time cells were resuspended in a plating media consisting of Ham's F-10 (Invitrogen (Sigma, N-6635) supplemented with 10% FBS, 10% horse serum and 1 x Penstrep. Myocytes were either plated on glass coverslips or 60 mm plastic culture dishes (Corning, 430166) coated with rat tail collagen (Upstate Biotechnology, 08-115) at a density of 1 million cells/60 mm diameter plate or 0.65-85 million/35 mm plate.

2.2.6b Non myocytes

Non myocytes were obtained from the upper portion of the Percoll gradient mentioned in the previous section. Cells were treated as described for the myocytes however the final pellet was resuspended in 20 ml of plating media and placed in two 100 mm diameter culture dishes. After 18 hours, cells were washed with HEPES buffer and fed Defined Media. Defined Media consisted of DMEM F-12 (Gibco Invitrogen Corp. 12500-062) supplemented with 0.5% FBS, 0.66% by volume albumen (Sigma, A-8327), 1 x penstrep, 115 μM ascorbic acid (Sigma, A-0278), and 1 x insulin transferrin selenium (ITS) (Gibco Invitrogen Corp., 51300-044). Cells were split every 24 hours 1:2 in the same way previously described for HEK 293 cells with the exception that they were fed the Defined Media described.

2.3 Assays

2.3.1 Oxidative Stress

Following plating, myocytes were washed with HEPES buffer and fed Defined Media (see section 2.2.6b for details). Cells were grown for 48 hours before switching to a Defined Media containing only 0.1 x ITS. Cells were cultured for an additional 24 hours before they were subjected to media with and without 200 μM H_2O_2 in media conditioned with transfected HEK 293 cells expressing either empty vector ($pcDNA_3$) for positive and negative controls and FGF-16 ($pcDNA_3$-FGF-16). Cells were then harvested after 24 hours by fixation with 1% paraformaldehyde solution which was prepared in advance and cooled to 4°C prior to use. The solution contained 154 mM NaCl, 0.4 mM KCl, 0.2 mM KH_2PO_4, 1.2 mM Na_2HP_4, 1% paraformaldehyde (Fisher, UN-2213) and 0.01 N NaOH and was prepared by heating to 55°C in order to dissolve solutes. The solution was then adjusted to a pH of 7.4 with HCl after it had cooled to room temperature. Samples were fixed for 15-20 min before treatment with 0.1% Triton X-100 in PBS for 15 min at 4°C, rinsed with PBS and stained with Hoechst stain. Coverslips were then mounted on slides with Crystal mount (Biomeda corp.).

Digital pictures were taken of the nuclei at low magnification (0.31 mm^2 per field). Using Image Tool software (University of Texas Health Science Center), the objects (nuclei) were segregated into 4 groups based on surface area. Those with sizes >180 or <50 μm^2 were dismissed as being more than one nuclei or debris, while those within the specified range consisted of both condensed, <135 μm^2 and normal sized nuclei, <135μm^2.

2.3.2 MAPK

Cells treated with recombinant FGF-16 were incubated for 48 hours in defined media, then a further 22 hours with defined media containing only 0.1 X ITS. To further reduce basal MAPK activity, cells were incubated in only DMEM-F12 for 2 hours prior to the treatment to be tested. This was achieved by the addition of a cocktail which made up 20% of the final culture dish volume (ie 1 of 5 ml). The cocktail contained the proper ratio of growth factor and/or vehicle to ensure that the only difference between plates was the amount of growth factor added. Heparin was also included in the cocktail to achieve a final concentration of 5 μg/ml. Cells subjected to conditioned media from HEK 293 cells were treated slightly different in that the switch to DMEM-F12 was omitted and the time extended by 2 hours. The intention being to minimize the serum response when the HEK 293 conditioned media was added as it already contained 5% serum. This translated to an increase of 0.5% serum in all samples including the control.

Samples were treated with the appropriate stimulus and then washed two times with ice cold PBS-CMF and frozen with liquid nitrogen. For processing, samples were thawed by adding 0.5 ml of lysis buffer which consisted of 50 mM Tris HCl pH 6.8, 10 mM NaF, 2% SDS, 25 mM β-glycerophosphate, 1mM Na_3O_4V, 1mM EDTA, 1mM ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA) and 1 protease inhibitor tablet/10 ml. Cells were scraped with a rubber policeman and boiled for 5 min. Samples were then sonicated briefly (~5-10 seconds) and centrifuged to remove insoluble material. The supernatant was then supplemented with a loading buffer and boiled prior to running in a sodium dodecyl sulphate poly acrylamide gel elecrophoresis (SDS-PAGE) gel.

Analysis of MAPK phosphorylation/activation was conducted by protein blotting using a phosphospecific MAPK sampler kit (Cell Signaling Technology, 9910). Protein blots were probed as described for the FGF-16 antibodies in section 2.3.6. Antibodies were diluted 1:1000 for phospho-p42/p44 ERK, phospho-p38 and phospho-JNK1/2 (Cell Signalling, 9106, 9216 and 9255, respectively).

2.3.3 Live dead assay

A cocktail of 500 µl IL-$3^{(-)}$ RPMI feeding media containing 5 µg/ml heparin and the appropriate components to be tested (ie. Media conditioned with transfected HEK 293 cells, recombinant FGF-16 protein or IL-3) were placed in 35 mm diameter culture dishes (Corning, 3516). BAF-3 cells were concentrated by centrifugation (3 min 500 x g), washed by resuspending in RPMI, centrifuged again and then resuspended in IL-$3^{(-)}$ feeding media. Cells were diluted to 0.5 million/ml and 2 ml of the cell suspension was then added to each culture dish. The plates were rocked side to side 3-4 times to mix the samples and then placed in an incubator at 37°C with 5% CO_2 for 17 hours. Cell suspensions were then removed by gentle titration and placed in an appropriate tube for flow cytometry. Cells were centrifuged at 500 x g for 3 min, the supernatant was then aspirated leaving approximately 150 µl behind. Cells were then washed with 5ml of PBS and the centrifugation and aspiration process repeated. A 50 µl cocktail containing components of the live/dead assay (Molecular probes, L3224) was added to the cells to yield a final concentration of 4 µM calcein and 4 µM ethidium bromide. After a 1.5 hour incubation, 1 ml of PBS containing 4 µM ethidium bromide was added to the tube to provide enough volume for flow cytometry analysis.

2.3.4 RNA isolation and blotting

Tissue samples were dissected from animals as soon as possible after death. Samples were frozen on dry ice and stored at -70°C until processed. RNA was isolated by means of one of 3 methods. 1) RNA isolated from heart samples used to clone the FGF-16 gene was isolated using a Trizol RNA extraction kit (Gibco Invitrogen Corp., 5596UA). 2) Tissue samples processed for nothern blots were done using the Qiagen Fibrous tissue RNA extraction kit (Qiagen, 75742). 3) Cell cultures were processed using the Sigma Genelute total RNA miniprep kit (Sigma, RTN-70).

RNA concentration was measured by optical density at 260 nm and aliquots frozen at –70°C until needed. Samples used for blotting were loaded on a 1.5% formaldehyde agarose gel as previously described (Maniatis et al., 1982). Samples were run overnight at 25V and transferred by capillary action to a nylon membrane (Pall Corp., 60207) using 20 x sodium chloride and citrate buffer (SSC). The Blot was then hybridized with the appropriate probe using NorthernMax Prehybridization/Hybridization Buffer (Ambion, 867-500) as per the manufacturers directions. Briefly after 4 hours prehybridization, the appropriate probe was boiled and added to the solution at 65°C and the hybridization oven temperature reduced to 42 °C where hybridization was allowed to occur for 24 hours. The blot was then washed 1 x with 6x SSC solution followed by 2 washes with 0.1 x SSC containing 0.1% SDS. The blot was then placed in a plastic bag and exposed to Biomax film (Kodak, 165-1454) for a period of 2-7 days. The 20 x SSC

solution consisted of 3.0 M NaCl and 0.3M $NaC_6H_5O_7$-$2H_2O$ which was filtered with a 0.2μm filter.

2.3.5 Heparin enrichment of samples

2.3.5a Conditioned media from cultures

Media from myocytes, non myocytes and transfected HEK 293 cells was allowed to condition for 48 hours prior to harvest. The media was frozen and stored at -20°C until needed. Media was supplemented with 0.2% Tween 20 (Biorad) and 1 protease inhibitor cocktail tablet (Roche Diagnostics, 1-836-170) per 50 ml media. A slurry of washed heparin sepharose beads (CL-6, Pharmacia) were then added to the media (100 μl/50 ml) and incubated on a rocking platform for 2 hours at room temperature. The mixture was then centrifuged to pellet the beads, after which they were transferred to an 1.5 ml centrifuge tube using a 1 ml pipette gun to remove the pellet and some media for a final volume of no more than 1.5 ml. The sample was then centrifuged for 15 seconds at 1000 x g in order to pellet the beads. The supernatant was removed by aspirating with a 32 gauge needle which left the beads behind. The beads were then washed by adding 1 ml of 250 mM NaCl containing 10 mM Tris HCl pH 7.0, 1 mM EDTA and 1 protease inhibitor tablet/50 ml and inverting the tube four times to dislodge the beads. This washing process was repeated with a buffer containing 100 mM NaCl. After removing the final wash solution, 50 μl of 1 x loading buffer was added to the beads. The pellet was subjected to vortex and boiled for 5 min prior to loading on a 15% SDS-PAGE gel.

Samples that were deglycosylated after extraction were done so by treatment with Endo HF glycosidase enzyme (New England Biolabs, P0703S). Briefly, the final 0.1M NaCl wash of the heparin beads with the bound protein was done in the absence of protein inhibitors. To the beads, the appropriate volume of diluted buffer and enzyme (1 µl per 50 µl sample) was added and then incubated for 1 hour at 37°C. Afterwards the appropriate amount of 5 x loading buffer (see section 2.3.6) was added for SDS-PAGE and the sample boiled before loading the liquid portion.

2.3.5b Extractions from heart tissues

Heart tissues were frozen with liquid nitrogen and stored at –70°C until processed. Hearts where homogenized in extraction buffer at a ratio of 1g tissue / 4ml buffer. The extraction buffer (as well as subsequent buffers with some variation) was composed of 1M NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma, P7626), 10 mM Tris HCl (pH7.0), 1 mM EDTA and 1 protease inhibitor tablet/50 ml. The homogenate was centrifuged at 45000 x g for 20 min at 20°C. The supernatant was removed and supplemented with 1% Tween-20, mixed and diluted with a buffer similar to that of the extraction buffer but without NaCl. The solution is diluted to a final concentration of 0.25M NaCl and centrifuged a second time to remove any precipitates. A slurry of 1:1 0.25M NaCl buffer and hydrated heparin sulfate beads was then added to the mixture at a ratio of 20µl for every 10 ml of supernatant. The mixture was then gently agitated for 2 hours before centrifuging at 500 x g to pellet the beads. The beads were washed once with 0.25 M NaCl containing buffer but without PMSF, centrifuged again, followed by a 0.1 M NaCl wash. After removal of as much liquid as possible using a 32 gauge needle,

an equal volume of loading buffer (1 x) was added to the beads. The beads were then boiled before loading the liquid portion on a SDS-PAGE gel.

2.3.5c Exctractions from cell cultures

Cells were washed twice with PBS to remove any remaining culture media. An extraction solution as described in the previous section was then applied to the cells (0.5 ml/ 60mm plate) with the exception that the NaCl concentration was 2M. The mixture was then frozen (-70 °C) until processed. The solution was thawed and sonnicated briefly (~10-15 seconds), followed by centrifugation at room temperature for 10 minutes at 15,000 x G. The supernatant was then removed and diluted to 0.25 M NaCl and incubated with heparin sepharose beads, washed and loaded as described in the previous section regarding tissue extractions.

2.3.6 Protein blotting

All samples were mixed 1:5 with 5x loading buffer of (10% SDS, 1.5M dithiothreitol, 0.3M Tris HCl pH 6.8) and boiled for 5 min before loading on a SDS-PAGE gel. SDS-PAGE gels (12-15%) were transferred to a polyvinylidine fluoride (PVDF, Immobilon, IPVH-00010) at 100 V for 2 hours using a buffer containing 20% methanol, 192 mM glycine, and 25 mM Tris. Membranes were stored in a Tris buffered solution (TBS) consisting of 10 mM Tris HCl pH 8.0 or 7.6 and 150 mM NaCl. Before probing,, blots were checked for equal protein loading between lanes using Ponceau S stain. After transfer to PVDF membrane, blots were soaked in the presence of 0.01% Ponceau S powder (Sigma, P3504) with 0.15% trichloroacetic acid for 10 minutes followed by a brief wash in water to visualize the proteins.

Membranes were blocked by incubating the membrane in a TBS solution containing 5% dried non fat milk for a period of 2 hours. The membrane was then washed for 5 min in TBS-T which was comprised of TBS solution supplemented with 0.5% by volume Tween-20 (Biorad Corp., 170-6531). The blot was then placed in TBS with 1% non fat milk powder for 5 min, followed by the same solution containing 0.13 μg/ml antibody Ab60 or Ab61, 0.3 μg/ml commercial FGF-16 antibody from AbPeprotech (Peprotech, 500-P160) or 1.0 μg/ml AbSantaCruz (Santa Cruz Biotechnology, SC168020). Control antibodies consisted of normal rabbit IgG at 10 μg/ml (Sigma, I-5006) for 1 hour at room temperature. The membrane was then washed 4 times with TBS-T with 1% milk for 5 min. Secondary antibody consisted of either anti rabbit horseradish peroxidase (HRP) (Biorad Corp., 170-6515) or anti-goat HRP (Sigma, A-5420) was then applied with 1% TBS-T 1% milk and incubated for 1 hour. The membrane was then washed 4 times in TBS-T solution for 5 min followed by a 5 min incubation with Pico West Supersignal (Pierce, 34080). Exposures of film (Amersham Biosciences, 120662) ranged from only a few seconds to over 20 min depending on samples.

Gels stained with coomassie G-250 stain were soaked in a staining solution consisting of 10 % acetic acid, 50 % methanol and 250 mg/100 ml coomassie G-250 (Feinbiochemica, 35050) for 1 hour at room temperature. The gel was then washed several times in 5% methanol 7.5 % acetic acid to remove the excess stain. Destaining required 24 hours.

2.3.7 Detection of FGFR-2c

Samples of RNA isolated using the methods described (section 2.4.4) were subjected to RT-PCR in order to amplify a region of the transcript unique to the FGFR-2c splice isoform. The protocol was similar to that previously described by Ford and colleagues (1997). Briefly, primers used consisted of Forward 5′-CCCATCCTCCAAGCTGGACTGTCCT-3′ and Reverse 5′-CTCCTTCTCTCTCACAGGTGCTGG-3′which flanked a unique region of the FGFR-2c splice isoform. The RT-PCR product was generated by using the following sequence of temperatures and times; 1) 95°C for 4 min to denature, 2) 59°C for 1 min to anneal, 3) 72°C for 1.5 min for elongation, 5) 95°C for 1 min to denature, repeating steps 2-5 for 29 cycles before ending with 59°C for 1 min, 72°C for 10 min and storing at –20°C until use. Products were probed by Southern blotting as previously described (Jin et al., 1999). Briefly, products were then run (equivalent to 40 ng RNA starting material) in a 1.5% agarose gel and transferred to nylon membrane where they were probed using an end labelled (^{32}P) oligonucleotide, 5′-GGTGTTAACACCACGGACAAAGAG-3′ probe homologous to a region of the expected product.

2.3.8 Immunohistochemistry

Immunofluorescent detection was performed on myocytes after transfection with either $pcDNA_3$ vector (Gibco Invitrogen) or $pcDNA_3$ with FGF-16 cDNA inserted into the vector. In addition vector expressing the enhanced green fluorescent protein (EGFP), pEGFP-C1 (BD Biosciences) was also used as well as a derivative containing the FGF-16

cDNA which produced a fusion protein (pEGFP-FGF-16). Myoctyes were plated at a density of 0.75 million/35 mm diameter culture dish containing a 22 mm diameter glass coverslip (Fisher Cat # 12-545-85) and were fed D-MEM with 10% FBS and 1 x Penstrep. Cells were treated with 8.75 µl/plate Lipofectamine (Invitrogen) and 8.5 µl/plate Plus reagent (Invitrogen) to a final volume of 2 ml with 2.25 µg/ml DNA plasmid. Co transfections were conducted by using a ratio of reporter (EGFP-C1) to expression vector ($pcDNA_3$ or $pcDNA_3$-FGF-16) of 1 to 3. Cells were allowed to take up the DNA for a period of 16 hours and then fed D-MEM with 10% FBS and 1 x Penstrep for a period of 24 hours before harvesting.

Cryosections of adult mouse heart (8-10 weeks) were prepared by freezing pieces of tissue in optimal cutting temperature solution (Sakura Finetek, 4583) in a dry ice ethanol slurry. Samples were then sectioned by cryostat to a thickness of 7 µm and placed on glass slides where they were fixed and treated as follows.

Fixation of harvested cells on coverslips and heart cryosections were as follows: Samples were fixed with 1% paraformaldehyde solution as described in section 2.3.1. Samples were washed 4 times and then the appropriate antibodies applied. Primary antibodies used included rabbit anti FGFR-1, FGFR-2, FGFR-3, and FGFR-4 at 1 µg/ml, Ab60 at 1 µg/ml, Ab61 at x 1µg/ml as well as goat AbPeprotech at 10 µg/ml and goat IgG at x 10 µg/ml (Sigma, 15256). After incubation overnight at 4°C, samples were washed with PBS 4 x and secondary antibodies applied. Secondary antibodies included anti rabbit Texas Red or Biotin (Amersham, RPN2034, RPN1004), anti goat Texas Red (12 µg/ml) (Jackson Labs, 705-155-147). For sections treated with anti-rabbit biotin, samples were washed 4 times with PBS and incubated with Streptavidin Fluorescein

(Amersham, RPN1232). Samples were then washed 4 times with PBS and either mounted with crytsal mount or stained with Hoecsht dye and then mounted.

2.3.8a Preabsorption of antibodies for cryosections

In order to preabsorb antibodies recombinant hFGF-16 (10 ng/μl) or the peptide (0.8 μg/μl) used to generate the custom antibodies were fused to a fixed substrate. Briefly, 200 μl of either FGF-16 protein, FGF-16 peptide or an amount of peptide equal to the molar concentration of the protein used (0.09 μg/μl) PBS with 2% BSA as a control were added to wells of a 96 well immunoassay microtitre plate (Fisher), where they were allowed to bind overnight at 4°C. The wells were then washed 4 times with PBS and blocked by filling (~250 μl) with PBS containing 2% BSA. After overnight incubation at 4°C, the wells were washed 4 times and the antibodies to be used added at 2 x (200 μl) the concentrations to be used. After an overnight incubation at 4°C, the antibodies were carefully removed and used on cryosections after dilution to the concentrations mentioned.

2.3.9 Statistical analysis

Statistical analysis of groups was performed using either Dunnet multiple comparisons test or Bonferroni one way analysis of variance. Statististical significance is indicated by * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

Chapter 3

3.0 Development of detection assays for FGF-16

3.1 Cloning of mouse FGF-16 cDNA

FGF-16 is a member of the FGF family that was reported to have high levels of transcript in the adult rat heart in comparison with other adult tissues (Miyake et al., 1998). However, the distribution of FGF-16 transcript has not been investigated in the mouse heart, nor has the endogenous protein been characterized. As a first step towards the assessment of mouse FGF-16 RNA, it was necessary to clone the FGF-16 cDNA from mouse heart RNA. This had two important purposes: 1) to serve as a probe for RNA blotting and 2) to predict the amino acid sequence of the protein, which can then be used for designing antibodies against FGF-16 protein. Cloning was achieved through the use of RT-PCR (section 2.2.1), which yielded a product of the expected size (636 bp). The nucleotide sequence closely resembled that of human and rat FGF-16 with 95% and 97% nucleotide sequence similarity (Figure 3.1). Predicted protein sequence revealed 99% conserved amino acid sequence between that of both human and rat FGF-16 with a calculated pI of 9.36.

3.2 Detection of FGF-16 transcript in adult mouse heart

Only limited information has been reported thus far in regards to FGF-16 transcript and its distribution. Initially, all information was obtained from the investigation of rat tissues (Miyake et al., 1998) and only recently has there been

evidence for the presence of FGF-16 transcript in embryonic mouse hearts (Lavine et al., 2005). With the possibility of generating a transgenic mouse model to further the study of FGF-16 function, it was necessary to first investigate endogenous FGF-16 transcript in the mouse. Although there is a high degree of sequence identity between FGF-16 versus FGF-9 and FGF-20, a difference in transcript size has been reported: 1.8 kb for FGF-16 and 2.4 kb for FGF-9 and FGF-20 (Miyake et al., 1998, Miyamoto et al., 1993, Kirikoshi et al., 2000). The FGF-16 cDNA was used to probe a blot containing RNA isolated from adult mouse heart and kidney (Figure 3.2). Equal amounts of RNA were loaded by calculating the concentration based on the absorbance at 260 nm and verified by ethidium bromide staining under ultraviolet light after running in an agarose gel. Thus, the kidney served as a negative control as it has been shown to contain elevated levels of FGF-9 transcript as compared to the heart (Miyamoto et al., 1993). The FGF-16 cDNA probe resulted in a prominent signal from mouse heart RNA (and not the kidney) which corresponded to a 1.8 kb transcript relative to the 28 and 18 S RNA (Figure 3.2). This is similar to that previously reported (Miyake et al., 1998, Konishi et al., 2000, Sontag and Cattini 2001), and is shorter than the 2.4 kb previously reported for FGF-9 (Miyamoto et al., 1993) and FGF-20 transcript (Kirikoshi et al.,2000). Therefore, FGF-16 transcript can be detected in adult mouse heart via northern blotting using FGF-16 cDNA as a probe.

3.3 Detection of FGF-16 protein

3.3.1 Generation of FGF-16 antibody

Examining some of the fundamental properties of FGF-16 (such as protein size and distribution) requires the use of antibodies. More specifically, these techniques are dependent on the antibody's ability to bind the protein of interest under the conditions employed. For example, epitope presentation is often dependent on whether a protein is 1) in its native conformation, 2) cross linked (i.e., fixation) to surrounding proteins or 3) is denatured due to the presence of detergents. It is therefore important to consider primary and secondary structure of a protein when selecting an immunogen for antibody generation. More specifically, an antibody that has been generated against a particular region of a protein may behave differently than one generated using the entire protein. These differences can often result in one antibody being better suited for one application as opposed to another. In addition other variables affecting binding may include changes to proteins as a result of posttranslational modification such as phosphorylation or glycosylation as they may interfere with epitope recognition. Therefore it is often best to generate and test several antibodies.

Initially, we proceeded with antibody construction by synthesizing a peptide which corresponded to a region of FGF-16. Using the FGF-16 amino acid sequence as a template, a region predicted as having a high probability of antigenicity (A^{164} to K^{182}, refer to section 2.1.2) was chosen and a corresponding peptide synthesized. This region was also compared for similarity to that of closely related FGF-9 and FGF-20 and found to differ by several amino acids (Figure 3.3). Although there were regions that were perhaps more unique in terms of differences in sequence they were predicted to be less

immunogenic and thus less likely to result in an antibody. Therefore the highly immunogenic peptide mentioned was the initial choice for the immunization of two rabbits (61660 and 61661) from which serum was later harvested for testing and affinity purification, resulting in the generation of two rabbit polyclonal antibodies designated Ab60 and Ab61. Subsequently, two independent commercial sources of FGF-16 antibody (from goat) raised against the full length protein (Peprotech Cat# 500-P160BT, or AbPeprotech) and an N-terminal peptide of FGF-16 also became available Santa Cruz (Cat # SC16820, or AbSantacruz).

3.3.2 Testing of antibody specificity

The high degree of homology between FGF-16 and FGF-9 raised concerns as to any given antibody's specificity for FGF-16. Therefore, antibodies were assessed for specificity by comparing their ability to detect recombinant human FGF-16 (hFGF-16, Peprotech) over recombinant human FGF-9 (hFGF-16, Peprotech) for sequence comparison see (Figure 3.4). Probing protein blots containing serial dilutions of hFGF-16 and hFGF-9 revealed that both Ab60 and Ab61 were able to detect FGF-16 at levels greater than 2.5 ng/lane while both commercial antibodies were not nearly as sensitive. Commercial antibody obtained from Santa Cruz was unable to detect any hFGF-16 or hFGF-9 even after prolonged exposures. While three of the antibodies could detect hFGF-16 with ease, hFGF-9 protein was also detectable when levels were above 7.5 ng per lane. This indicated that the antibodies have a limited specificity toward hFGF-16 as compared to that of hFGF-9 with a difference in detection of approximately four fold.

Commercial antibody (Peprotech) against FGF-9 were also tested but were unable to detect recombinant FGF-9 or FGF-16 even after prolonged exposures (data not shown).

3.4 Production of FGF-16 protein

The generation of pure FGF-16 is desirable for use in assays both to test antibodies and to investigate the biological function of FGF-16 *in vitro*. Initially, there was no commercial source of recombinant FGF-16 protein available, so a bacterial expression system was employed to generate and purify the protein. The cDNA for FGF-16 was subcloned into an expression vector (pET19b) containing a polyhistidine tag on the N-terminus of FGF-16. In this system, the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) results in the induction of T7 RNA polymerase via the lac operon LaCV5, and the polymerase in turn transcribes the his-tagged FGF-16. The accumulation of recombinant protein prior to purification can be seen over time with coomasie blue staining, as shown in Figure 3.5A. Following purification with a nickel affinity column, Coomasie blue staining revealed a single prominent band with an apparent molecular weight of 26.3 kDa (Figure 3.5B), comparable to that of the predicted size for his-tagged FGF-16 (26.8 kDa).

Since the development of the his-tagged expression system, commercially produced recombinant hFGF-16 became available from Peprotech, making it difficult to justify the use of his-tagged protein when pure, recombinant, wild-type protein was available. However, our system has been catalogued and set aside for future use where a polyhistidine tag may be advantageous, such as in the study of protein-protein

interactions where antibody binding may interfere with the interaction being studied and where an alternative method of FGF-16 identification and extraction is necessary.

His-tagged or not, recombinant FGF-16 produced in bacterial systems is not subject to post-translational modifications as is normally the case in eukaryotic cells. COS-1 cells transfected with Myc-tagged FGF-16 produce a secreted, glycosylated protein (Miyakawa and Imamura, 2003). Given that we now have antibodies raised against an FGF-16 peptide, the use of a Myc tag is no longer necessary and so we sought to express wild-type FGF-16 in human embryonic kidney (HEK 293) cells with a view to using the secreted product in assays of biological function. Using this secreted product alongside commercially produced recombinant protein allows for the comparison of function with and without post-translational modifications (Chapter 5). In addition, the production of a fusion protein consisting of the enhanced green fluorescent protein (EGFP) fused to the N-terminus of FGF-16 (EGFP-FGF-16) was undertaken with a view to investigating the subcellular localization of FGF-16 (Chapter 4).

Thus, the FGF-16 was cloned into expression vectors to produce both FGF-16 (in the vector pcDNA3) and EGFP-FGF-16 (in pEGFP-C1), and the transfection of HEK 293 cells resulted in the production of detectable protein product in heparin-extracted conditioned media after 48 hours (Figure 3.6). Only cultures containing FGF-16 cDNA produced a signal after protein blotting and antibody detection; non-transfected cells as well as those transfected with vector alone did not produce a signal. Media from cells transfected with pcDNA3-FGF-16 produced two bands with apparent molecular weights of 25.4 and 21.1 kDa. These were slightly different from the predicted size (23.7 kDa) or recombinant protein used for comparison (22.9 kDa), which also sometimes yielded a

second band corresponding to what is believed to be the dimer of the protein (~45 kDa). The pEGFP-FGF-16 plasmid also produced two bands in conditioned media: a 47.9 kDa product, approximate to the predicted size of the fusion protein (50.7 kDa), and a much smaller 21.7 kDa protein, likely the result of proteolytic cleavage of the fusion protein (Figure 3.6).

The discrepancy between the predicted and apparent molecular weight of FGF-16 could be explained by post-translational modification: in particular, a larger than expected size may be due to glycosylation of the secreted product. In Figure 3.7, the addition of glycosidase enzyme to heparin enriched samples from conditioned media of transfected HEK 293 cells expressing FGF-16 resulted in a decrease in size of the predominant band to that of the predicted protein size. This result was independent of the antibody used to probe the protein blot. These data support the hypothesis that FGF-16 protein secreted from cells is glycosylated, consistent with that reported for COS-1 cells (Miyakawa and Imamuara, 2003).

3.5 Summary

The mouse FGF-16 cDNA is 624 bp long and closely resembles that of rat and human FGF-16 with 95% and 97% sequence identity respectively. The cDNA codes for a protein which when expressed in a HEK 293 cell line results in the secretion of a glycosylated form of the protein, a feature which distinguishes it from commercially available recombinant FGF-16. Antibodies generated against the protein are able to detect both the glycosylated and non glycosylated form of the protein suggesting a possible means of detecting and characterizing endogenous FGF-16.

Fig 3.1

```
mouse   ATGGCGGAGGTCGGGGGCGTCTTTGCCTCCTTGGACTGGGACCTGCAAGGCTTCTCCTCC 60
rat     ATGGCGGAGGTCGGGGGCGTCTTTGCCTCCTTGGACTGGGACCTGCAAGGCTTCTCCTCC 60
human   ATGGCAGAGGTGGGGGGCGTCTTCGCCTCCTTGGACTGGGATCTACACGGCTTCTCCTCG 60

mouse   TCTCTGGGGAACGTGCCCTTAGCTGACTCCCCGGGTTTCCTGAACGAGCGCCTGGGCCAG 120
rat     TCTCTGGGGAACGTGCCCTTAGCTGACTCCCCGGGTTTCCTGAACGAGCGCCTGGGCCAG 120
human   TCTCTGGGGAACGTGCCCTTAGCTGACTCCCCAGGTTTCCTGAACGAGCGCCTGGGCCAA 120

mouse   ATCGAGGGGAAGCTGCAGCGCGGCTCGCCCACAGACTTCGCCCACCTGAAGGGGATCCTG 180
rat     ATCGAGGGGAAGCTGCAGCGCGGCTCGCCCACAGACTTCGCCCACCTGAAGGGGATCCTG 180
human   ATCGAGGGGAAGCTGCAGCGTGGCTCACCCACAGACTTCGCCCACCTGAAGGGGATCCTG 180

mouse   CGGCGCCGCCAGCTCTACTGCCGCACCGGCTTCCACCTTGAGATCTTCCCCAACGGCACG 240
rat     CGGCGCCGCCAGCTCTACTGCCGCACGGGCTTCCACCTTGAAATCTTCCCCAATGGCACG 240
human   CGGCGCCGCCAGCTCTACTGCCGCACCGGCTTCCACCTGGAGATCTTCCCCAACGGCACG 240

mouse   GTGCACGGCACCCGCCACGACCACAGCCGCTTCGGAATTCTGGAATTTATCAGCTTGGCT 300
rat     GTGCATGGCACCCGCCACGACCACAGCCGCTTCGGAATTCTGGAATTTATCAGCTTGGCT 300
human   GTGCACGGGACCCGCCACGACCACAGCCGCTTCGGAATCCTGGAGTTTATCAGCCTGGCT 300

mouse   GTGGGGCTGATCAGCATCAGGGGAGTGGACTCTGGCCTGTACCTAGGAATGAATGAGCGA 360
rat     GTGGGGCTGATCAGCATCCGGGGAGTAGACTCTGGCCTATACCTAGGAATGAATGAGCGA 360
human   GTGGGGCTGATCAGCATCCGGGGAGTGGACTCTGGCCTGTACCTAGGAATGAATGAGCGA 360

mouse   GGAGAGCTCTATGGATCGAAGAAACTCACACGTGAATGTGTTTTCCGGGAACAGTTTGAA 420
rat     GGAGAGCTGTTTGGATCGAAGAAACTCACACGAGAATGTGTTTTCCGGGAACAGTTTGAA 420
human   GGAGAACTCTATGGGTCGAAGAAACTCACACGTGAATGTGTTTTCCGGGAACAGTTTGAA 420

mouse   GAAAACTGGTACAACACCTATGCCTCCGCCTTGTACAAACACTCGGACTCGGAGAGACAG 480
rat     GAAAACTGGTACAACACCTATGCATCCACCTTGTACAAACACTCGGACTCGGAGAGACAG 480
human   GAAAACTGGTACAACACCTATGCCTCAACCTTGTACAAACATTCGGACTCAGAGAGACAG 480

mouse   TATTATGTGGCCCTGAATAAAGACGGCTCACCCCGGGAGGGATACAGGACTAAACGACAC 540
rat     TATTATGTGGCCCTGAATAAAGACGGCTCACCCCGGGAGGGATACAGGACTAAACGACAC 540
human   TATTACGTGGCCCTGAACAAAGATGGCTCACCCCGGGAGGGATACAGGACTAAACGACAC 540

mouse   CAGAAATTCACTCACTTTTTACCAAGGCCAGTAGATCCTTCTAAGTTGCCCTCCATGTCC 600
rat     CAGAAATTCACTCACTTTTTACCCAGGCCAGTAGATCCTTCTAAGTTGCCCTCCATGTCC 600
human   CAGAAATTCACTCACTTTTTACCCAGGCCTGTAGATCCTTCTAAGTTGCCCTCCATGTCC 600

mouse   AGAGACCTCTTCCGCTATAGGTAA 624
rat     AGAGACCTCTTCCGCTATAGGTAA 624
human   AGAGACCTCTTTCACTATAGGTAA 624
```

Figure 3.1 Sequence comparison of FGF-16 cDNA between species. Alignment of mouse (AF292104), rat (NM 21867) and human (AB 003868) FGF-16 cDNAs. Regions corresponding to differences in nucleotide identity are denoted in red while conserved regions are in black. Mouse FGF-16 has 95% and 97% sequence identity when compared to that of human and for rat respectively.

Fig 3.2

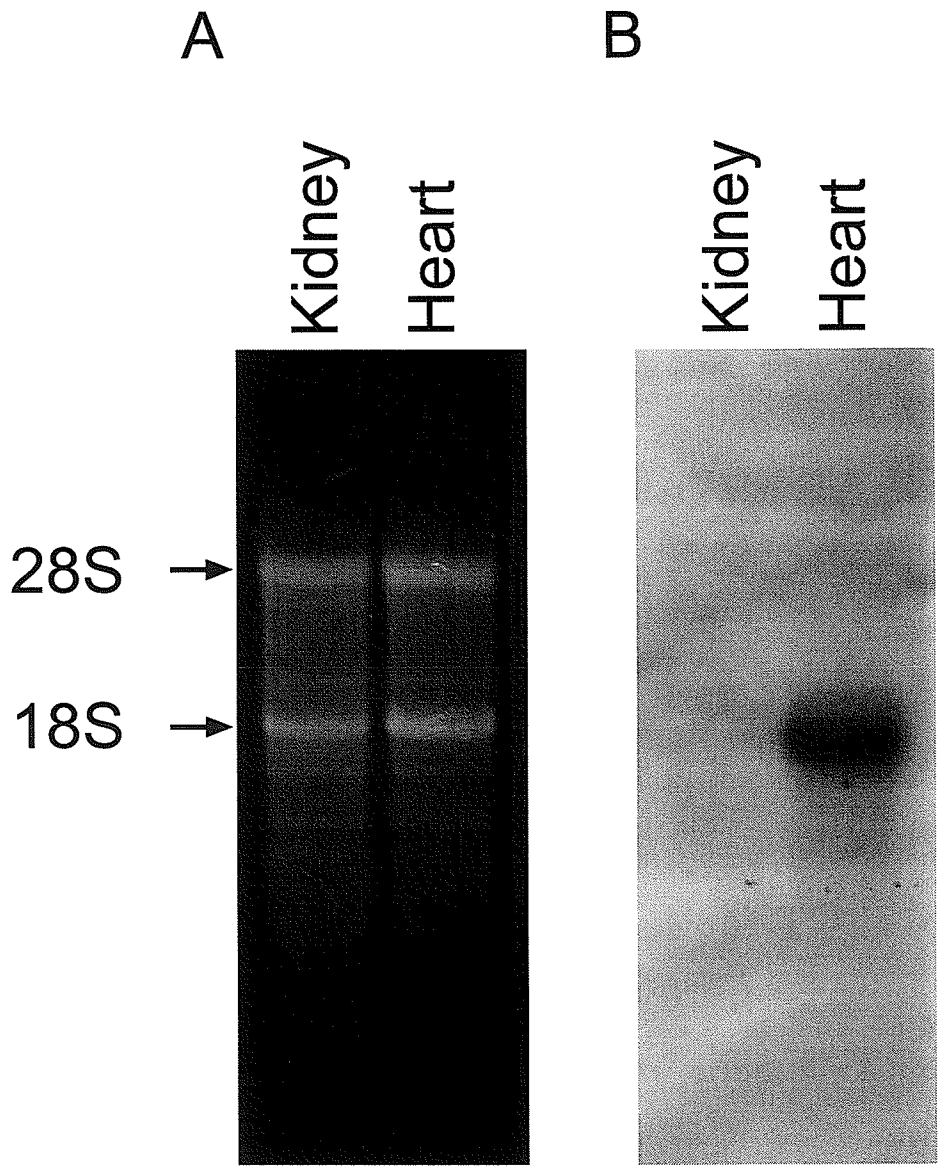


Figure 3.2 RNA blotting detects FGF-16 transcript (1.8 kb) in mouse heart, but not kidney RNA. A) Total RNA (60 μg) isolated from mouse kidney and heart run in a 1.5% agarose gel and visualized via ethidium bromide stain under UV light. B) The same samples after transfer to nylon membrane and probed with radiolabelled FGF-16 cDNA. The signal obtained revealed a band with an apparent size of 1.8 kb relative to the 28S (4.71 kb) and 18S (1.87 kb) ribosomal RNA bands. Data shown are representative of the result obtained from three different blots.

Fig. 3.3

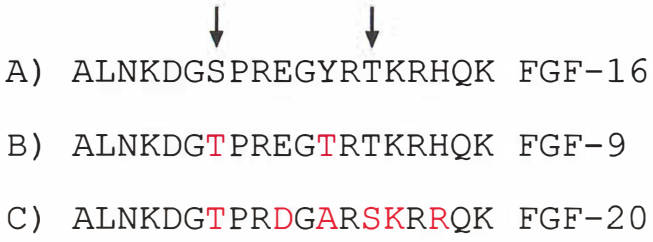

A) ALNKDGSPREGYRTKRHQK FGF-16
B) ALNKDGTPREGTRTKRHQK FGF-9
C) ALNKDGTPRDGARSKRRQK FGF-20

Figure 3.3 Comparison between the peptide antigen used to generate the FGF-16 antibody and corresponding region in closely related FGF family members. The amino acid sequence of the A) peptide used for FGF-16 antibody generation (A^{164} to K^{182}). Closest family members B) FGF-9 and C) FGF-20 are aligned for comparison with mismatches in red. Note arrows identifying potential phosophorylation sites by protein kinase C at residues S^{170} and T^{177}.

Fig 3.4

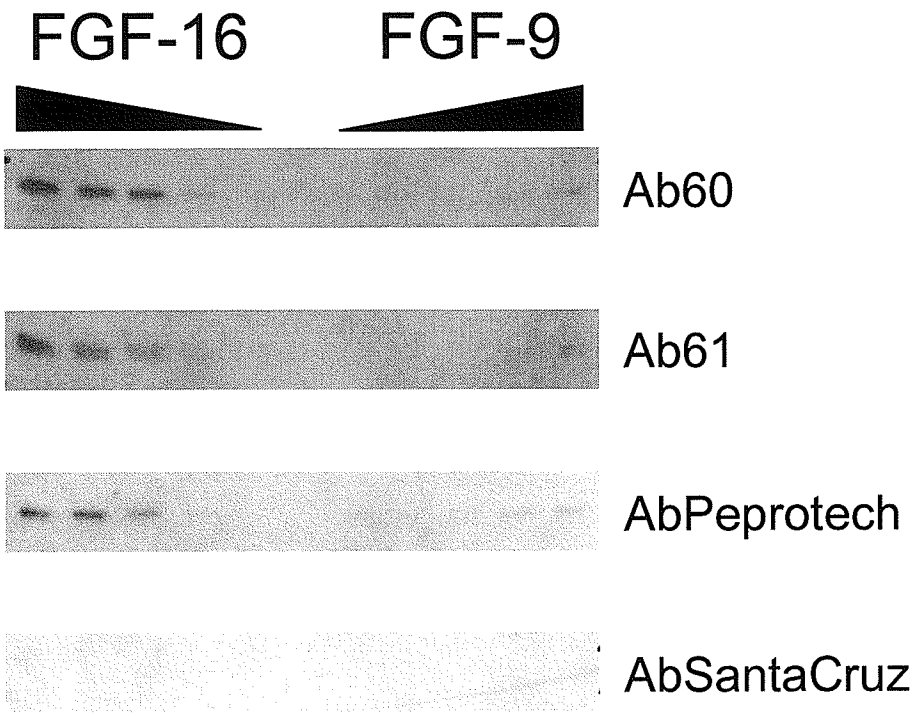


Figure 3.4 Protein blotting demonstrates preferential detection of FGF-16 over FGF-9 by FGF-16 antibodies. Serial dilutions of recombinant FGF-16 (10, 7.5, 5, 2.5 and 1.25 ng) compared to that of increasing concentrations of recombinant FGF-9 (1.25, 2.5, 5, 7.5, and 10 ng) run in 15% SDS-PAGE. Ab60 (0.13 μg/ml), Ab61 (0.13 μg/ml), and AbPeprotech (0.3 μg/ml) resulted in a signal for FGF-16 while AbSantacruz (2 μg/ml) did not.

Fig. 3.5

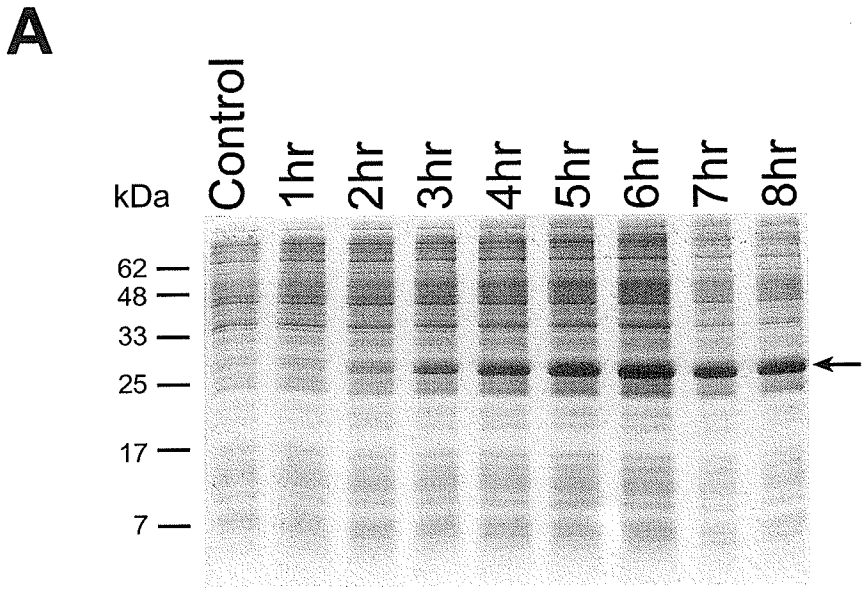

A
kDa
Control
1hr
2hr
3hr
4hr
5hr
6hr
7hr
8hr
62
48
33
25
17
7
B
kDa
175
83
62
48
33
25
17
7
15% SDS Page
his-mFGF-16

Figure 3.5 Bacterially expressed his-tagged FGF-16 protein following induction. A) Samples of bacterial lysate run by SDS-PAGE and stained for total protein via Coomasie brilliant blue. Samples were taken from the same culture at set time points following induction of his-FGF-16 expression via the addition of IPTG. The band indicated by the arrow identifies the protein of interest, which corresponds to the predicted size of his-FGF-16. B) The protein isolated from the bacterial expression system after elution from a nickel affinity column. The sample was run via SDS-PAGE with a 15% gel and stained with Coomasie brilliant blue to resolve the protein. A band of 26.3 kDa was observed, consistent with that of the expected size for the his-FGF-16 protein. Modified from Sontag and Cattini (2003).

Fig. 3.6

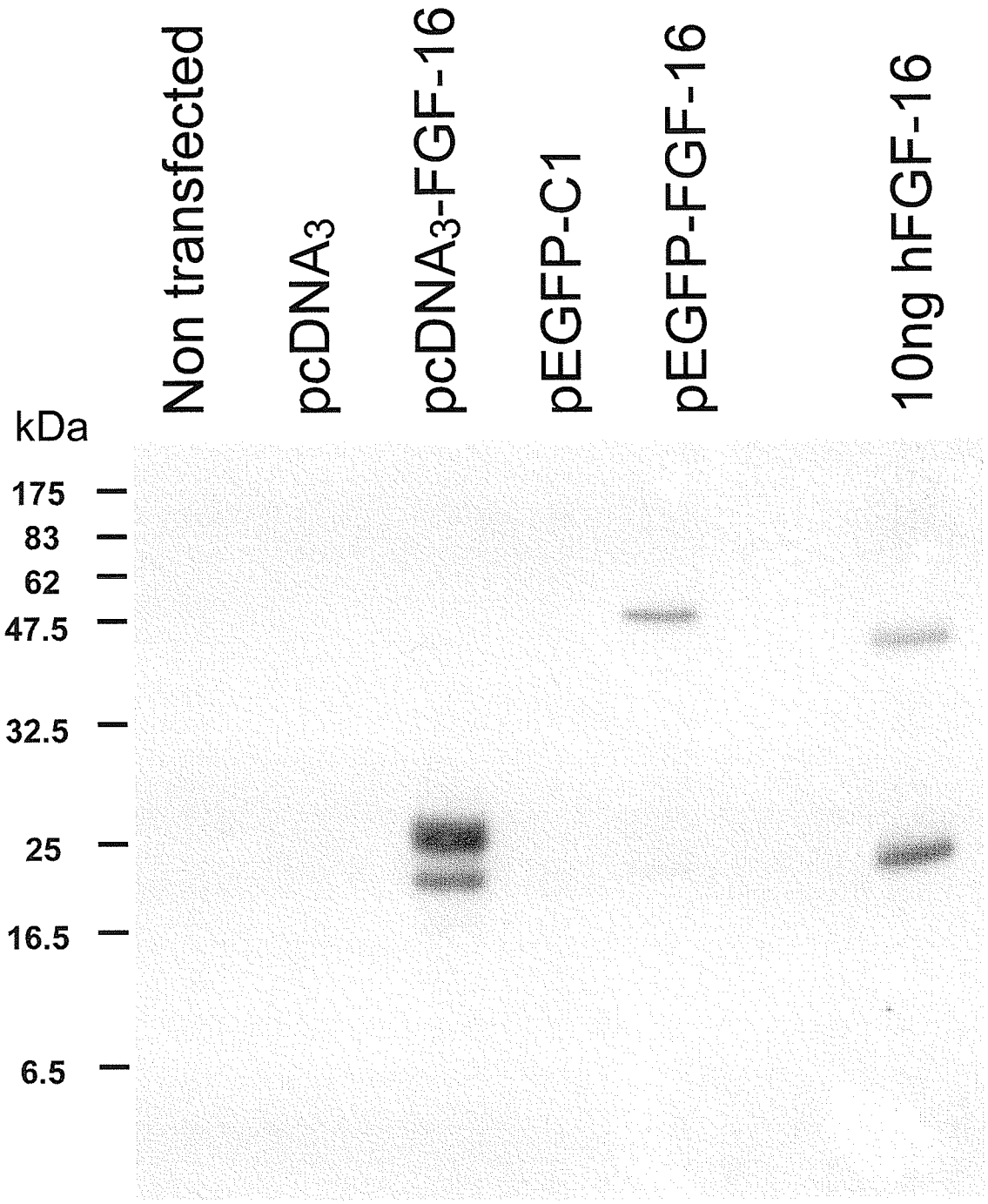
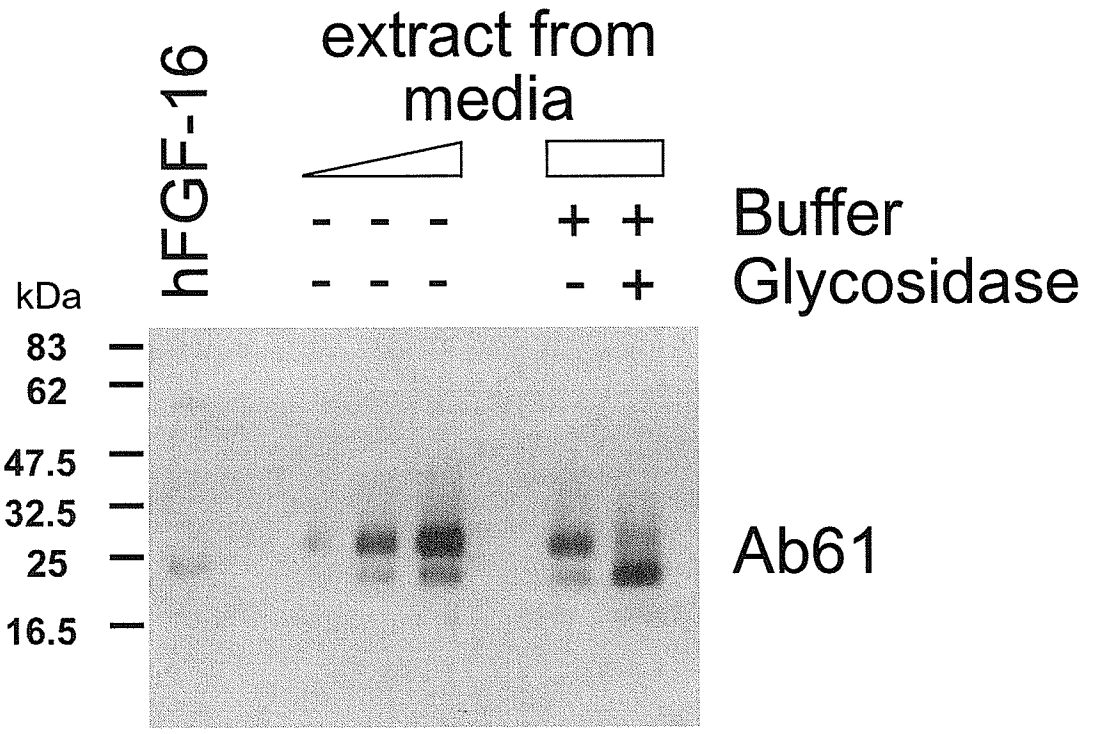

Non transfected
pcDNA3
pcDNA3-FGF-16
pEGFP-C1
pEGFP-FGF-16
10ng hFGF-16
kDa
175
83
62
47.5
32.5
25
16.5
6.5

Figure 3.6 Detection of FGF-16 in the media of transfected human embryonic kidney (HEK) 293 cells. Conditioned media from transfected HEK 293 cells was examined for the presence of FGF-16 via western blotting. Expression vectors used included empty vector ($pcDNA_3$), wild type FGF-16 ($pcDNA_3$-FGF-16), enhanced green fluorescent protein (EGFP-C1) and EGFP-FGF-16 fusion protein (pEGFP-FGF-16). Heparin binding proteins were extracted from the media by incubating with heparin sepharose beads under physiological salt concentrations. The beads were then washed with 0.5 M NaCl followed by a less stringent 0.1 M NaCl to reduce excess salt. The protein was then eluted from the beads by boiling with a standard loading buffer containing SDS and run via 15% SDS-PAGE. The gel was then transferred to PVDF membrane where it was probed with AbPeprotech.

Fig 3.7


hFGF-16
extract from media
- - - + +
- - - - +
Buffer
Glycosidase
kDa
83
62
47.5
32.5
25
16.5
Ab61

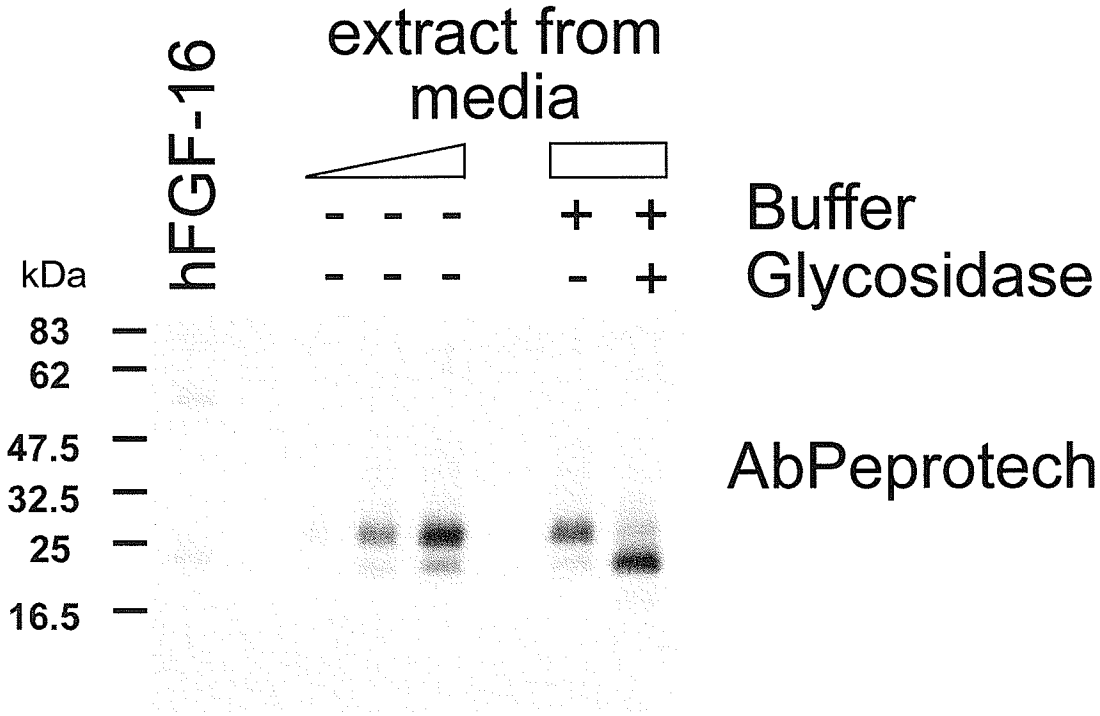

hFGF-16
extract from media
- - - + +
- - - - +
Buffer
Glycosidase
kDa
83
62
47.5
32.5
25
16.5
AbPeprotech

Figure 3.7 FGF-16 expressed and secreted from HEK 293 cells is glycosylated. Western blotting of a 15% SDS-PAGE probed with two independent antibodies. Samples included both purified protein (recombinant hFGF-16), serial dilutions of FGF-16 extracted from the media of transfected HEK 293 cells (equivalent to 1, 5 and 10 ml of media), extracted protein treated with the glycosidase buffer as well as glycosidase buffer in combination with the glycosidase enzyme.

Chapter 4

4.0 The detection of endogenous cardiac FGF-16 mRNA transcript and protein.

4.1 Tissue specificity and FGF-16 mRNA expression

FGF-16 has been reported to be preferentially expressed in the adult rat heart as opposed to various other tissues. To determine whether the expression pattern was similar in other species, the mouse was used as a model. Using the FGF-16 cDNA fragment as a probe, RNA blots were examined. Total RNA (30 μg) from various adult mouse tissues revealed a signal corresponding to a transcript of the expected size (1.8 kb) only from adult heart (data not shown). The result was also confirmed using an independent blot containing 2 μg of poly A mRNA isolated from adult mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis, which once again confirmed that only the adult mouse heart contained the 1.8 kb transcript (Figure 4.1). Subsequent reprobing of the same blot with cDNA from FGF-9 (Figure 4.2) revealed that the kidney had the highest concentration of FGF-9 with apparent sizes of 2.7, and 4.3 kb. Comparisons by densitometry and taking in to account the length and strength of the probe estimate that the signal obtained from FGF-16 is at least one hundred fold greater than that of FGF-9 in the heart. FGF-16 transcript is therefore most prevalent in the adult mouse heart as compared to other adult tissues, which is consistent with that reported for rat (Miyake et al., 1998).

4.2 Developmental regulation of FGF-16 expression in the heart

4.2.1 Cardiac FGF-16 mRNA accumulation during development

Although FGF-16 mRNA levels were reported to increase during embryonic development, their relative levels in the postnatal and adult heart are not known. Of particular interest was whether FGF-16 mRNA levels were static or continued to increase with age after birth. Therefore it was of interest to determine the developmental profile of FGF-16 expression in the heart. Hearts from mice of various ages were collected from embryonic day 19, postnatal day 1, day 4 and adult 8 weeks. Hearts were further dissected into right and left ventricles which were then processed for RNA extraction. Blots containing total RNA (30 μg) from each ventricle were probed using the cDNA for FGF-16 (Figure 4.3). Transcript levels were highest in the day 4 and adult hearts with much lower levels of transcript detectable from day 1 samples (3 of 3 blots examined). A signal was detected in only one of the embryonic samples in one of the 3 blots examined, which corresponded to the right ventricle. Thus, FGF-16 mRNA levels are much lower in the embryonic heart but increase after birth..

4.2.2 Cardiac FGF-16 protein accumulation in development

In order to investigate whether the protein level of FGF-16 follows the same expression profile as the RNA, mouse hearts from animals of various ages were examined via western blotting. Initial attempts to detect FGF-16 via western blotting through the use of crude lysates proved unsuccessful (data not shown). This is not unreasonable as FGF-2, another member of the FGF family, yields only 30-600 ng/g of

total protein when extracted from various tissues (Gospodarowicz 1987). Assuming complete extraction, this would translate to less than 10 picograms of FGF-2 per 20 μg total protein sample in a SDS-PAGE gel, beyond the sensitivity of the antibodies tested (as demonstrated in Figure 3.4). However, by taking advantage of the ability of FGF-16 to bind heparin (Danilenko et al., 1999, Deepa et al., 2002), heparin sepharose was used to enrich samples for FGF-16 as well as other heparin binding proteins (section 2.4.5). An equal amount of heart tissue from mouse embryonic day 17, postnatal day 4, and adult 8 week old animals were extracted for heparin binding proteins and probed for FGF-16 protein via western blotting. Upon investigation, several bands were detected, however, samples from the adult heart (Figure 4.4) revealed two distinct bands (26.5 and 19.5 kDa) similar in size to FGF-16 expressed in transfected HEK 293 cells as was discussed in section 3.4. Although both bands were not always apparent when small amounts (<0.4 g) of starting material were used, larger amounts (> 0.4 g) of adult mouse heart or pig heart provided a clear signal with either Ab60, Ab61 or commercial antibody AbPeprotech (Figure 4.5).

4.3 Immunofluorescent detection of FGF-16 in adult mouse heart

Given that both RNA and protein blotting supported the expression of FGF-16, it was of interest which cells might be producing the protein and/or where it might be stored in the heart. To determine the cellular and subcellular localization of FGF-16, cryosections of adult mouse heart were examined by immunofluorescence microscopy with the antibodies generated against the FGF-16 protein. Primary antibodies examined included Ab60, Ab61 and AbPeprotech. The use of AbPeprotech, however, did not

produce a signal above that of the control background when compared to normal IgG alone (Figure 4.6). However, both Ab60 and Ab61 detected a dull and diffuse signal above background, which was verified for specificity by preabsorbing the antibodies. Briefly, antibodies (Ab60 and Ab61) were incubated with either recombinant human FGF-16 (hFGF-16) or peptide fused to fixed substrate prior to application to tissue sections (as described in Section 2.4.7a). As a control, bovine serum albumen (BSA) was used in place of peptide or hFGF-16. Control samples had a brighter fluorescence as compared to antibody preabsorbed with either peptide or recombinant hFGF-16 (Figures 4.7 and 4.8). In addition, the signal was predominantly associated with the striated cells and less so with the vasculature. Higher magnification (Figure 4.9) revealed no obvious association with either the extracellular matrix nucleus or cellular membrane. Although there was some indication of FGF-16 signal in the vaculature the intensity relative to that of the striated muscle cells was less.

4.4 Cell type(s) expressing FGF-16

4.4.1 FGF-16 mRNA in myocytes versus non myocytes

Thus far the data suggest that there is not only transcript but also FGF-16 protein present in the heart. However, there are many cell types within the heart and any one of them may be responsible for FGF-16 production. Attempts at detecting FGF-16 through the use of immunofluorescence microscopy did not provide a clear answer as to its location. Furthermore, a positive signal would only have indicated where the protein was located and not necessarily where it was produced. In order to gain some insight as to

which cell type(s) were responsible for FGF-16 synthesis, primary cultures of cardiac cells were examined for FGF-16 production. Neonatal rat cardiac cell cultures were chosen based on their ease of culture and based on information obtained from the developmental northern blots. As was indicated earlier (section 4.2.1), FGF-16 transcript levels are present within 24 hours of birth and reach a plateau within 4 days. It was hypothesized that because cells would be at least this age by the time of harvest, FGF-16 should be present. Therefore, RNA extracted from cultures enriched for myocytes or non myocytes was examined (as described section 2.2.6). Populations of myocyte cultures consisted of greater than >90% myocytes whereas non myocyte cultures contained <5% myocytes as represented in Figure 4.10 (personal observations based on the percentage of cells also staining positive for α-actinnin). Total RNA was then loaded equally according to amount (40 μg) measured by the OD_{260} and verified visually using ethidium bromide under UV light (Figure 4.10). When RNA samples were transferred to nylon membrane and probed with radiolabeled FGF-16 cDNA, only that isolated from myocytes yielded a positive signal of 1.8 kb which was consistent for FGF-16 transcript. The signal observed from myocytes was highly reproducible, as it was detectable in 4 of 4 samples examined from independent cell isolations.

4.4.2 Protein blotting to detect FGF-16 in neonatal rat cardiac cultures

Given that myocytes were found to contain FGF-16 transcript, protein blotting was used to investigate whether the protein could be detected and if its properties were similar to those observed with transfected HEK 293 cells. Therefore, cells and media from both neonatal rat cardiac myocytes and non myocytes were processed to enrich for

FGF-16 through the use of heparin sepharose. Samples were then run via SDS-PAGE and examined by protein immuno blotting for the presence of FGF-16 protein using three different antibodies (Figure 4.11). Several bands were detected from the myocyte cell lysates with Ab60 (35.3, 23.12 and 14.14 kDa) but not with AbPeprotech, suggesting that they were perhaps below the threshold for detection with AbPeprotech. In particular, the 23.1 kDa band was of interest as it was close to the predicted size (23.7 kDa) of the protein and similar to that of the deglycosylated protein observed with the transfected HEK 293 cells (22.0 kDa). Samples from non myocytes, however, produced no signal from either the media or cell lysates which was consistent with data obtained from the RNA, suggesting that they were not responsible for FGF-16 production. The conditioned media from myocytes (but not from non myocytes) contained a signal that corresponded to a band with an apparent molecular weight of 26.5 kDa, which was detectable with either antibody tested.

The size of the secreted protein was consistent with a glycosylated product as was observed in HEK 293 cells (Figure 3.8). Heparin binding proteins were therefore extracted from the conditioned media of neonatal cardiac myocytes and exposed to a glycosidase enzyme as was described for the HEK 293 cell media (section 3.4). The glycosidase treatment resulted in a shift of the protein band to that of a lower molecular weight (26.5 to 24.1 kDa) as represented in Figure 4.12. Treatment with and without reaction buffer for the glycosidase enzyme had no effect on the apparent molecular weight of the band observed. These data demonstrate that myocytes not only produce FGF-16, but that it is secreted as a glycosylated molecule. This is of interest as not all

FGF molecules are readily secreted, thus suggesting that its site of action may be extracellular.

4.5 Immunofluorescent detection of FGF-16 in neonatal cardiac myocytes.

Evidence of mRNA transcript in rat neonatal cardiac myocytes suggested that cells were producing FGF-16 protein. Therefore an investigation was made of whether the protein could be detected through the use of immunofluorescence microscopy using antibodies Ab60, Ab61 and AbPeprotech. Only a faint and diffuse signal was observed, which suggested one of two possibilities: 1) The antibodies were incapable of FGF-16 detection under the conditions used, or 2) that cellular levels of FGF-16 protein were much too low for detection by the antibody. In order to resolve this issue, it was decided that myocytes would be transfected with FGF-16 expression vectors to increase FGF-16 expression in hopes of being better able to detect the protein.

Given that myocytes have a low transfection efficiency (<5% with transient transfection using Lipofectamine®, personal observation using EGFP as a reporter), it was necessary to be able to identify those cells which were transfected. The EGFP-FGF-16 fusion protein construct described in section 3.4 was employed to this end, allowing for identification of transfected cells as well as identification of FGF-16 through co localization with the EGFP tag. The use of pEGFP-C1 vector alone (which produces only the EGFP protein) was used as a negative control for antibody staining. Our immediate observation was that cells transfected with the pEGFP-C1 plasmid had a fluorescent green signal which did not seem to associate with any particular cellular compartment in particular. On the other hand, the pEGFP-FGF-16 plasmid resulted in a green

fluorescence associated not only with the cytoplasm but had an even stronger signal associated with what appeared to be the nucleus of the cells (15 of 16 cells as opposed to 3 of 20 for EGFP alone). In addition, after looking at Ab60 and Ab61 (Figure 4.13 and 4.14 respectively) the staining observed correlated directly with that of the EGFP-FGF-16 fusion protein (n = 15 for each). At no time was there anything more than a dull staining of cells expressing the EGFP alone, which was similar to that of surrounding non transfected cells. The commercially available Peprotech antibody however was unable to detect EGFP-FGF-16 fusion protein (Figure 4.15).

The apparent subcellular localization of the fusion protein to the nucleus was interesting, since transfection of COS-1 cells with an FGF-16 fusion protein resulted in subcellular localization to the mitochondria (Miyakawa et al., 2003). As this reported staining pattern was completely different from that observed with our EGFP-FGF-16 fusion protein, this suggested that either the EGFP was interfering with FGF-16 localization and/or secretion and/or perhaps the protein was being processed differently between the two cell types. Therefore, FGF-16 protein was expressed in addition to pEGFP as a separate protein to investigate FGF-16 localization alone. The FGF-16 expression vector (pcDNA$_3$-FGF-16) in combination with the EGFP expression vector (pEGFP-C1) were cotransfected at a ratio of 3 to 1, after which cells were fixed and processed for immunofluorecence using various antibodies.

After using the green fluorescence to identify transfected cells, those cells were also examined for FGF-16 immunofluorescence (Figure 4.16). Almost half the cells that were pEGFP positive did not have any discernable FGF-16 immunofluorescence above that of neighboring non transfected cells (7 of 16 cells with Ab61, and 4 of 9 cells with

Ab60). Although some signal was observed, the intensity was highly variable in intensity and had the appearance of small punctate spots. These were very similar in appearance to that previously reported for its closest relative FGF-9 (Revest et al., 2000), as well as FGF-16 when expressed in transfected COS-1 cells (Miyakawa and Imamura, 2003). Therefore, cellular localization of the wild type FGF-16 protein on its own appears to behave differently from that of the EGFP tagged FGF-16 protein. A characteristic which has been reported elsewhere with at least one other protein (Hanson et al. 2004) The data also suggest that the level of FGF-16 present within the cells is below the threshold for detection with the antibodies used.

4.6 Summary

In a survey of the major tissues, the transcript for FGF-16 was detectable in RNA samples only from adult mouse heart, which is consistent with that reported for the rat. The transcript levels peak within four days after birth in both the left and right ventricles of mice, although western blot analysis suggests that the levels of protein require further time to accumulate. In addition, FGF-16 transcript is present in neonatal cardiac myocytes after 4 days of culture and results in the production of a secreted and glycosylated form of the protein. Cultures of non myocytes produced no evidence of FGF-16 transcript or protein protein. Evaluation of antibodies raised against FGF-16 or a portion of the protein revealed that two of the antibodies (Ab60 and Ab61) were useful in the detection of endogenous FGF-16 protein by protein blotting. Furthermore, only the custom made antibodies Ab60 and Ab61 were capable of detecting a signal when used for immunofluorescence microscopy. The tagging of FGF-16 protein with EGFP is

different from that of wild type FGF-16, which appears to be secreted from neonatal cardiac myoyctes.

Fig 4.1

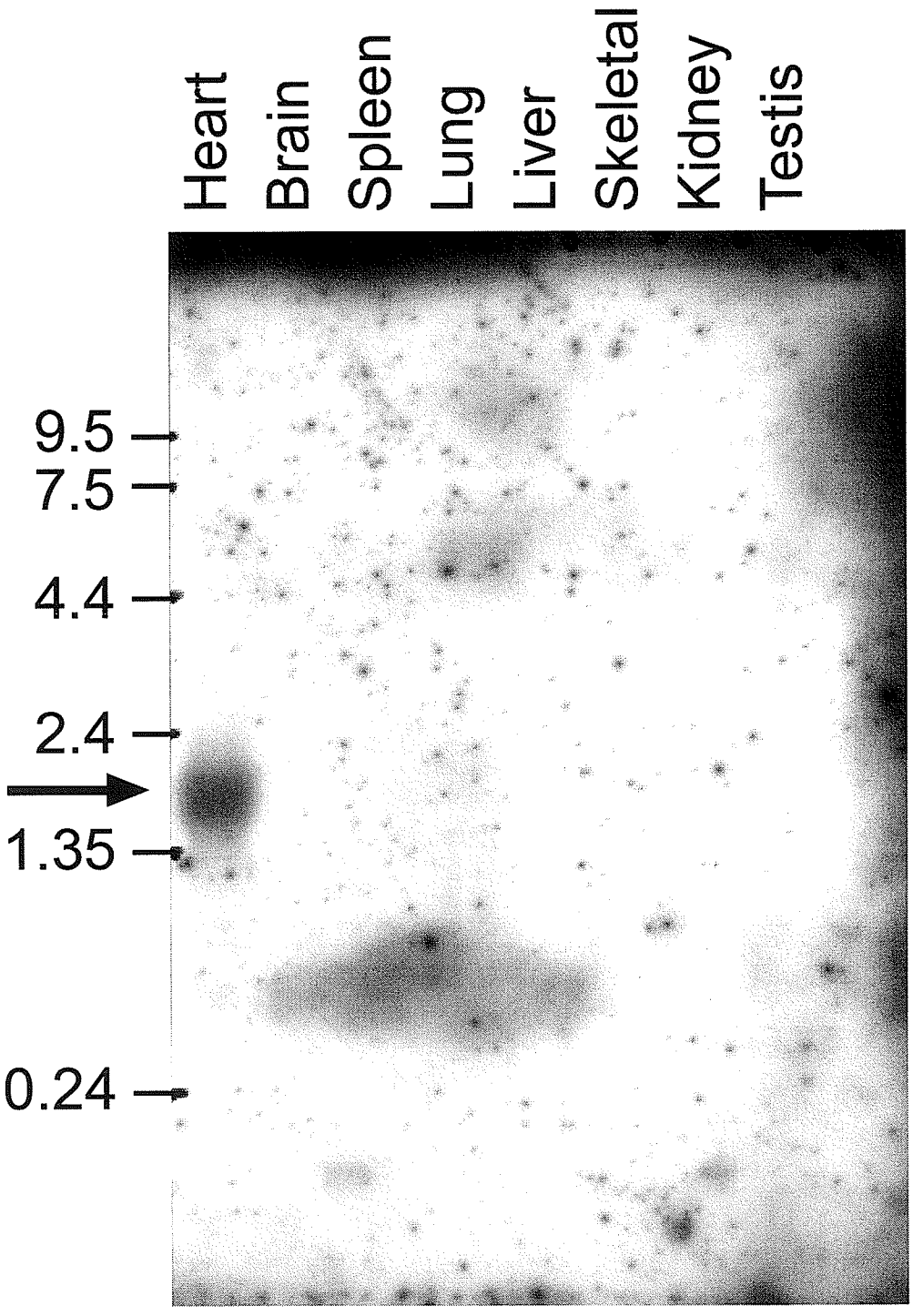

Heart
Brain
Spleen
Lung
Liver
Skeletal
Kidney
Testis
9.5
7.5
4.4
2.4
1.35
0.24

Figure 4.1 Tissue distribution of FGF-16 transcript. A commercial blot (Clontech) of poly A mRNA (2 μg) isolated from various mouse tissues was probed with radiolabeled FGF-16 cDNA. A prominent signal (arrow) corresponds to FGF-16 with apparent size of 1.8 kb relative to molecular markers (kb). Transcript was detected only in the heart.

Fig 4.2

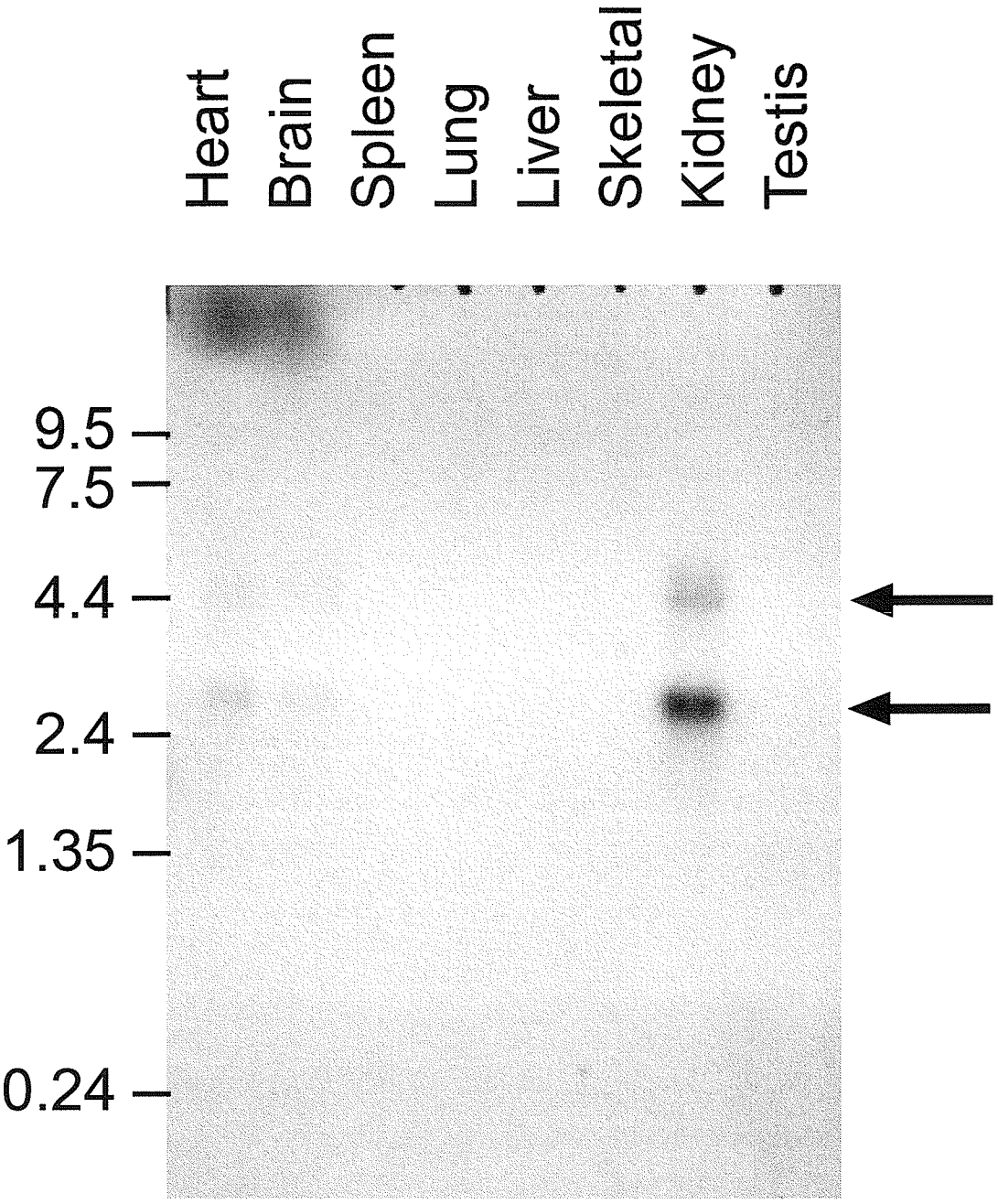

Heart
Brain
Spleen
Lung
Liver
Skeletal
Kidney
Testis
9.5
7.5
4.4
2.4
1.35
0.24

Figure 4.2 Tissue distribution of FGF-9 transcript. A commercial blot (Clontech) of poly A mRNA (2 μg) as in Figure 4.1 probed with radiolabeled FGF-9 cDNA. A prominent signal (arrow) corresponds to FGF-16 with apparent size of 2.7 and 3.4 relative to molecular markers (kb). Transcript was detected only in the heart, kidney and brain.

Fig 4.3

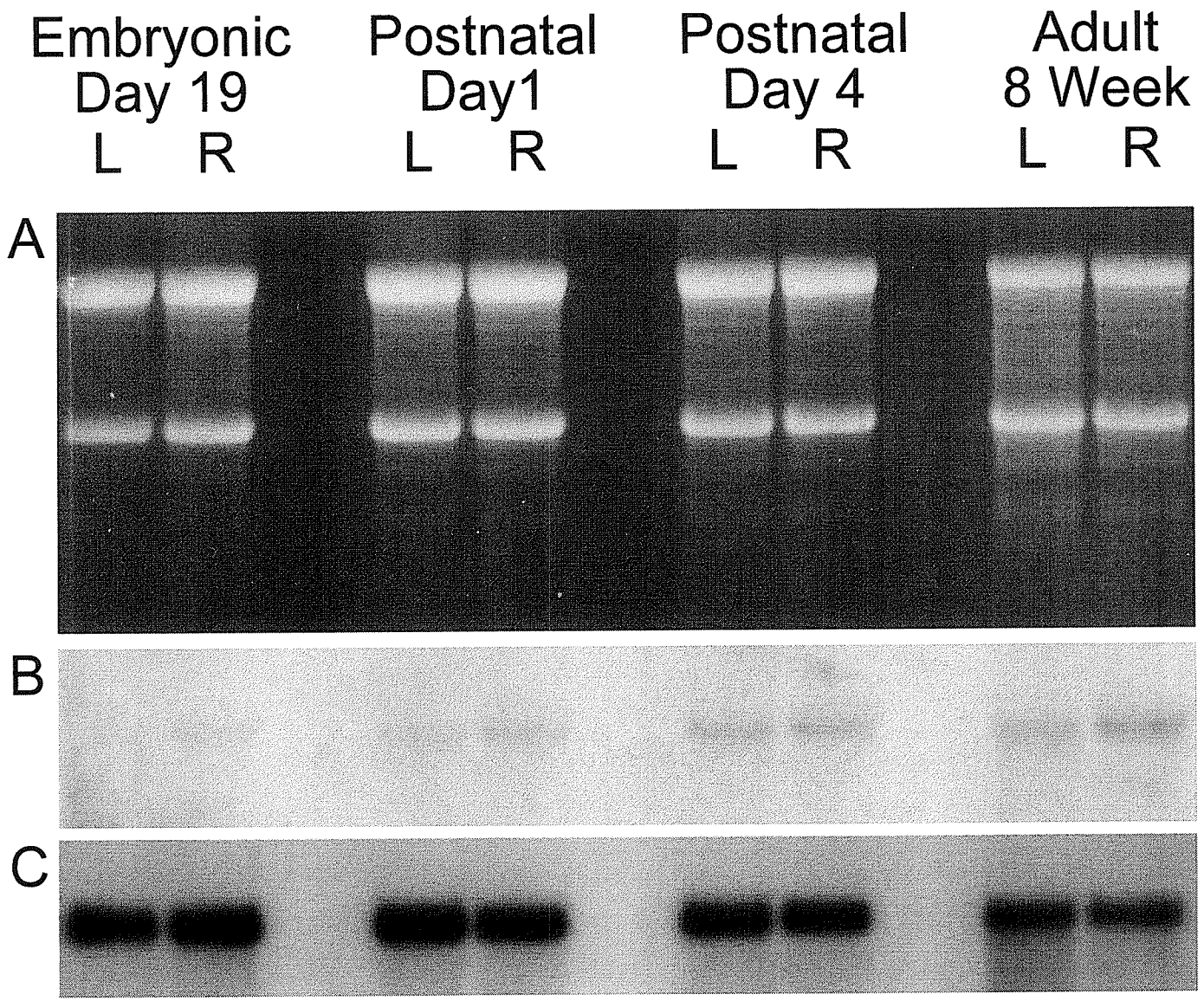

Embryonic Day 19
L R
Postnatal Day1
L R
Postnatal Day 4
L R
Adult 8 Week
L R
A
B
C

Figure 4.3 Developmental profile of FGF-16 mRNA expression in ventricles from mouse hearts. Total RNA (30 μg) was isolated from the left (L) or right (R) ventricles of mouse hearts of various ages. Samples were A) visualized by ethidium bromide, B) transferred to nylon membrane and probed with radiolabeled FGF-16 cDNA which resulted in a signal corresponding to a 1.8 kb band, then C) reprobed with GAPDH to check for differences in loading. Similar results were observed with three independent blots.

Fig 4.4

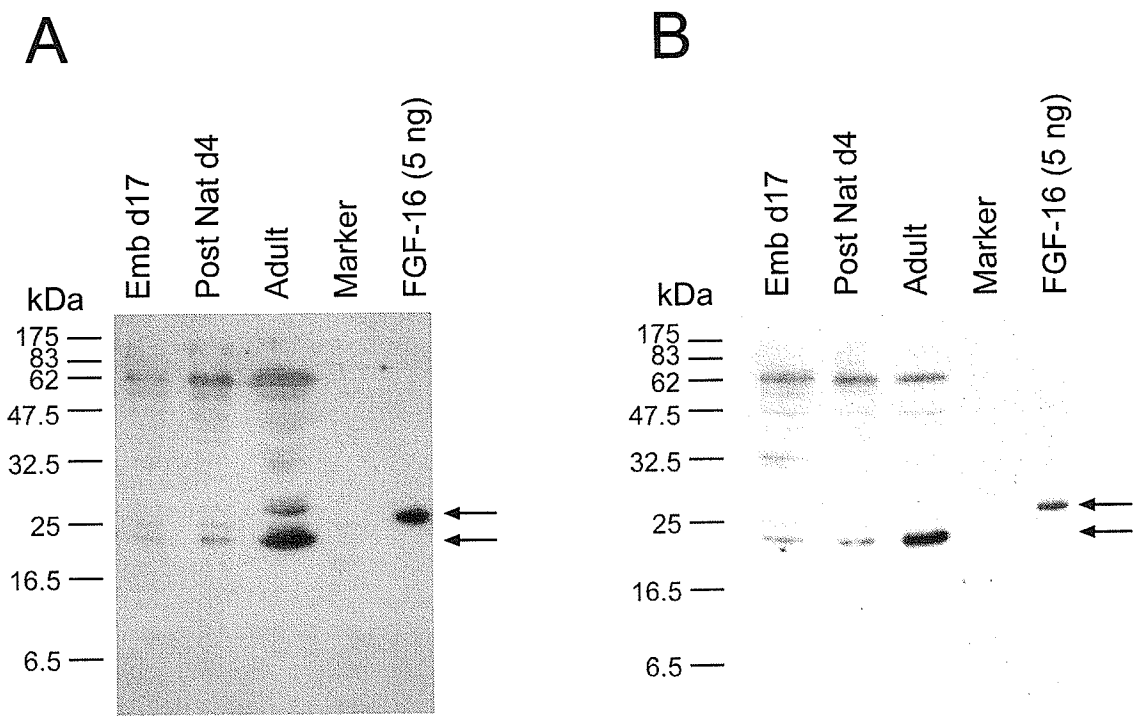

A
B
kDa
Emb d17
Post Nat d4
Adult
Marker
FGF-16 (5 ng)
175
83
62
47.5
32.5
25
16.5
6.5

Figure 4.4 Developmental profile of FGF-16 protein accumulation in mouse heart. Protein blot of heparin binding proteins extracted from embryonic day 17, postnatal day 4 and adult 8 week mouse hearts (0.2 g tissue) run by 15% SDS-PAGE. Positive controls consisted of 5 ng of recombinant human FGF-16 (hFGF-16). Blots were probed with anti-FGF-16 A) Ab60 and B) AbPeprotech. Prominent signals were detected in the adult heart corresponding to proteins with an apparent molecular weights of 19.5 and 26.5 kDa with Ab60 while the Peprotech antibody revealed only a 19.5 kDa protein. Molecular weight markers are indicated in kDa.

Fig 4.5

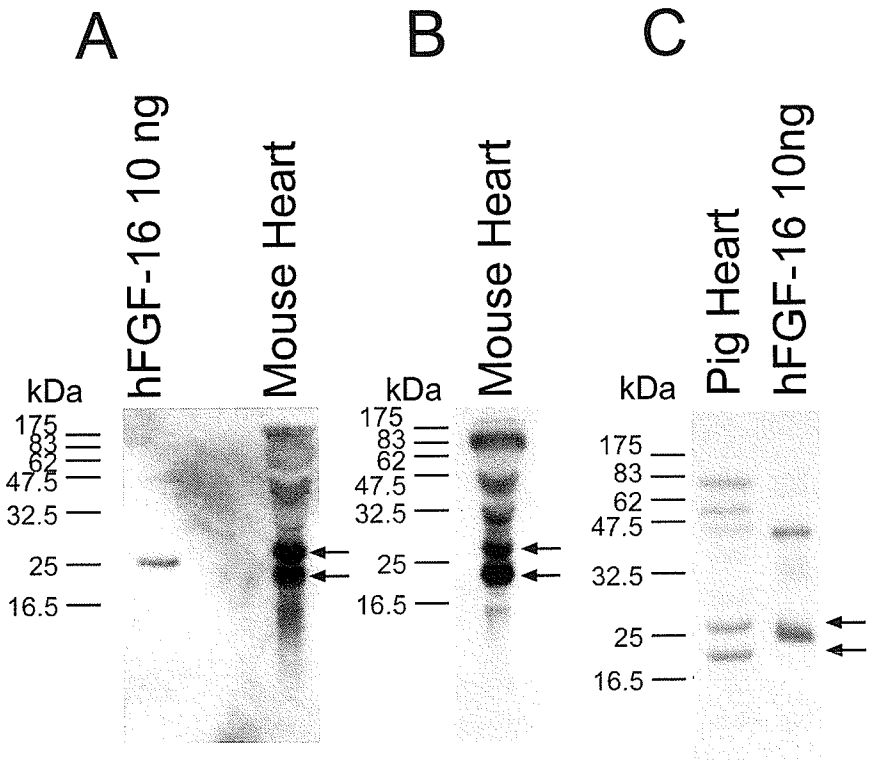

A
B
C
hFGF-16 10 ng
Mouse Heart
Mouse Heart
Pig Heart
hFGF-16 10ng
kDa
175
83
62
47.5
32.5
25
16.5

Figure 4.5 Protein blots of cardiac FGF-16 protein recognized by three independent FGF-16 antibodies. Heparin binding proteins extracted from (1.2 g) adult mouse heart (15% SDS-PAGE) and probed with A) Ab61 and B) AbPeprotech anti FGF-16. C) Heparin binding proteins extracted from adult pig heart (0.5g) (12.5 SDS-PAGE) were probed with Ab60. Positive controls consisted of recombinant hFGF-16. Two prominent bands were observed with extracts from each of the samples and with both antibodies corresponding to proteins with apparent molecular weights of 19.5 and 26.5 kDa. Molecular weight markers are indicated in kDa.

Fig 4.6

Goat IgG

AbPeprotech

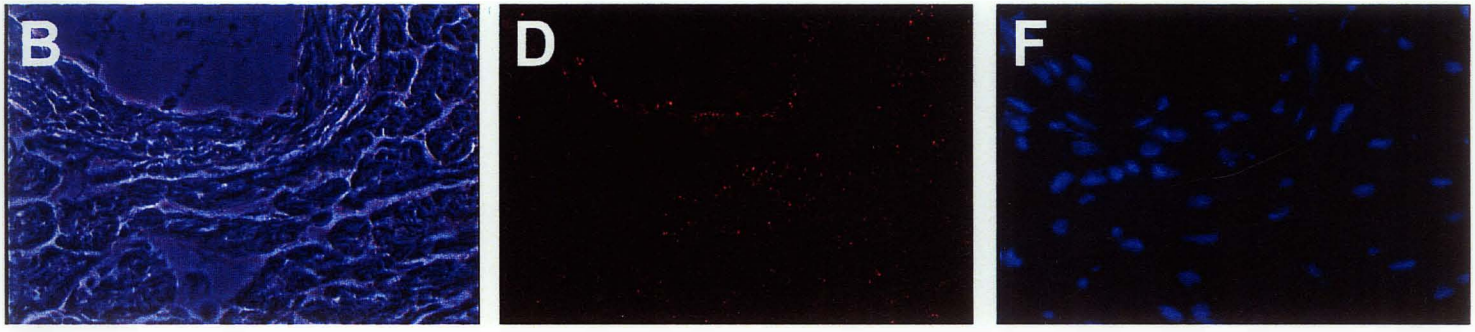


Figure 4.6 Commercial FGF-16 antibody (Peprotech) does not detect FGF-16 by immunofluorescence microscopy in adult mouse heart. A) and B) represent light micrographs of cryosections taken from adult mouse heart and treated with either C) IgG or D) goat anti FGF-16 (Peprotech). Sections were also stained for nuclei with Hoechst stain E) and F).

Fig 4.7a

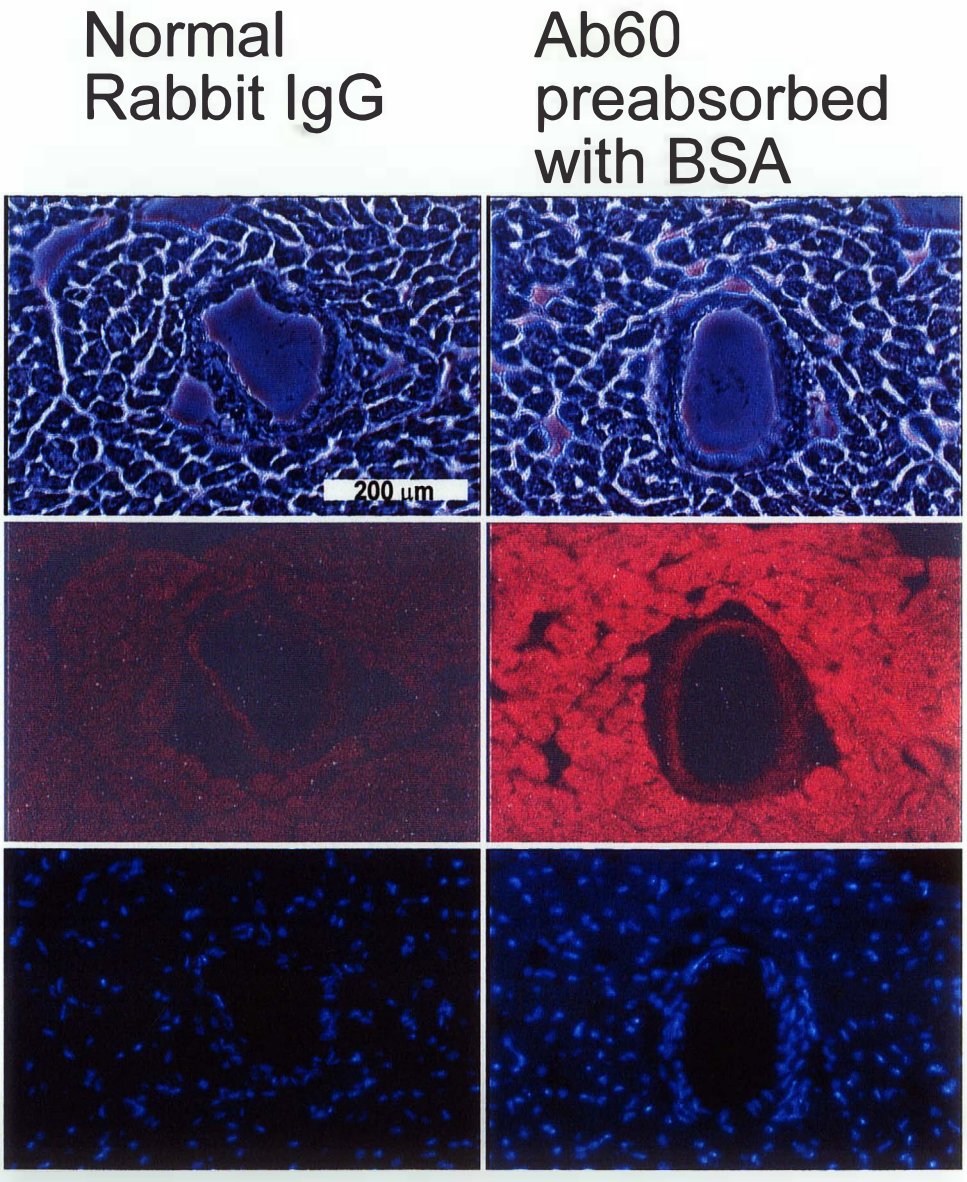


Fig 4.7b

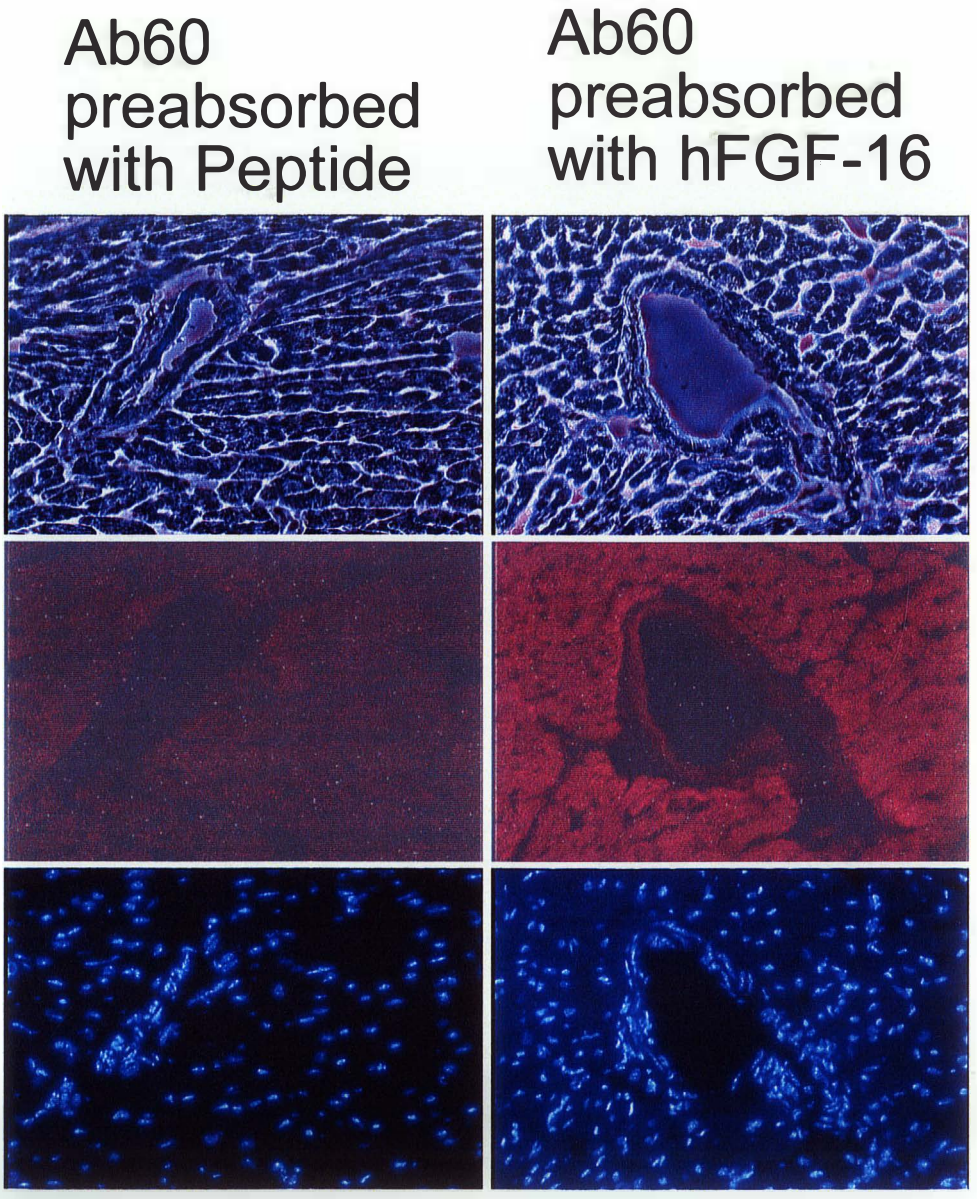

Ab60
preabsorbed
with Peptide
Ab60
preabsorbed
with hFGF-16

Figure 4.7 Examination of FGF-16 in adult mouse heart by immunofluorescence microscopy using Ab60. From top to bottom, light micrographs (top panels) of cryosections taken from adult mouse hearts treated with respective antibody treatment as labeled (middle panels) and stained for nuclei with Hoechst stain (bottom panels). From left to right, cryosections were treated with normal rabbit IgG pre incubated with BSA as a control, anti FGF-16 (Ab60) pre incubated with BSA, anti-FGF-16 antibody pre incubated with either the peptide used as an antigen for generating the antibody or recombinant hFGF-16. A fluorescent signal above background (normal IgG) was observed in striated cells which was less intense when the antibody was preabsorbed with either recombinant hFGF-16 or the peptide antigen.

Fig 4.8

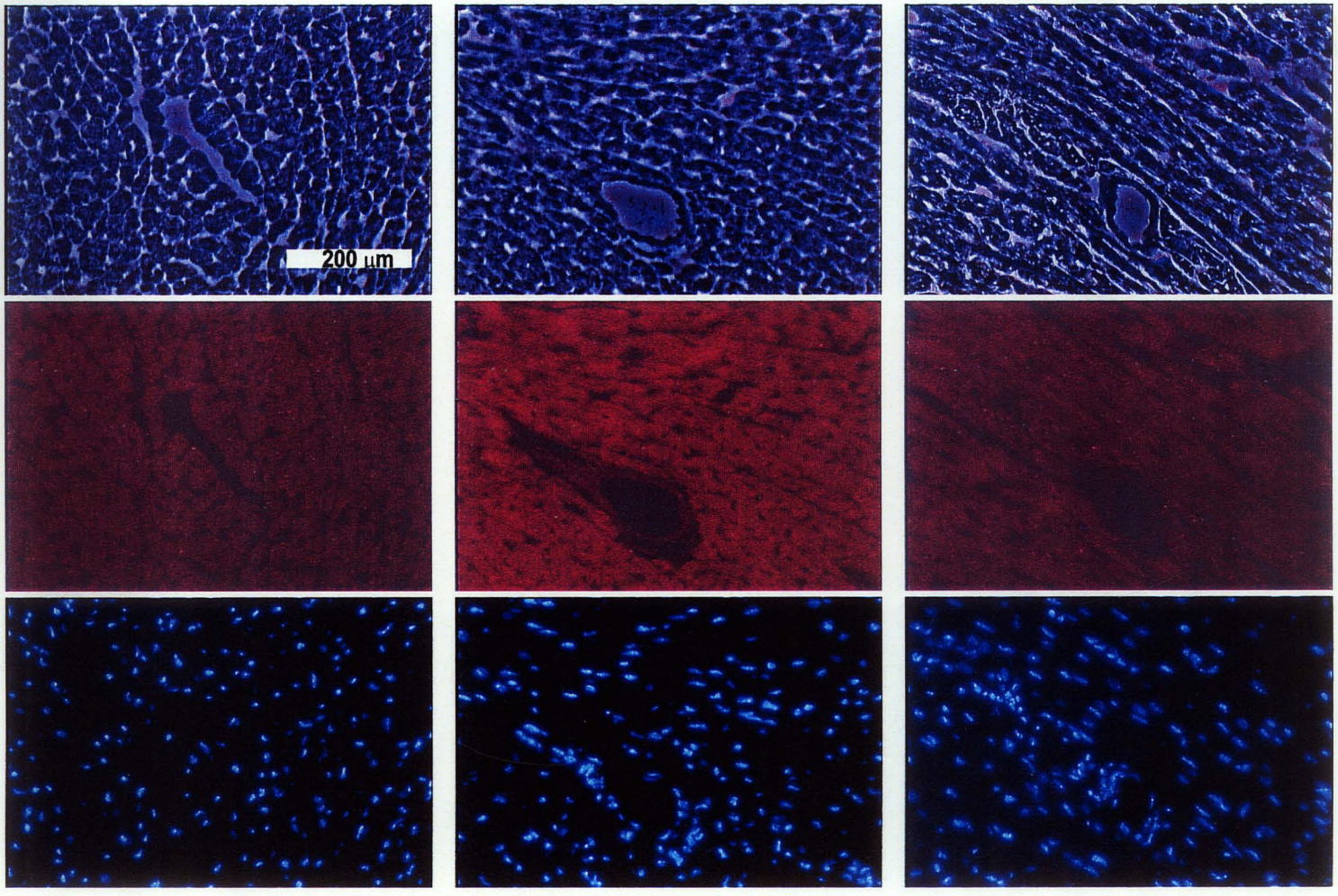
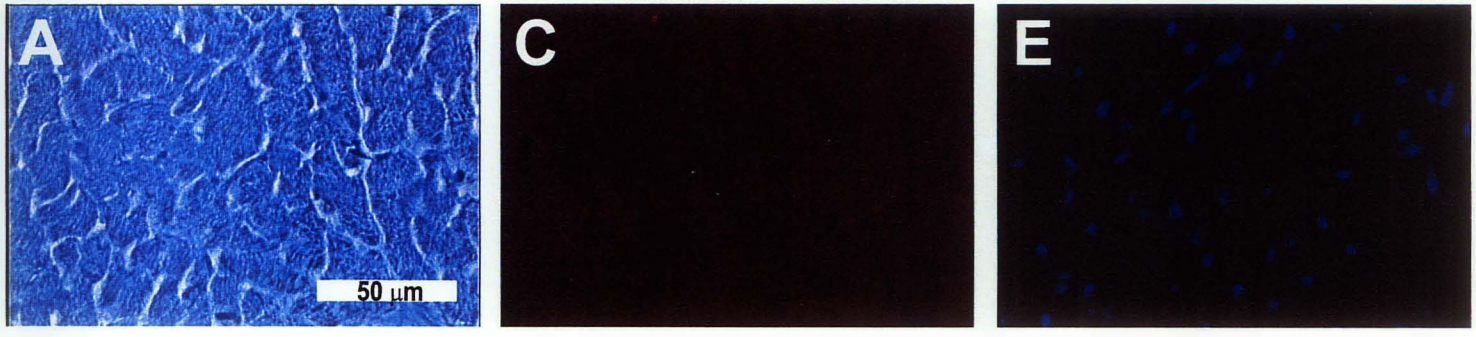

Normal
Rabbit IgG
Ab61
preabsorbed
with BSA
Ab61
preabsorbed
with Peptide
200 µm

Figure 4.8 Examination of adult mouse heart by immunofluorescence microscopy using Ab61. From top to bottom, light micrographs (top panels) of transverse cryosections taken from adult mouse hearts treated with respective antibody treatment as labeled (middle panels) and stained for nuclei with Hoechst stain (bottom panels). From left to right, cryosections were treated with normal rabbit IgG pre incubated with BSA as a control, anti FGF-16 (Ab61) pre incubated with BSA, anti-FGF-16 antibody pre incubated with the peptide used as an antigen for generating the antibody. A fluorescent signal above background (normal IgG) was observed in striated cells which was less intense when the antibody was preabsorbed with the peptide antigen.

Fig 4.9

Rabbit IgG



Ab60

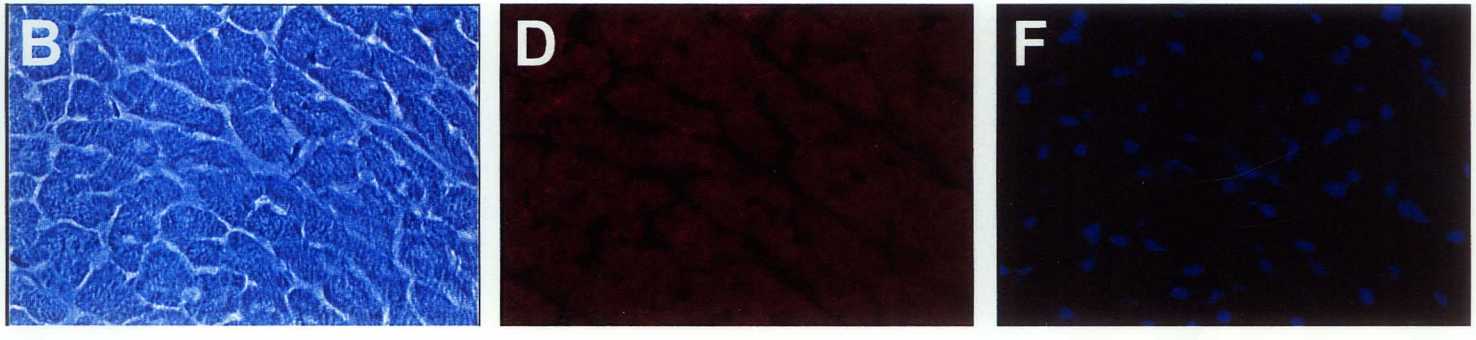


Figure 4.9 Examination of FGF-16 in adult mouse heart by immunofluorescence microscopy using Ab60 at higher magnification. A) and B) represent light micrographs of cryosections taken from adult mouse heart and treated with either C) IgG or D) rabbit anti FGF-16 (Ab60). Sections were also stained for nuclei with Hoechst stain E) and F).

Fig 4.10

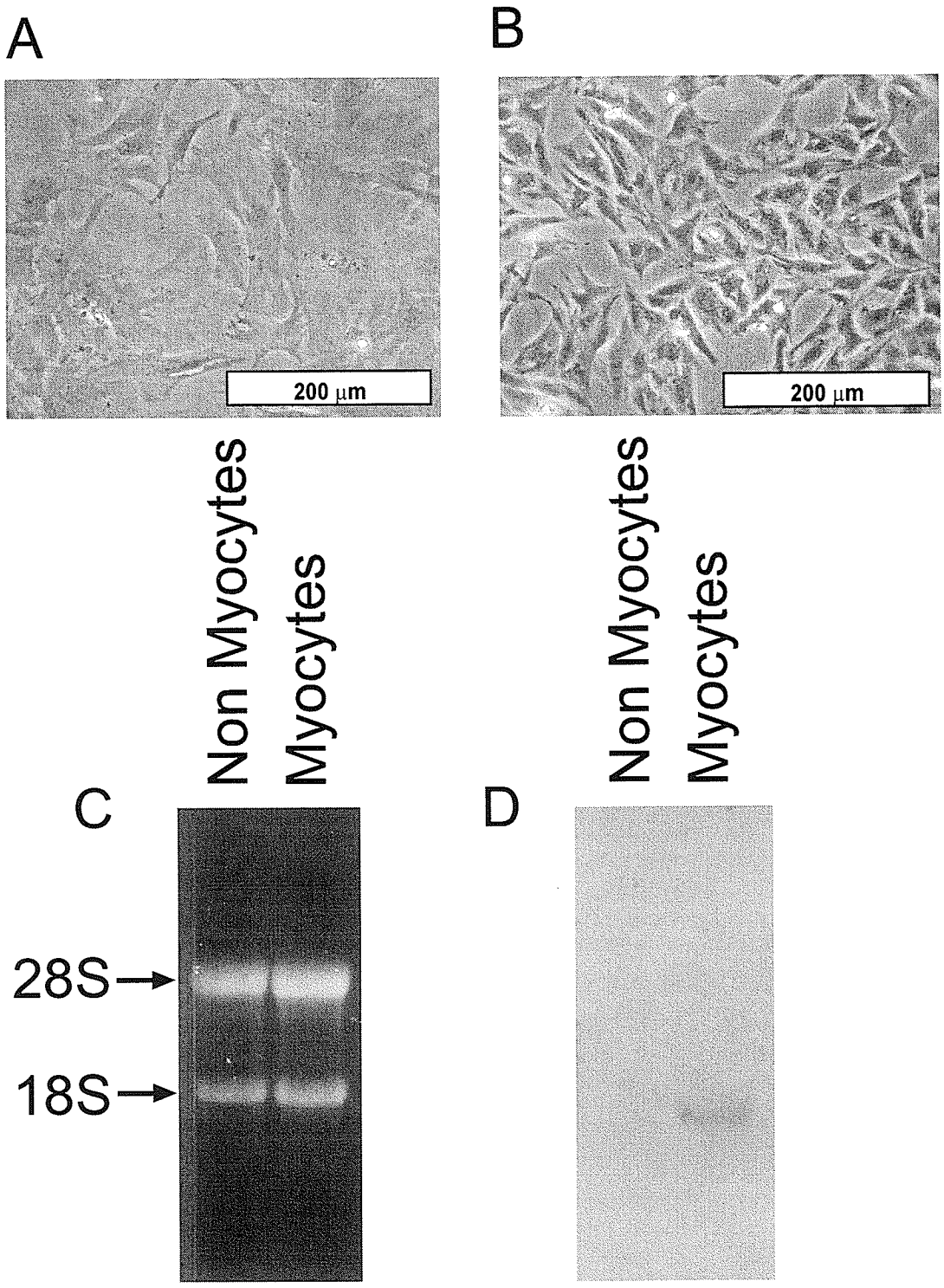
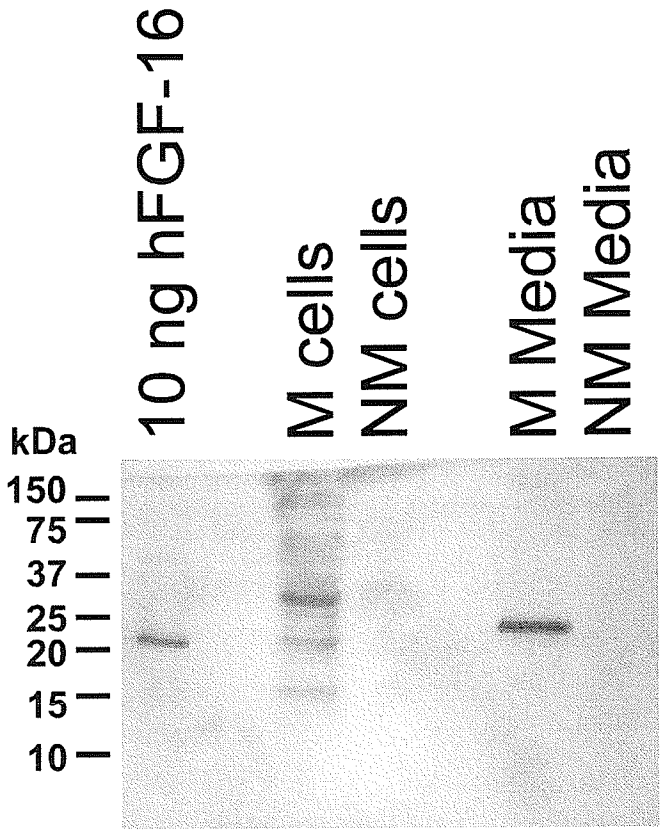
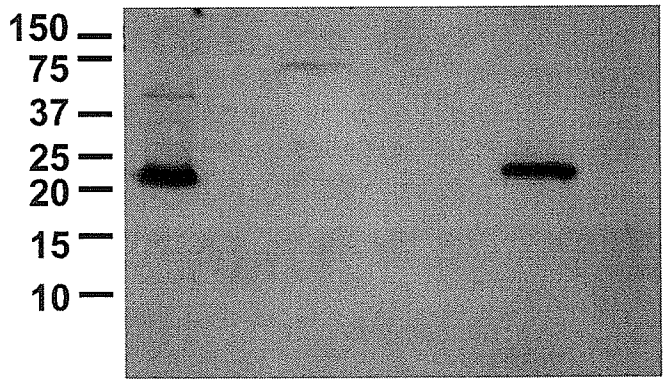

A
B
200 μm
200 μm
Non Myocytes
Myocytes
Non Myocytes
Myocytes
C
D
28S
18S

Figure 4.10 RNA blot of FGF-16 transcripts detected in neonatal rat cardiac myocytes. A) Light micrograph of non myocytes and B) myocytes. C) Total RNA (40 μg) isolated from cell cultures enriched for either myocytes or non myocytes visualized with ethidium bromide. D) Blots were probed using radiolabeled FGF-16 cDNA. RNA isolated from myocytes but not non myocytes yielded a band corresponding to a 1.8 kb FGF-16 transcript (arrow) relative to the 28S and 18S ribosomal RNA bands.

Fig 4.11



Ab61



AbPeprotech

Figure 4.11 Protein blot of FGF-16 detected from primary neonatal rat cardiac cultures. Myocytes (M) as well as non myocyte (NM) cultures were processed and samples enriched for heparin binding proteins. Enriched samples from both cells types as well as the conditioned media were run in a 15% SDS-PAGE gel and then probed using two independent antibodies as indicated. The signal obtained from the conditioned media from myocytes was 26.5 kDa, which was larger than the expected size for FGF-16 (23.7 kDa). The mobility of molecular weight markers are indicated on the left in kDa.

Fig 4.12

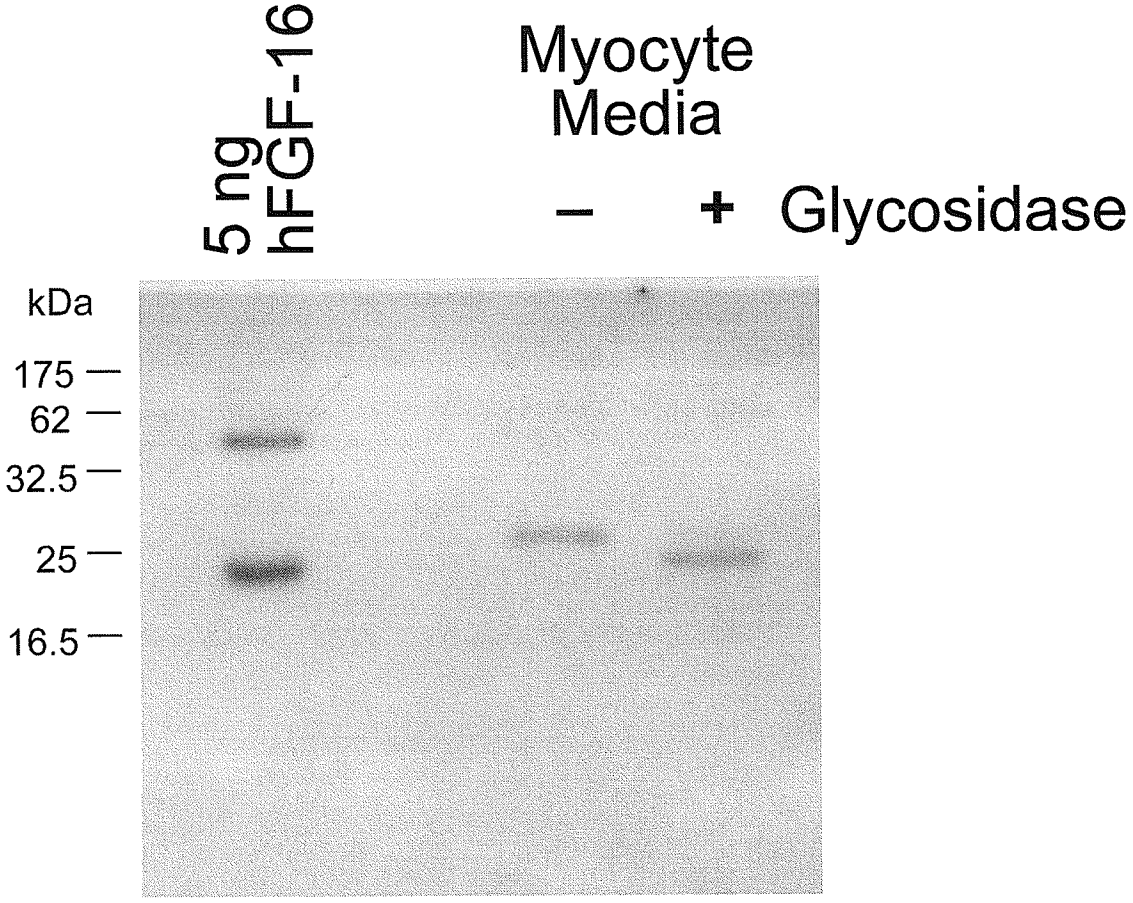


Figure 4.12 Neonatal rat cardiac myocytes secrete glycosylated FGF-16. Protein blotting of recombinant hFGF-16 as well as samples from conditioned media of neonatal rat cardiac myocytes enriched for heparin binding proteins. Samples were treated with glycosidase buffer with and without glycosidase enzyme. When the blot was probed using anti-FGF-16 antibody (AbPeprotech) a distinct shift in the apparent molecular weight of the protein detected was observed with samples treated with the glycosidase enzyme. The mobility of molecular weight markers are shown on the left in kDa.

Fig 4.13

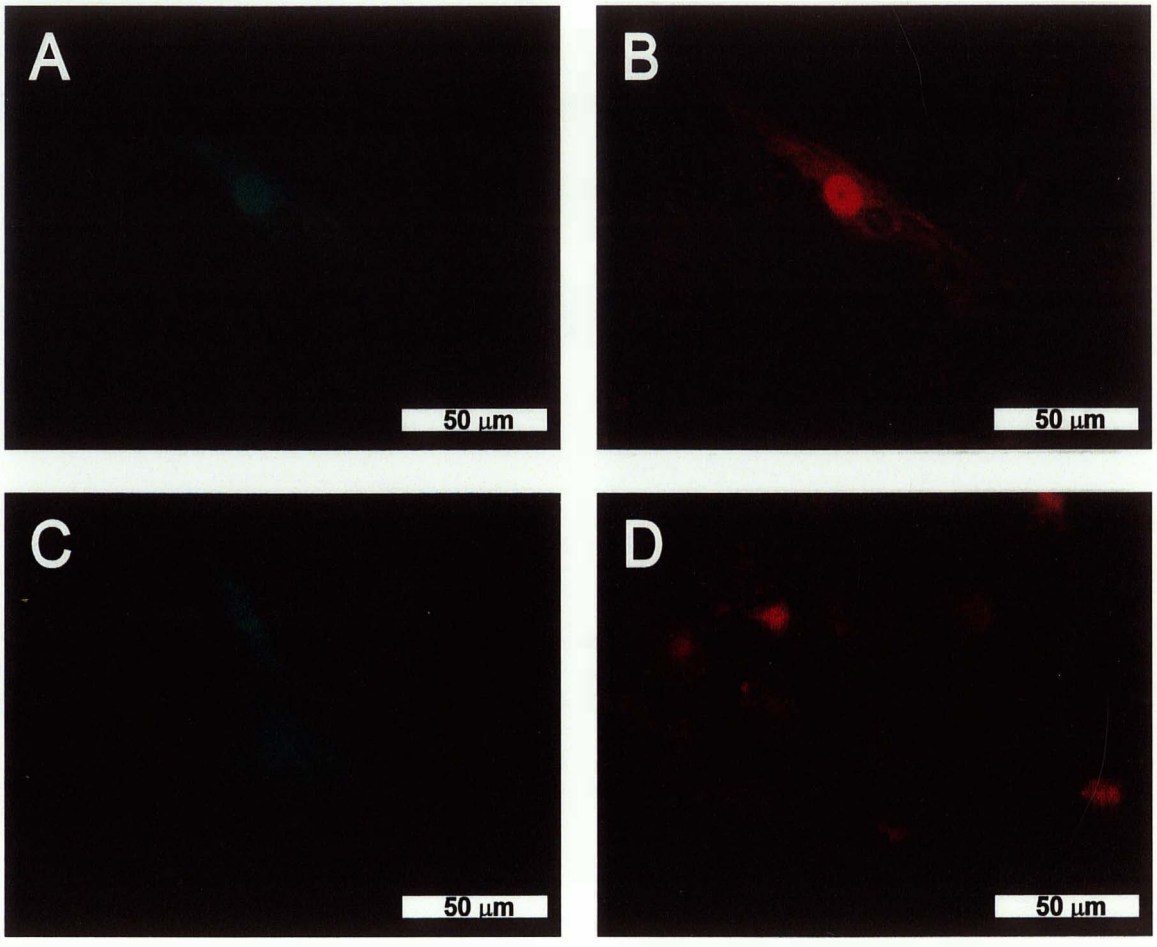


Figure 4.13 FGF-16 antibody (Ab60) detects cells expressing EGFP-FGF-16 fusion protein. A) Fluorescence signal of rat neonatal cardiac myoyctes transfected with EGFP tagged FGF-16, and stained B) with anti FGF-16 (Ab60) and Texas red secondary antibody. C) Neonatal rat cardiac myocytes transfected with GFP expression vector and D) stained with anti-FGF-16 (Ab60) and Texas red secondary antibody.

Fig 4.14

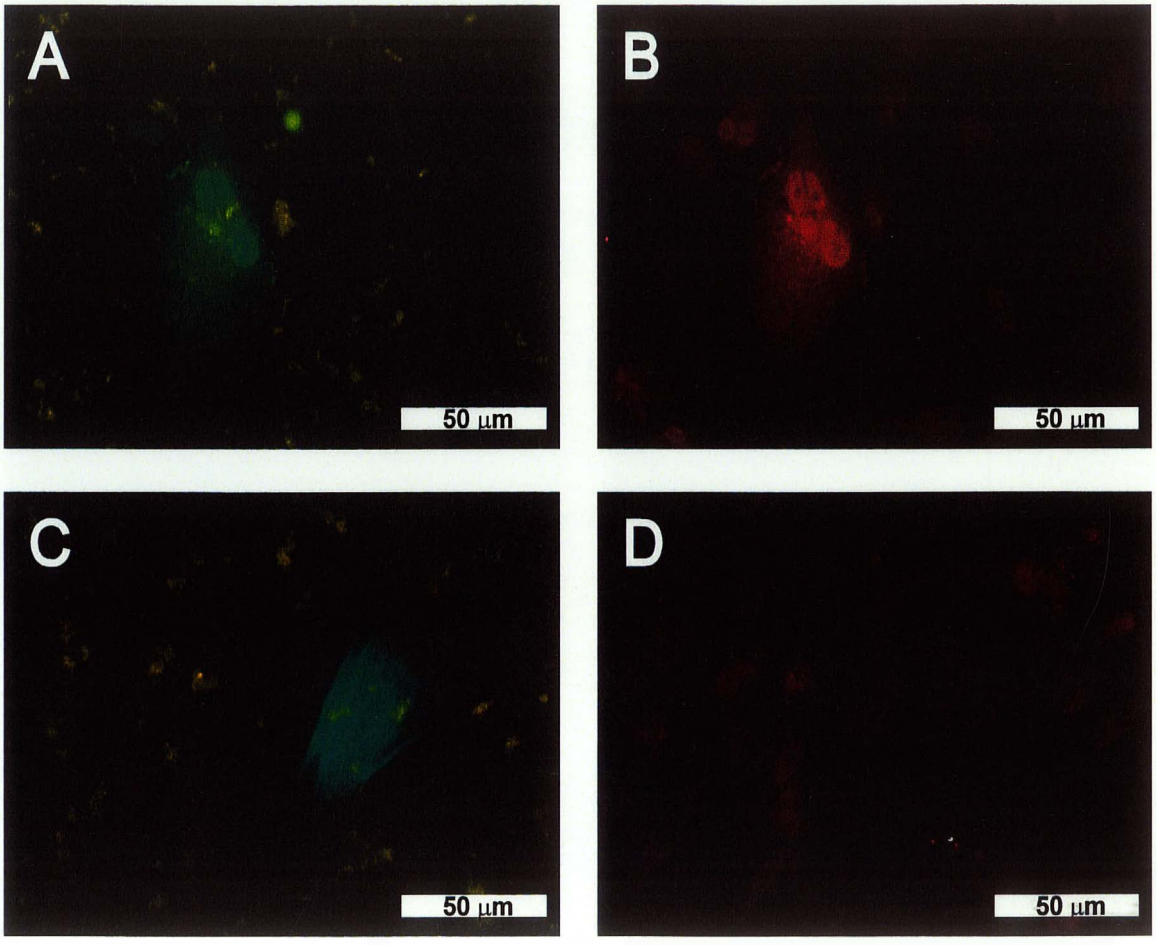


Figure 4.14 FGF-16 antibody (Ab61) detects cells expressing EGFP-FGF-16 fusion protein. A) Fluorescence signal of rat neonatal cardiac myoyctes transfected with GFP tagged FGF-16, and stained B) with anti FGF-16 (Ab61) and Texas red secondary antibody. C) Neonatal rat cardiac myocytes transfected with GFP expression vector and D) stained with anti-FGF-16 (Ab61) and Texas red secondary antibody.

Fig 4.15

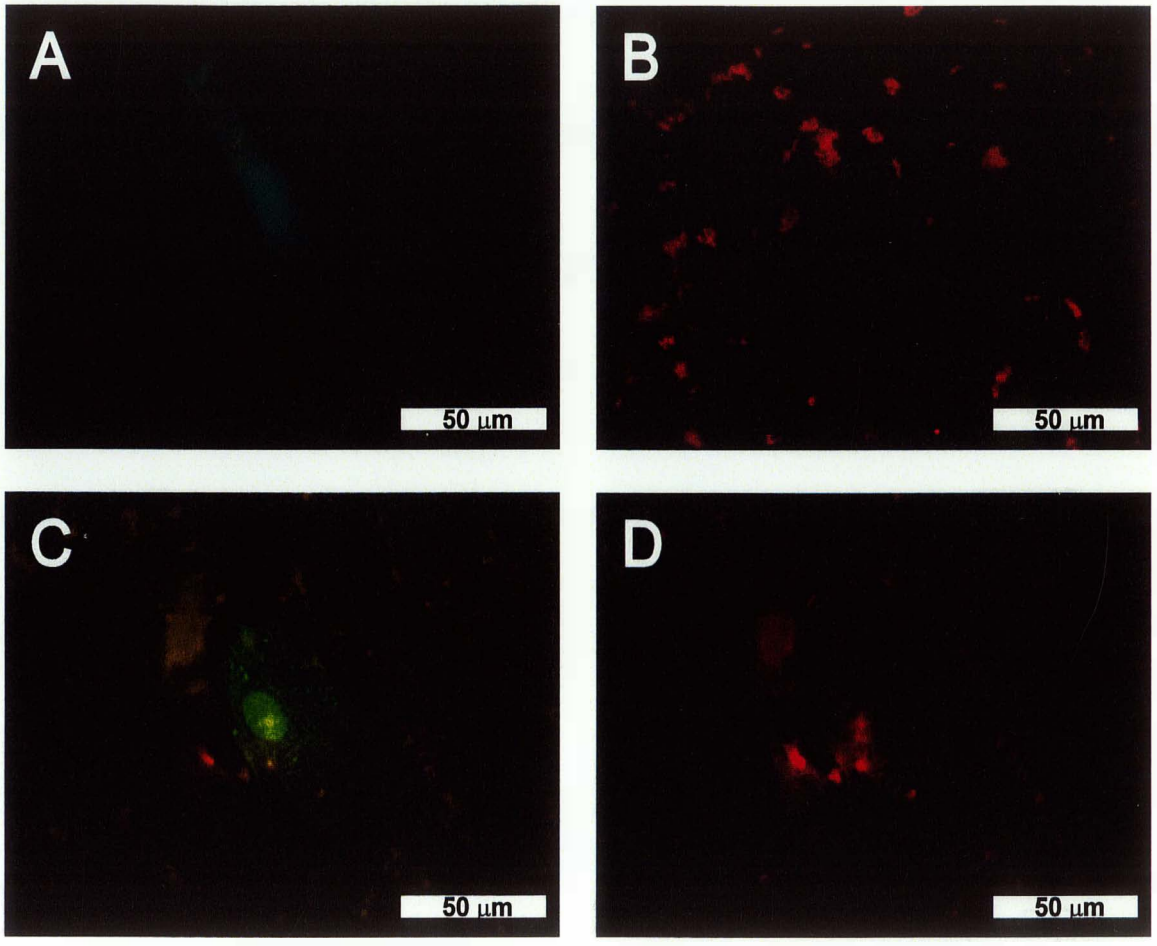


Figure 4.15 FGF-16 antibody (AbPeprotech) does not detect cells expressing GFP-FGF-16 fusion protein. A) No apparent fluorescent signal could be observed from rat neonatal cardiac myocytes transfected with GFP tagged FGF-16, and stained B) with anti FGF-16 (Ab61) and Texas red secondary antibody. C) Neonatal rat cardiac myocytes transfected with GFP expression vector and D) stained with anti-FGF-16 (Ab61) and Texas red secondary antibody.

Fig 4.16

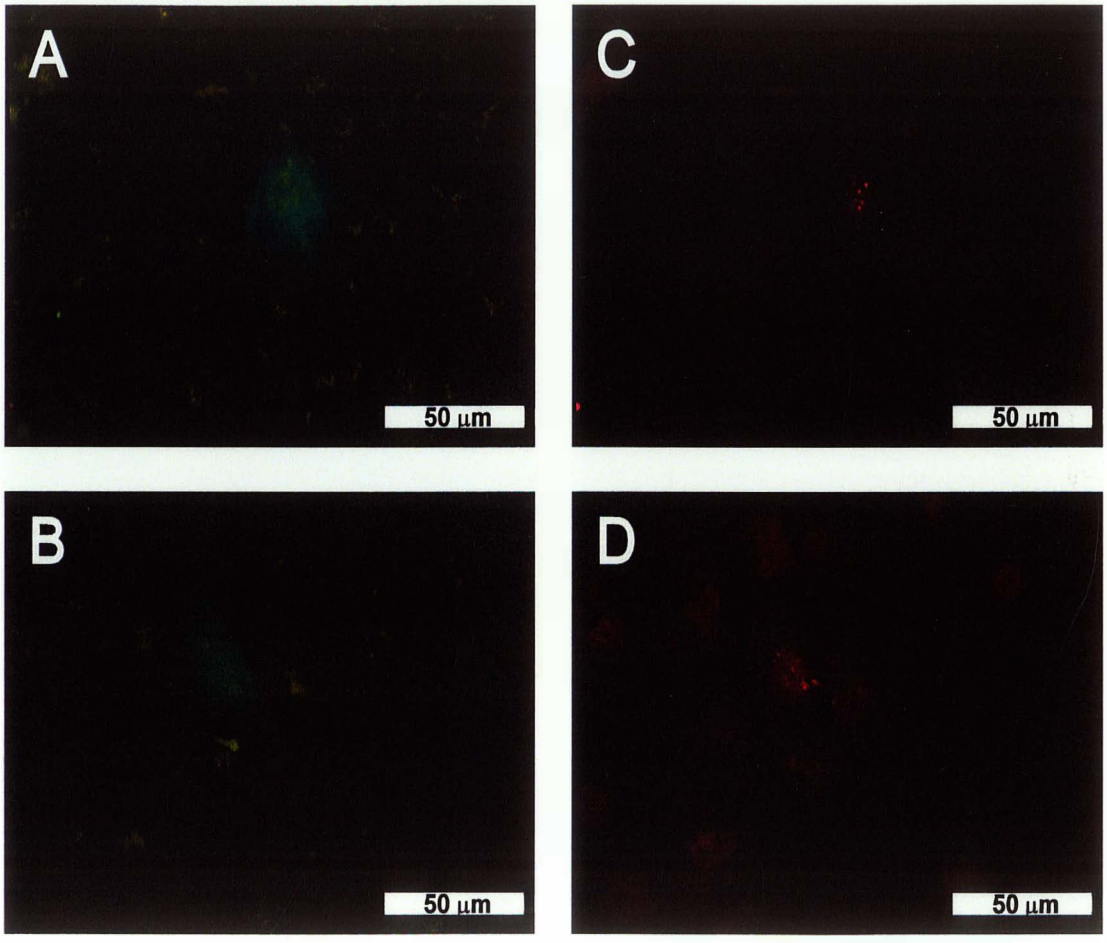


Figure 4.16 Two independent antibodies detect cells co-transfected with pEGFP-C1 and pCDNA$_3$-FGF-16 expression vector. A) and B) EGFP fluorescence of transfected cells treated with anti FGF-16 C) Ab60 and D) Ab61 visualized with Texas Red conjugated secondary antibody. The antibodies resulted in fluorescent signal in only 45 % of the cells which produced a positive signal for the EGFP protein. Cells shown are representative of those which were positive for antibody staining.

Chapter 5

5.0 Assessment of FGF-16 bioactivity and signaling pathways in cardiac myocytes and non myocytes.

5.1 Testing the biological activity of FGF-16 in BAF-3 cells

FGF-16 is a glycosylated molecule secreted by cardiac myocytes (Chapter 4). The fact that FGF-16 is a secreted molecule immediately suggests possible autocrine and/or paracrine effects on cardiac cells. However, before examining such possibilities it was necessary to determine if the secreted and glycosylated protein was biologically active. This was accomplished through the use of a bone marrow progenitor cell line (BAF-3) modified to express a predetermined FGF receptor isoform. BAF-3 cells are normally dependent on interleukin-3 (IL-3) for cell survival and division, but can respond to FGF ligand with increased DNA synthesis (as measured by tritiated thymidine incorporation) if expressing a suitable FGFR (Ornitz et al., 1996). Although there are many different FGF receptors to choose from, a BAF-3 cell line modified to express the FGFR-2c splice isoform was selected as it had already been found to respond consistently to FGF-16 (Dr. David Ornitz Washington University Medical School, University of Washington, St. Louis, personal communication).

Although tritiated thymidine incorporation is often used as an indicator of DNA synthesis/proliferation, an alternative method of assaying cell proliferation which did not require the use of radioactive materials was investigated. Rapraeger and colleagues (Allen et al., 2001) had previously shown that the response of BAF-3 cells expressing FGFR could be reported in terms of increases in cell number, or cell division. However,

initial attempts to use this assay as a model for investigating the biological activity of FGF-16 were unsuccessful. Upon removal of IL-3, cells began to undergo apoptosis as previously described (Palacios and Steinmetz, 1985; Rodrigues-Tarduchy et al., 1990), despite the addition of FGF-16. Although cell death occured by 48 hours, the number of cells living after 17 hours was noticeably greater in the presence of FGF-16. Thus, there was a delay in apoptosis in the presence of FGF-16. An assay was therefore developed which measured the survival rate of BAF-3 cells after IL-3 removal, both in the presence and absence of FGF-16.

Cells were stained as described (Section 2.3.3) and scored using flow cytometry. The nuclei of cells with compromised membranes (ie., apoptotic) stained red/orange with a membrane impermeant ethidium homodimer, while living cells with intact membranes absorbed and retained calcein through an enzymatic process that labeled cells green (Figure 5.1). This resulted in two populations of cells, which could be scored according to colour using flow cytometry. It is also worth noting that live versus dead cells could also be discerned when their measured values for side scatter were plotted against their forward angle light scatter. This resulted in two distinct clusters of cells, which also coincided with the calcein and ethidium fluorescence. (Figure 5.2).

When BAF-3 cells were starved of IL-3 for 18 hours, 28.4% of the cells were scored as live, as opposed to 89.6% of cells supplied with IL-3. FGF-16 was supplied to IL-3 starved cells in two forms: 1) in varying concentrations of recombinant hFGF-16 and 2) in varying amounts of conditioned media from HEK 293 cells transfected with pcDNA$_3$-FGF-16. Both sources of FGF-16 (representing non-glycosylated and glycosylated protein, respectively) increased BAF-3 cell survival (from 30% to almost

60%) in a dose-dependent manner (Fig 5.3). These results therefore confirmed the biological activity of not only the recombinant but also the FGF-16 secreted from transfected HEK 293 cells. Furthermore, the data also show that FGF-16 has pro-survival characteristics with the FGFR-2c pathway being a logical candidate..

5.2 Detection of cardiac FGF receptor

Evidence obtained thus far confirmed the production and secretion of FGF-16 from cardiac myocytes (section 4.4.2) and biological activity of FGF-16 in cells expressing FGFR-2c. Therefore, an investigation was performed to establish which FGFRs were available in the heart for interaction with FGF-16. Cryosections of mouse heart were treated with antibodies against FGFR-1-4 followed by fluorescent labeling (Fig 5.4A and B representing sections from two separate regions of the heart). Using normal rabbit IgG as a control for background, signal was observed in samples treated with anti FGFR-1, FGFR-2 and FGFR-3 (Figure 5.4A). The staining observed was most intense for FGFR-1 and FGFR-3 in the larger vascular structures (Figure 5.4B) and tended to have a strong association with the nuclei of not only the vascular cells but the striated muscle. FGFR-2 was also detected in some of the nuclei of the striated muscle cells but did not show the same association with the vascular structures (Figure 5.4). In contrast there did not appear to be any obvious staining pattern with the FGFR-4 antibody.

The FGFR antibodies however, are not able to distinguish between receptor isoforms. In particular the FGFR-2c isoform, which was shown in the previous section to be activated by FGF-16 in BAF-3 cells. A recent study by Ornitz and colleagues (Lavine

et al., 2005) also confirmed the activity of FGF-16 in a BAF-3 assay with FGFR-2c, FGFR-3b and FGFR-3c. However, responses to FGFR-2c were roughly ten fold or higher than that observed with FGFR-1b, FGFR-1c, or FGFR-2b. Therefore it was of particular interest to determine whether FGFR-2c, which shows the strongest activation in response to FGF-16 of all the receptors examined, was present in the heart as opposed to FGFR-2b (Lavine et al., 2005).

As mentioned, the difference between FGFR-2b and FGFR-2c lies in alternate splicing of the third immunoglobulin domain (Figure 1.2). Using an assay previously reported (Ford et al., 1997, see section 2.3.7 for details), primers were chosen based on sequence flanking the alternatively spliced region of mouse FGFR-2c (Figure 5.5). This consisted of a primer matching a region before the alternative splice site shared by both FGFR-2c and FGFR-2b and a second primer further downstream matching only that of the FGFR-2c splice variant (Figure 5.5). The identity of the RT-PCR product was also confirmed through Southern blotting using a sequence within the expected product as a probe.

RNA isolated from BAF-3 cells expressing the FGFR-2c isoform served as a positive control while reverse transcriptase cocktail substituted with H_2O in place of enzyme served as a negative control. RT-PCR of RNA isolated from adult mouse heart as well as primary cell cultures of rat neonatal myocytes and non myocytes resulted in a product of the expected size (339 bp) for FGFR-2c. A Southern blot containing the RT-PCR products also tested positive when probed using the internal sequence for FGFR-2c (Figure 5.6), thus confirming the presence of the FGFR-2c splice variant.

5.3 FGF-16 function in cardiac cells

Once it was established that both forms of FGF-16 possessed biological activity and that cardiac myocytes produce both ligand (FGF-16) and receptor (FGFR-2c), this raised two separate yet related questions regarding FGF-16 function:

1) Does the pro-survival action of FGF-16 in BAF-3 cells translate to a cytoprotective effect on cardiac myocytes?

2) What are the signal transduction pathways activated by FGF-16 in cardiac myocytes?

5.3.1 Assessment of pro-survival action of FGF-16 in cardiac myocytes.

To address whether FGF-16 promotes cell survival in myocytes following conditions of stress, neonatal rat cardiac myocytes were subjected to a well characterized model of oxidative stress using hydrogen peroxide (H_2O_2). At concentrations between 50 and 200 μM, H_2O_2 induces apoptosis in cardiac myocytes while causing necrosis at higher doses (von Harsdorf et al., 1999, Aikawa et al., 2000, Kwon et al., 2003). One of the characteristics of apoptosis is that of nuclear condensation which has been supported by other apoptotic markers such as TUNEL stain (Aikawa et al., 2000, Mizukami et al., 2001, Kwon et al., 2003). In addition, the apoptotic effect caused by the addition of H_2O_2 is reported to be reduced by the presence of insulin (Aikawa et al., 1997). Using nuclear condensation or nuclear size as an indicator of myocyte health, cells treated with H_2O_2 displayed a marked condensation of nuclei (Figure 5.7). Segregating nuclei based on calculated surface area from photographs revealed a binomial distribution. Cells were

therefore scored as being >135 μm^2 (normal nuclei) or <135 μm^2 (compacted nuclei). The addition of a media supplement containing insulin selenium and transferrin (ITS) was effective in preventing the condensation caused by H_2O_2 insult, while the addition of FGF-16 in the form of conditioned media from transfected HEK 293 cells did not reduce the percentage of compacted nuclei (Figure 5.8). This assay produced similar results in two experiments with each treatment group (n = 3). Therefore, under the conditions tested, FGF-16 did not appear to prevent the nuclear condensation caused by H_2O_2 insult.

5.3.2 MAPK activation by FGF-16

Many FGF receptors including the FGFR-2c isoform have been documented as exerting their effects through mitogen activated protein kinase (MAPK) activation (Agrotis et al., 2001, Nakamura et al., 2001, Jang et al., 2002). This signaling pathway has been implicated in many different cardiac responses (covered in section 1.3.3.4). As previously demonstrated, myocytes and non myocytes both possess transcript for the FGFR-2c splice isoform which is known to be activated by FGF-16. It was therefore decided to investigate whether these cells would respond to FGF-16 stimulus in the form of MAPK activation.

Purified cultures of neonatal rat cardiac myocytes and non myocytes were examined for MAPK activation following addition of FGF-16 in the form of either recombinant hFGF-16 (non glycosylated) or conditioned media from transfected HEK 293 cells (glycosylated and secreted form of FGF-16). It is important to note that cells were serum starved prior to stimulation to reduce background MAPK phosphorylation levels. However, the protocols used for FGF-16 in either the recombinant or conditioned

media form were slightly different. Serum was completely removed from cells two hours prior to stimulation with recombinant hFGF-16, while cells treated with FGF-16 in the form of conditioned media were starved in 0.5% serum prior to stimulation. The reason being that the conditioned media contained 5% serum and the increase of 10% by volume by the addition of conditioned media resulted in a 0.5% increase in serum concentration. Complete removal of serum was therefore avoided in the group treated with conditioned media in order to minimize the possibility of MAPK induction by the serum alone. For future reference, samples from either of the two different serum starved groups will be referred to as either belonging to the recombinant (testing hFGF-16) or conditioned media (with glycosylated FGF-16) groups.

Cells were assayed after a 10 minute stimulation with either control (vehicle), FGF-2 (10ng/ml), serum (10% by volume) or three different concentrations of FGF-16 (either 1, 10 and 100 ng/ml or 0.1 hFGF-16, 1 and 10% by volume HEK 293 conditioned media). Cell lysates were examined via protein blotting with antibodies specific to the phosphorylated form of various MAPK. These consisted of; p38, extracellular receptor kinases (ERK 1/2) and p46/p54 or c-Jun N-terminal kinases (JNK). The activation of the kinases were scored by densitometry. Those samples with a signal between 130-180% of control (arbitrarily set to 100%) were considered a moderate response while anything greater than 180% was considered a strong response.

5.3.2a Non myocyte MAPK activation.

The addition of either form of FGF-16 to non myocyte cells did not result in increased MAPK phosphorylation in either the recombinant or conditioned media group

(0 of 3 and 0 of 3 respectively, as represented in Figure 5.9 and summarized in Table 5.1). However, both recombinant bovine FGF-2 and 10% serum each resulted in strong increases in ERK1/2 from both groups (3 of 3 for recombinant and 3 of 3 for conditioned media). The response of p38 was also strong for both groups treated with either FGF-2 or serum (3 of 3 and 3 of 3, respectively, for recombinant, or 3 of 3 and 3 of 3, respectively, for conditioned media). Strong responses of p46 JNK were observed in both groups (3 of 3 for recombinant and 3 of 3 for conditioned media), with only moderate activation of p54 JNK in the recombinant group (2 of 3).

5.3.2b Myocyte MAPK activation

The addition of either FGF-2 or serum to cardiac myocytes in culture resulted in strong increases of ERK1 activation (3 of 3 and 3 of 3, respectively, for the recombinant group) and ERK2 (3 of 3 and 3 of 3, respectively for the conditioned media group, as represented Figure 5.10 and summarized in Table 5.2). Responses of ERK 1 to FGF-16 were moderate in the recombinant group (3 of 9, all FGF-16 concentrations combined) and similar with ERK 2 (5 of 9, all FGF-16 concentrations combined). No increases in ERK 1 or 2 were observed in the conditioned media group.

Strong responses of p38 (as represented in Figure 5.10 and summarized in Table 5.2) were observed from the recombinant group after treatment with FGF-2 (3 of 3) or serum (2 of 3). The addition of recombinant hFGF-16 produced variable activation of p38 with both strong and moderate responses (2 strong and 3 weak of 9, from all three hFGF-16 concentrations combined). The conditioned media group however, showed no indication of p38 activation in response to FGF-16 (0 of 9).

Activation of p46 JNK by the addition of either positive control in the recombinant group were strong (3 of 3 for serum and 3 of 3 for FGF-2). Similar strong effects were observed with p54 JNK (2 strong and 1 moderate of 3 for serum and 3 of 3 strong for FGF-2). The addition of recombinant hFGF-16 however produced mostly moderate effects (6 of 9 for all recombinant hFGF-16 treatments combined).

A similar effect was observed with the positive controls for the conditioned media group in that activation of p46 JNK was strong (3 of 3 for serum and 3 of 3 for FGF-2). The activation of p54 JNK was more variable for FGF-2 (1 strong and 1 moderate of 3) and serum (1 moderate of 3). However, activation by FGF-16 for the conditioned media group was isolated (1 moderate response of all 9 FGF-16 treatments combined).

5.3.2c Testing for a synergistic effect of FGF-2 with FGF-16 on MAPK.

The results from the previous sections revealed that FGF-16 did not activate MAPK to the same degree as that of FGF-2 or serum. However, normally both growth factors are present at the same time. A recent study by Ornitz and colleagues (Lavine et al., 2005) proposed that a combination of FGFs may be necessary to promote proper cardiac development. In fact, this study resulted in the hypothesis that the effects of FGF-2 may be dependent on the presence of at least one or possibly several other FGFs. Therefore, an interactive or even synergistic effect of FGF-2 and FGF-16 on MAPK activation is a distinct possibility and was therefore tested.

Samples from both the recombinant and conditioned media group treated with FGF-16 were examined in combination with FGF-2 to see if MAPK activation was any different from that of FGF-2 alone (as represented in Figure 5.11 and summarized in

Table 5.3). FGF-16 from neither the recombinant nor conditioned media groups resulted in further activation of ERK1/2 (0 of 12 from the recombinant group and 0 of 15, with results from all three FGF-16 concentrations combined) as compared to FGF-2 alone.

Examination of recombinant hFGF-16 with FGF-2 as opposed to FGF-2 alone revealed only moderate activation of p38 (3 of 12, for all three FGF-16 concentrations combined). In contrast, the effects of conditioned media containing FGF-16 in combination with FGF-2 resulted in strong responses (9 of 15, for all three FGF-16 concentrations combined). For comparison, only moderate responses were observed in 2 of 5 samples treated with FGF-2 alone.

The addition of FGF-16 in the form of either recombinant protein or conditioned media resulted in only moderate activation p46 JNK in some samples (3 of 12 for the recombinant group and 2 of 15 for the conditioned media group, results from all three FGF-16 concentrations combined in each group). At no time did either form of FGF-16 in combination with FGF-2 result in a strong response compared to that of FGF-2 alone.

The combination of FGF-2 and FGF-16 revealed strong activation of p54 JNK (2 moderate and 5 strong out of 12, compared with results from all three FGF-16 concentrations combined) which superceeded that of either FGF-2 or FGF-16 alone. With conditioned media, the combination of FGF-2 with FGF-16 also resulted in a moderate to strong response (2 moderate and 7 strong of 15 with results from all thre concentrations of FGF-16 combined). Therefore, the effects of combining FGF-2 with FGF-16 were greatest with p54 JNK as compared to any of the other MAPK.

5.4 Summary

Both glycosylated as well as non glycosylated FGF-16 were biologically active and prolonged the survival of IL-3 deprived BAF-3 cells expressing the FGFR-2c isoform. Cryosections of adult mouse heart were positive for immunohistochemical stain of FGFR-1, FGFR-2 and FGFR-3 but not FGFR-4. RNA from adult mouse heart, as well as cultured neonatal rat cardiac myocytes and non myocytes all gave signal for the FGFR-2c splice isoform by RT-PCR. Treatment of neonatal cardiac myocytes with H_2O_2 resulted in nuclear condensation which could be prevented by the addition of supplemental insulin containing ITS but not FGF-16. When neonatal cardiac myocytes and non myocytes were examined for responses to FGF-16 by changes in MAPK activity, the effects were subtle at best compared to those of FGF-2 or serum. However, when both FGF-2 and FGF-16 were combined, there was a increased activation of both the p38 and p54 kinases.

Table 5.1 Summary of neonatal rat cardiac non myocyte culture MAPK activation in response to FGF-16. Note: Numbers in each column represent responses for each of the kinases detected. For example, there were 3 strong responses toward 10% serum for ERK1 and 3 strong responses for ERK2, hence 3-3 for ERK1 – ERK2.

Recombinant Group (addition of recombinant hFGF-16)

Non Myocytes	ERK 1- ERK 2		p38		p46 - p54	
Treatment n = 3	Strong response	Moderate response	Strong response	Moderate response	Strong response	Moderate response
1 ng hFGF-16/ml	0-0	0-0	0	0	0-0	0-0
10 ng hFGF-16/ml	0-0	0-0	0	0	0-0	0-0
100 ng hFGF-16/ml	0-0	0-0	0	0	0-0	0-0
Serum 10%	3-3	0-0	3	0	3-0	0-2
10 ng/ml FGF-2	3-3	0-0	3	0	3-0	0-2

Conditioned Media Group (addition of conditioned media with secreted form of FGF-16)

Non Myocytes	ERK 1- ERK 2		p38		p46-p54	
Treatment n = 3	Strong response	Moderate response	Strong response	Moderate response	Strong response	Moderate response
0.1 % FGF-16 CM	0-0	0-0	0	0	0-0	0-0
1 % FGF-16 CM	0-0	0-0	0	0	0-0	0-0
10 % FGF-16 CM	0-0	0-0	0	0	0-0	0-0
Serum 10%	3-3	0-0	3	0	3-0	0-0
10 ng/ml FGF-2	3-3	0-0	0	3	3-0	0-0

Table 5.2 Summary of neonatal rat cardiac myocyte culture MAPK activation in response to FGF-16.

Recombinant Group (addition of recombinant hFGF-16)

Myocytes	ERK 1- ERK 2		p38		p46-p54	
Treatment n = 3	Strong response	Moderate response	Strong response	Moderate response	Strong response	Moderate response
1 ng hFGF-16/ml	0-0	1-2	1	1	0-0	0-2
10 ng hFGF-16/ml	0-0	2-2	1	1	0-0	0-2
100 ng hFGF-16/ml	0-0	1-1	0	1	0-0	0-2
Serum 10%	3-3	0-0	2	0	3-2	0-1
10 ng/ml FGF-2	3-3	0-0	3	0	3-3	0-0

Conditioned Media Group (addition of conditioned media with secreted form of FGF-16)

Myocytes	ERK 1- ERK 2		p38		p46-p54	
Treatment n = 3	Strong response	Moderate response	Strong response	Moderate response	Strong response	Moderate response
0.1 % FGF-16 CM	0-0	0-0	0	0	0-0	0-0
1 % FGF-16 CM	0-0	0-0	0	0	0-0	0-0
10 % FGF-16 CM	0-0	0-0	0	0	0-0	0-1
Serum 10%	3-3	0-0	1	1	3-0	0-1
10 ng/ml FGF-2	3-3	0-0	1	1	3-1	0-1

Table 5.3 Summary of neonatal rat cardiac myocyte culture MAPK activation in response to FGF-16 and FGF-2 as opposed to FGF-2 alone.

Recombinant Group (addition of recombinant hFGF-16)

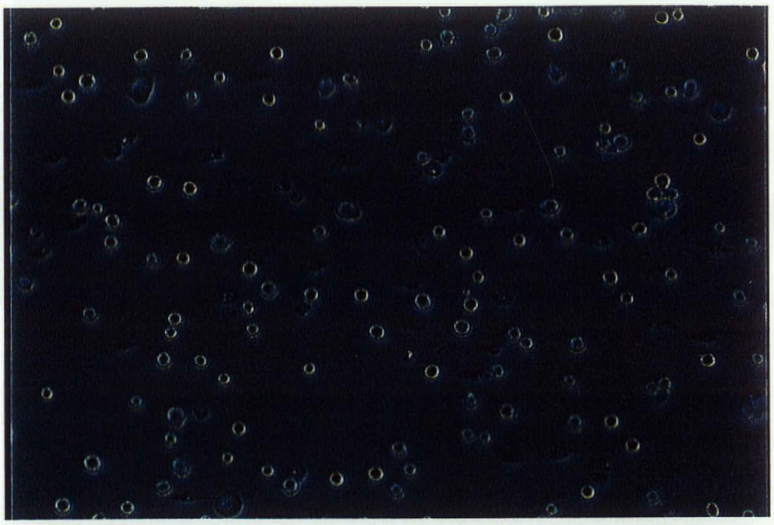
Myocytes	ERK 1- ERK 2		p38		p46-p54	
Treatment n = 4	Strong response	Moderate response	Strong response	Moderate response	Strong response	Moderate response
100 ng hFGF-16	0-0	0-0	0	0	0-0	0-0
1 ng hFGF-16/ml +10 ng FGF-2/ml	0-0	0-0	0	1	0-3	2-0
10 ng hFGF-16/ml + 10ng FGF-2/ml	0-0	0-0	0	1	0-2	1-0
100 ng hFGF-16 +10 ng FGF-2/ml	0-0	0-0	0	1	0-0	0-2

Conditioned Media Group (addition of conditioned media with secreted form of FGF-16)

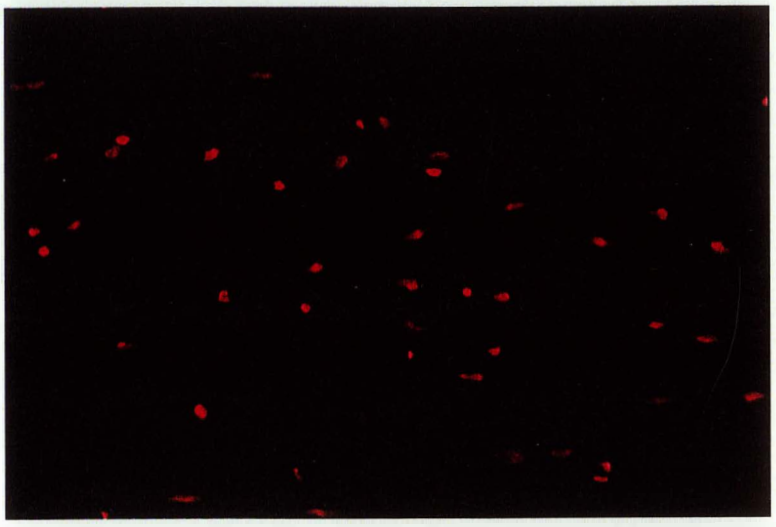
Myocytes	ERK 1- ERK 2		p38		p46-p54	
Treatment n = 5	Strong response	Moderate response	Strong response	Moderate response	Strong response	Moderate response
10 % FGF-16 CM	0-0	0-0	0	0	0-0	0-0
0.1 % FGF-16 CM +10 ng FGF-2/ml	0-0	0-0	3	0	0-3	1-1
1 % FGF-16 CM +10 ng FGF-2/ml	0-0	0-0	3	0	0-2	1-1
10 % FGF-16 CM +10 ng FGF-2/ml	0-0	0-0	3	0	0-2	0-0

Fig 5.1

A) Phase Contrast



B) Ethidium



C) Calcein

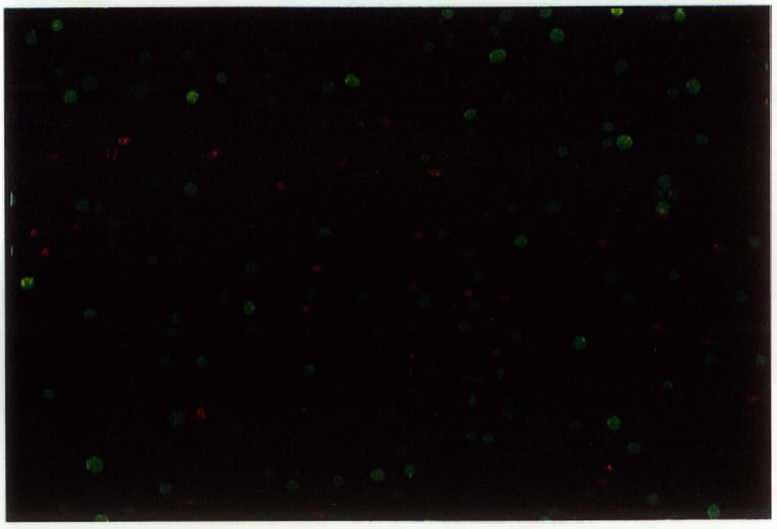
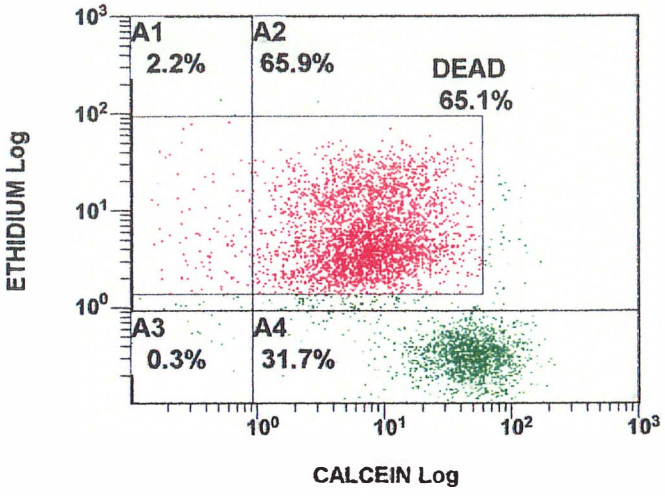


Figure 5.1 Fluorescent stain identifying live versus dead BAF-3 cells. A) Light micrograph of cells stained with B) ethidium bromide (red) which stains the nuclei of dead cells with compromised membrane integrity while C) cells which have trapped calcein (green) through an enzymatic process, represent the living population of cells.

Fig 5.2

A


A1
2.2%
A2
65.9%
DEAD
65.1%
A3
0.3%
A4
31.7%
ETHIDIUM Log
CALCEIN Log
10^0
10^1
10^2
10^3

B

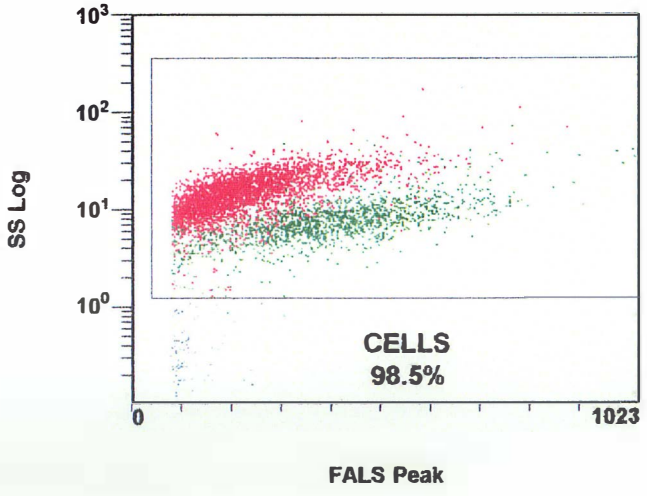

CELLS
98.5%
SS Log
FALS Peak
0
1023
10^0
10^1
10^2
10^3

Figure 5.2 Representative data of fluorescence activated cell sorting identifying live versus dead BAF-3 cells. Cells were sorted according to A) calcein fluorescence (green) representing live cells and ethidium fluorescence (red) representing dead cells. A threshold for each stain was established using positive and negative controls (live versus dead cells). B) Measurements of forward angle light scatter (FALS) and side scatter (SS) were then plotted against one another. Each data point is coloured to represent its classification as in panel A. Cells could be classified as live or dead based on either the live dead assay stain A) or light scatter B).

Fig 5.3

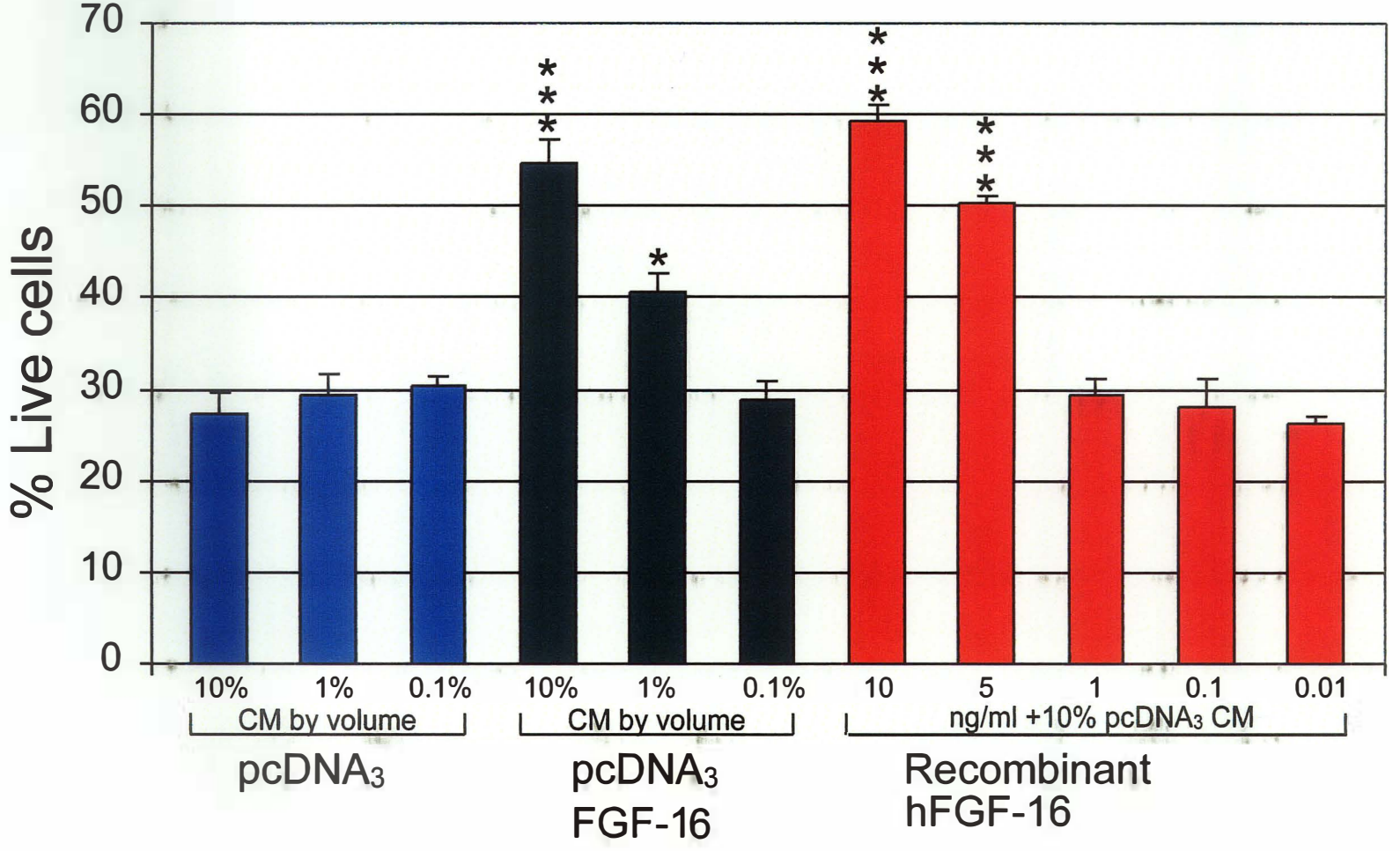

% Live cells
70
60
50
40
30
20
10
0
***
*
***
***
10%
1%
0.1%
10%
1%
0.1%
10
5
1
0.1
0.01
CM by volume
CM by volume
ng/ml +10% pcDNA3 CM
pcDNA3
pcDNA3 FGF-16
Recombinant hFGF-16

Figure 5.3 Summary of FGF-16 biological activity on BAF-3 cell survival. BAF-3 cells were starved of IL-3 and supplemented with conditioned media from HEK 293 cells as indicated. Media from HEK 293 cells transfected with the empty expression vector $pcDNA_3$ served as a control. Conditioned media from HEK 293 cells transfected with the $pcDNA_3$ FGF-16 construct increased cell survival in a dose dependent fashion. Supplementing the $pcDNA_3$ conditioned media (10% by volume) with recombinant hFGF-16 also increased cell survival. Conditioned media samples were compared using Bonferroni one way analysis of variance and statistical significance indicated by * = $p<0.05$ and *** = $p<.001$ with respect to their corresponding control.

Fig. 5.4a

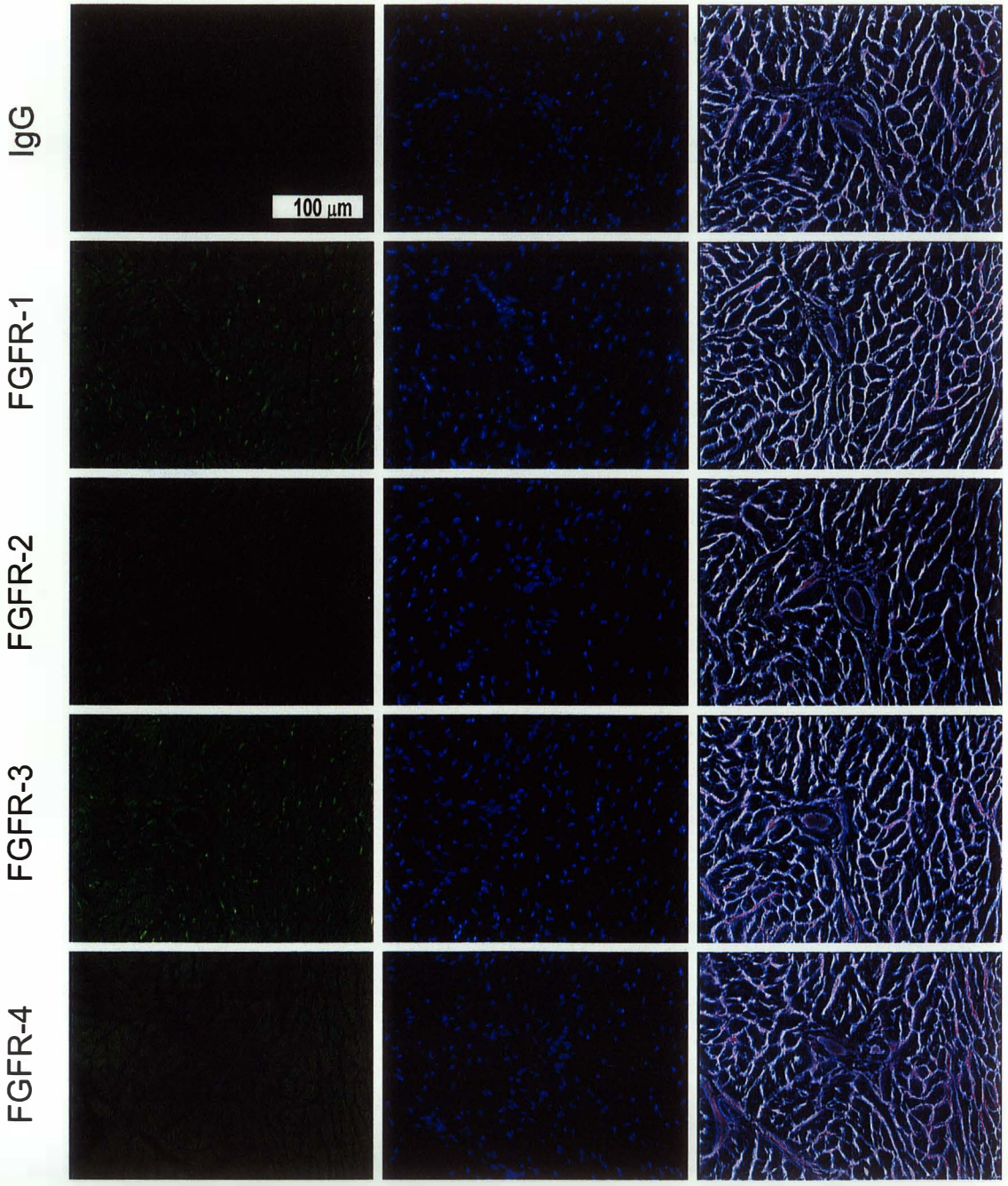


Fig. 5.4b

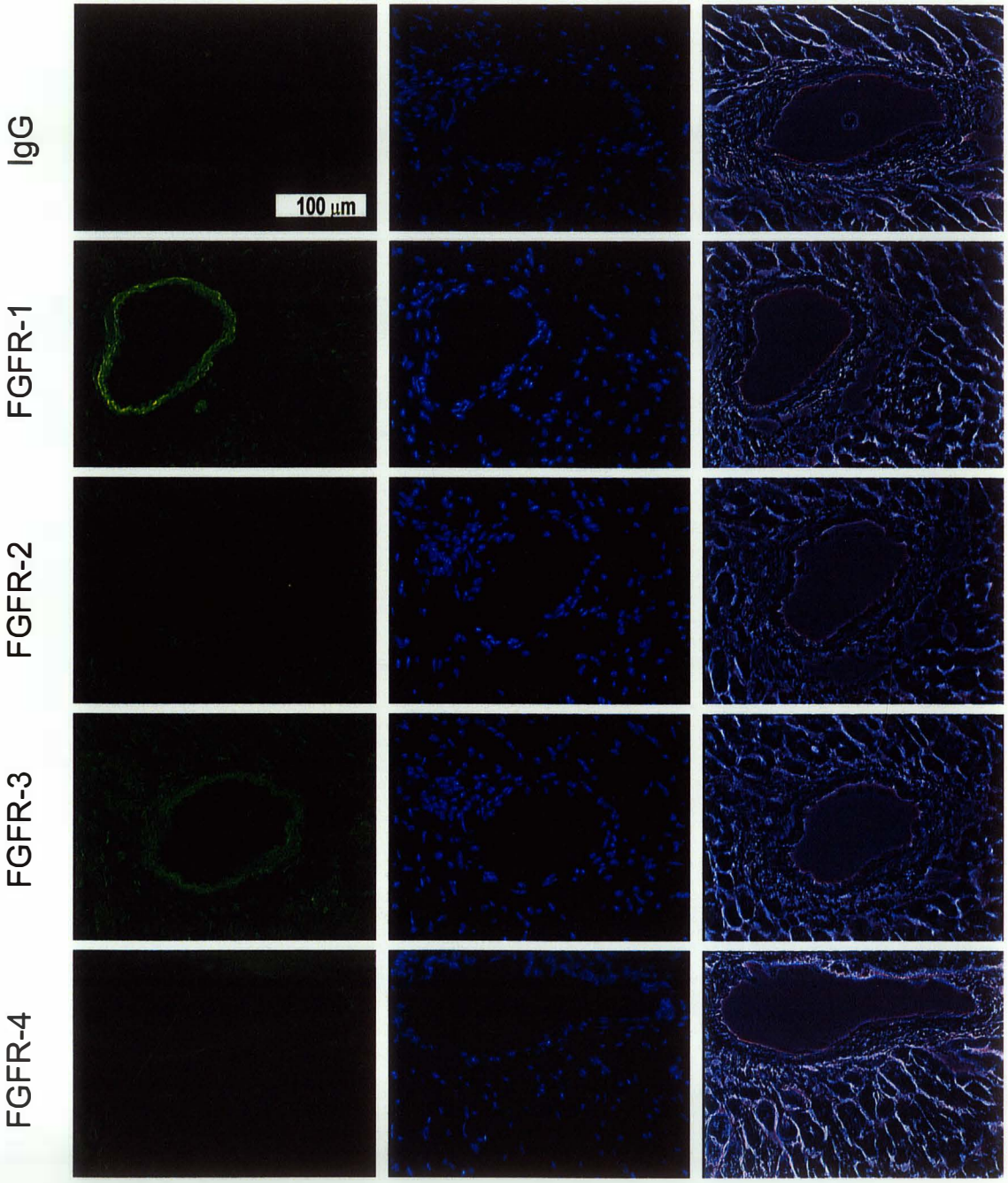


Figure 5.4 Detection of FGFR in adult mouse heart by immunofluorescence microscopy. Cryosections (7 μm) were paraformaldehyde fixed and treated with primary antibodies consisting of normal rabbit IgG, anti-FGFR-1, FGFR-2, FGFR-3 and FGFR-4. Sections were then incubated with secondary anti rabbit biotin followed by STREP-FITC (green). Signal for FGFR-1, FGFR-2 and FGFR-3 was observed in the striated cells and appeared to be associated primarily with the nuclei as represented in A). Staining for FGFR-1 and FGFR-3 also appeared to associate with the vasculature as represented in B). No clear signal for FGFR-4 could be identified. Sections were also stained for nuclei using Hoechst dye (blue) and photographed using phase contrast.

Fig 5.5

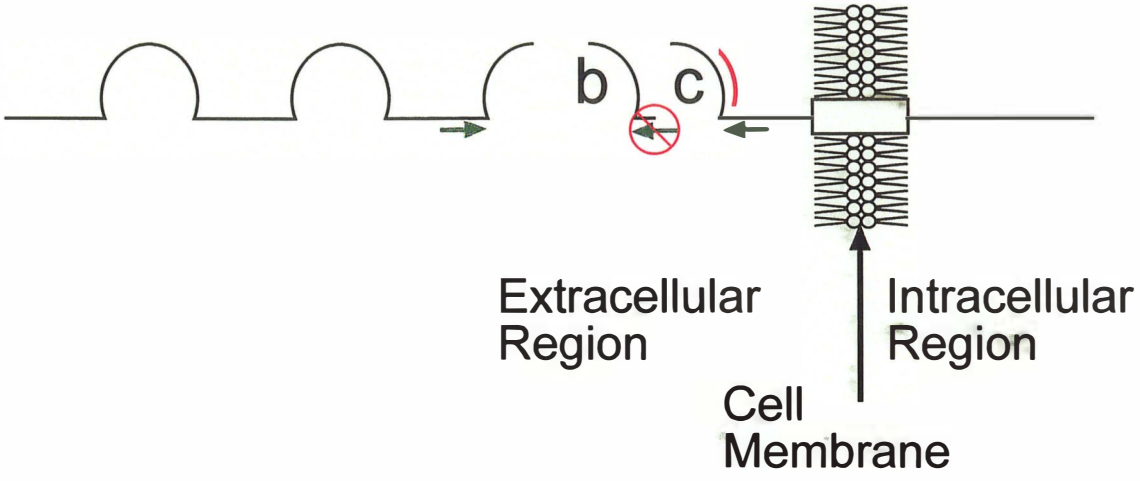

b
c
Extracellular Region
Intracellular Region
Cell Membrane

Figure 5.5 Schematic representing the location of the primers designed to detect FGFR-2c. Alternatively spliced regions for the two receptor isoforms are labeled as "b" and "c". Green arrows depict locations of forward and reverse primers that amplify a 339 bp product from the FGFR-2c but not the FGFR-2b splice variant. The region indicated in red is unique to the FGFR-2c splice isoform and can be used as a probe to identify FGFR-2c RT-PCR product by Southern blotting.

Fig 5.6

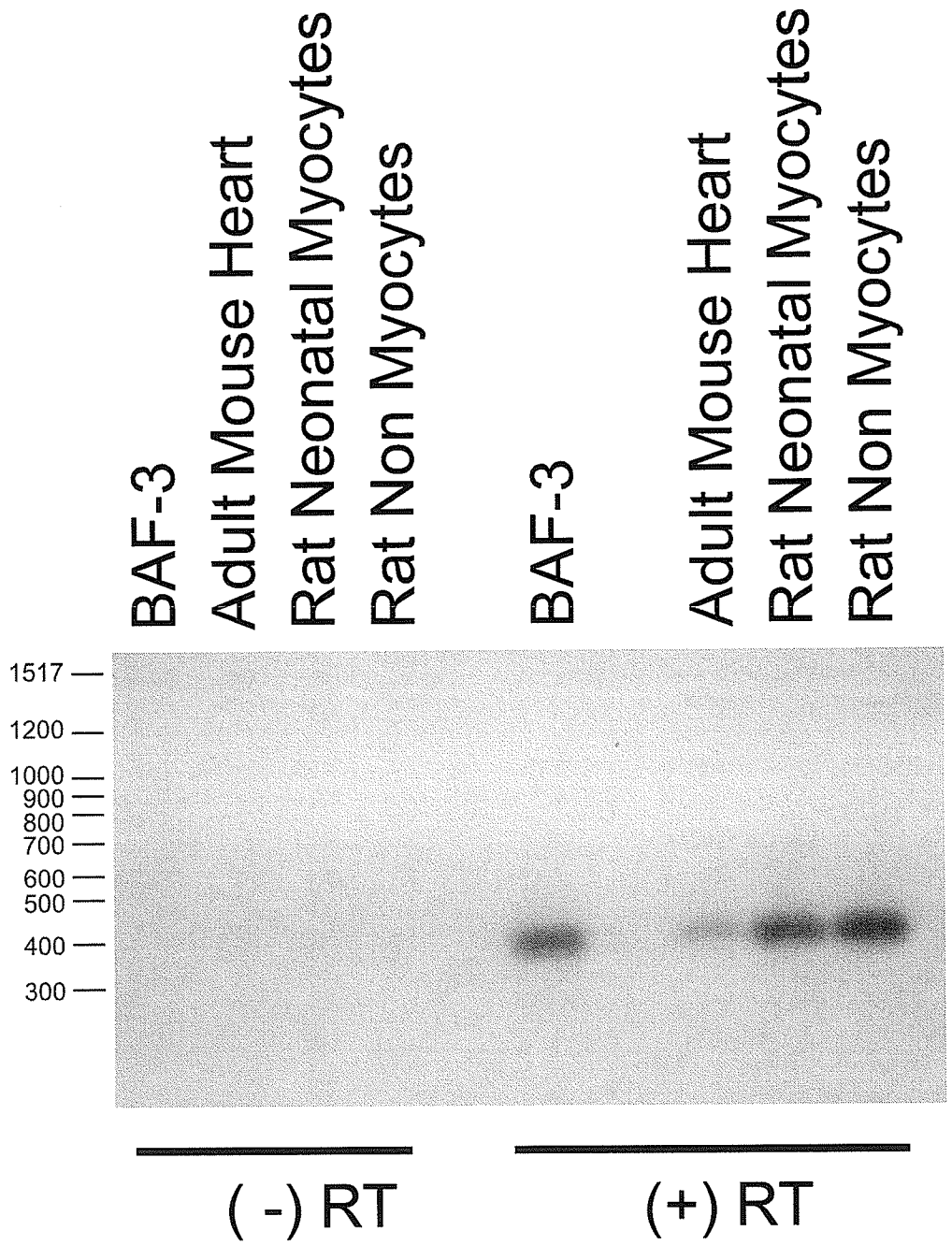

BAF-3
Adult Mouse Heart
Rat Neonatal Myocytes
Rat Non Myocytes
BAF-3
Adult Mouse Heart
Rat Neonatal Myocytes
Rat Non Myocytes
1517
1200
1000
900
800
700
600
500
400
300
(-) RT
(+) RT

Figure 5.6 Southern blot identifying FGFR-2c from RT-PCR reactions with RNA samples from heart tissue and cell cultures. BAF-3 cells expressing the FGFR-2c were used as a positive control and resulted in a product of the expected size (339 bp). RT-PCR products amplified from the RNA of adult mouse heart and primary cultures of neonatal rat cardiac myocytes and non myocytes also yielded products of the expected size. A probe designed to recognize a specific region of the expected FGFR-2c RT-PCR product yielded a clear signal as represented by Southern blot. Size markers are indicated on the left side in base pairs.

Fig 5.7

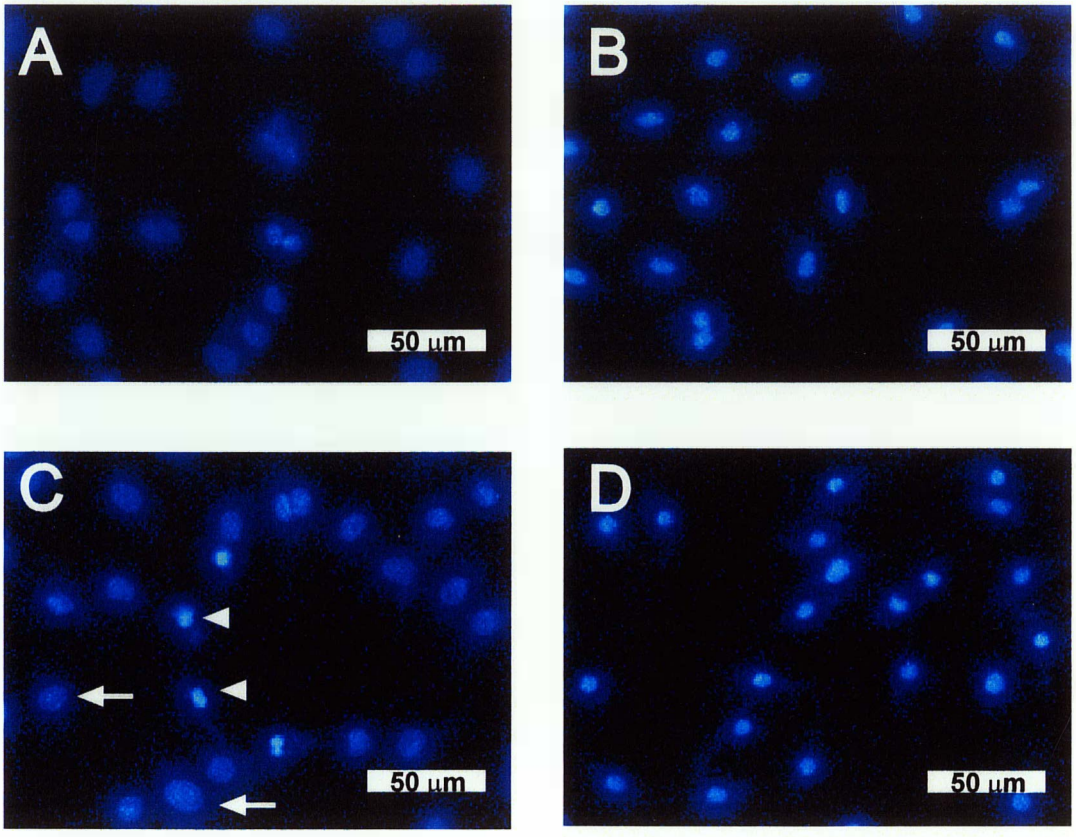


Figure 5.7 Fluorescence microscopy of normal and condensed nuclei of myocytes. Conditioned media from HEK 293 cells transfected with $pcDNA_3$ and supplemented with either A) Control media, B) H_2O_2 (200μM), C) ITS with H_2O_2 or D) conditioned media from HEK 293 cells transfected with FGF-16-$pcDNA_3$ with H_2O_2. Nuclei of cells were stained with Hoechst dye (blue) and visualized by fluorescence microscopy. The addition H_2O_2 induced nuclear condensation (in panel C, arrowheads and arrows represent condensed and normal nuclei, respectively) which was reduced by the addition of insulin transferrin selenium (ITS) but not FGF-16.

Fig 5.8

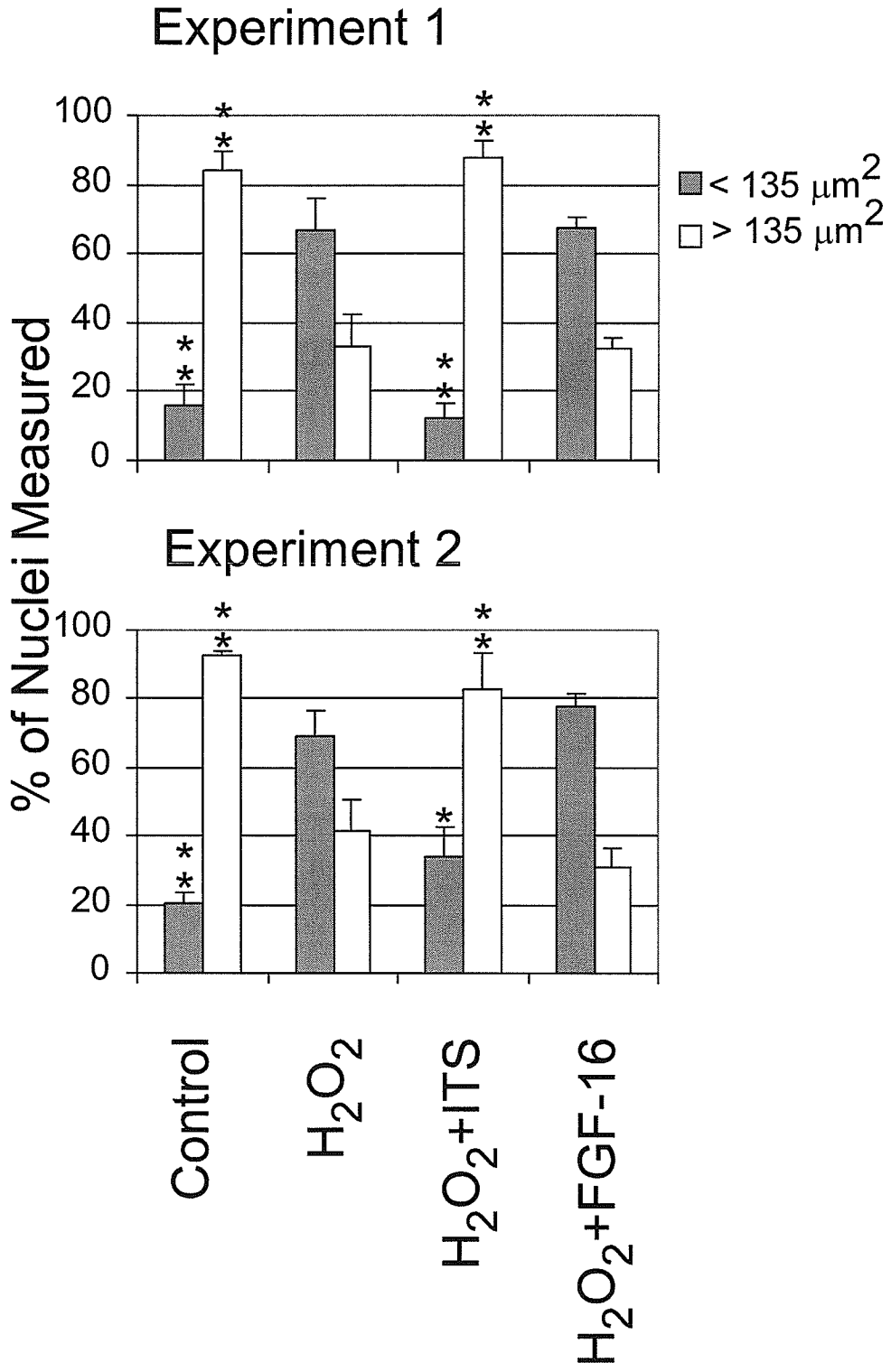

Experiment 1
Experiment 2
% of Nuclei Measured
100
80
60
40
20
0
< 135 μm2
> 135 μm2
Control
H2O2
H2O2+ITS
H2O2+FGF-16

Figure 5.8 Bar chart representing the percentage of condensed versus normal nuclei as depicted in Figure 5.7. Nuclei were scored and classified as being larger (normal) or smaller (compacted) than 135 μm^2. Averages represent the nuclei counted from 3 separate plates having counted 5 fields from each treatment as indicated. Statistical significance (**=p<.01 and *=p<.05) is indicated after using a Dunnett multiple comparisons test between the normal or condensed nuclei from the control group (H_2O_2 treated cells) as compared to that of other groups.

Fig 5.9

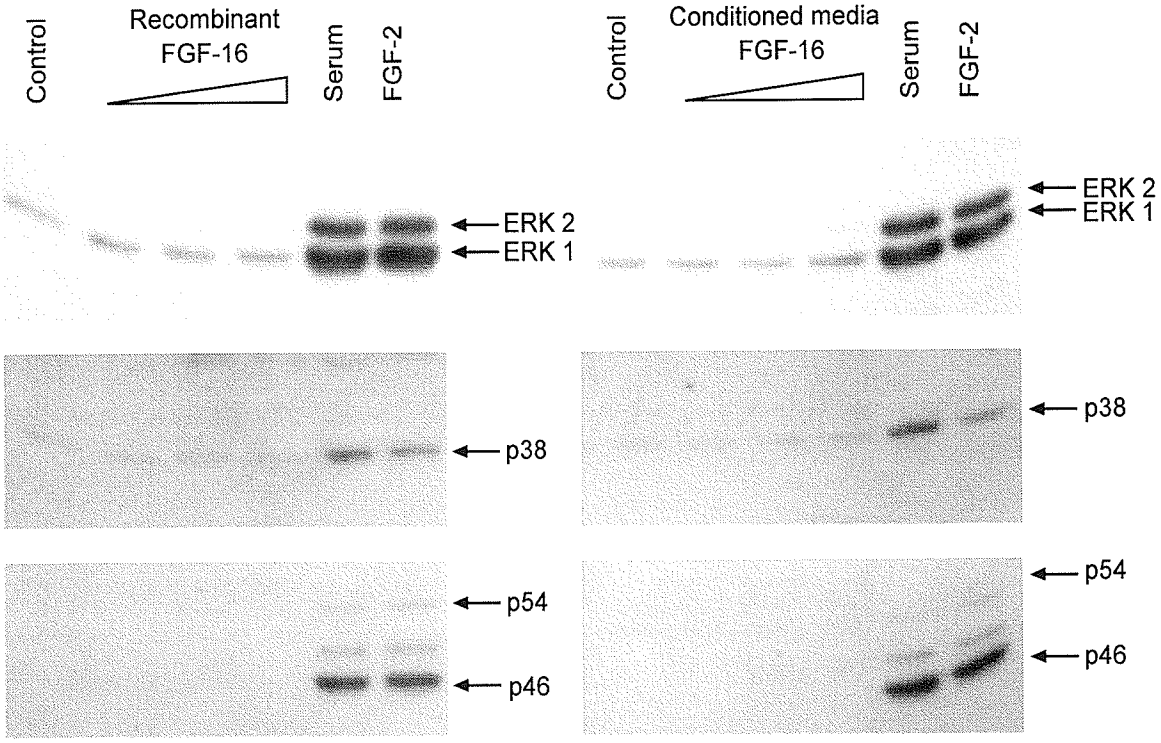

Control
Recombinant FGF-16
Serum
FGF-2
Control
Conditioned media FGF-16
Serum
FGF-2
ERK 2
ERK 1
ERK 2
ERK 1
p38
p38
p54
p46
p54
p46

Figure 5.9 Western blots representing phosphorylated MAPK in neonatal rat cardiac non myocyte cultures after treatment with FGF-16. Blots were probed with an antibody against the phosphorylated form of ERK 1 and ERK 2 (top panels), p38 (middle panels) and p54 and p46 JNK (bottom panels). Panels on the left side represent cells treated with recombinant hFGF-16 protein (1, 10 or 100 ng/ml) while those on the right were treated with conditioned media from transfected HEK 293 cells expressing FGF-16 (0.1, 1, or 10% by volume). Control samples consisted of either vehicle or conditioned media from HEK 293 cells transfected with $pcDNA_3$ (empty vector). Cells were also stimulated with 10% serum or FGF-2 as positive controls. All samples were harvested after 10 minutes of stimulation.

Fig 5.10

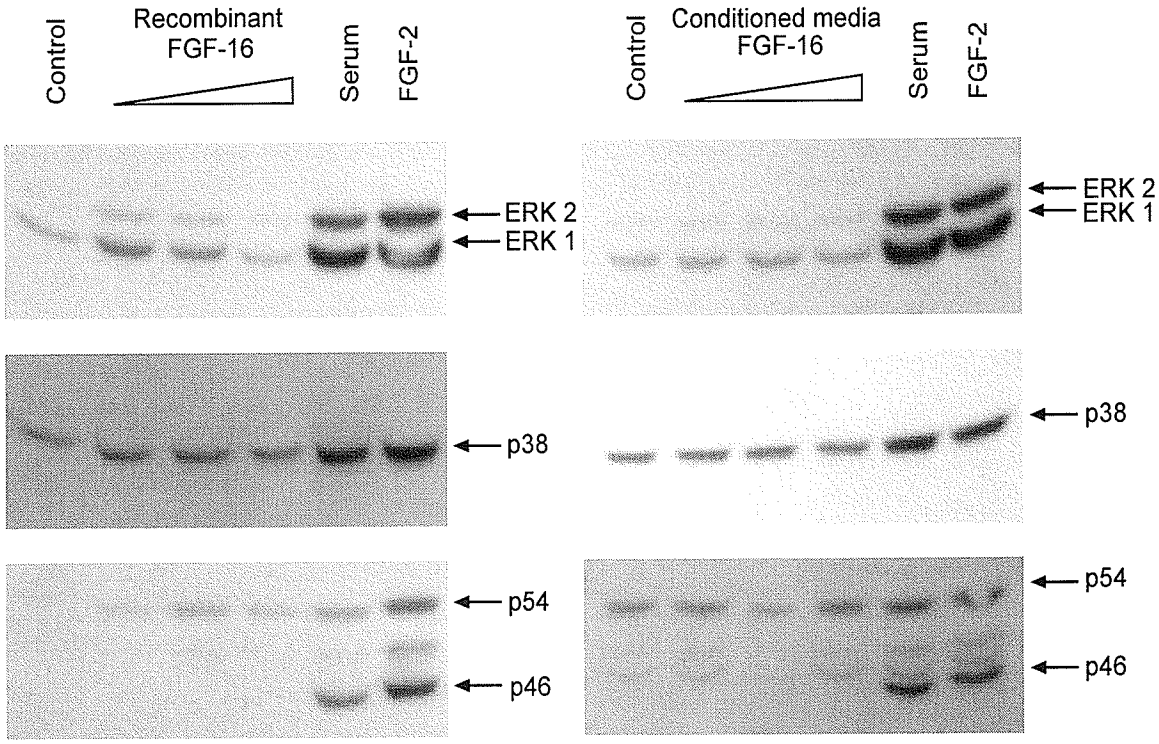

Control
Recombinant FGF-16
Serum
FGF-2
Control
Conditioned media FGF-16
Serum
FGF-2
ERK 2
ERK 1
ERK 2
ERK 1
p38
p38
p54
p46
p54
p46

Figure 5.10 Western blots representing phosphorylated MAPK in myocyte cultures after treatment with FGF-16. Blots were probed with an antibody against the phosphorylated form of ERK 1 and ERK 2 (top panels), p38 (middle panels) and p54 and p46 JNK (bottom panels). Panels on the left side represent cells treated with recombinant hFGF-16 protein (1, 10 or 100 ng/ml) while those on the right were treated with conditioned media from transfected HEK 293 cells expressing FGF-16 (0.1, 1, or 10% by volume). Control samples consisted of either vehicle or conditioned media from HEK 293 cells transfected with $pcDNA_3$ (empty vector). Cells were also stimulated with 10% serum or FGF-2 as positive controls. All samples were harvested after 10 minutes of stimulation.

Fig 5.11

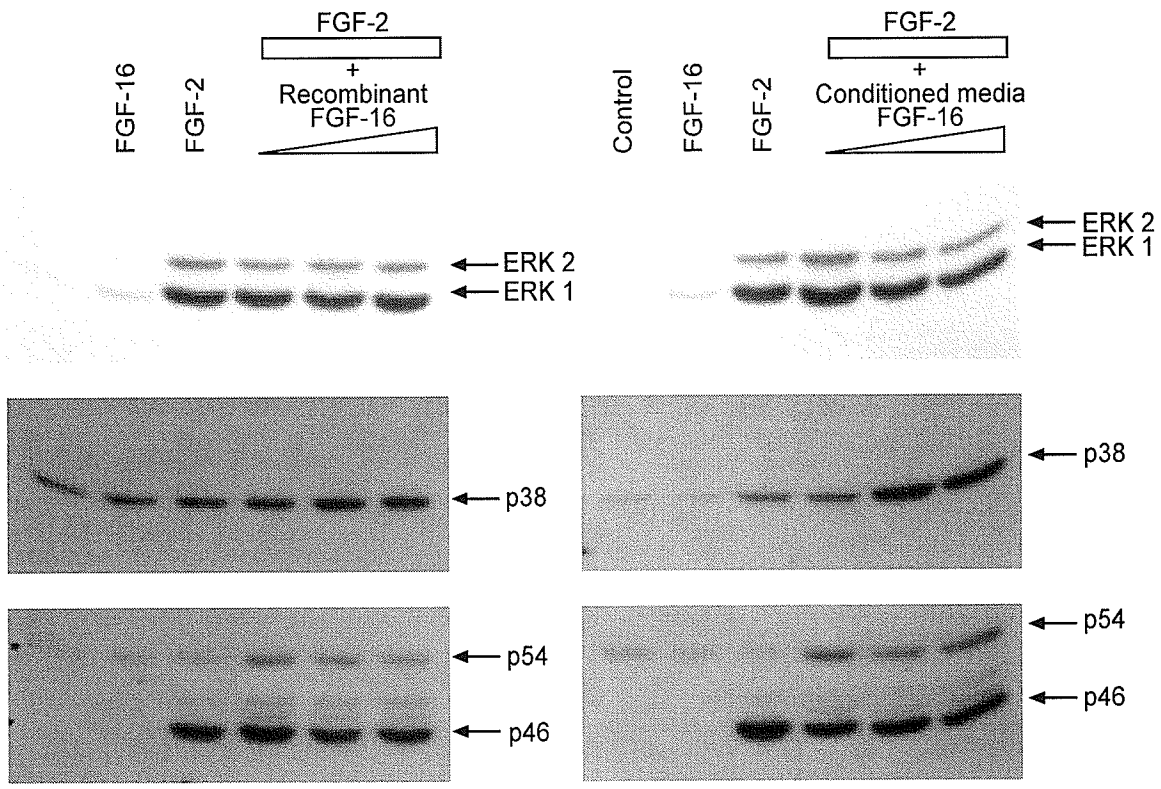

FGF-16
FGF-2
FGF-2
+
Recombinant
FGF-16
Control
FGF-16
FGF-2
FGF-2
+
Conditioned media
FGF-16
ERK 2
ERK 1
ERK 2
ERK 1
p38
p38
p54
p46
p54
p46

Figure 5.11 Western blots representing phosphorylated MAPK in myocyte cultures after treatment with a combination of FGF-16 and FGF-2. Blots were probed with an antibody against the phosphorylated form of ERK 1 and ERK 2 (top panels), p38 (middle panels) and p54 and p46 JNK (bottom panels). Panels on the left side represent cells treated with recombinant hFGF-16 protein (1, 10 or 100 ng/ml) while those on the right were treated with conditioned media from transfected HEK 293 cells expressing FGF-16 (0.1, 1, or 10% by volume). Control samples consisted of either vehicle or conditioned media from HEK 293 cells transfected with $pcDNA_3$ (empty vector). Increases were observed in most cases by the addition of FGF-2 alone. The addition of FGF-16 with FGF-2 further increased the phosphorylation of both p54 and p38 as opposed to FGF-2 alone. All samples were harvested after 10 minutes of stimulation.

Chapter 6

6.0 Discussion

6.1 FGF-16 mRNA detection and tissue distribution

The successful cloning and sequencing of FGF-16 from mouse heart revealed a sequence with 95% and 97% sequence identity when compared to human and rat FGF-16, respectively (Figure 3.1). Testing revealed that the use of FGF-16 cDNA as a probe for RNA blotting was an effective means for the detection of FGF-16 mRNA. Probe specificity was evaluated based on two criteria. First, the size of the transcript detected in the heart (1.8 kb) coincided with that previously reported for FGF-16 as opposed to closely related FGF-9 or FGF-20 which both have a similar but larger transcript size (2.4 kb) (Miyamoto et al., 1993, Miyake et al., 1998, Kirikoshi et al., 2000). Secondly, RNA from the kidney, which is known to have the highest levels of FGF-9 transcript of all adult tissues examined (Miyamoto et al., 1993), did not produce a signal when probed with the FGF-16 cDNA, indicating that the FGF-16 cDNA probe does not recognize FGF-9 transcript. In addition, no evidence of FGF-16 transcript was present in tissues examined other than the heart (Figure 4.1). Therefore the signal detected in adult mouse heart is consistent with that previously reported for rat (Miyake et al., 1998).

6.1.1 FGF-16 transcript levels during development

Miyake and colleagues depicted FGF-16 transcript levels as switching from being primarily restricted to brown adipose tissue in the embryonic rat to becoming

predominantly cardiac specific in the adult. Although transcript was detected FGF-16 in embryonic heart through the use of RT-PCR and/or in situ hybridization (Miyake et al., 1998 and Lavine et al., 2005), these methods of detection are more sensitive than RNA blotting and no direct comparison between the relative levels of cardiac FGF-16 transcript during development was reported. Using RNA blotting, a comparison between hearts of various age groups revealed that the levels of FGF-16 transcript increase dramatically after birth (Figure 4.3).

Although FGF-16 transcript has been reported in the embryonic heart, there are differences in the reported distribution for the transcript (Miyake et al., 1998, Lavine et al., 2005), for instance, Miyake et al., (1998) described that FGF-16 transcript signal was diffuse and coincided with cardiac myocytes. However, they did not specifically indicate which age they were referring to. This is different from the observation by Lavine et al., (2005), who reported regional differences of FGF-16 transcript within the heart, whereby mouse embryonic day 10.5 was positive for FGF-16 transcript in the endocardium and epicardium. While older embryonic day 12.5 hearts also revealed a positive signal in the endocardium, it was not explicit whether FGF-16 signal was no longer present in the epicardium or it had not been studied. One possibility that would be consistent with the findings of both groups is that the regions of the heart that produce FGF-16 transcript may change during embryonic development. Another possibility is that the distribution is different between the mouse and rat, although this argument is less compelling given the similarities between the species such as conserved sequence identity and the similar tissue expression patterns.

Careful examination of the methods for FGF-16 transcript detection revealed minor differences in the *in situ* hybridization techniques from both groups (Miyake et al., 1998, Lavine et al., 2005). However, both used FGF-16 cDNA to create an antisense RNA probe for detection. The only difference being, that one group used radiolabelled probe as opposed to immunofluorescence to identify regions of tissue containing the transcript. Although there is no clear way of testing the specificity of the probes used for FGF-9 versus FGF-16 there were indications that they did at least function differently. The spatial distribution revealed by each probe was different, suggesting different transcripts. In terms of verifying the identity of the transcript, there was no mention of the probes being tested with tissues known to contain FGF-9 or absent for FGF-16. Thus there are clear differences in terms of the quality and limitations between using *in situ* hybridization and RNA blotting as assays for determining transcript amount and distribution.

The overall levels of cardiac FGF-16 transcript were found to increase, however, we do not know at this time whether this is the result of a greater number of cells producing the transcript or increased production by a certain population of cells. Indications from the present study are that the increases are not due to asymmetrical expression, as both left and right ventricles appeared to have similar levels of FGF-16 transcript at any given developmental stage examined. If regional gradients exist, in terms of FGF transcription, they would probably be more related to that of a transmural difference as was described by Ornitz and colleagues (Lavine et al., 2005) for the early embryonic heart.

Future studies will necessitate a closer examination of specific regions of the embryonic and adult heart. In particular, a closer examination into the transmural expression levels of the FGF-16 transcript is warranted. This could be achieved by the dissection of the ventricular wall into different transmural zones which could be processed and assessed for FGF-16 transcript levels either through RNA blotting or quantitative PCR. Alternatively, an in situ method could be employed as was previously used by Ornitz and colleagues (Lavine et al., 2005).

6.2 Detection of FGF-16 protein.

6.2.1 Antibody specificity

Several antibodies were created and tested in order to gain a better understanding of the distribution and properties of FGF-16 protein. Evaluation of the sensitivity of the antibodies as well as their specificity revealed that they were capable of detecting low levels (<5 ng) of recombinant hFGF-16 protein via western blotting. Both of the custom made (Ab60 and Ab61) as well as one of the commercially available antibodies (AbPeprotech) demonstrated a higher degree of detection and selectivity for hFGF-16 as opposed to hFGF-9 when tested via protein blotting (Figure 3.4).

Although none of the FGF-16 antibodies tested were 100% selective for the detection of hFGF-16 over hFGF-9 (Figure 3.4), there were two observations which are strongly in favor of the signal from cardiac sources being primarily due to FGF-16 and not FGF-9. First, FGF-16 transcript demonstrates highest transcript levels in adult cardiac tissue as compared to other organs (as determined by RNA blotting, Figure 3.2 and

Figure 4.1), while the detection of FGF-9 transcript reveals much lower levels (Figure 4.2). Secondly, the signal for FGF-16 protein (as detected with the antibodies), coincides with the increases in FGF-16 transcript during development.

6.2.2 FGF-16 production and subcellular distribution in cardiac cells

Cultures of neonatal cardiac myocytes and non myocytes were examined for FGF-16 protein using the FGF-16 antibodies. Immunoblotting detected a signal in conditioned media from cardiac myocytes after enrichment via heparin affinity (Figure 4.11). The signal detected possesses many of the characteristics of that observed with FGF-16 produced by transfected HEK 293 cells. These include secretion and glycosylation, as well as having comparable sizes in both glycosylated and deglycosylated states (Figure 3.7 and 4.12). Examination of conditioned media from non myocytes however, did not result in the detection of an FGF-16 signal (Figure 4.11). These results correlate precisely with RNA data, together demonstrating that FGF-16 is produced and secreted from cardiac myocytes isolated from postnatal rat hearts.

Immunofluorescence microscopy was also used to determine if FGF-16 could be detected in neonatal rat cardiac myocytes or cryosections from adult mouse heart. However, in both cases only a faint and diffuse signal slightly above that of background was observed with only minor staining of the vasculature (Figure 4.7 and 4.8). Although it is not clear at this time whether the signal observed in the vasculature originates from the vasculature or is a result of that secreted from myocytes.. Given the evidence obtained from transfected HEK 293 cells and neonatal cardiac myocytes suggesting that the protein is secreted, it is possible that FGF-16 is not retained by the cells and that the vast

majority of FGF-16 protein synthesized is secreted. Further evaluation of the antibodies for immunofluorescence microscopy revealed that both Ab60 and Ab61 were capable of detecting the fusion protein EGFP-FGF-16 as well as wild type FGF-16 expressed by transfected myocytes. The punctate pattern detected with cells over expressing the wild type FGF-16 was similar to that recently reported for a poly histidine tagged version of the FGF-16 protein (Miyakawa et al., 2003) as well as closely related FGF-9 (Revest et al., 2000) expressed in a non cardiac cell line. However, cells expressing the EGFP-FGF-16 fusion protein demonstrated a nuclear and cytoplasmic expression pattern. Thus, the EGFP tag appeared to have adverse effects on the normal cellular trafficking of FGF-16 and most notably the secretion of the protein. Furthermore, blotting revealed that the FGF-16 signal detected from the media of HEK 293 cells transfected with $pcDNA_3$-FGF-16 were approximately four fold more intense than that from cells expressing EGFP-FGF-16 (Figure 3.6).

6.2.3 Detection of FGF-16 in the intact mouse heart

Initial attempts to detect endogenous FGF-16 protein from total protein lysates of adult heart were unsuccessful. This was not surprising given that the maximum amount of protein loaded on SDS gel was approximately 40 μg per lane and the minimum amount of FGF-16 required for detection was in the range of nanogram quantities. This meant that FGF-16 would have to make up at least 0.01% of the total protein loaded. For comparison, the amount of FGF-2 reportedly extracted from various tissues represents only 0.00006% of total protein by weight (Gospodarowicz 1987). Thus, the relative abundance of FGFs is low compared to that of total protein. Heparin sepharose was

therefore used to enrich for FGF-16 from larger amounts of tissue so that it could be detected via protein blotting.

Samples enriched for heparin affinity from both adult mouse and pig heart resulted in the detection of several bands when examined by western blotting. Two bands corresponding to sizes of 26.5 and 19.5 stood out as they were similar to that of the glycosylated (26.5 and 25.4 kDa for myocytes and transfected HEK 293 cells, respectively) and non glycosylated (24.1 and 21.1 kDa for myocytes and transfected HEK 293 cells, respectively) forms of FGF-16 (Figure 3.7 and 4.4). Given the similar sizes these signals are believed to be FGF-16 in both forms. Additionally there is also the possibility that the smaller (19.5 kDa) signal is the result of proteolytic cleavage of either form of the protein. Further studies are therefore needed to confirm the identity of the signals as well as properties such as glycosylation.

Attempts to sequence proteins obtained from heparin extraction via mass spectrophotometry have thus far been unsuccessful. This may be due to the amount of FGF-16 relative to other heparin extracted proteins is very low. This possibility is further supported by a failure of FGF-16 in addition to other cardiac FGFs.(see Figure 1.1 for complete list) Thus, the abundance of FGF molecules within the heparin extracts are below the threshold for detection under the conditions used. Future detection of FGF-16 will therefore require more stringent extraction procedures. There have been several attempts at creating affinity columns using cyanogen bromide or Affigel (Biorad) as a matrix. However, loss of antibody function and shedding of the antibody from the column have prevented successful isolation of even purified recombinant protein. Future investigation will therefore require other possible avenues for isolation of the protein.

These may include the use of alternate substrates for antibody fixation or the use of an ion exchange column for further enrichment of FGF-16. The use of a two dimensional gel may also serve to further enrich and isolate the protein in question.

6.3 FGF-16 after secretion

Other members of the FGF family (such as FGF-2) are reportedly secreted by myocytes and stored in the extracellular matrix (ECM) by binding to heparan sulphate proteoglycans (Folkman et al., 1988). While FGF-16 is also clearly secreted from cardiac myocytes (Figure 4.11), evidence to support it being stored in the ECM is not compelling. Adult heart samples enriched for heparin binding proteins showed indications of FGF-16 when examined by protein blotting (Figure 4.5). However, the amounts of tissue required for the extraction were quite large, suggesting a low abundance of protein or poor extraction efficiency. In addition, examination of adult mouse heart cryosections did not reveal FGF-16 being associated with the ECM. This observation was based on our previous experience with FGF-2, whereby the localization pattern coincides with staining for collagen IV and areas surrounding the myocytes (Sheikh et al., 2001). Furthermore, detection of endogenous protein in the cryosections of adult mouse heart (Figure 4.7 and 4.8) and cultures of neonatal rat cardiac myocytes (Figure 4.11 and 4.12) is slight at best. Thus, while FGF-16 is efficiently secreted it does not appear to be associated with the ECM, and detection of the protein may be limited to whatever protein is retained, reabsorbed and/or synthesized by the cells themselves. Of course, this does not preclude the possibility that the epitopes recognized by the FGF-16 antibody are somehow masked while bound to extracellular matrix. It is conceivable that

FGF-16 is not retained by the heart after secretion, as not all ECM components have a strong interaction with all FGFs. Selectivity and specificity for particular FGFs and ECM has previously been reported (Allen et al., 2001). For example, while exogenous FGF-2 can bind to the ECM of various tissue sections in a heparan sulphate dependent manner, FGF-4 does not bind to the ECM of tissues such as heart and blood vessels (Allen et al., 2001). Attempts to detect FGF-16 within the blood by protein blotting were unsuccessful (data not shown). However, future attempts will therefore necessitate the development of new methods. First, although the antibodies tested (Ab60, Ab61 and AbPeprotech) demonstrated a degree of selectivity for FGF-16 over FGF-9, they are not completely specific and therefore unable to discern between the two proteins. Secondly, if we were to use the levels of circulating FGF-2 in the plasma as an indication of what to expect for FGF-16, the basal levels of FGF-2 are less than 10 ng/l, making enrichment through heparin affinity difficult. This would require enrichment of at least a half a liter of plasma in order to extract an amount of FGF-16 that could be detected by protein blotting with the antibodies currently available. Thirdly, the more sensitive assays (enzyme linked immunosorbent assay) used for the detection of FGF-2 require that the antibodies used be absolutely specific for the protein in question, relating back to the concerns of antibody specificity already mentioned.

6.4 Biological activity of glycosylated FGF-16

The protein detected from the media conditioned by transfected HEK 293 cells was predominantly of the glycosylated form (Figure 3.7). This was contrary to that of the recombinant hFGF-16 obtained from a commercial source. Therefore it was of interest to

determine if both forms were biologically active. This issue was addressed through the use of a BAF-3 cell (expressing FGFR-2c) survival assay, which confirmed biological activity for FGF-16 from both sources (Figure 5.3). Although the concentration of FGF-16 within the conditioned media was not accurately measured, an estimation was performed by comparing the signal on protein blots of heparin extractions from conditioned media with that of media supplemented with known concentrations of recombinant protein. Interestingly, the given survival rate in the BAF-3 assay corresponded well between the known concentrations of recombinant hFGF-16 and the estimated concentrations of glycosylated FGF-16 (Figure 5.3). This result is similar to that previously reported for other FGFs, where glycosylation does not appear to influence the biological activity of the molecules (Bates et al., 1991, Bellosta et al., 1993, Clements et al.,1993).

6.4.1 FGF-16 affects cell survival

The addition of FGF-16 in either the recombinant or conditioned media form resulted in increased BAF-3 cell survival. As mentioned, using cell proliferation as a measurement of biological activity was not feasible given that BAF-3 cells died in the absence of IL-3. This finding was unexpected, as others (Allen et al., 2001) have demonstrated that BAF-3 cells are able to overcome IL-3 dependence when appropriate FGFR and ligand are supplied. Possible reasons for the differences may have been due to something as subtle as washing the cells to remove traces of IL-3 prior to stimulation (although this is speculative). Another more plausible explanation is that the survival/cell division is a response that depends on the potency of FGFR activation. Rapraeger and colleagues (Allen et al., 2001) were able to demonstrate increased cell density (as

measured relative to optical density) when cells were stimulated with FGF-4 or FGF-2. However, a comparison between relative activities of the different FGFs for FGFR-2c reveals that activation by FGF-16 is only 30% that of FGF-1 (Lavine et al., 2005) while both FGF-2 and FGF-4 have higher relative activity (64% and 94%, respectively) (Ornitz et al., 1996). Therefore, the activity of FGF-16 may not be sufficient to allow complete independence of BAF-3 cells from IL-3. Alternatively, the biological activity of FGF-16 may be limited. If so, frequent replacement of media with fresh FGF-16 may allow for prolonged cell survival and eventually even cell division. Future study is therefore required to determine if this is the case.

There is also the possibility that activation of the receptor at the cell surface alone is not the only mechanism by which these molecules exert their signaling. For instance, there are several reports of FGF/FGFR complex being internalized after receptor activation (Marchese et al., 1998, Roghani and Moscatelli 1992, Sorokin et al., 1994, Fannon and Nugent 1996). Although internalization is not believed to be a requirement for inducing DNA synthesis (Fannon and Nugent 1996) nuclear localization sequences within the associated FGF play a role in intracellular trafficking (Immamura et al., 1990, 1994, Friedman et al., 1994, Wiedlocha et al., 1994). Therefore, it is conceivable that although two different FGFs can activate the same receptor, the internalization and intracellular trafficking may differ thus resulting in different responses.

Stimulation with FGF-16 did result in a significant reduction in the number of dead BAF-3 cells when examined 17 hours after removal of IL-3. This prompted an investigation as to whether the effects of FGF-16 were similar toward cardiac myocytes. In order to induce apoptosis in cardiac myocytes, a model of hypoxic injury was chosen.

However, addition of FGF-16 was unable to prevent or reduce the number of apoptotic cells (using nuclear condensation as an indicator of apoptosis) following hypoxic injury (Figure 5.7 and 5.8). Although both situations (removal of IL-3 for BAF-3 cells or H_2O_2 induced injury for myocytes) induce apoptosis, they are none the less different models. It is possible that FGF-16 may serve more as a trophic factor for myocyte survival rather than protection. In addition, the extent of the injury caused by H_2O_2 may have been beyond the abilities of FGF-16 to influence cell survival. Future studies to determine whether FGF-16 possesses cytoprotective properties will therefore require a range of doses of H_2O_2 in addition to other forms or models of injury.

6.5 MAPK activation by FGF-16

One of the prominent cellular signaling pathways induced by other FGF's is that of the MAPK pathway (Klint et al., 1999, Powers et al., 2001). In order to determine some of the biological/cellular processes or targets of FGF-16, responses in the form of MAPK activation were investigated in both neonatal rat cardiac myocytes and non myocytes. These results are summarized in Tables 5.1-3. Non myocyte cultures responded well to stimulation by either FGF-2 or serum by activation of various MAPKs (Figure 5.9). With the exception of ERK1/2 however, responses observed for cells from the group treated with the recombinant hFGF-16 protein appeared to be stronger than those treated with conditioned media. As mentioned previously, the stimulation of cells with conditioned media was carried out such that the overall serum concentration for each treatment increased from 0.5 to 1% serum by volume with the addition of conditioned media. Cells in that particular data set were starved with 0.5% serum to

minimize activation by the additional serum in the conditioned media. However, by doing so, this may have resulted in a decreased responsiveness overall. This observation must be taken into consideration pertaining to myocyte stimulation as well. Regardless of these minor differences in the degree of activation with positive controls, FGF-16 did not appear to result in MAPK activation in the non myocytes under any of the conditions used. Although non myocytes contained FGFR-2c it is possible that either the expression level for the protein is not high enough to elicit a response that can be measured by the assay used or that the receptor may be involved in pathways other than MAPK. Alternatively, there is also the possibility that the serum or conditioned medium itself contains inhibitors or antagonists which prevent the actions of FGF-16 on the cells in question.

Rat neonatal cardiac myocytes responded to the addition of either FGF-2 or serum with increases in ERK1/2 and p38 MAPK activation (Figure 5.10, 5.11 and Tables 5.2 and 5.3). Activation was also observed by the addition of various concentrations of recombinant hFGF-16 protein. Comparable amounts of FGF-16 (~0.1, 1.0 and 10ng/ml) in the form of conditioned media however, did not evoke ERK1/2 and p38 activation. Although one possible reason for the difference in the response may have been the glycosylation of FGF-16, this in unlikely in light of the decreased responsiveness of the positive controls mentioned above. Furthermore, similar responses were observed between the two forms of FGF-16 in the BAF-3 cell survival assay (Figure 5.3).

The effects of FGF-16 on p46 and p54 JNK in myocytes were distinct from ERK1/2 or p38. Cells from either group (recombinant or conditioned media containing FGF-16) did not respond with p46 JNK activation; however, moderate changes in p54

JNK activation were observed in those cells stimulated with recombinant hFGF-16 (Figure 5.10). In fact, although the response was variable, FGF-16 appeared to surpass FGF-2 in its activation of p54 JNK (Table 5.2). Thus, it would appear that FGF-16 is somewhat less effective than FGF-2 at stimulating ERK1/2, but apparently more effective at stimulating p54 JNK. This is interesting, as ERK1/2 is implicated in cell survival following various forms of cardiac injury, such as ischemia-reperfusion, oxydative stress and hypoxia (Zhu et al., 1999). Furthermore, the protective effects of FGF-2 on cultured cardiac myocytes treated with H_2O_2 require the activation of ERK1/2 (Iwai-Kanai et al., 2002).

When the effects of FGF-16 and FGF-2 were examined in combination, different patterns of MAPK activation from the addition of FGF-2 alone began to emerge (Figure 5.11 and Table 5.3). Treatment with both growth factors resulted in a stronger response in terms of p54 and p46 JNK activation in comparison with that of FGF-2 alone. This is interesting as JNK activation has been demonstrated not to be involved in the suppression of apoptosis in the BAF-3 cells but rather cell proliferation (Smith et al., 1997). However, the previous study did not specifically target p54 but both p46 and p54 JNKs. Evidence to the contrary using deletion studies has revealed that p54 JNK is involved in the prevention of apoptosis in the developing brain (Sabapathy et al., 1999, Kuan et al., 1999). This more recent observation may provide a key as to the mechanism by which FGF-16 was able to reduce the mortality in BAF-3 cells. However, as mentioned in section 5.1, BAF-3 cells eventually underwent apoptosis regardless of FGF-16 after prolonged periods. Perhaps the actions of FGF-16, which delayed the apoptosis of BAF-3 cells, were due to p54 JNK activation. In addition, there is also the possiblity that the half

life for FGF-16 activity is too short to allow for a sustained response. This would also explain the reason for the differences between activation of FGFR-2c in BAF-3 cells by FGF-2 (Allen et al 2001) as opposed to FGF-16. In order to investigate this possibility, repeated doses of FGF-16 could be used to investigate whether BAF-3 survival could be prolonged further.

Although activation of ERK1/2 was very prominent with the addition of FGF-2, no further activation was observed by the addition of FGF-16. One cannot discount the possibility that the activation was already maximal and that lower concentrations of FGF-2 may reveal a shift in the activation upon the addition of FGF-16. To determine whether this is the case, would require that the experiment be carried out with various concentrations of FGF-2 to establish a dose response curve.

MAPK are involved in many cellular processes and have a tremendous potential for cross talk between pathways. This complexity enables the same MAPK to be involved in effects that can be either acute or chronic. In the current study, MAPK activation was assessed 10 minutes after stimulation. The results from the positive controls indicate that this time period was sufficient to evoke MAPK activation, at least for FGF-2 and serum. However, one cannot discount the possibility that the time course of MAPK activation may be different for FGF-16. The activation may have peaked within minutes of stimulation and quickly returned to basal levels before the 10 minute time point. Alternatively, activation may take place some time after the 10 minutes, or perhaps even a combination of both making the response biphasic. Further study into the activation profile or time course of MAPK following FGF-16 stimulation is therefore required to

answer these questions and to lead to a better understanding of FGF-16 signaling and function.

6.6 Differences between FGF-2 and FGF-16 MAPK activation

Positive controls consisting of either serum or FGF-2 resulted in a very strong and prominent activation of ERK1/2 MAPK in particular, while the effects of FGF-16 were much weaker. Reasons for the differences in activation are most likely due to differences in the types of FGFR each FGF activates and/or its affinity for them. FGF-2 is very promiscuous in terms of receptor activation (Ornitz et al., 1996), while examination of the receptors reported to respond to FGF-16 reveals a somewhat more limited or restricted choice of receptors which can be activated. Ornitz and colleagues (Lavine et al., 2005) reported that FGF-16 is capable of activating both FGFR-2c and FGFR-3c variants but has a much lesser effect on FGFR-3b, FGFR-1c and FGFR-4 with little to no activation of FGFR-1b and FGFR-2b. However, FGF-2 is reported to activate all of the receptors mentioned (Ornitz et al., 1996). In terms of which receptors are expressed in the adult heart, there has not been a complete analysis. The present study however, suggests the presence of FGFR-1, FGFR-2 and FGFR-3 receptors through immunofluroescence (Figure 5.4). FGFR-1, which has also been previously reported in myocytes (Jin et al., 1994, Kardami et al., 1995, Liu et al., 1995), is a prime candidate for the differences observed between FGF-2 versus FGF-16 as neither b or c isoforms are strongly activated by FGF-16 (Lavine et al., 2005). Although both FGF-2 and FGF-16 activate FGFR-2c, which was identified as being present in neonatal cardiac myocytes (Figure 5.6), this may not have been the receptor used by FGF-2 to activate the MAPK pathways observed.

Differences between the pathways activated and the mitogenicity between FGFR have also been reported. For instance, activation of the FGFR-4 receptor expressed in BAF-3 cells by FGF-1 or FGF-2 has limited signaling capacity that does not activate ERK (Wang et al., 1994).

Another possible explanation for the discrepancies in MAPK activation involves the role of HSPGs. As mentioned in section 1.2, not only is there a requirement for heparin or heparan sulfate for the activation of certain receptors, but sometimes a particular type of heparan sulfate is required to get receptor activation (Allen et al., 2001). Given that cell culture systems are only an approximation of what happens *in vivo*, they may not contain the proper heparan sulfate for FGF and receptor interaction. Therefore, in order to increase the chances of receptor activation, heparin was added to each of the treatments (Ornitz et al., 1996, Allen et al., 2001). While this may alleviate the need for the proper heparan sulfate, it does not compensate for other limitations in the cell culture system. Other possible reasons for not seeing full or even partial FGF-16 receptor interaction may be due to the fact that the cells are grown as a monolayer. For example, it has been reported that while myocytes grown in cardiac tissue explants responded to FGF-2 by increasing DNA synthesis, those myocytes isolated and plated as a monolayer did not respond (Armstrong et al., 2000). The authors' explanation for the discrepancy was the need of a three-dimensional matrix or the need for other cell attachment or adhesion proteins in order to observe proper FGF signaling. While one cannot discount the possibility that the interactions are not necessarily a direct result of the growth factor but may be due to paracrine effects from other cell types, this does reveal some of the limitations of cell culture assays. The fact that cultured myocytes do

not take on the rod-like morphology of cells *in vivo* is a prominent reminder that the organization within the cells (and quite possibly receptor distribution on the cell membrane) may not be the same.

An analysis of adult mouse heart, neonatal cardiac myocytes and non myocytes revealed the presence of FGF-2c RNA transcript (Figure 5.6), which is a receptor known to be activated by FGF-16 (Lavine et al., 2005). It was also determined that FGF-16 is secreted from neonatal cardiac myocytes (Figure 4.11) and the addition of FGF-16 in either form is capable of activating certain MAPK pathways in these same cells (Figure 5.10). Furthermore, the addition of FGF-16 resulted in moderate activation of ERK2, p38, and p54 JNK in certain instances. In addition, activation of p38 and p46 and p54 were further increased by the combination of FGF-16 and FGF-2 as opposed to FGF-2 alone. It is important to note however that although effects are reported as activation of particular MAPKs, one cannot discount the possibility that the activation was perhaps indirect. For instance, if FGF-16 affects the actions of one of the phosphatases associated with dephosphorylation of a MAPK(s) this may upset the balance between kinases and associated MAPK. For example, potential candidates include the dual specificity MAPK phosphatases, in particular those belonging to subgroups III and IV as recently reviewed by Farooq and Zou (2004), which preferentially act on p38 and JNK MAPK. Further studies are therefore required to clarify the exact mechanism by which these MAPK(s) are regulated by FGF-16.

The activation of p38 has been associated with forms of cardiac injury such as ischemia reperfusion and increased hemodynamic load (Bogoyevitch et al., 1996, Cook et al., 1999, Fischer et al., 2001, Liao et al., 2001, Lochner et al., 2003). However,

inhibition of p38 has been found to increase cell survival and decrease remodeling following injury. Given this evidence one would think that activation of p38 by FGF would be disadvantageous, however other FGF's also known to activate p38 have been proven cardioprotective by means of other pathways (Jiang et al., 2002, Palmen et al., 2004). As for the effects of p46/p54 JNK activation by FGF-16 in terms of cardiac injury, there is still controversy in the literature. While the inhibition of p46 JNK has been linked to increased apoptosis (Dougherty et al., 2002), others report the contrary (Hreniuk et al., 2001). Reasons for these discrepancies may be the methods used to inhibit p46 JNK (antisense oligonucleotides versus dominant negative adenovirus), which translate to an absence of p46 JNK as opposed to competition with a non-functional p46 JNK with substrates. With a decrease in p46, as would be expected through the addition of antisense oligonucleotides for p46, this frees up molecules that would otherwise interact with p46 JNK. On the contrary, with the second scenario, there exists the possibility that certain molecules become otherwise preoccupied with the dominant negative molecule that would otherwise be free to interact in the first scenario. Further investigation is required in order to clarify the role of p46 JNK. As there is some indication that p46 JNK may be involved in the prevention of cardiac injury it is only logical that FGF-16 may somehow also be implicated. Future studies as to the effects of FGF-16 on recovery following cardiac injury would therefore be of interest.

Current data seems to indicate that alterations in p54 JNK activation do not affect myocyte survival the same way p46 JNK does (Hreniuk et al., 2002). However, recent data have made a link between p54 JNK and the inhibition of cell proliferation (Sabapathy and Wagner 2004). This observation coincides perfectly with what is known

about myocyte proliferation and the association with increased FGF-16 expression following birth as was demonstrated in the current study. At birth the mitotic rate of myocytes is reported to decrease dramatically (Soonpaa et al., 1996). However, as was found in the current study, the transcript and protein for FGF-16 increase in the postnatal heart (Figure 4.3 and Figure 4.4). This raises the possibility that FGF-16 may actually inhibit cellular proliferation. However, Ornitz and colleagues (Lavine et al., 2005) demonstrated that FGF-16 increased DNA synthesis (BrdU incorporation) in explants from embryonic hearts suggesting that it plays a proliferative role. Of course, DNA synthesis is not only associated with mitosis but also binucleation, which is another factor associated with myocyte differentiation in the postnatal heart (Soonpaa et al., 1996). Other growth factors, FGF-2 in particular, have also been associated with DNA synthesis and stimulating cell proliferation (Kardami, 1990, Hortala et al., 2005) as well as binucleation of cardiac myocytes (Pasumarthi et al., 1996). Furthermore ERK1/2 is well known to be associated with cell proliferation. However, findings of the current study revealed relatively little effect on ERK1/2 activation with FGF-16 in comparison with that of FGF-2 (Figure 5.10). Therefore, it is not clear at this time whether the changes that occur in the postnatal heart such as binucleation and decreased cell division are linked to the increases in FGF-16 expression. Future studies regarding the potential role of FGF-16 in these processes is therefore required.

6.7 Summary

At the onset of this study very little was known about FGF-16. Development of tools for the detection, production and purification of FGF-16 RNA and protein were therefore needed in order to determine some of the fundamental characteristics of this molecule. Several advances in the characterization of FGF-16 were made and have allowed basic hypotheses to be generated regarding the function of this molecule. These advances are listed as follows:

1) Cloning of the sequence for mouse FGF-16 revealed that it has 95 and 97% shared sequence identity between that of the human and rat FGF-16 cDNA, respectively. The similarities between amino acid identity are even closer between mouse as compared to human and rat with 99% identity between each.
2) The cDNA was determined to be a specific probe for FGF-16 in the detection of FGF-16 as opposed to that of closely related FGF-9. Transcript levels for FGF-16 are highest in the heart as compared to other tissues examined in the adult mouse.
3) Transcript levels for FGF-16 rapidly increase at the time of birth and plateau within 4 days post partum.
4) There does not appear to be any difference in the relative level of FGF-16 transcript between that of the left and right ventricle.

5) Antibodies generated against the protein (custom and some commercial) are capable of detecting recombinant as well as endogenous cardiac FGF-16. However, of the antibodies capable of FGF-16 detection by protein blotting, none of them have 100% selectivity toward FGF-16 over FGF-9.

6) The levels of FGF-16 protein increase with age and are highest in the adult heart as compared to prenatal and newborn mouse hearts. FGF-16 transcript and protein is produced and expressed in cultured neonatal rat cardiac myocytes and not non myocytes.

7) Endogenous FGF-16 expressed by neonatal rat cardiac myocytes is efficiently secreted from cells as a glycosylated protein.

8) The secreted as well as recombinant forms of the FGF-16 are both biologically active as determined by increased cell survival in BAF-3 cells expressing the FGFR-2c splice variant.

9) Myocyte cell survival is no different with the addition of FGF-16 following hypoxic injury (as measured by nuclear condensation following administration of 200 μM H_2O_2).

10) Recombinant hFGF-16 activates ERK1/2, p38 and p54 JNK MAPKs in rat neonatal cardiac myocytes.

11) Combinations of FGF-16 with FGF-2 resulted in further stimulation of p38 and p54 JNK MAPKs as compared to FGF-2 alone.

Chapter 7

7.0 Future directions

The basic characterization of FGF-16 has revealed many interesting findings. However, with new information brings new questions. For instance, FGF-16 was could evoke response in BAF3 cells expressing FGFR-2c. Despite the presence of FGFR-2c in non myocytes, FGF-16 was only found to activate certain MAPK within myocytes. FGF-16 however, did not increase cell survival but result in the activation of certain MAPK in neonatal rat myocytes which also contained transcript for FGFR-2c. This begs the question of whether the actions of FGF-16 on myocytes are in fact due to an interaction with FGFR-2c or whether other receptors such as FGFR-3 or FGFR-4 are involved.Therefore further study is required to not only identify which receptors and isoforms are present in the heart but their relative level of expression between cell types. Perhaps the best method of addressing these questions would be through the use of real time PCR so as to identify not only identify the receptor isoforms present but their relative abundance. The immunofluorescece signal for other receptors, for instance FGFR-3 was also detected in vessels suggesting a possible interaction with smooth muscle and/or endothelial cells through FGFR-3c.

Other models of injury also need be examined to determine whether FGF-16 may protect against other aspects of ischemic injury, for example the generation of primarily hydroxyl free radicals by the addition of ferrous iron (Josephson et al., 1991). In addition, ischemic injury is primarily a condition associated with adult myocytes. Therefore, additional work examining for protective effects of FGF-16 on adult myocytes may prove

to be more rewarding. As adult cardiac myocytes may exhibit differences between the receptor isoforms as well as their relative abundance or ratios. Therefore another important avenue requiring further study is determining thedifferent FGF receptor isoforms. Perhaps through the use of real time PCR to amplify specific regions of the receptors.

The activation of various MAPKs were examined following 10 minutes of stimulation. As previously discussed (section 6.5), this time point was chosen based on the response of ERK1/2 toward FGF-2. Further studies are therefore needed to determine the time course required for optimal activation of various MAPKs by stimulation with FGF-16 as well as combinations with other FGF's present in the heart. In addition to MAPK, other prominent pathways such as protein kinase C and phospholipase C are known to be activated by FGFs and require examination for responses toward FGF-16.

Examination of FGF-16 transcript and protein levels revealed dramatic increases after birth, but there was no indication of differences between levels in the left and right ventricles. However, recent data from *in situ* hybridization of embryonic tissue suggests regional differences in expression such as longitudinal as well as transmural gradients in the embryonic heart (Lavine et al., 2005). Future studies will necessitate a closer examination of whether such gradients also exist in the postnatal and adult heart.

Currently, no information is available regarding the regulation of FGF-16 protein expression other than its association with the transition from embryonic to postnatal heart. Further study is required to determine if the level of FGF-16 transcript and protein remains constant throughout life. Of particular interest are the level of FGF-16 and its association with events prior to and after cardiac failure. The heart is known to undergo a

process of hypertrophy in at least three different conditions. The first and foremost is that which occurs after birth and coincides with the timing of increased FGF-16 transcript levels. The second scenario which leads to hypertrophy is that which results from exercise and is believed to be beneficial for cardiac function. However, the third condition which leads to hypertrophy is that caused by the maladaptive response to cardiac injury which can ultimately lead to cardiac failure. Given the close association between cardiac hypertrophy at birth and the increased expression of FGF-16, it is of interest to determine whether FGF-16 is involved in regulating cardiac hypertrophy. In addition, other stimuli which result in hypertrophy such as exercise and injury need to be addressed.

Of tremendous importance would be the generation of transgenic animals for testing the hypothesis that FGF-16 regulates hypertrophy. The study of transgenic animals that either lack (knockout) or overexpress FGF-16 may reveal a phenotype which is indicative of FGF-16 function. However, of more interest would be the conditional expression or knockout of FGF-16 prior to or after subjecting animals to stimuli that induce cardiac hypertrophy such as pressure overload, exercise etc. This could be accomplished through the use of systems such as a tetracyclin inducible and/or tissue and a developmental specific promoter to overexpress FGF-16. Similarly, a conditional knockout model could be designed using a combination of Cre recombinase and loxP sites at either end of the FGF-16 gene. By either overexpressing or removing FGF-16 at specific times such as before or after cardiac injury this would allow one to assess the effects of FGF-16 on cardiac function/recovery.

However, in addition to hypertrophy, other processes occur shortly after birth that also coincide with increases in FGF-16 transcript and protein. For example, there is a marked increase in myocyte binucleation after birth (Soonpaa et al., 1996) which may be related to FGF-16 expression. This suggests that FGF-16 may play a role in the differentiation of myocytes into an adult cardiac phenotype, in which case cells enter a state of senescence and stop dividing. However, the addition of FGF-16 has been associated with increased DNA synthesis in embryonic heart explants (Lavine et al 2005). Thus, the DNA synthesis may not have been indicative of cell division but rather binucleation. Therefore, a closer examination FGF-16 and its effects on DNA synthesis and binucleation are required. Either stimulation of primary cardiac cell cultures with FGF-16 may be used to address this question or *in vivo* studies making use of the transgenic models previously mentioned.

Thus, at this time it is not clear whether FGF-16 promotes or prevents cardiac repair following injury. Therefore, there is little doubt that the experiments mentioned are only the initial steps toward a better understanding FGF-16 and its role in cardiovascular development and disease.

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