EXPRESSION AND RELEASE OF HIGH MOLECULAR WEIGHT FIBROBLAST GROWTH FACTOR-2 BY CARDIAC MYOFIBROBLASTS

BY

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A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Physiology

Faculty of Medicine

University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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MASTER OF SCIENCE

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For

Dina Eufemia

Daniello-Santiago

Acknowledgements

It is my pleasure to express my deepest gratitude to my supervisor and teacher, Dr. Elissavet Kardami for her true mentorship and unwavering support throughout my degree. I want to thank you, Vetta, for your guidance, heartfelt commitment, and profound belief in my abilities. Your passion for science is evident and will always be a lifelong influence on my research. You have always motivated me to think critically specially in my own work; and encouraged me to participate, collaborate, network, and embrace University life. I thank you enormously for building a solid foundation of my future career.

I would also like to thank my advisory committee: Dr. Peter Zahradka, Dr. Nasrin Mesaeli, and Dr. Thomas Netticadan. Your unfailing commitment, encouragement, and never ending questions or inquiries have truly propelled not only my knowledge but also my research. Thank you for keeping your lab doors open for me and for the numerous reference letters you have provided. I would also like to give my appreciation to Dr. Ian Dixon, whom I collaborated during the characterization studies of myofibroblasts. I am grateful for all your help and I am looking forward to having you as part of my advisory committee during my future studies. In addition, I would also like to extend my acknowledgement to Dr. Janice Dodd for her kindness, support, and continued interest in my training. Thank you for keeping your office door open to me for advice and for that, I am really grateful.

This master thesis would not have been possible without the help from the members of the Kardami Lab and the team-oriented environment that the lab

practices each and every day. I am especially grateful to my laboratory colleagues, who have actually become my dear friends. Special thanks to Robert Fandrich (Frank), Barb Nickel (Mom), and Xitong Dang (Z) for their outstanding technical expertise and wisdom that I'm sure every lab would love to have around. Also, I would not have been able to achieve so much without the constant encouragements, sincere friendship, and great energy from Sarah Jimenez (Genius), Madhu Jeyaraman (Enya), Xin Ma (Machine), and Wattamon Srisakuldee (Drama Queen). You guys made the lab environment very enjoyable and interesting. I am very lucky to be part of the Kardami lab.

I am also grateful to St. Boniface General Hospital Research Centre, CIHR, and the University of Manitoba and all its affiliates for the awards, stipend, and financial assistance to conduct the research.

Finally, I would particularly like to thank my parents (Mommy Lucy and Daddy Jorge), the many members of my immediate family, and my wife, Dina. Thank you guys for your unconditional love, patience, and understanding throughout my studies. You guys have always given me the support I needed through the most difficult times. Thank you LORD for blessing me with such wonderful people and for always reminding me what is truly important in this world.

Abstract

Maladaptive cardiac hypertrophy, following a compensatory trophic response to myocardial infarction (MI), is a major predictor of poor outcome in heart disease, leading to heart failure. It is therefore essential to identify factors that regulate the quality of the repair response as well as those that contribute to an adaptive versus maladaptive phenotype. Fibroblast growth factor-2 (FGF-2) is one such factor that can have both beneficial as well as detrimental effects, depending on the particular isoform expressed as well as its extracellular or intracellular mode of action. FGF-2 exists as high molecular weight, CUG-initiated, 22-34 kDa (hi-FGF-2), or low molecular weight, 18 kDa (lo-FGF-2) isoforms, products of differential translation from the same mRNA. Because of the widely held belief that only lo-FGF-2 is released to the environment, and thus is the only FGF-2 species that can act in an autocrine or paracrine fashion on cells, the vast majority of studies have reported on lo-FGF-2. It is therefore well established that lo-FGF-2 is a potent mitogen, angiogenic as well as cytoprotective agent, and a very important component of cardiac repair, regeneration and developmental growth. The role of hi-FGF-2 on the other hand is not as well understood.

Our laboratory recently documented that administration of different FGF-2 isoforms exerted different effects on cardiomyocytes. Specifically, exogenous hi-FGF-2, but not lo-FGF-2, stimulated significant cardiomyocyte hypertrophy in culture and *in vivo*, suggesting that endogenous hi-FGF-2 may play a similar role if released by cells to the extracellular environment. Because cardiac fibroblasts

represent a significant cellular source of cardiac FGF-2 and other cytokines, a series of studies was undertaken to examine the hypothesis that hi-FGF-2 is expressed and released by a major cardiac cell population, the fibroblasts, in a manner regulated by the pro-hypertrophic Angiotensin II peptide.

Primary cultures of neonatal rat cardiac myofibroblasts were used as our primary model. Selected studies were also performed using human cardiac myofibroblasts obtained from commercial sources. Firstly we established that the first plating of cells (P0) isolated from one-day-old Sprague-Dawley rat pup hearts were positive for vimentin, a marker of fibroblasts. At later passages (P1-P3), these cells showed increasing expression of focal adhesion proteins, non-muscle heavy chain Myosin, (SMemb), and α-smooth muscle actin (α-SMA), indicating that they phenotypically modulated to myofibroblasts. We then determined that cardiac myofibroblasts accumulate predominantly (over 90% of total) hi-FGF-2, detected as 22-23 kDa (for the rat) and 22-34 kDa (for the human) bands by Western blotting with FGF-2-specific antibodies. Cells were then treated with Angiotensin II at concentrations between 10^{-10} M to 10^{-6} M for up to 48 hours. Western blot analysis showed that Angiotensin II significantly stimulated cell-associated hi-FGF-2 accumulation in a time (24-48 hours) and dose-dependent (10^{-8}-10^{-6} M) manner. Secondly, the 22-23 kDa bands (hi-FGF-2) did not represent a glycosylated version of lo-FGF-2 since it was not affected by endoglycosidase treatment. Furthermore, Angiotensin II stimulated the release of hi-FGF-2 to the extracellular space. Finally, the effects of Angiotensin II on myofibroblast hi-FGF-2 accumulation were found to be exerted by binding to

the AT_1 but not AT_2 receptors, as determined with the selective receptor antagonists, losartan (10^{-6} M) and PD123319 (10^{-6} M), respectively. Similar responses were obtained when using human myofibroblasts.

The Angiotensin II-stimulated accumulation and release of hi-FGF-2 to the extracellular environment by a cardiac cell population in culture suggest a similar *in vivo* scenario in which pro-hypertrophic agents act, at least in part, by upregulating hi-FGF-2 expression and release by these cells. The regulation of hi-FGF-2 expression and release by cardiac myofibroblasts provides a possible therapeutic target for reversal or management of heart disease. Conversely, down-regulation of hi-FGF-2 may contribute to the known beneficial effects of inhibiting Angiotensin II signaling in heart disease.

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List of Abbreviations

Ang II	angiotensin II
ANOVA	analysis of variance
AT_1	angiotensin type 1 receptor
AT_2	angiotensin type 2 receptor
α-SMA	alpha-smooth muscle actin
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BSA	bovine serum
cAMP	cyclic adenosine 5'-monophosphate
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethlenediaminetetraacetic acid
Egr-1	early growth response-1
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF-2	fibroblast growth factor-2

FGFR	fibroblast growth factor receptor
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
hi-FGF-2	high molecular weight fibroblast growth factor-2
hnRNP A1	heterogeneous nuclear ribonucleoprotein A1
HSPG	heparan sulfate proteoglycan
IgG	immunoglobulin
IL-1	interleukin-1
IRES	internal ribosome entry site
kDa	kilodalton
LMP1	latent membrane protein 1
lo-FGF-2	low molecular weight fibroblast growth factor-2
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NLS	nuclear localization sequence
N.S.	not significant
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PIC	protease inhibitor cocktail
PKC	protein kinase C
PLC	phospholipase C

PMSF	phenylmethylsulfonylfluoride
PPIC I	phosphatase inhibitor cocktail I
PPIC II	phosphatase inhibitor cocktail II
PVDF	polyvinylidene difluoride
RAS	renin-angiotensin system
rpm	rotations per minute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SMemb	non-muscle heavy chain Myosin
Sp1	stimulating protein 1
TBS-T	tris-buffered saline with Tween
TE	translational enhancer
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
U	units

Chapter 1 Review of the Literature

1.1 Fibroblast Growth Factor-2: general introduction

Fibroblast growth factor-2 or FGF-2, first purified from bovine pituitary (Abraham et al, 1986) over 30 years ago, is a prototypic member of a large family of heparin-binding proteins composed of 23 members (Yamashita et al, 2000). Characterized as a basic protein based on its isoelectric point, FGF-2 is a ubiquitous, multifunctional growth factor, well conserved throughout the eukaryotic world (Ornitz et al, 2001). This protein exists as five isoforms, products of regulation at the translational level, arising from a single-copy gene within chromosome 4 in the region of q26-q27 of the human genome (Yu et al, 2007). The isoforms include the 18 kDa low molecular weight FGF-2 (lo-FGF-2), and the 22-34 kDa high molecular weight FGF-2 (hi-FGF-2) (Kardami et al, 2004). The biological activities of FGF-2 are mediated by binding to plasma membrane tyrosine kinase receptors (FGFR1-4) (Klint et al, 1999; Sorensen et al, 2006). Because of the widely held belief that only lo-FGF-2 can be released to the extracellular environment and activate signal transduction pathways by binding to FGFR, most of the studies up-to-date have used and/or implicated lo-FGF-2 in cell proliferation, migration, differentiation, and angiogenesis. Increasing evidence however suggests that hi-FGF-2 may also be released into the extracellular space, and thus act on plasma membrane FGFR. The notion that hi-FGF-2 can be released to the extracellular environment is the main investigational subject of this thesis.

1.2 FGF-2: gene expression

The fgf-2 gene is highly conserved among vertebrates and has been shown to give rise to multiple polyadenylated sense and antisense mRNAs via bidirectional transcription (Delrieu, 2000). The FGF-2 promoter region is devoid of classical TATA or CAAT boxes but it contains GC-rich regions which are transcription factor (Sp1 and Egr-1) binding sites and mediate transcription of fgf-2 gene (Jin et al, 2000). Several studies have shown that transcription of the fgf-2 gene can be induced by various stress stimuli including ischemia, cytokines and growth factors; for example, IL-1 in vascular smooth muscle cells (Gay et al, 1991); TGFβ in AKR-2B cells (Pertovaara et al, 1993); TNFα in HOME cells (Okamura et al, 1991); and bioactive molecules such as Angiotensin II or Ang II and Endothelin-1 or ET-1 (Peifley et al, 1998). The fgf-2 gene can also be induced by ligands that stimulate β-adrenergic and acetylcholine receptors, and requires protein kinase C (PKC) and cyclic adenosine 5'-monophosphate (cAMP) as secondary messengers. Moreover, the FGF-2 protein can autoregulate its own expression by activating the fgf-2 gene promoter (Jimenez et al, 2004). There are also reports that intracellular FGFR1 plays a role in the activation of fgf-2 gene by binding directly to the FGF-2 promoter (Maher, 1996; Stachowiak et al, 1996; Peng et al, 2001).

1.3 FGF-2: translational regulation

All isoforms of FGF-2 are derived from a single mRNA. The various isoforms of FGF-2 are generated by translation initiation either from the

conventional AUG start site, generating the 18 kDa species, or from several (four in human) CUG start sites, generating various species of hi-FGF-2 (Yu et al, 2007). In addition, FGF-2 translation can occur via both cap-dependent as well as cap-independent, internal ribosome entry site (IRES) pathways. The conventional cap-dependent model involves the binding of ribosomes to the 5'-end capped structure of the mRNA that subsequently scans the mRNA in search of the conventional Kozak start codon (AUG) corresponding to methionine. In the case of FGF-2 isoforms, the 18 kDa FGF-2 is translated via non-classical, cap-independent, internal ribosome entry process due to the presence of IRES located in the first 176 nucleotides of the FGF-2 mRNA, upstream of the 18 kDa AUG start codon (Vagner et al, 1995). Similarly, hi-FGF-2, in particular 22, 22.5, and 24 kDa, are translated by an internal ribosome entry site mechanism that allows for their synthesis, however, starting from non-canonical CUG (leucine) start codon (Vagner et al, 1995). The largest human isoform (34 kDa), in contrast to other hi-FGF-2 isoforms, is synthesized by a cap-dependent and IRES-independent mechanism (Delrieu, 2000). It is of interest that cap-dependent translation is inhibited under stress conditions and thus IRES-mediated FGF-2 translation results in FGF-2 accumulation in stressed cells (Prats et al, 2002).

Several cis-acting or trans-acting elements affect translation of the FGF-2 mRNA. Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), involved in pre-mRNA processing, has been shown to bind to the hairpin-loop structures within the FGF-2 mRNA and control the initiation of translation of FGF-2 in IRES-mediated fashion (Bonnal et al, 2005). Interestingly, p53 induces conformational

changes in mRNA that inhibit FGF-2 translation independent of p53's role in fgf-2 gene activation, thus generating a translation blockade (Galy et al, 2001). Touriol et al (2000) have shown that the alternative translation initiation of human FGF-2 mRNA can be controlled by its 3'-untranslated region involving a Poly(A) switch and a Translational Enhancer (TE), which favors the relative expression of CUG-initiated hi-FGF-2 isoforms.

Despite all the studies that have been conducted up to date, translational regulation of FGF-2 is still not completely understood. There is no doubt that there is some tissue-specific regulation, resulting in the accumulation of hi- or lo-FGF-2 isoforms predominantly in some tissues such as the adult brain, or heart, respectively (Liu et al, 1993; Coffin et al, 1995; Touriol et al, 2003; Teshima-Kondo et al, 2004).

1.4 FGF-2: tissue distribution and subcellular localization

Expression of FGF-2 and relative ratios between different isoforms vary accordingly to the cell type, tissue type, and developmental stage (Riese et al, 1995). In terms of cell types, several studies show that hi-FGF-2 is predominantly associated with transformed cell lines whereas some primary cells such as skin fibroblasts mainly produce lo-FGF-2 (Vagner et al, 1996). Nonetheless, hi-FGF-2 is rapidly produced when normal cells like skin fibroblasts are exposed to heat shock, oxidative stress, and γ-irradiation (Vagner et al, 1996; Cohen-Jonathan et al, 1997; Delrieu, 2000). Thus preferential or increased translation of hi-FGF-2 varies with different cellular conditions and stimuli.

Hi-FGF-2 levels also vary in a tissue specific manner due to the fact that the FGF-2-IRES has a tissue-specific activity (Gonzalez-Herrera et al, 2006). In the brain, hi-FGF-2 is constitutively accumulated and is the predominant FGF-2 species (Delrieu, 2000). Relative levels of hi-FGF-2 reach maximum levels in the adult brain, while lo-FGF-2 is more predominant in the adult heart (Liu et al, 1993). However, hypothyroidism promotes hi-FGF-2 accumulation in rat hearts but not in brain tissues (Liu et al, 1993). Hyperglycemia has also been shown to upregulate IRES-dependent CUG-initiated FGF-2 expression in mouse aorta but not the brain (Teshima-Kondo et al, 2004). Further studies are necessary to examine if such pathophysiological conditions have a direct or indirect effect on IRES trans-acting factors such as p53 and hnRNP A1.

The relative ratios and cellular distribution of the different FGF-2 isoforms vary accordingly to the stage of development during embryogenesis as well (Riese et al, 1995). Studies have shown that FGF-2-IRES activity levels off at E11 (embryonic day 11) and begin to decrease at E16 (embryonic day 16) in most tissues except for the brain and testis in mouse models (Gonzalez-Herrera et al, 2006). Furthermore, as previously mentioned, adult rat heart expresses lo-FGF-2 predominantly, while hi-FGF-2 predominates in the neonatal rat heart (Liu et al, 1993). In adult mouse heart, both hi- and lo-FGF-2 isoforms are present (House et al, 2003).

Hi- and lo-FGF-2 exhibit different subcellular compartmentalization. Lo-FGF-2 is widely reported as predominantly cytosolic, while hi-FGF-2 isoforms localize mainly to the nucleus (Bikfalvi et al, 1997; Arese et al, 1999; Delrieu,

2000) due to the presence of putative nuclear localization sequence (NLS) at the N-terminal extension. The translocation of the 22-34 kDa FGF-2 isoforms into the nuclear matrix is accompanied by methylation of the arginine residues at the N-terminal extension. Inhibition of methyltransferase, involved in the methylation of hi-FGF-2, has resulted in blockade of hi-FGF-2 translocation and accumulation within the nucleus (Pintucci et al, 1996).

Lo-FGF-2 can also be found in the nucleus as seen in studies of rat cardiomyocytes (Pasumarthi et al, 1994; Pasumarthi et al, 1996) and rat Schwann cells (Claus et al, 2003). In this neuronal cell type, the investigators showed that lo- and hi-FGF-2 localize to different sub-nuclear domains. Whereas lo-FGF-2 was found in the nucleoli, nucleoplasm, and Cajal bodies; the hi-FGF-2 exhibited a punctuate pattern and was found in association with mitotic chromosomes. As well, they and others (Foletti et al, 2003), identified an additional non-canonical NLS in the C-terminal region of the protein within the lo-FGF-2 core, in particular, Arg149 and Arg151, which play a role in the nuclear localization of FGF-2 isoforms.

FGF-2 is also found at the extracellular space, associated with heparan sulfate proteoglycans or HSPGs (Moscatelli et al, 1987). Binding to HSPGs protects FGF-2 from proteolytic degradation, serves as a local reservoir for FGF-2 (Vlodavsky et al, 1991), and facilitates interaction with the plasma membrane FGFR. It is also been found that cell-surface HSPGs are essential components of the unconventional, non-classical transport of FGF-2 across the plasma membrane (Zehe et al, 2006) that can act as a molecular trap for FGF-2

molecules and help to extract FGF-2 from the membrane. However, it is widely believed that only lo-FGF-2 can be found outside the cell. Nevertheless, as will be presented in the following section, there is some evidence that hi-FGF-2 may also be found in the extracellular environment (Trudel et al, 2000; Taverna et al, 2003), either associated with HSPGs or released to the circulation.

1.5 FGF-2: release to the extracellular space

FGF-2 lacks a secretory sequence (signal peptide) and thus it is not exported from cells by a conventional secretory mechanism. Various cell types have, however, been shown to release FGF-2 to the environment, including extracellular matrix (ECM), basal lamina, plasma, and body fluids (Delrieu, 2000). Other proteins that are devoid of secretory signals, such as interleukin 1β (IL-1β) and the muscle lectin L-14, have been shown to be released via exocytosis, independently of the classical ER-Golgi pathway (Mignatti et al, 1991). Similarly, FGF-2 is externalized in an energy-dependent and ER-Golgi-independent mechanism (Piotrowicz et al, 1997); this was shown by using brefeldin, an inhibitor of protein secretion via the ER-Golgi complex, which did not block FGF-2 release.

Cell death or injury by any kind of trauma, mechanical damage, endotoxins and/or irradiation, as well as sub-lethal plasma membrane disruptions, have been described as major mechanisms for the release of FGF-2 (Yu et al, 2007). Adult cardiomyocytes release FGF-2 on a beat to beat basis via mechanically-induced transient disruptions of the sarcolemma (Clarke et al,

1995), and this release is[el] implicated in the induction of either pathological, pressure overload induced hypertrophy (Schultz et al, 1999) or in physiological, exercised induced hypertrophy (unpublished data). In lung cancer, cisplatin induced-apoptosis or heat-induced necrosis in non-small cell lung cancer (NSCLC) cells resulted in significantly increased levels of released FGF-2 (Kuhn et al, 2005).

However, injury or cell death is not the sole mechanism that controls the release of FGF-2. Mignatti et al (1991) have shown that viable, single NIH 3T3 cells overexpressing all FGF-2 forms actively release FGF-2 that promotes cell migration through an autocrine mechanism. The process of secretion involves the α-subunits (alpha1-4) of the Na^+/K^+ ATPase (Florkiewicz et al, 1998; Dahl et al, 2000; Wakisaka et al, 2002) because inhibition of ion transport by ouabain also inhibits FGF-2 release. FGF-2 release in this manner is an energy dependent process facilitated through the 27 kDa heat shock protein (Piotrowicz et al, 1997) which acts as a chaperone for FGF-2.

Taverna and co-investigators (2003) have reported that FGF-2 can be released from human SK-Hep 1 hepatoma and NIH 3T3 cells through plasma membrane vesicle shedding. Viable cells release membrane vesicles in an energy-dependent manner in the presence of serum; this decreased dramatically when cells are serum-starved but can be rapidly rescued upon re-addition of serum. Analysis of the shed vesicles showed the presence of 18, 22, and 24 kDa FGF-2 species, a finding that points to a possible release of hi-FGF-2 to the

environment. This relatively new observation has not yet been studied or examined in cardiac cell type populations.

Most recently, Zehe et al (2006) have proposed that FGF-2 secretion is linked to low affinity cell-surface heparan sulfate proteoglycans or HSPGs. To negate the effect of high affinity FGF-2 receptors (FGFRs) in this study, they made use of Chinese Hamster Ovary (CHO) cell lines because CHO cells lack FGFRs yet are still capable of secreting FGF-2 (Engling et al, 2002; Backhaus et al, 2004). The investigators have also made use of a mutant FGF-2, incapable of binding to HSPGs and cells with impaired HSPG biosynthesis. In this study, they clearly showed that wild type but not mutant FGF-2 could be detected in the extracellular environment. Interestingly, FGF-2 secretion from HSPG-deficient cells was rescued by co-cultivation with cells expressing HSPGs on their surface and cells that are in close proximity. They have speculated that the heparan sulfate chains of proteoglycans may act as a molecular trap for FGF-2 molecules, extracting FGF-2 from the membrane to the cell surface. The interaction between FGF-2 and HSPGs depends on the proper folded 3D structure of FGF-2 (Faham et al, 1996; Raman et al, 2003), and this suggests that FGF-2 translocation occurs in a folded state, as indicated by other studies as well (Backhaus et al, 2004).

At this point there is no evidence that different mechanisms might exist for the release of different FGF-2 isoforms to the environment. Thus one might assume that isoform composition of released FGF-2 mirrors that of intracellular FGF-2, in other words, if a cell expresses predominantly hi- or lo-FGF-2 it is likely

to also release hi- or lo-FGF-2. FGF-2 isoform composition is cell and tissue-specific, and can also be influenced by environmental conditions and stimuli.

In human coronary artery endothelial cells (HCAEC), release of lo-FGF-2 is enhanced by matrix proteins (type IV collagen, laminin, or fibronectin) and β-estradiol, a process requiring the PKC signaling pathway and cell surface estrogen receptor (Albuquerque et al, 1998). In HSP27-transfected-bovine arterial endothelial cells (BAEC), the same treatment, i.e. β-estradiol, induces preferential hi-FGF-2 accumulation and release into the conditioned media (Piotrowicz et al, 1997; Piotrowicz et al, 2001). In contrast, ectopic expression of the viral oncoprotein E1A into BAEC, which upregulated FGF-2 both at the mRNA and protein levels, stimulated release of only lo-FGF-2 in the conditioned media (Giampietri et al, 2000). Another viral oncoprotein known as latent membrane protein 1 or LMP1, induces the expression of FGF-2 mRNA and protein via NF-κB signaling, leading to the release of 18 kDa FGF-2 by epithelial cells (Wakisaka et al, 2002). In this study, the release of 18 kDa FGF-2 protein was partially suppressed by ouabain, but not by brefeldin, which is consistent with the fact that FGF-2 secretion is via a Na^+/K^+ ATPase-dependent and ER-Golgi-independent pathway. Other cancer related studies show the role of protein kinase C-Ras-MAPK p44/42 in the secretion of lo-FGF-2 from folliculostellate cells treated with ethanol and transforming growth factor-β3 (Chaturvedi et al, 2005).

Several laboratories have reported that proliferation of smooth muscle cells treated with platelet-derived growth factor-BB is mediated by externalized

FGF-2 (Pintucci et al, 2005). Similarly, proliferation of pulmonary interstitial fibroblasts treated with transforming growth factor-β1 is also mediated by released FGF-2 (Khalil et al, 2005). The above and numerous other studies employed an enzyme-linked immunosorbent assay (ELISA) kit to detect FGF-2 in the conditioned medium. This method uses antibodies that cannot discriminate between the different FGF-2 isoforms, therefore studies which report on the presence of FGF-2 in the conditioned medium need to be re-evaluated to identify which FGF-2 isoform was detected, since some of the biological effects of FGF-2 are isoform-specific.

A large number of studies on FGF-2 release, including some that reported release of hi-FGF-2 to the environment, are based on cells that have been engineered to overexpress hi- and/or lo-FGF-2, and thus the physiological relevance of their findings may be in question. There is scant information on the properties of endogenously expressed hi-FGF-2. It is therefore important to re-evaluate the question of endogenous hi-FGF-2 release, in physiological and pathophysiological conditions. It is also noteworthy to examine hi-FGF-2 release using primary, non-transformed cells.

1.6 FGF-2: signaling pathways

The different FGF-2 isoforms can act via autocrine/paracrine (extracellular) as well as intracrine (nuclear) pathways and can have different and sometimes opposing effects on cells (Delrieu, 2000; Kardami et al, 2004). The biological activities of extracellular FGF-2 are mediated by interacting with high

affinity FGFR(1-4) and low affinity HSPGs. Some reports have shown that the interaction of FGF-2 with HSPG, on its own, can also lead to activation of extracellular signal-regulated kinases 1 and 2 or ERK 1/2 (Chua et al, 2004). In the conventional signaling pathway, upon binding of FGF-2, FGFR dimerizes and become autophosphorylated on tyrosine residues (Klint et al, 1999). Phosphorylation of tyrosine residues leads to activation of the FGFRs which then recruit and phosphorylate other signaling molecules that result in the activation of major signal transduction pathways including all branches of mitogen activated protein kinase (MAPK), phospholipase C-protein kinase C, and Src-dependent pathways (Kardami et al, 2007).

In addition to the pathway described in the preceding section, FGF-2, after binding to its receptor, is capable of being internalized as a complex or separately, to reach the cytosol and nucleus of cells (Bikfalvi et al, 1989; Malecki et al, 2004). Activated nuclear FGFR may activate various genes directly (Maher et al, 1996; Reilly et al, 2001) that are associated with many cellular processes including those related to cell proliferation and differentiation (Stachowiak et al, 2003). The mechanism of nuclear translocation upon internalization of the FGF-2/FGFR complex is mediated by β-importin, which is a major component of cellular nuclear import mechanism that recognizes nuclear localization signals (Stachowiak et al, 2003). In the case of extracellular-acting lo-FGF-2, nuclear translocation is mediated by binding to translokin, a ubiquitous cytosolic protein, found to co-localize with the microtubule network; translokin does not bind to hi-FGF-2 (Bossard et al, 2003). Altogether, the effects of extracellular-acting FGF-2

are mediated by two pathways: (a) by signals downstream of the plasma membrane FGF receptors; and (b) by internalized FGF-2 and FGF-2/FGFR complex that translocate to the nucleus and activate gene expression directly (Kardami et al, 2004).

Besides the extracellular-acting FGF-2, the non-externalized nuclear high molecular weight FGF-2 has also been shown to have an intracellular (intracrine) mode of action. Studies on cardiomyocytes, based on ectopic expression approaches, have reported that while hi- and/or lo-FGF-2 can stimulate the proliferation of neonatal rat cardiac myocytes via an autocrine or paracrine mechanism, overexpression of hi-FGF-2 resulted in a significant increase in cell binucleation and chromatin compaction. These effects were not blocked by extracellular acting neutralizing antibodies against FGF-2, strongly supporting the notion of intracrine action (Pasumarthi et al, 1996). Intracrine effects of hi-FGF-2 in cardiomyocytes include, in addition to non-mitotic chromatin compaction, inhibition of cell DNA synthesis, and eventual cell death in an apoptotic manner (Hirst et al, 2003); all of these are in direct contrast to the effects of intracellular lo-FGF-2 (see review Kardami et al, 2007). More recently, Ma and colleagues (2007) demonstrated that chromatin compaction induced by rat or human hi-FGF-2 results in apoptosis in HEK293 cells; and that these effects were absolutely dependent on nuclear localization of hi-FGF-2, activation of the MEK1-ERK pathway, and mitochondrial participation (Ma et al, 2007).

1.7 Biological activities associated with extracellular-acting FGF-2

FGF-2, either administered as a 'treatment', or endogenously expressed and acting in an autocrine or paracrine fashion, plays a major role in various physiological and pathophysiological processes including angiogenesis, hypertrophy, tumor growth, vascular remodeling, and bone development (Yu et al, 2007). FGF-2 is also a potent cytoprotective agent, as testified by several studies particularly with respect to neuroprotection and cardioprotection from ischemic injury. Administration of FGF-2 into coronary arteries displays beneficial effects on patients with myocardial infarction (Simons et al, 2003) by stimulating revascularization in ischemic regions. However, these effects have been deemed as transient. Pintucci et al (2002) have shown that FGF-2 plays a critical role in angiogenesis by controlling endothelial cell proliferation and migration at sites of wound healing. Furthermore, administration of FGF-2 before, during, and after ischemia, has been shown to exert acute and direct cardioprotective effects in the heart (Kardami et al, 2007), by a mechanism that requires the activity of plasma membrane FGFR, downstream PKC activation, and may also include the phosphorylation of a major gap junction in the heart known as connexin-43 present at the intercalated discs (Srisakuldee et al, 2006). Both lo- and hi-FGF-2 isoforms act as acute cardioprotective agents (Kardami et al 2007; and unpublished observations) when administered by perfusion to the isolated rat heart.

1.8 FGF-2 and myofibroblasts

Fibroblast cells are a major source of FGF-2 in the heart (Akimoto et al, 1999). They represent the largest cardiac cell populations in terms of numbers, and are largely found throughout normal cardiac tissue (Camelliti et al, 2004). Fibroblasts bridge the voids between myocardial tissue layers and in essence, remain closely related, in proximity, to cardiomyocytes. Fibroblasts are also well known for the production of extracellular matrix proteins, namely collagen (types I, III, and VI); and thus, they provide the proper support, shape, and architecture of the heart (Camelliti et al, 2004). Fibroblasts have also been shown to contribute to cardiac development, cell signaling, and electro-mechanical functions (Kohl et al, 1996; MacKenna et al, 2000; Sun et al, 2002). During cardiac remodeling, fibroblasts infiltrate the damaged area; modulate towards the myofibroblastic phenotype with the acquisition of specialized contractile features (Tomasek et al, 2002); and form scar tissue to fill the voided and damaged zones. However in many instances, due to mechanisms not yet understood, myofibroblasts, which are the key elements in the production of extracellular matrix, show inappropriate synthesis of collagen that often leads to a process called fibrosis (Tomasek et al, 2002). It has been proposed that the onset of hypertrophic-scar formation may be the result of the lack of apoptotic process of myofibroblast during the remodeling phase (Schurch et al, 1997). Thus, myofibroblasts that do not undergo apoptosis after wound healing remain viable in the scar tissue and may serve as source of released growth factors that can initiate a maladaptive response during cardiac remodeling. FGF-2 is one such

factor that may play an active role in this process via paracrine or autocrine fashion. FGF-2 has been shown to increase both fibroblast and myofibroblast proliferation during the process of scar formation (Galzie et al, 1997; Hoerstrup et al, 2000); and as well contribute to the induction of cardiac hypertrophy (Jiang et al, 2007).

It is often difficult to differentiate myofibroblasts from fibroblasts. Morphologically, myofibroblasts resemble that of fibroblasts by having sheet-like extensions, extensive rough endoplasmic reticulum and a prominent Golgi apparatus (Gabbiani et al, 2003). However, the modulation of fibroblasts into myofibroblasts involves the formation of extensive stress fibers, supermature focal adhesion proteins, and expression of contractile proteins, for example, α-smooth muscle actin (α-SMA) and embryonic isoform of myosin heavy chain (SMemb) (Tomasek et al, 2002). Several recent studies have shown that cells of subsequent passages (P1-P2) after the first plating (P0) were positive for reported markers of myofibroblasts including α-SMA and SMemb detected by immunofluorescent-based assessment (Freed et al, 2005; Raizman et al, 2007). This supports the notion that passage number, possibly due to increase mechanical stress, may influence the phenotypic change of fibroblastic cells into myofibroblasts. The role of FGF-2 in this manner is not well understood and needs further investigation.

1.9 FGF-2 and cardiac hypertrophy

Cardiac hypertrophy, characterized as an increase in heart mass relative to body weight, evolves in response to extrinsic stress, including excess hemodynamic load brought about by increase pressure and volume (Schultz et al, 1999), or in response to intrinsic factor, as in familial hypertrophic cardiomyopathy (Frey et al, 2003). Cardiac hypertrophy can be classified on the basis of myocardial architecture, function, and whether the outcome progresses into disease state or not. For example, hypertrophy can be categorized based on the following: (a) concentric (when a pressure load leads to growth in cardiomyocyte thickness) or eccentric chamber remodeling (when a volume load results in myocyte lengthening); (b) adaptive or maladaptive functional outcome; and (c) physiological, as in intermittent training exercise, or pathophysiological, as in the presence of hypertension and/or aortic stenosis (Opie et al, 2006). Nevertheless, compensatory cardiac hypertrophy normalizes the increase in wall tension and ejection fraction that occurs in the face of excess workload. However, during sustained or prolonged hemodynamic overload, hypertrophy becomes decompensatory, where progressive systolic dysfunction ultimately occurs and leads to heart failure (Hunter et al, 1999). This pathological hypertrophy has to be distinguished from physiological hypertrophy, as the latter is considered benign and contributes to enhanced cardiac performance without the development of heart failure (Dorn et al, 2007). Thus, it is of medical significance to identify the underlying molecular triggers and events leading to the various types of cardiac hypertrophy in order to develop a proper clinical

approach towards preventing the hypertrophic phenotype from transitioning to subsequent heart failure (Kardami et al 2004).

Multiple signal transduction pathways have been implicated in the hypertrophic response of the heart to the increased work load; each with its own potential as targets for novel anti-hypertrophic strategies. One of the major signaling pathways of cardiomyocyte hypertrophy that has been implicated involves the G-protein coupled receptors (GPCRs). Angiotensin II or Ang II (besides its hemodynamic effects) and others (e.g. endothelin-1) have been shown to be sufficient to induce cardiomyocyte hypertrophy through an activation of GPCRs coupled to the GTP-binding protein, $G_{q/11}$ (Sadoshima et al, 1993; Dostal et al, 1997; Frey et al, 2003).

Ang II is a product of the renin-angiotensin system (RAS), which is involved in blood pressure homeostasis and contributes to sustain elevated pressure in hypertensive patients (Pellieux et al, 2001). Briefly, hepatic-derived angiotensinogen is cleaved by renal-derived renin to produce a decapeptide Angiotensin I, which is subsequently converted into the octapeptide Ang II mediated by angiotensin-converting enzymes or ACEs (Berry et al, 2001). ACE inhibitors not only normalize blood pressure, but also have been shown to inhibit left ventricular hypertrophy (Waeber et al, 1990). In addition to ACEs, an alternative pathway for the conversion of Ang II from Angiotensin I involves chymases that are released from mast cells (Belova, 2000). Chymase is a chymotrypsin-like serine protease stored in secretory granules and activated immediately upon secretion into the interstitial tissues following vascular injury

(Bacani et al, 2006). Inhibition of chymase activity during vascular insult shows promosing results in the prevention of multiple cardiovascular disorders in several animal models (Miyazaki et al, 2006).

Ang II is a pleiotrophic peptide that can act as pressor agent, growth factor, and cytokine (Touyz et al, 2002). It has many biological activities influencing cell growth, apoptosis, cell migration, and differentiation (Kim et al, 2000). In regards to hypertrophy, the trophic action of Ang II may be mediated in part by the release of factors with paracrine activities (Pellieux et al, 2001). Interestingly, pro-hypertrophic Ang II has been shown to upregulate the expression and release of FGF-2 (Kardami et al, 1989; Fischer et al, 1997); and studies done on animal models have implicated FGF-2, of unknown isoform, in the development of overload- and Ang II-induced cardiac hypertrophy.

Overall, besides hemodynamic stress, several growth promoting factors, like Ang II, have been implicated in hypertrophy including endothelin-1, interleukin-6, insulin-like growth factor-1, and more recently, FGF-2 (Kardami et al, 2004). In rat ventricular cardiomyocytes that are paced at a consistent rate, cells exhibited hypertrophic response characterized by increase in cell surface area and protein synthesis (Kaye et al, 1996). This biological effect was abrogated in the presence of neutralizing antibody to FGF-2 indicating that FGF-2 is released by paced cardiomyocytes. Thus, released FGF-2 can activate plasma membrane receptors via an autocrine mechanism that leads to the induction of hypertrophy. Pericardial fluids with high levels of FGF-2 have also been shown to induce cardiomyocyte hypertrophy *in vitro* (Corda et al, 1997).

Earlier *in vitro* studies had shown that when lo-FGF-2 is added to neonatal cardiac myocytes directly, these cells switch from the adult to a fetal program of gene expression with respect to contractile proteins, natriuretic peptides, and early response genes. Because these changes in gene expression also occur during induction of hypertrophy, lo-FGF-2 was considered as pro-hypertrophic (Parker et al, 1990). Nevertheless, similar patterns of gene activation can occur during exit of cells from a quiescent to a proliferative state; at that time neonatal cardiomyocytes were erroneously considered incapable of a proliferative response. Thus their response to lo-FGF-2 was automatically assumed as pro-hypertrophic.

In experiments involving FGF-2 knock-out mice, Pellieux et al (2001) demonstrated that the Ang II-induced cardiac hypertrophy observed in wild type mice was completely prevented. This paper also pointed to the importance of cardiac fibroblasts as "integrators" of the hypertrophic response: in the presence of Ang II, cardiac fibroblasts are stimulated to release growth promoting factors like FGF-2 (of unknown isoform composition) that can have paracrine pro-hypertrophic actions on neighboring cardiac cells. Similar results were obtained by another group, who showed that FGF-2 knock-out mice were incapable of a hypertrophic response to pressure overload obtained by transverse aortic coarctation (Schultz et al, 1999). Although the knock-out studies provide compelling evidence for the involvement of FGF-2 in pressure overload hypertrophy, they could not discriminate between the different FGF-2 isoforms.

Recent findings in our laboratory, demonstrated that hi-FGF-2, but not lo-FGF-2, is responsible for the induction of cardiomyocyte hypertrophy, using both *in vivo* and *in vitro* studies (Jiang et al, 2007). Recombinant hi- and lo-FGF-2 were added intracardially into the ischemic zone during acute evolving myocardial infarction. The results revealed that both hi- and lo-FGF-2 were effective in reducing infarct size acutely, however at later time points, between 6-8 weeks, the hi-FGF-2 group presented substantial and significant hypertrophy. The hypertrophic mechanism likely involves cardiotrophin-1, a member of the IL-6 family of cytokines well known to induce hypertrophy (Kuwahara et al, 1999; Latchman et al, 1999; Aoyama et al, 2000; Freed et al, 2003), presumably released from cardiac fibroblasts and affecting the surrounding cardiac myocytes. This provides key evidence, along with several others, that interaction of non-myocyte cells and cardiac cells is required to induce hypertrophic responses by cardiomyocytes in the presence of various stimuli. *In vitro*, addition of recombinant hi- but not lo-FGF-2 promoted increased protein synthesis; and increased surface area per cell (Jiang et al, 2007). Furthermore, administration of hi- but not lo- FGF-2 upregulated cardiotrophin-1 accumulation and release by fibroblasts *in vitro* (Jiang et al 2007)

1.10 Rationale for study

According to the majority of literature, hi-FGF-2 is believed to remain intracellular and is not released from cells. Thus, the physiological relevance of findings reporting on a pro-hypertrophic role for endogenous hi-FGF-2 can be

questioned. If, on the other hand, hi-FGF-2 is released by a cardiac cell type population, such as fibroblasts or myofibroblasts, in response to known pro-hypertrophic stimuli, it is logical to expect that endogenous hi-FGF-2 plays a pro-hypertrophic role *in vivo*. This is the core subject of this thesis. We hypothesized that pro-hypertrophic agent, Ang II, stimulates hi-FGF-2 accumulation and release by cardiac myofibroblasts. During the studies, we have identified a number of specific unknowns which needed to be investigated. Our objectives included the following: (a) to identify the nature of FGF-2 isoform composition in cardiac myofibroblasts, (b) to determine whether hi-FGF-2 can be released by cardiac myofibroblasts to the extracellular environment, and (c) to examine whether the pro-hypertrophic agent, Ang II, would stimulate hi-FGF-2 release from cardiac myofibroblasts. The above questions and/or objectives were addressed using both rodent and human myofibroblast *in vitro* models, because of a degree of dissimilarity between rat and human hi-FGF-2 isoforms.

Chapter 2 Materials and Methods

2.1 MATERIALS

2.1.1 Reagents

Culture media used for neonatal cardiac myofibroblasts (Ham's Nutrient Mixture F-10) was purchased from Sigma-Aldrich Inc; fetal bovine serum (FBS), antibiotics (penicillin and streptomycin) and trypsin-EDTA were purchased from GIBCO. Culture plates of 100 mm diameter were obtained from Corning Inc. Broad-range molecular markers used in Western blotting were purchased from BioRad, Benchmark pre-stained protein ladder was obtained from Invitrogen, and the Polyvinylidene difluoride (PVDF) membranes were purchased from Roche Diagnostics. Other reagents purchased from Sigma-Aldrich Inc. included the following: (1) Bicinchoninic Acid (BCA) used for protein assay; (2) insulin, ascorbic acid, transferrin/selenium, and Bovine Serum Albumin (BSA) used as supplements when cells were incubated in low serum; (3) Protease Inhibitor Cocktail (PIC), Phosphatase Inhibitor Cocktail I (PPIC I), and Phosphatase Inhibitor Cocktail II (PPIC II), used for total cell lysates extraction; (4) phenylmethylsulfonylfluoride (PMSF), leupeptin, and pepstatin, used as inhibitors in heparin-Sepharose enrichment of FGF-2 experiments; and (5) Ang II used as stimulus or treatment for cells. Drugs that block the effect of Ang II (losartan, AT_1 blocker, obtained from Merck Frost, and PD123319, AT_2 blocker, purchased from Sigma-Aldrich Inc.) were gifts from Dr. Ian Dixon and Dr. Peter Zahradka of the University of Manitoba. The enhanced chemiluminescence (ECL Plus) detection system was purchased from Amersham Biosciences. Heparin-Sepharose CL-6B

beads used for enrichment of FGF-2 were also obtained from Amersham Biosciences. Endoglycosidase H_f enzyme used to cleave the chitobiose core of high mannose and hybrid oligosaccharides from N-linked glycoproteins was purchased from New England BioLabs.

2.1.2 Antibodies

A mouse monoclonal antibody preparation, recognizing all isoforms of fibroblast growth factor-2 (FGF-2) was purchased from Upstate Cell Signaling Solutions and was used for both Western blotting and immunofluorescence. Also purchased from Upstate Cell Signaling Solutions was anti-vinculin antibody, a mouse monoclonal IgG_1. Other antibodies immunoreactive to proteins within focal adhesion complexes were purchased from the following: anti-paxillin antibody, a rabbit polyclonal immunoglobulin, was obtained from Cell Signaling Technology; anti-tensin antibody, a mouse monoclonal antibody, was purchased from BD Transduction Laboratories; site specific monoclonal antibody to the extra domain of cellular fibronectin (EDA sequence) was purchased from Chemicon International; and lastly, polyclonal anti-FAK phosphospecific antibody, purified from rabbit serum, was obtained from Biosource.

A well-characterized mouse monoclonal anti-vimentin antibody, a marker of fibroblasts, was purchased from Sigma-Aldrich Inc. Monoclonal antibody specific for α-SMA was also purchased from Sigma-Aldrich Inc. A mouse monoclonal antibody to SMemb, a marker of myofibroblasts, was obtained from Abcam and was a gift from Dr. Ian Dixon of the University of Manitoba. Another

antibody obtained from Abcam was a mouse monoclonal anti-GAPDH antibody used as loading control.

Secondary antibodies for immunofluorescence (anti-mouse Texas red) were purchased from Amersham Biosciences. Secondary antibodies for Western blotting (anti-mouse IgG and anti-rabbit IgG, conjugated to horseradish peroxidase) were purchased from BioRad.

2.1.3 Human cardiac myofibroblasts

Human cardiac myofibroblasts derived from embryonic heart tissue were purchased from Cell Applications Inc. These cells were cryopreserved at first passage and can be cultured and propagated for at least 8 population doublings. The cells were grown in Basal Medium, 10% Growth Supplements, both obtained from Cell Applications Inc., and 1% antibiotics (penicillin and streptomycin). The cells tested negative for HIV, Hepatitis B and Hepatitis C, and negative for mycoplasma, bacteria, yeast, and fungi. In the human sample study, cells no greater than P3 were used for Ang II treatment experiments.

2.2 METHODS

2.2.1 Cell culture

Primary cultures of neonatal cardiac fibroblasts were isolated from one-day-old Sprague-Dawley rat pups (36 pups/preparation) according to standard protocols (Doble et al, 1996; Doble et al, 2000). The hearts were placed into a Petri dish with cold Phosphate-Buffered Saline (PBS) containing 3.5 g/L of

glucose. The hearts were minced with scissors until the pieces were about 1 mm^3 or small enough to pass through the tip of 10 mL pipette. The heart tissue was digested in a water-jacketed spinner flask at 37°C containing collagenase (740 U/digest), trypsin (370 U/digest), and DNase I (2880 U/digest). The digestion was repeated six times, and the cells were pooled in a bottle with 10 mL of High Clone FBS (GIBCO) to inactivate the enzymes. After the 6^{th} digestion, the cells were filtered through a nytex membrane using a syringe filter; and centrifuged at 2000 rpm for 5 minutes. The pellet was resuspended in 1x ADS buffer (6.8 g NaCl, 4.76 g HEPES, 0.14 g $NaH_2PO_4 \bullet H_2O$, 1.0 g glucose, 0.4 g KCl, 0.2 g $MgSO_4 \bullet 7H_2O$, pH to 7.35 $\pm$ 0.5 with 1 N NaOH in 100 mL to make 10x ADS, then diluted to 1x ADS); and once again filtered through a nytex membrane. The cell suspension was layered onto a discontinuous Percoll gradient (Amersham Biosciences) and centrifuged at 3500 rpm for 30 minutes. The upper band of cells was removed and plated in 100 mm cell culture dish using Ham's Mixture Nutrient F-10 medium in 10% FBS and 1% penicillin/streptomycin; and labeled as P0 cells. The cells were passaged once (P1), two (P2) or three (P3) times. Most studies presented here were done using cells no greater than third passage (P0-P3). Cells were also cryopreserved in 10% DMSO and 40% FBS; and stored at -140°C in an ultra cold freezer for future experiments.

2.2.2 Angiotensin II and receptor blocker treatments

The cells were grown to 100% confluency and starved for 24-48 hours in low serum (0.5% FBS) with supplements and antibiotics. Myofibroblasts, *in vitro*,

maintained in less than 2% fetal bovine serum, require supplements to keep them viable. Supplements added to neonatal cardiac myofibroblasts and human embryonic cardiac myofibroblasts were 10 µg/mL insulin, 10 µg/mL transferrin/selenium, 20 µg/mL Ascorbic Acid and 0.2% BSA in Ham's Mixture Nutrient F-10 media. After the starvation period, the cells were treated with Ang II at different concentrations (10^{-10} M - 10^{-6} M) for up to 48 hours. In experiments using drugs, cells were pre-incubated for 45 minutes with the selective AT_1 and AT_2 receptor antagonists, losartan at 10^{-6} M and PD123319 at 10^{-6} M, respectively, prior to the start of the experiment. The cells were incubated with each drug separately. Experiments were terminated by collecting the conditioned media and rinsing the cells three times with ice-cold PBS. The plates and the conditioned media were frozen using liquid nitrogen and stored in -80°C to be assessed by Western blotting.

2.2.3 Cell surface and extracellular matrix-associated FGF-2

In studies where salt extraction of cell surface and extracellular matrix-associated FGF-2 was done, the cells were washed with 2 mL (per 100 mm dish) of 2 M NaCl with 20 mM Tris-HCl pH 7.2 (Khalil et al, 2005) and 0.5% BSA to prevent FGF-2 (a very basic molecule) from sticking to the side walls of the tubes. High salt washes (repeated twice) were done prior to rinsing the cells with ice-cold PBS. The high salt extracts were diluted to 0.5 M NaCl, 10 mM Tris-HCl pH 7.2; and the FGF-2-enriched fraction was obtained by absorption to heparin-Sepharose beads (addition of a 100 µL of 1:1 slurry).

2.2.4 FGF-2-enriched fractions by heparin-Sepharose

Heparin-Sepharose CL-6B beads were washed at neutral pH with distilled water (200 mL per gram of freeze-dried powder, added in several aliquots) in a sintered glass filter to remove any preservatives or additives. After washing, the beads were collected into a tube containing wash buffer (0.6 M NaCl, 10 mM Tris-HCl pH 7.0). The beads were centrifuged at 1000 rpm for 5 minutes. The excess wash buffer was discarded, leaving a 1:1 ratio of beads to wash buffer (about 1.0 g freeze-dried powder gives about 4 mL final volume of slurry). The equilibrated beads (100 μL of 1:1 slurry) were added to the conditioned media or to the salt extracts solution containing 0.5 M NaCl and 10 mM Tris-HCl pH 7.2 for 2 hours with inhibitors (1 mM PMSF, 5 μg/mL pepstatin, and 5 μg/mL leupeptin). After the incubation period, the beads were pelleted and washed with 1x PBS plus inhibitors; this was repeated twice. After the last wash, all liquids were removed with a Hamilton syringe. The bound proteins were then eluted using 3x SDS buffer (10% SDS, 50% glycerol, 0.5 M DTT, 300 mM Tris-HCl pH 6.8, 0.005% bromo phenol blue in 5x SDS, then diluted to 3x SDS with ddH_2O). The samples were boiled for 10 minutes and the proteins were separated by 15% SDS-PAGE. The blot was probed with monoclonal anti-FGF-2 antibodies.

2.2.5 Total cell lysates protein extraction and protein assay

Total cell lysates were obtained by scraping cells directly into 1x SDS buffer (1% SDS, 50 mM Tris-HCl pH 6.8, and 10% glycerol) with 1:100 dilutions of protease (PIC) and phosphatase (PPIC I, and PPIC II) inhibitor cocktails.

Lysates were boiled for 5 minutes and sonicated briefly, setting the frequency rate at 40 Hz. The samples were then centrifuged at 14,000 rpm for 5 minutes at 4°C with a micro-centrifuge. The insoluble pellets were discarded and the supernatants were transferred into autoclaved Eppendorf microcentrifuge tubes. Bicinchoninic Acid (50 mL) with copper sulfate (1 mL) was used to determine the protein concentration of the supernatants (BCA technique) (Smith et al, 1985). Different known concentrations of BSA were used to obtain a standard curve. The samples were measured in triplicates and the absorbance was read using a spectrophotometer at a wavelength of 562 nm.

2.2.6 Endoglycosidase treatment

For the endoglycosidase experiment, total cell lysates were obtained by scraping the cells directly into 1x glycoprotein denaturing buffer (0.5% SDS, 40 mM DTT) with 1:100 dilutions of PIC, PPIC I, and PPIC II. The protein extraction and protein assay were done as described above except that a Protein Assay Kit purchased from BioRad was used instead of the BCA/$CuSo_4$ solution because the BCA method gave a false positive reaction due to the denaturing buffer containing 40 mM of DTT detergent. Like other protein assays, BCA assay is prone to influence from non-protein sources, particularly detergents like DTT. The BCA assay (based on the reduction of Cu^{2+} to Cu^{1+} by amides) can only tolerate 1 mM of DTT (Smith et al, 1985); while the BioRad Protein Assay (based on Bradford method) can tolerate up to 1 M of DTT (Bradford, 1976). Therefore, the BioRad Protein Assay Kit was employed (λ=595 nm). The samples (100 µg

of protein) were boiled for 10 minutes in 1x glycoprotein denaturing buffer. The samples were then incubated in 1x reaction buffer (50 mM Sodium Citrate, pH 5.5) with gentle flicking of the tubes to mix the solutions. The reaction proceeded with the addition of the Endoglycosidase H_f enzyme (5 μL per reaction) and the samples were incubated at 37°C for different time periods (0, 1, and 4 hours). For positive control, RNase B (Sigma-Aldrich Inc.), a known glycoprotein was used in the same conditions as described for the total cell lysates. The samples were analyzed by SDS-PAGE and Western blotting for FGF-2.

2.2.7 Western blot analysis of target proteins

For the total cell lysates, the samples were mixed in a 1:5 ratio (v/v) with 5x SDS loading buffer (10% SDS, 50% glycerol, 0.5 M DTT, 300 mM Tris-HCl pH 6.8, 0.005% bromo phenol blue). The samples (50-100 μg of proteins) were boiled for 5 minutes before loading onto a 15% SDS-PAGE gel. Heparin-Sepharose-bound proteins were eluted by boiling directly in 3x SDS and then loaded onto the gel. Broad-range molecular weight standards and pre-stained molecular weight markers were also included in the gel.

Separated proteins were electrophoretically transferred onto a 0.45 μM PVDF membrane at 200 V for 1 hour using a buffer containing 20% methanol, 192 mM glycine, and 25 mM Tris base. Membranes were stored in a Tris-Buffered Saline (10 mM Tris-HCl pH 7.6 or 8.0 and 150 mM NaCl) with 0.1% Tween-20 (TBS-T). Before probing, blots were checked for equal protein loading between lanes using Ponceau S stain. Also, the molecular weight standards

were marked while the blots were stained with Ponceau S. The membranes were blocked in a TBS-T solution containing 10% dried non-fat (skim) milk powder for a period of 1 hour at room temperature on an orbital shaker. The membranes were briefly rinsed with two changes of TBS-T. The blots were then incubated with primary antibody in 1% non-fat skim milk powder in TBS-T overnight at 4°C with constant agitation or for one hour at room temperature. The antibodies used were diluted as follows: (a) monoclonal anti-FGF-2 antibody was used at 1:1000 dilution; (b) monoclonal anti-vimentin antibody was used at 1:1000 dilution; (c) monoclonal anti-α-smooth muscle actin antibody was used between 1:500-1:1000 dilutions; (d) monoclonal anti-SMemb antibody was used at 1:1000 dilution; (e) polyclonal anti-paxillin antibody was used at 1:1000 dilution; (f) monoclonal anti-vinculin antibody was used at 1:1000 dilution; (g) monoclonal anti-tensin antibody was used at 1:250 dilution; (h) polyclonal anti-FAK antibody was used at 1:1000 dilution; (i) monoclonal anti-ED-A fibronectin antibody was used at 1:1000 dilution; and lastly, (j) monoclonal mouse anti-GAPDH antibody was used at 1:5000 dilution.

Membranes were briefly washed with two changes of 1% milk in TBS-T. Then the membranes were washed again for 15 minutes once, followed by 3 washes for 5 minutes at room temperature on an orbital shaker. Secondary antibody consisted of anti-mouse and anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP) was then applied at 1:10000 dilution in TBS-T containing 1% skim milk and incubated for 1 hour at room temperature on an orbital shaker. Following the secondary antibody, the membranes were washed

again following the same methods as described for the primary antibodies except the membranes were washed in TBS-T only. Protein bands on Western blots were visualized by ECL Plus according to the manufacturer's instructions, and developed on film. Exposures of film (Kodak) ranged from only a few seconds to over 20 minutes depending on the samples. The bands were normalized against GAPDH signal; and used to confirm even protein loading.

2.2.8 Immunofluorescence

Primary rat and human cardiac myofibroblasts were plated on glass coverslips in 35 mm six-well plates, and allowed to grow for 24 hours in Ham's Nutrient Mixture F-10 containing 10% FBS plus 1% antibiotics (penicillin and streptomycin). For the human cardiac myofibroblasts, glass coverslips were coated with poly-lysine, purchased from Sigma-Aldrich Inc. After 24 hours of incubation, cell growth was arrested by the addition of low serum media for another 24 hours before treatment with or without Ang II. After three washes with ice-cold PBS, the cells were fixed with cold 4% paraformaldehyde in PBS for 15 minutes at 4°C. Cells were then permeabilized with cold 0.1% Triton X-100 (Roche Diagnostics) in PBS for another 15 minutes at 4°C, followed by extensive washing with PBS for a total of 5 changes of fresh PBS. After removing the excess buffer, the cells were incubated overnight at 4°C with the primary monoclonal anti-vimentin antibody diluted at 1:500 or with monoclonal anti-FGF-2 antibody diluted at 1:100 in 1% BSA in PBS. After washing 4 times with PBS over 5 minutes, the cells were incubated with secondary anti-mouse immunoglobulin

conjugated to Texas red diluted (1:100) in 1% BSA in PBS for 1 hour at room temperature. The coverslips were once again washed 4 times with PBS over 5 minutes and stained with 2.5 mM Hoechst 33342 (Calbiochem) for 1 minute for visualization of nuclei, followed by 5 washes with fresh PBS. Prolong Antifade Kit from Molecular Probes was used to mount the coverslips onto slides. An epifluorescent microscope (Carl Zeiss Axiovert 200) and Nikon Diaphot were used to obtain the fluorescent images.

2.2.9 Statistical analysis

Quantified densitometric analysis of each band was done using a computer program (Quantity One 1-D Analysis Software) connected to a scanner (GS-800 Calibrated Densitometer). All isoforms of hi-FGF-2 were combined as one band and analyzed separately from lo-FGF-2. In cases where total FGF-2 was analyzed, all band intensities were included. Within the program, the Background Subtraction Method was set to local and the Regression Method was set to linear. Data were then normalized using GAPDH as loading control. All values were expressed as means ± SEM. Two-way Analysis of Variance (ANOVA) followed by Holm-Sidak Comparison Test was used for comparing the differences among multiple groups with two or more variables using SigmaStat 3.5 software program. One-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test was used for comparing the differences among multiple groups with single variable using GraphPad InStat 3.0 computer program. Significant differences among groups were defined between $P<0.05$ to $P<0.001$.

Chapter 3 Results

3.1 STUDIES ON NEONATAL RODENT CARDIAC MYOFIBROBLASTS

All of the myofibroblastic cells for *in vitro* studies were obtained from neonatal rat of cardiac origin, otherwise, the source of cells are specified.

3.1.1 Characterization of cardiac fibroblasts and myofibroblasts

In order to verify that the isolated cells from neonatal rat pups were fibroblasts, isolated cells were grown on glass coverslips and then probed for fibroblast and myofibroblast markers by immunofluorescence (Figure 1A) and Western blotting (Figure 1B). As shown in Figure 1A, all of the cells (100%) were positive for vimentin indicating that the isolated cells were of fibroblastic origin. Cells of later passages expressed α-smooth muscle actin, marker of myofibroblasts, in a manner independent of Ang II (10^{-8} M) addition (Figure 1B).

3.1.2 Modulation of fibroblasts into myofibroblasts

We examined phenotypic changes in cytoskeletal and focal adhesion proteins as a function of passage (P0-P3) of isolated cells. As shown in Figure 2, a significant increase in α-SMA was seen between P2-P3. Another reported marker of myofibroblasts, SMemb (embryonic isoform of myosin heavy chain isoform) showed a steady increase in expression from P1 to P3. Vimentin also showed a significant increase but only at the latest passage (P3). Figure 3 summarizes changes in focal adhesion proteins. Paxillin, vinculin, tensin, a splice variant of fibronectin (ED-A sequence), and focal adhesion kinase also showed

increases in later passages. This indicated that at later passages fibroblasts modulate to myofibroblasts by acquiring contractile features and accumulating focal adhesion complexes.

3.1.3 Expression of FGF-2 isoforms by neonatal cardiac myofibroblasts

We examined the effect of passage number (P0-P3) on levels of FGF-2. We quantitated relative levels of expression of hi- and lo-FGF-2 during the phenotypic modulation of neonatal rat cardiac fibroblasts into myofibroblasts; and results are shown in Figure 4. Levels of both hi- and lo-FGF-2 increased significantly in P1-P3 compared to their corresponding levels in P0, with a peak in expression during P2. We then examined relative expression of hi- and lo-FGF-2 isoforms in neonatal cardiac myofibroblasts at P2. Immunoreactive bands, migrating at 18 and 22-23 kDa, were detected to the expected sizes for lo- and hi-FGF-2, respectively (Figure 5A). As shown in Figure 5B, hi-FGF-2 represented about 90% of total FGF-2.

3.1.4 Effect of Angiotensin II on the accumulation of FGF-2 isoforms: time dependence

Neonatal cardiac myofibroblasts, grown to 100% confluency and starved for 24 hours in low serum, were treated with 10^{-8} M of Ang II for 0 (control), 12, 24, 36, and 48 hours. Relative levels of hi- and lo-FGF-2 at the various time points were determined by Western blotting, and results are shown in Figure 6. Ang II stimulated a steady increase in hi-FGF-2 compared to controls, becoming

significant at 24-48 hours. Lo-FGF-2 also showed a statistically significant increase at 36-48 hours compared to control levels. The increase in hi-FGF-2 (60-fold at 36 hours) was far greater than the increase in lo-FGF-2 (10-fold) at the same time point.

3.1.5 Effect of Angiotensin II on the accumulation of FGF-2 isoforms: dose-response

Neonatal cardiac myofibroblasts, grown to 100% confluency and starved for 24 hours in low serum, were treated with Ang II at different concentrations (10^{-10} - 10^{-6} M) for another 24 hours. Cell-associated FGF-2 protein accumulation was examined by Western blotting, and results are shown in Figure 7. Ang II, at concentrations between 10^{-8} M to 10^{-6} M, stimulated a significant upregulation of hi-FGF-2 compared to controls (Figure 7B). No significant upregulation of lo-FGF-2 was evident at any concentration (Figure 7C). Myofibroblasts obtained from adult rat hearts were also tested for expression of FGF-2 isoforms and for the effect of Ang II (10^{-8} M). As shown in Figure 8, hi-FGF-2 represented about 90% of total FGF-2 and Ang II stimulated a significant increase in cell-associated hi-FGF-2. No significant difference was observed with respect to lo-FGF-2.

3.1.6 Effect of Endoglycosidase H_f Treatment on hi-FGF-2 electrophoretic mobility

Total cell lysates of neonatal cardiac myofibroblasts treated with Ang II (10^{-8} M) for 24 hours were collected and incubated with 5.0 units of

Endoglycosidase H_f (an enzyme that cleaves hybrids of oligossacharides) for different time points (0, 1, and 4 hours) in a 37°C water bath. Pure RNase B, and pure recombinant hi-FGF-2, were subjected to similar treatment. FGF-2 was detected by Western blotting, while RNase B was detected by Comassie Blue staining. Results are shown in Figure 9. Endoglycosidase treatment resulted in the conversion of RNase B to a band of faster electrophoretic motility, consistent with de-glycosylation, and showing that the enzyme is active (Figure 9A). Endoglycosidase H_f had no effect on either recombinant hi-GF-2 (Figure 9B) or the relative levels of the 22-23 kDa immunoreactive bands in cell lysates (electrophoretic migration), indicating that the 22-23 bands are not glycosylated (Figure 9C). There is a slight increase in the 22 kDa hi-FGF-2 and 18 kDa lo-FGF-2 bands at 4 hours of incubation period. The explanation for such observation will be commented in the Discussion section.

3.1.7 Effect of AT_1 and AT_2 inhibitors on Angiotensin II stimulation of hi-FGF-2

We next investigated the effect of pharmacological inhibition of different Ang II receptors on its ability to stimulate hi-FGF-2 accumulation. Myofibroblasts were pretreated with 10^{-6} M losartan (AT_1 blocker) or 10^{-6} M PD123319 (AT_2 blocker) for 45 minutes prior to the start of the experiment. Cells were then stimulated, or not, with 10^{-8} M Ang II, for 24 hours in the presence of the inhibitors. FGF-2 accumulation was assessed by Western blotting and results are shown in Figure 10. Ang II stimulated the expected increase in hi-FGF-2. Ang II

and losartan but not PD123319 resulted in a significant decrease in hi-FGF-2 upregulation (from a 3.7-fold increase to a 2-fold increase). Thus, losartan block the Ang II effect but PD123319 did not.

3.1.8 Effect of Angiotensin II on hi-FGF-2 release by myofibroblasts

Conditioned media from cardiac myofibroblasts stimulated or not with Ang II (10^{-9}, 10^{-8} M) were analyzed for the presence of FGF-2 by Western blotting of the heparin-Sepharose-bound fraction. Results are shown in Figure 11. While no immunoreactive band was detected in control conditioned medium, a clear band at ~23 kDa, hi-FGF-2, was detected in conditioned medium from cells treated with 10^{-8} M Ang II. Trace amounts could also be seen at the lower Ang II concentration. To verify that our approach was appropriate for removing FGF-2 from culture medium, we also analyzed the heparin-Sepharose-bound fraction from non-conditioned culture medium which was supplemented with 1 ng of recombinant hi-FGF-2. A strong hi-FGF-2 band, serving as positive control, was detected in this sample.

3.1.9 Effect of Angiotensin II on the extracellular destination of exported/released FGF-2

After examining FGF-2 released into the conditioned medium (Figure 11), we also investigated the FGF-2 isoforms bound at the cell surface or extracellular matrix of cells maintained in the presence or absence of Ang II for 24 hours. This FGF-2 fraction was obtained by subjecting cells to a gentle salt (2 M NaCl) wash.

To be able to compare relative FGF-2 levels in the 2 M salt wash from different groups, equal volumes of the 2 M salt wash, deriving from same size plates and similar numbers of cells were absorbed to equal amounts of heparin-Sepharose beads; total eluate from these beads was loaded in each lane, and analyzed for FGF-2 by Western blotting. Results are shown in Figure 12A. Both hi- and lo-FGF-2 were detected in the 2 M salt wash, but hi-FGF-2 was the predominant species. Ang II treatment appeared to decrease FGF-2 (both hi- and lo-) levels in the 2 M salt wash, suggesting that it actually reduced relative levels of extracellular, cell and matrix-bound hi- and lo-FGF-2. We were unable to probe for an internal marker and thus could not correct for possible loading variations between samples. A non-specific 67 kDa band seemed to be present in all samples to a similar extent, including both negative and positive controls. This band is likely a contaminant from heparin-Sepharose beads and suggests that each lane contained eluates from similar amounts of heparin-Sepharose.

A complementary approach was used to determine the presence and levels of exported/released FGF-2 that remained bound to the cell surface/matrix: we analyzed total FGF-2 extracted from cells before and after the 2 M salt wash. Extracts from non-salt-treated cells are presumed to contain both intracellular FGF-2 as well as FGF-2 associated with the extracellular surface of the plasma membrane, while those from salt-treated cells contain mainly intracellular FGF-2. The difference represents the fraction of salt-released FGF-2. Results are shown in Figure 12B. FGF-2 levels, particularly those of the predominant hi-FGF-2 (Figure 12C), were significantly reduced in cells subjected to the 2 M salt wash.

This was evident in both unstimulated and Ang II stimulated cells, but the levels of 'freed' FGF-2 from the Ang II-treated cells appeared to be higher than those of the control cells. This finding is in contrast to data presented in Figure 12A and will be discussed in the Discussion section.

3.2 STUDIES ON HUMAN EMBRYONIC CARDIAC MYOFIBROBLASTS

All of the myofibroblastic cells of human cardiac origin used for *in vitro* studies were obtained commercially, otherwise, the source of cells are specified.

3.2.1 Expression pattern of FGF-2

Primary human embryonic cardiac myofibroblasts were grown to confluence and then examined for their FGF-2 content and isoform composition by Western blotting. Results are shown in Figure 13. Immunoreactive bands were obtained at 18 kDa (lo-FGF-2), and at 22-24 and 34 kDa (hi-FGF-2). Hi-FGF-2 isoforms represented 97% of the total human FGF-2 as shown in Figure 13B.

3.2.2 Qualitative analysis of FGF-2 in the presence and absence of Angiotensin II

Cells were grown on poly-lysine-coated glass coverslips in conditioned medium; and treated or not with 10^{-7} M Ang II for 48 hours after 24 hours of starvation in low serum. They were then processed for detection of total FGF-2 by immunofluorescence. Because hi-FGF-2 represents 97% of total cellular FGF-

2, it is expected that the antibodies are detecting mainly hi-FGF-2. Hoechst counter-staining was also used to visualize the nuclei. Results are illustrated in Figure 14. FGF-2 staining was seen in the cytosol, nuclei and perinuclear areas of control cells. Ang II-treated cells presented stronger anti-FGF-2 staining, especially in association with cell nuclei.

3.2.3 *Effect of Angiotensin II on hi-FGF-2 accumulation*

Cells were grown to 100% confluency, starved for 48 hours and treated with 10^{-7} M Ang II for another 48 hours. Cell-associated FGF-2 was analyzed by Western blotting and results are shown in Figure 15. Ang II stimulated a 2-fold increase in human hi-FGF-2 (Figure 15B); no significant increase was detected with lo-FGF-2 (Figure 15C).

3.2.4 Effect of Angiotensin II on the release of human FGF-2 to the conditioned medium

Conditioned media were collected after 48 hours of treatment with 10^{-7} M Ang II. The heparin-Sepharose-bound proteins were eluted and analyzed by Western blotting. Results are shown in Figure 16. Conditioned medium from non-treated cells contained trace amounts of 18 kDa lo-FGF-2 band, while conditioned medium from Ang II-treated cells contained strong immunoreactive hi-FGF-2 bands at 33-34 kDa.

3.2.5 Effect of Angiotensin II on cell-surface and matrix-bound FGF-2

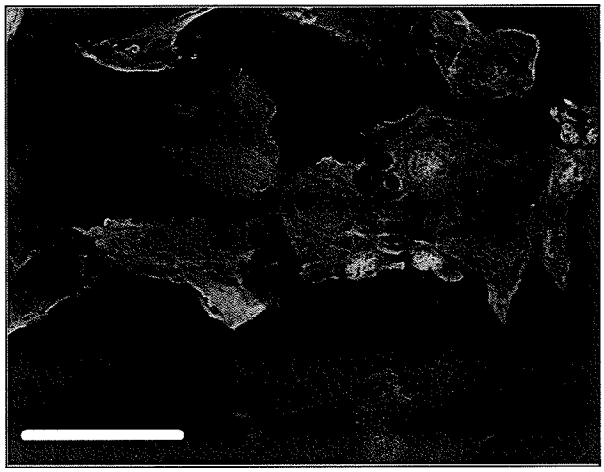
Our standard method for preparing cell lysates results in extracts containing both intracellular proteins and also proteins that are bound tightly to the cell surface. To detect any FGF-2 bound to the cell surface or the extracellular matrix, cells, maintained or not in the presence of Ang II for 48 hours, were subjected to a gentle salt (2 M NaCl) wash. Heparin-bound fractions from the 2 M salt wash were then analyzed for FGF-2 by Western blotting and results are shown in Figure 17. A strong 18 kDa immunoreactive band, lo-FGF-2, and trace amounts of hi-FGF-2 (22-24 kDa) were detected, irrespectively of Ang II treatment.

To differentiate between intracellular and cell-surface- or matrix-bound FGF-2 in another way, we measured FGF-2 content and isoform composition in cells, treated or not with Ang II, and subjected, or not, to the 2 M salt wash. Results are shown in Figure 18. Ang II treatment resulted in a significant increase in total FGF-2 as well as hi-FGF-2, in agreement with data shown in Figure 15. The salt wash resulted in a significant decrease of total cell-extracted FGF-2, both in cells maintained in the presence or in the absence of Ang II, suggesting that a substantial amount of FGF-2 extracted from human cells represents FGF-2 associated with the cell surface/matrix. Hi-FGF-2 was significantly decreased after the 2 M salt wash in both Ang II-treated and untreated cells; this would indicate that human hi-FGF-2 is exported/released by cells and remains in tight association with cells/matrix.

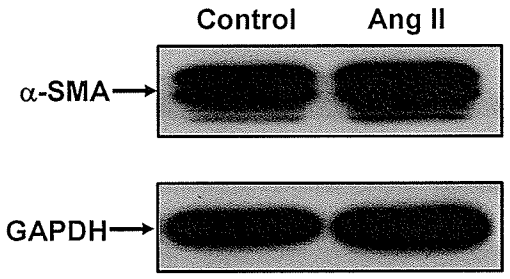
Results obtained from Figure 18 are in apparent contrast with those shown in Figure 17 in regards to the isoform composition of human FGF-2 bound to the cell surface/matrix: the latter identified mainly lo-FGF-2 while the former identified hi-FGF-2. This will be commented upon in the Discussion section.

Figure 1

A



B


Control
Ang II
α-SMA
GAPDH

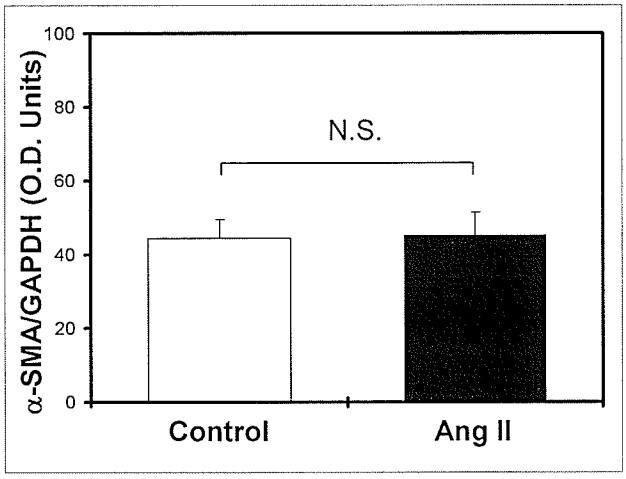

α-SMA/GAPDH (O.D. Units)
100
80
60
40
20
0
N.S.
Control
Ang II

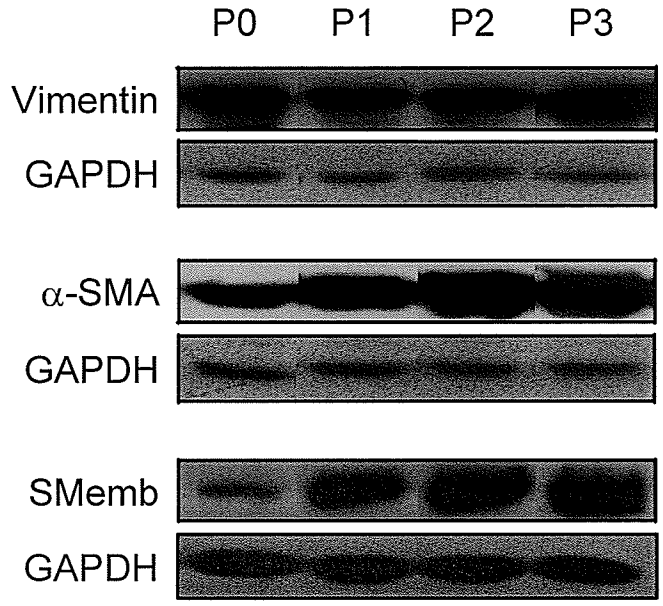
Figure 1: Characterization of cardiac fibroblasts and myofibroblasts: anti-vimentin and α-smooth muscle actin immunostaining

Panel A: Double fluorescence staining of cardiac fibroblasts for vimentin (red; a marker for fibroblasts) and nuclei (blue; Hoechst stain). All cells were positive for vimentin; sizing bar = 20 μm.

Panel B: Western blot analysis of 50 μg/lane of cardiac myofibroblast lysates for α-SMA (a marker of myofibroblasts and smooth muscle cells). Cells were treated for 24 hours with or without Ang II (10^{-8} M), as indicated. Statistical difference was calculated using Tukey-Kramer Multiple Comparisons Test. The two-tailed P value was calculated to be 0.89 ($P>0.05$), which is considered not significant (n=3 per group).

Figure 2

A


P0
P1
P2
P3
Vimentin
GAPDH
α-SMA
GAPDH
SMemb
GAPDH

B

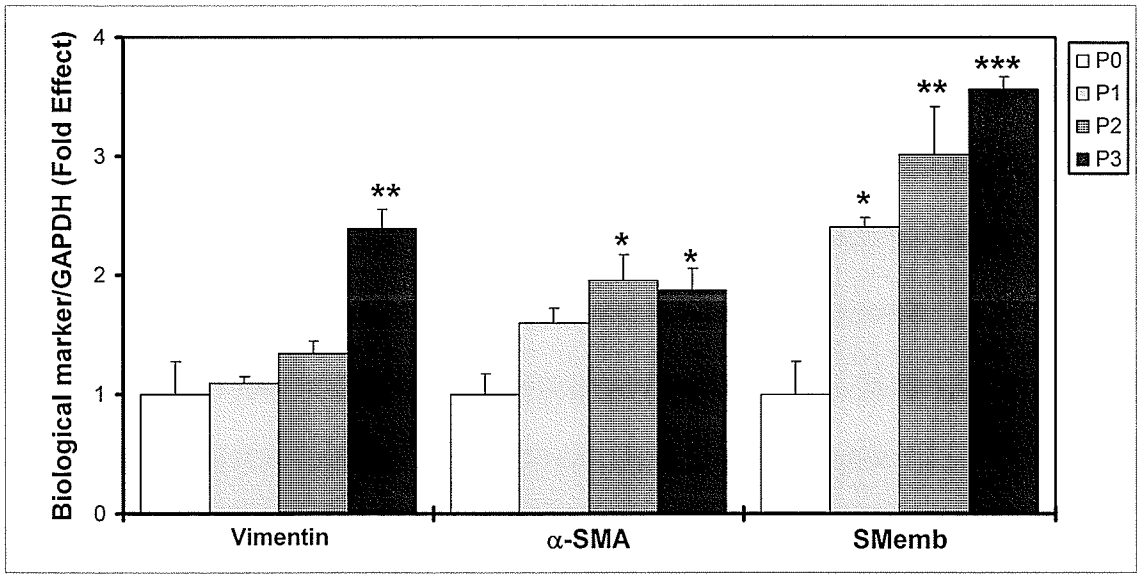

Biological marker/GAPDH (Fold Effect)
0
1
2
3
4
P0
P1
P2
P3
Vimentin
α-SMA
SMemb
*
**
***

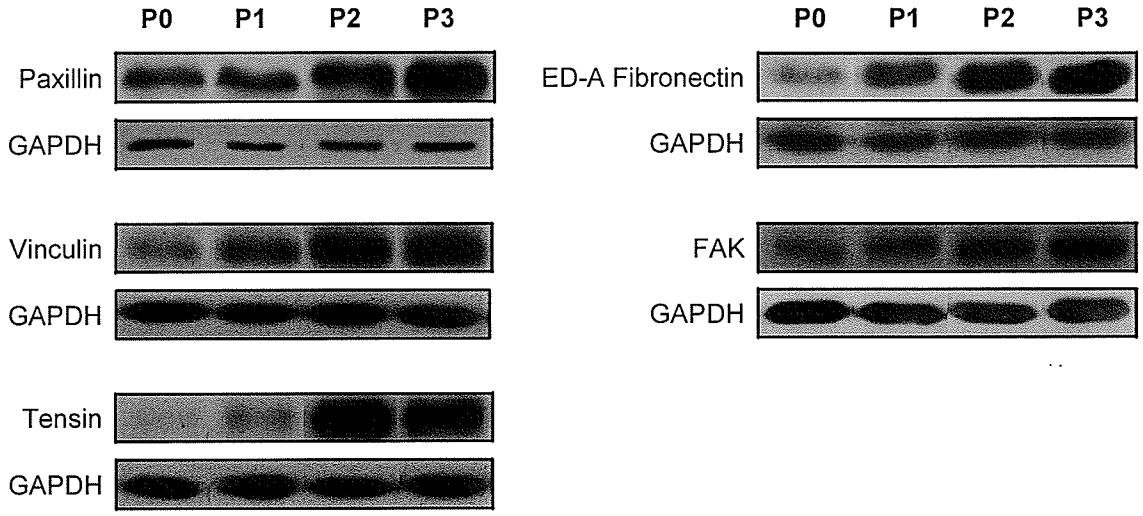
Figure 2: Primary cardiac fibroblasts phenotypically modulate to myofibroblasts

Panel A: Representative Western blots of cardiac fibroblast and myofibroblast lysates probed for corresponding biological markers (vimentin, at 58 kDa; α-SMA at 42 kDa; SMemb at 200 kDa). Each blot was probed for GAPDH to indicate relatively even protein loading among the lanes. Primary neonatal cardiac fibroblasts (P0) were passaged to P1, P2 and P3.

Panel B: Quantitation of vimentin, α-SMA, and SMemb accumulation in neonatal cardiac fibroblasts and myofibroblasts as a function of passage number. The bands were normalized against the corresponding GAPDH loading control, quantified by densitometric scanning (n=3/group); statistical difference is shown as *P<0.05 vs. P0, **P<0.01 vs. P0, and ***P<0.001 vs. P0 using Tukey-Kramer Multiple Comparisons Test. Data are expressed as mean ± SEM; and shown as fold effect where P0 expression level is set to one.

Figure 3

A


P0
P1
P2
P3
Paxillin
GAPDH
Vinculin
GAPDH
Tensin
GAPDH
ED-A Fibronectin
GAPDH
FAK
GAPDH

B

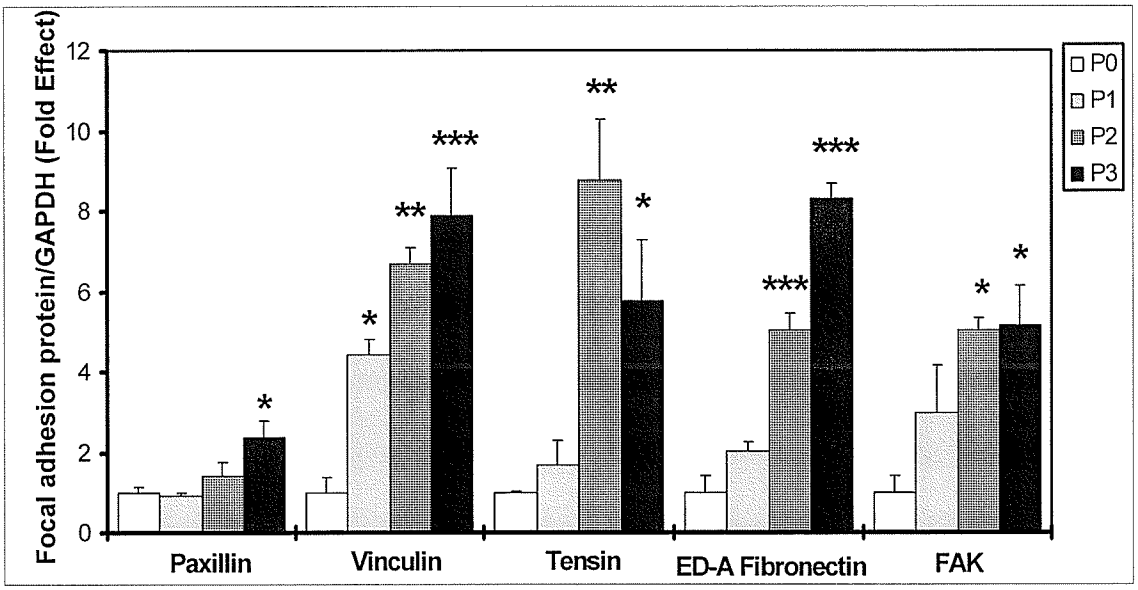

Focal adhesion protein/GAPDH (Fold Effect)
0
2
4
6
8
10
12
P0
P1
P2
P3
Paxillin
Vinculin
Tensin
ED-A Fibronectin
FAK

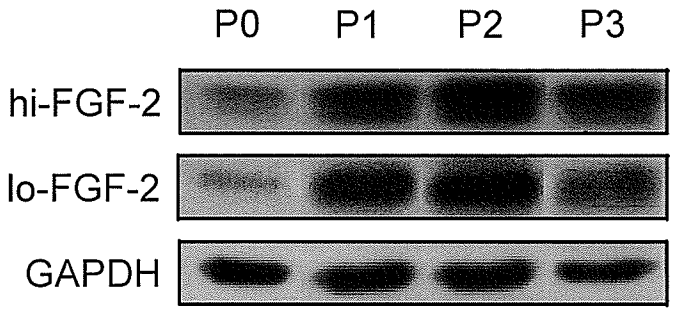
Figure 3: Upregulation of adhesion complexes in neonatal cardiac myofibroblasts

Panel A: Representative Western blots of focal adhesion complexes in neonatal cardiac fibroblasts and myofibroblasts. Western blots were probed for paxillin (68 kDa), vinculin (117 kDa), tensin (215 kDa), ED-A fibronectin (44-52 kDa), and focal adhesion kinase or FAK (125 kDa). To indicate even protein loading, the blots were probed and normalized with the corresponding GAPDH (40 kDa) as loading control.

Panel B: Quantitation of expression of focal adhesion proteins in neonatal cardiac fibroblasts and myofibroblasts. The bands were quantified by densitometric scanning; *$P<0.05$ vs. P0, **$P<0.01$ vs. P0 and ***$P<0.001$ vs. P0 using Tukey-Kramer Multiple Comparisons Test. The data shown are expressed as mean ± SEM of n=3 per group; and shown as fold effect where P0 expression level is taken as one.

Figure 4

A


P0
P1
P2
P3
hi-FGF-2
lo-FGF-2
GAPDH

B

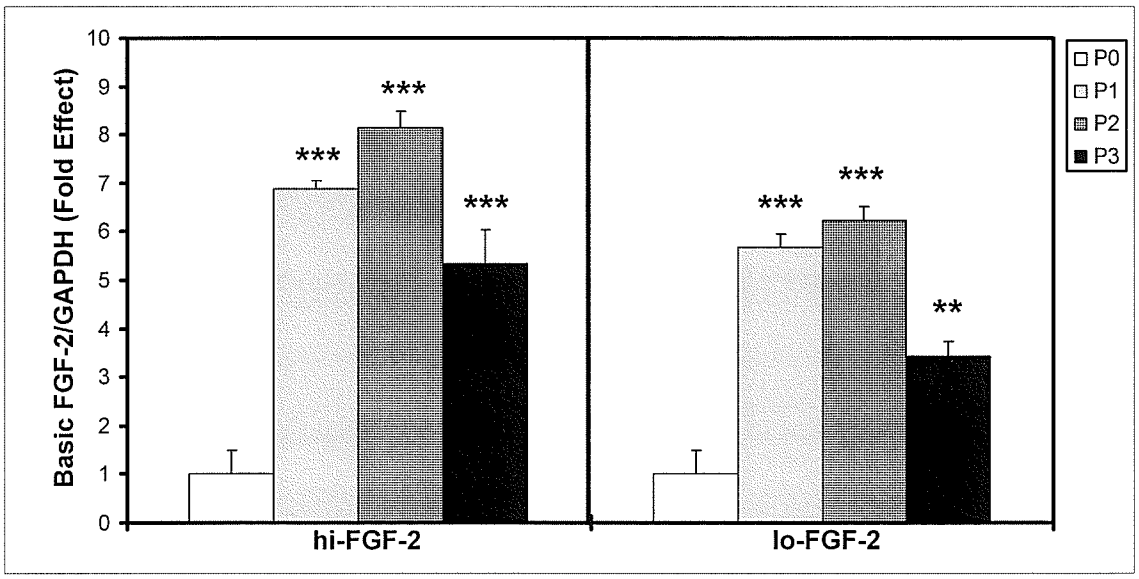

Basic FGF-2/GAPDH (Fold Effect)
10
9
8
7
6
5
4
3
2
1
0
***
***
***
***
***
**
hi-FGF-2
lo-FGF-2
P0
P1
P2
P3

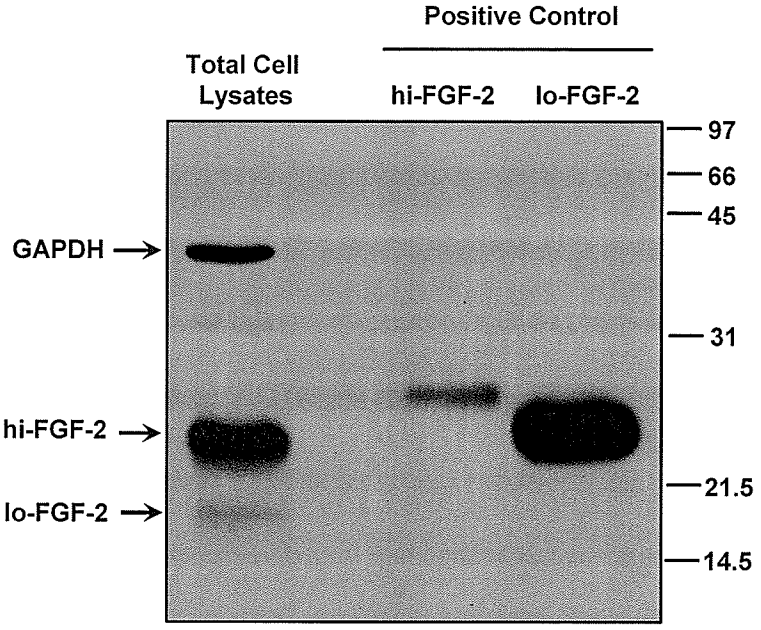
Figure 4: Effect of passage number (P0-P3) on levels of hi- and lo-FGF-2

Panel A: Representative Western blots of hi-FGF-2 and lo-FGF-2 isoforms in neonatal rat cardiac fibroblasts and myofibroblasts. GAPDH (40 kDa) was used as loading control. Please note that the bands corresponding to hi- and lo- FGF-2 have been exposed for different lengths of time and thus no comparison can be made here in regards to the relative levels of hi- versus lo-FGF-2.

Panel B: Quantitation of relative hi-FGF-2 or lo-FGF-2 expression as a function of passage number (n=3/group). Statistical difference (using Tukey-Kramer Multiple Comparisons Test) is shown as $^{**}P<0.01$ vs. P0 and $^{***}P<0.001$ vs. P0, where P0 expression level is set to one. Data are expressed as mean ± SEM; and shown as fold effect. The densities of the bands were normalized to the loading control.

Figure 5

A


Positive Control
Total Cell Lysates
hi-FGF-2
lo-FGF-2
97
66
45
31
21.5
14.5
GAPDH
hi-FGF-2
lo-FGF-2

B

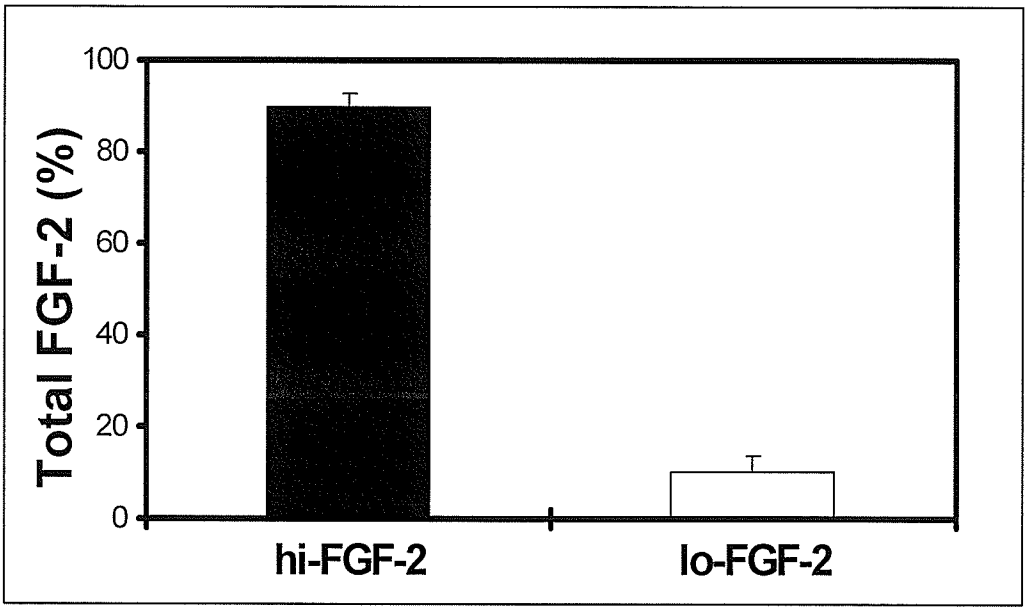

Total FGF-2 (%)
100
80
60
40
20
0
hi-FGF-2
lo-FGF-2

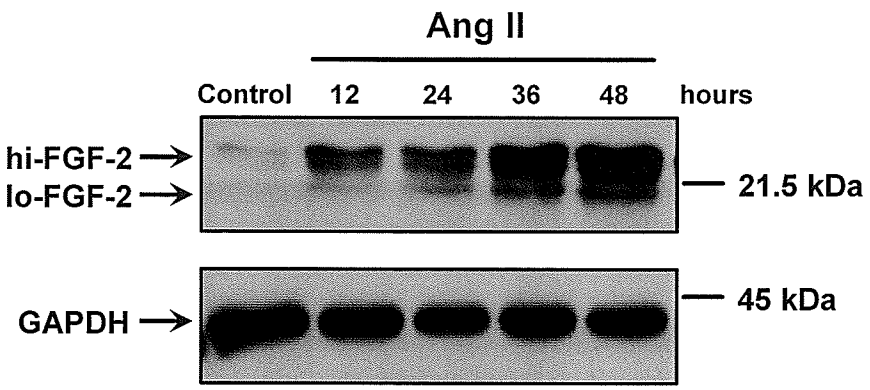
Figure 5: Relative levels of hi- versus lo-FGF-2 in neonatal cardiac myofibroblasts

Panel A: Representative Western blot of myofibroblast lysates (50 µg/lane) probed for FGF-2 and GAPDH (loading control). Recombinant hi- (1 ng) and lo-FGF-2 (4 ng) are included as positive controls and appeared higher than the corresponding endogenous FGF-2 due to the histidine-tags used for purification process. Migration of molecular weight markers is indicated on the right.

Panel B: Quantitative assessment of relative (% of total) hi- and lo-FGF-2 levels in cardiac myofibroblasts, as indicated (n=4). GAPDH has been used to correct for loading variations.

Figure 6

A


Ang II
Control
12
24
36
48
hours
hi-FGF-2
lo-FGF-2
21.5 kDa
GAPDH
45 kDa

B

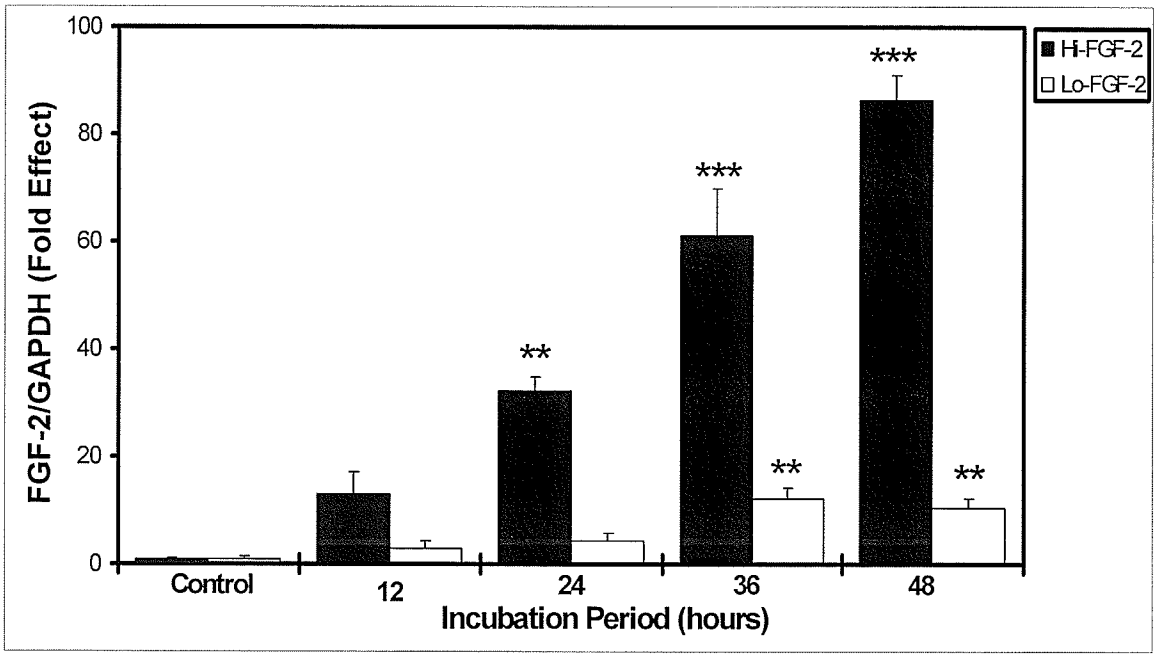

FGF-2/GAPDH (Fold Effect)
100
80
60
40
20
0
Control
12
24
36
48
Incubation Period (hours)
Hi-FGF-2
Lo-FGF-2
**
***
***
**
**

Figure 6: Angiotensin II stimulates hi-FGF-2 accumulation in cardiac myofibroblasts: Time dependence

Panel A: Representative Western blot images showing hi- and lo-FGF-2 in lysates from cardiac myofibroblasts treated with Ang II (10^{-8} M) for different time points as indicated. Western blot of GAPDH (40 kDa) indicated relatively even protein loading between lanes.

Panel B: Quantitation of hi- and lo-FGF-2 accumulation by Ang II-treated cardiac myofibroblasts as a function of time in culture (n=4/group). Isoforms of hi-FGF-2 were combined as one band and analyzed separately from lo-FGF-2. FGF-2 band intensity was normalized against GAPDH for each sample. **P<0.01 vs. control and ***P<0.001 vs. control using Tukey-Kramer Multiple Comparisons Test; data are expressed as mean ± SEM and control is set to one.

Figure 7

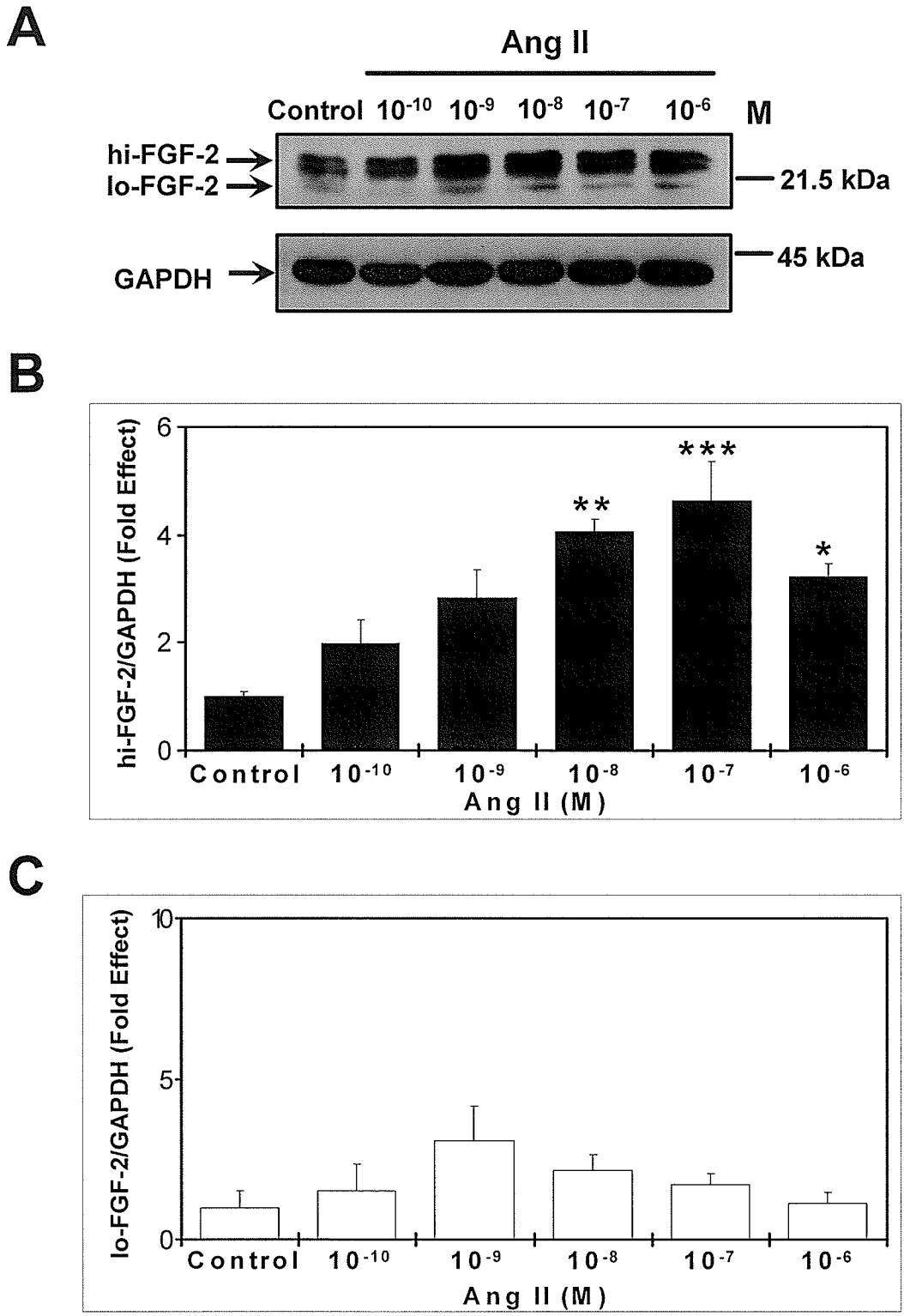

A
Ang II
Control 10-10 10-9 10-8 10-7 10-6 M
hi-FGF-2
lo-FGF-2
21.5 kDa
GAPDH
45 kDa
B
hi-FGF-2/GAPDH (Fold Effect)
0
2
4
6
*
**
***
Control 10-10 10-9 10-8 10-7 10-6
Ang II (M)
C
lo-FGF-2/GAPDH (Fold Effect)
0
5
10
Control 10-10 10-9 10-8 10-7 10-6
Ang II (M)

Figure 7: Effect of Angiotensin II on hi-FGF-2 accumulation in neonatal cardiac myofibroblasts: Dose response

Panel A: Representative Western blot images showing hi- and lo-FGF-2 in lysates from cardiac myofibroblasts treated with different concentrations of Ang II, as indicated, for 24 hours. Western blot of GAPDH (40 kDa) indicated relatively even protein loading between lanes.

Panels B and C: Quantitation of hi- and lo-FGF-2 accumulation, respectively, in cardiac myofibroblasts treated with increasing amounts of Ang II, as indicated (n=4/group). FGF-2 band intensity was normalized against GAPDH for each sample. $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control using Tukey-Kramer Multiple Comparisons Test; data are expressed as mean ± SEM and control is set to one.

Figure 8

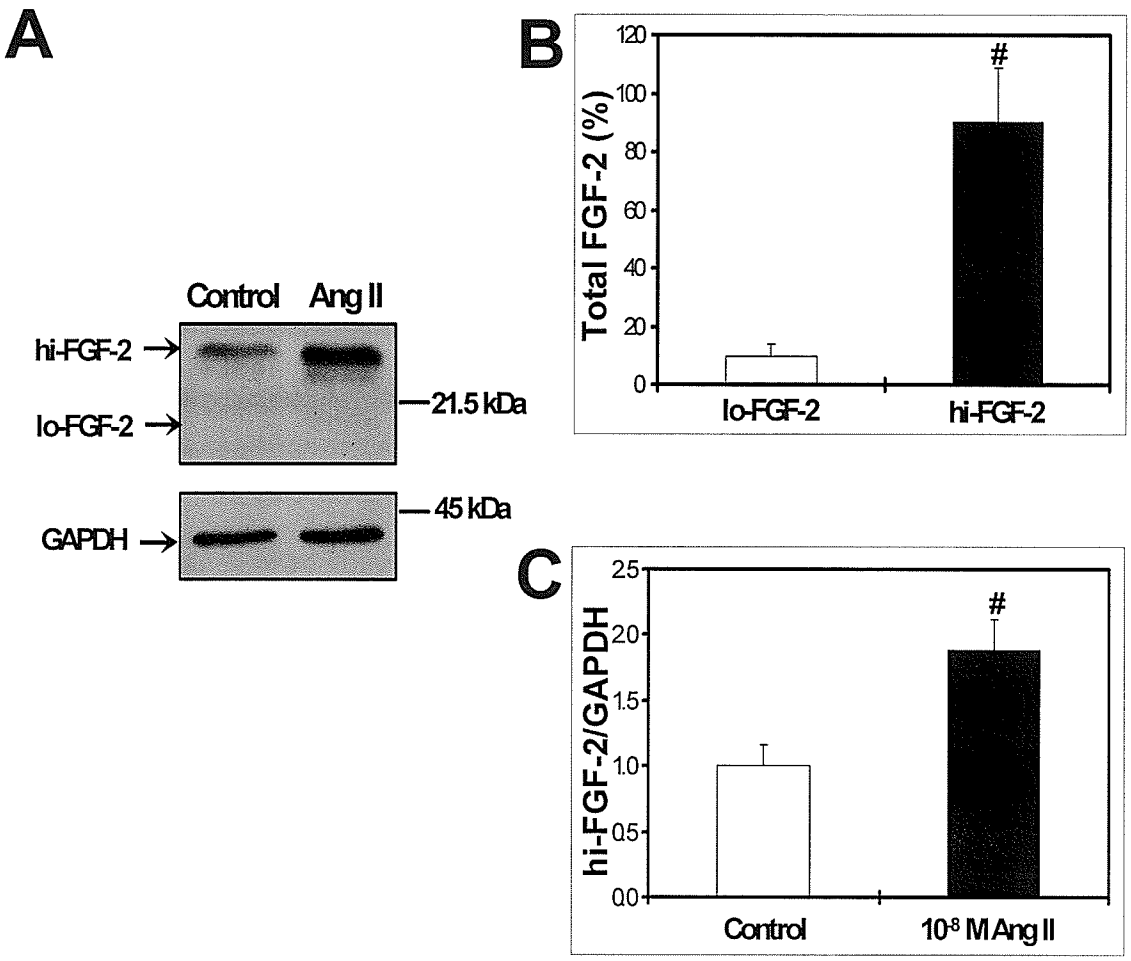

A
Control
Ang II
hi-FGF-2
lo-FGF-2
21.5 kDa
GAPDH
45 kDa
B
Total FGF-2 (%)
120
100
80
60
40
20
0
#
lo-FGF-2
hi-FGF-2
C
hi-FGF-2/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
#
Control
10-8 M Ang II

Figure 8: Expression of FGF-2 isoforms by adult cardiac myofibroblasts: Effect of Angiotensin II

Panel A: Representative Western blot of lysates from adult cardiac myofibroblasts, treated or not, with Ang II (10^{-8} M) and probed for FGF-2. The blots were also probed with GAPDH to verify relatively even loading.

Panel B: Quantitation of relative levels (% of total) of hi- and lo-FGF-2 in untreated adult cardiac myofibroblasts (n=5). $^{\#}P<0.05$ vs. lo-FGF-2.

Panel C: Quantitation of the effect of Ang II on hi-FGF-2 accumulation by cardiac myofibroblasts (n=5). $^{\#}P<0.05$ vs. control; data are expressed as mean ± SEM where control is taken as one. Isoforms of hi-FGF-2 were combined as one band and analyzed separately from lo-FGF-2. No significant difference was observed with respect to lo-FGF-2 (not shown).

Figure 9

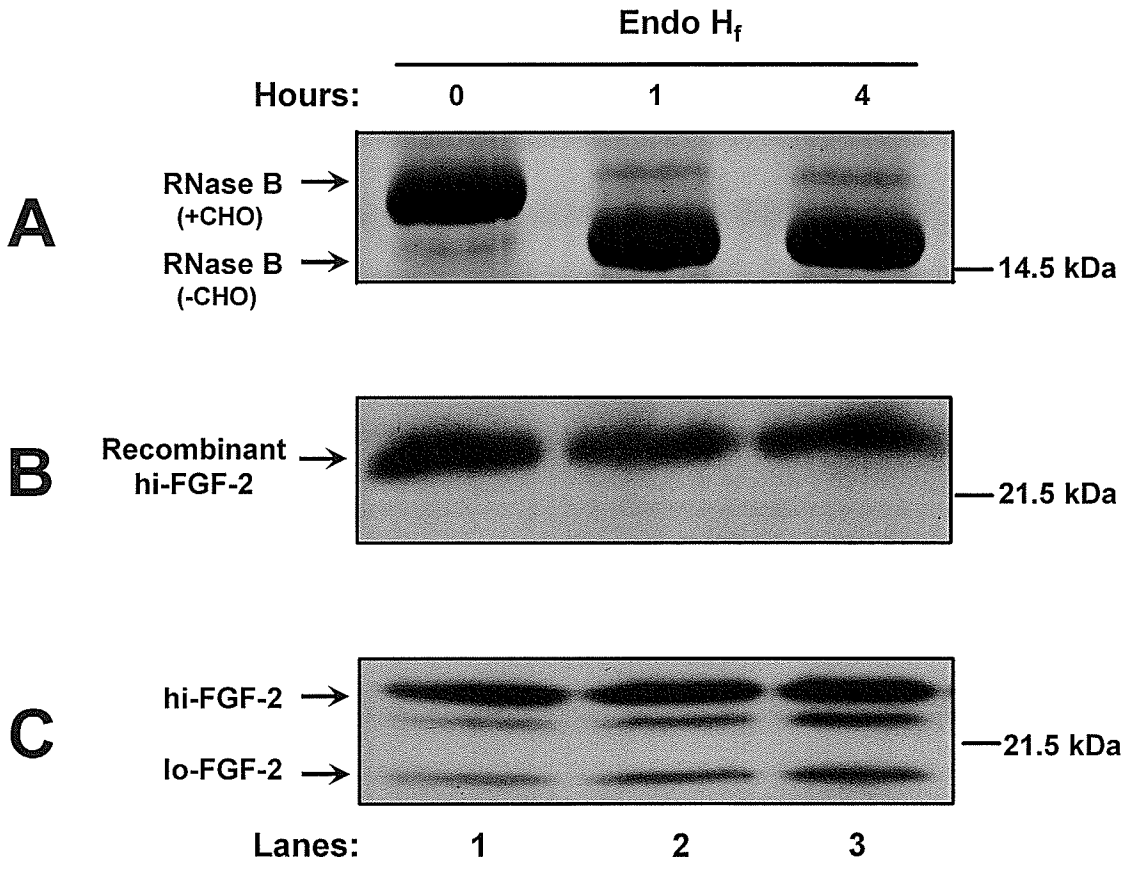

Endo H_f
Hours:
0
1
4
A
RNase B
(+CHO)
RNase B
(-CHO)
14.5 kDa
B
Recombinant
hi-FGF-2
21.5 kDa
C
hi-FGF-2
lo-FGF-2
21.5 kDa
Lanes:
1
2
3

Figure 9: Endoglycosidase treatment does not affect electrophoretic migration of hi-FGF-2

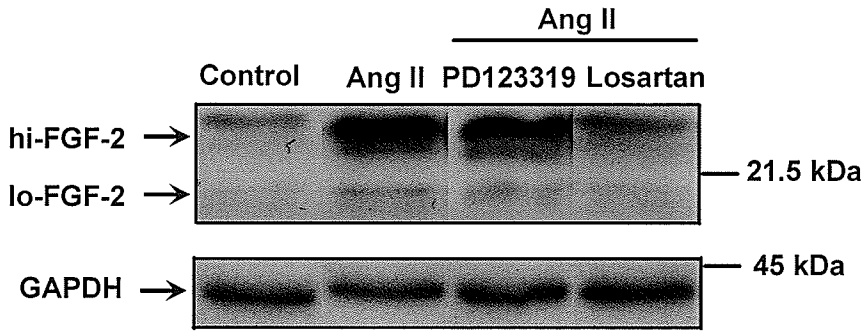
Panel A: Mobility shift assay showing deglycosylation of RNase B using 5.0 units of Endoglycosidase H_f. Comassie Blue stained gel showing control, untreated RNase B migrating at 17 kDa (lane 1) and RNase B treated with the enzyme for 1 (lane 2) and 4 hours (lane 3), migrating at 15 kDa. CHO represents carbohydrate (glucose polymer residues removed from proteins).

Panel B: Western blot analysis (for FGF-2) of His-tagged recombinant hi-FGF-2, untreated (lane 1) or treated (lanes 2 and 3) with 5.0 units of Endoglycosidase H_f for 1 and 4 hours, as indicated.

Panel C: Western blot analysis (for FGF-2) of myofibroblast cell lysates, untreated (lane 1) or treated (lanes 2 and 3) with 5.0 units of Endoglycosidase H_f enzyme for 1 and 4 hours, as indicated. Cells were treated with 10^{-8} M Ang II for 24 hours before enzyme treatment. The experiment was performed twice.

Figure 10

A


Ang II
Control
Ang II
PD123319
Losartan
hi-FGF-2
lo-FGF-2
21.5 kDa
GAPDH
45 kDa

B

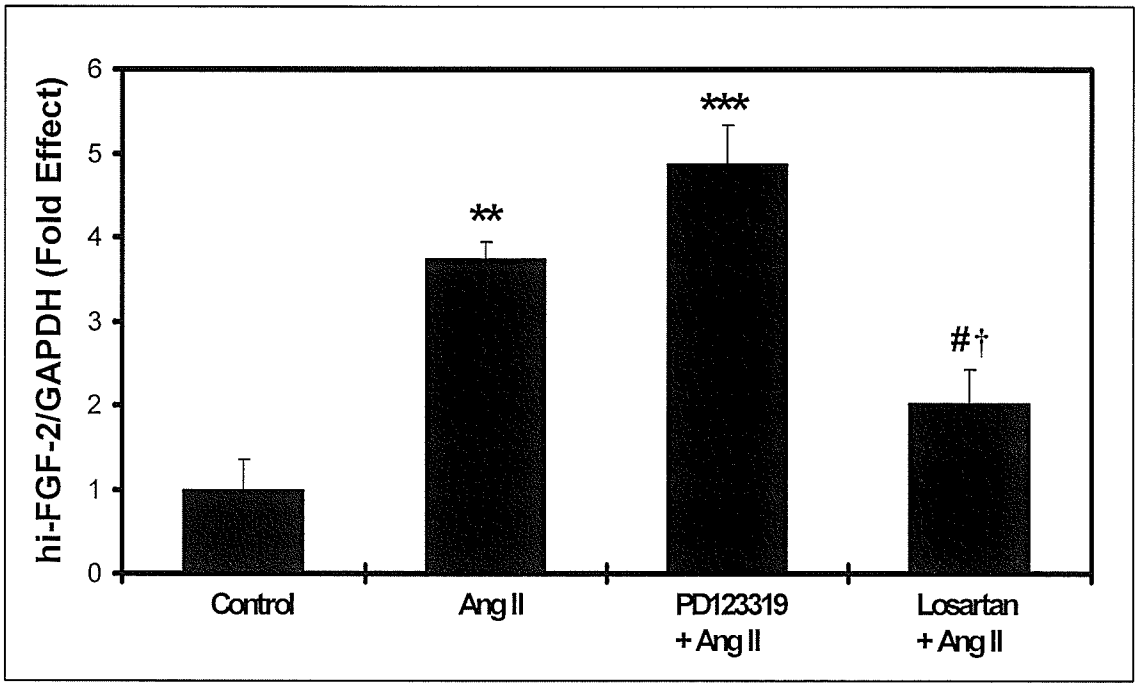

hi-FGF-2/GAPDH (Fold Effect)
0
1
2
3
4
5
6
**
***
#†
Control
Ang II
PD123319 + Ang II
Losartan + Ang II

Figure 10: Upregulation of hi-FGF-2 in response to Angiotensin II is blocked by losartan (AT_1 blocker) and not by PD123319 (AT_2 blocker)

Panel A: Representative Western blot of FGF-2 expression in cardiac myofibroblasts treated with 10^{-8} M Ang II and pre-treated with either 10^{-6} M losartan (antagonist of Angiotensin type 1 receptor) or 10^{-6} M PD123319 (antagonist of Angiotensin type 2 receptor). The blot was also probed with GAPDH to ascertain even loading.

Panel B: Quantitation of the effect of Ang II receptor inhibitors (losartan or PD123319, as indicated) on the Ang II-stimulation of hi-FGF-2 accumulation by cardiac myofibroblasts. Statistical comparison is shown as $^{**}P<0.01$ vs. control, $^{***}P<0.001$ vs. control, $^{\#}P<0.05$ vs. Ang II treatment, †$P<0.05$ vs. PD123319 + Ang II using Two Way ANOVA followed by Holm-Sidak Comparison Test. Data are expressed as mean ± SEM (n=3/group) and control is set to one. The band intensities are normalized to GAPDH, the loading control.

Figure 11

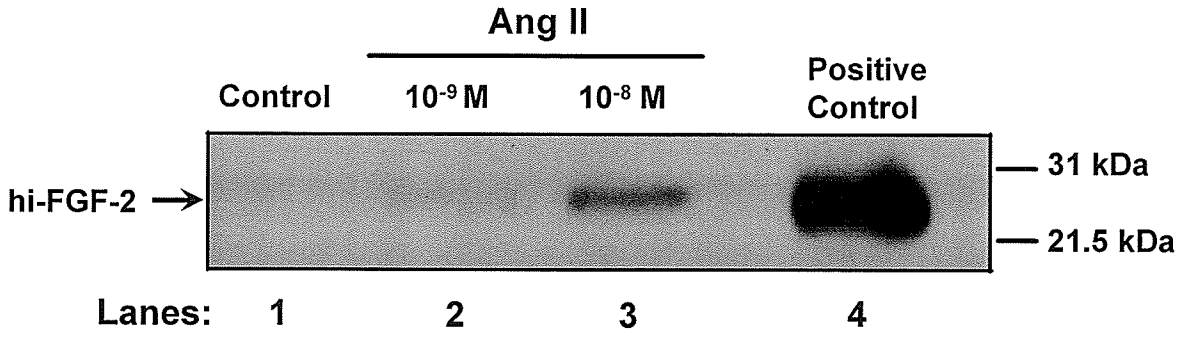


Figure 11: Angiotensin II stimulates release of hi-FGF-2 to the conditioned medium

Western blot analysis showing the presence of hi-FGF-2 (23 kDa) in conditioned media from Ang II-treated cardiac myofibroblasts. Lanes 1, 2, and 3 contain the heparin-bound fraction from conditioned media of control, untreated cultures, or from cultures treated with Ang II, as indicated. Lane 4 contains the heparin-bound fraction from non-conditioned medium (Ham's Nutrient Mixture F-10) supplemented with 1 ng of recombinant hi-FGF-2, serving as positive control. The experiment was performed two times.

Figure 12

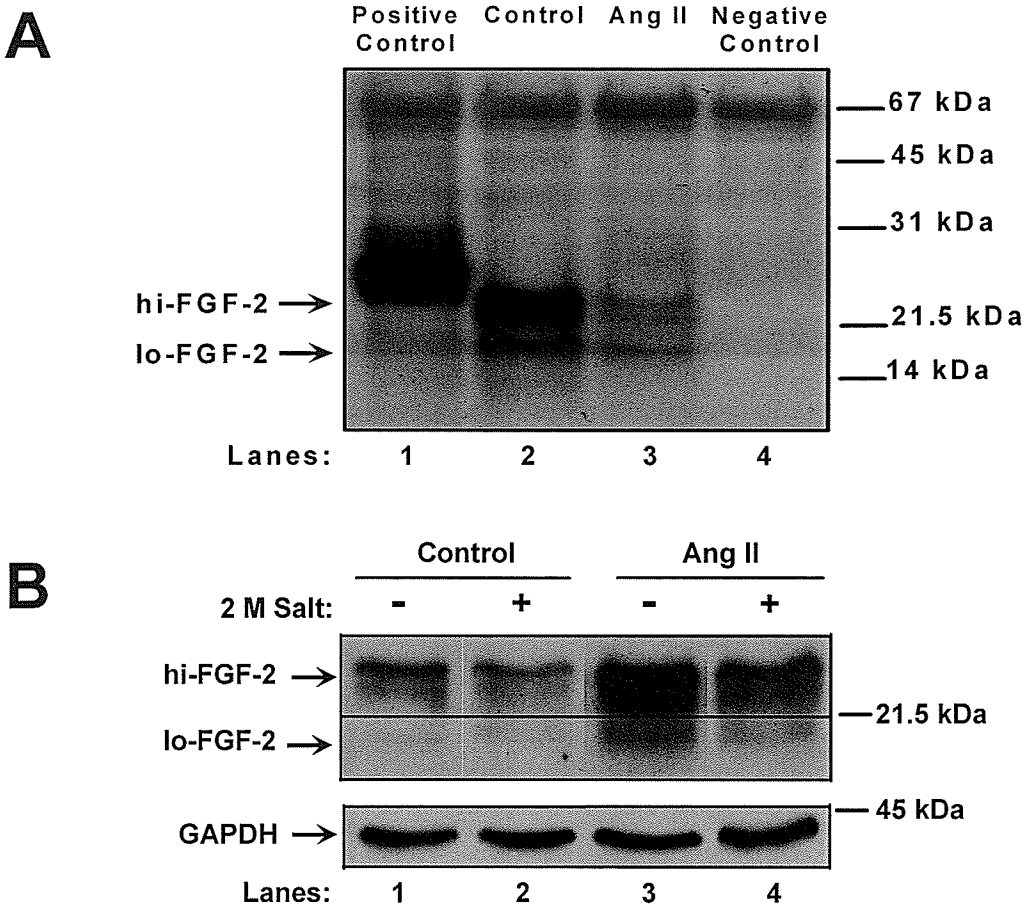

A
Positive Control
Control
Ang II
Negative Control
67 kDa
45 kDa
31 kDa
21.5 kDa
14 kDa
hi-FGF-2
lo-FGF-2
Lanes: 1 2 3 4
B
Control
Ang II
2 M Salt: - + - +
hi-FGF-2
lo-FGF-2
21.5 kDa
GAPDH
45 kDa
Lanes: 1 2 3 4
C
FGF-2/GAPDH (Densitometric Units)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Hi-FGF-2
Lo-FGF-2
*
*
#
Control
Control + 2 M Salt
Ang II
Ang II + 2 M Salt
Control
Control + 2 M Salt
Ang II
Ang II + 2 M Salt

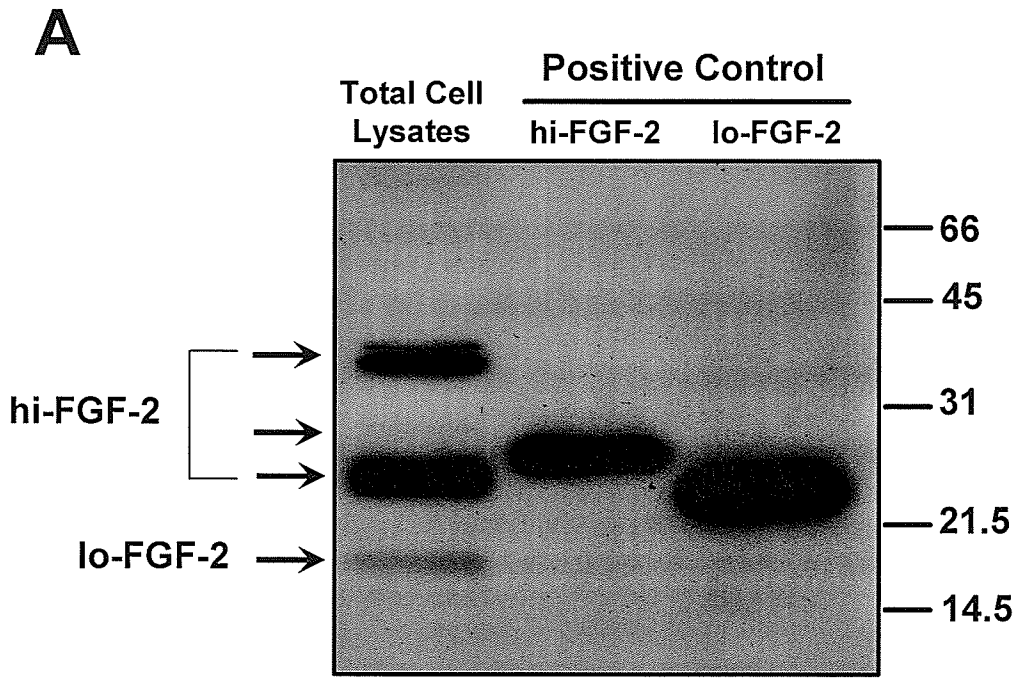
Figure 12: Cell extracted and cell surface-associated FGF-2 isoforms in rat cardiac myofibroblasts

Panel A: FGF-2 bound to the cell surface and extracellular matrix was extracted with a 2 M salt wash. The heparin-Sepharose-bound fraction of the 2 M salt wash was analyzed by Western blotting for FGF-2. Lane 1 contains the heparin-bound fraction from the salt extraction buffer supplemented with recombinant hi-FGF-2 (1 ng) as positive control. Lane 2 contains the heparin-bound fraction from the salt wash of untreated cells. Lane 3 contains the heparin-bound fraction from the salt wash of Ang II-treated cells. Lane 4 represents heparin-bound fraction of equal volume of salt extraction buffer as negative control.

Panel B: Representative Western blot for hi- and lo-FGF-2 present in cell associated lysates obtained from rat myofibroblast cell cultures subjected (+) or not (-) to a 2 M salt wash, and treated or not with Ang II (10^{-6} M) for 24 hours, as indicated. Lane 1 represents untreated cells not washed with 2 M salt. Lane 2 represents untreated cells washed with 2 M salt. Lane 3 represents Ang II-treated cells not washed with 2 M salt. Lane 4 represents Ang II-treated cells washed with 2 M salt. GAPDH indicated even protein loading between lanes.

Panel C: Quantitation of hi- and lo-FGF-2 present in lysates obtained from rat myofibroblast cell cultures subjected (+) or not (-) to a 2 M salt wash, and treated or not with Ang II (10^{-6} M) for 24 hours, as indicated. The bands were normalized against GAPDH as the loading control. Data are expressed as mean ± SEM. Statistical difference using Tukey-Kramer Multiple Comparisons Test is shown as $^{*}P<0.05$ vs. control and $^{\#}P<0.05$ vs. Ang II (n=4 per group).

Figure 13


A
Positive Control
Total Cell Lysates
hi-FGF-2
lo-FGF-2
66
45
31
21.5
14.5
hi-FGF-2
lo-FGF-2

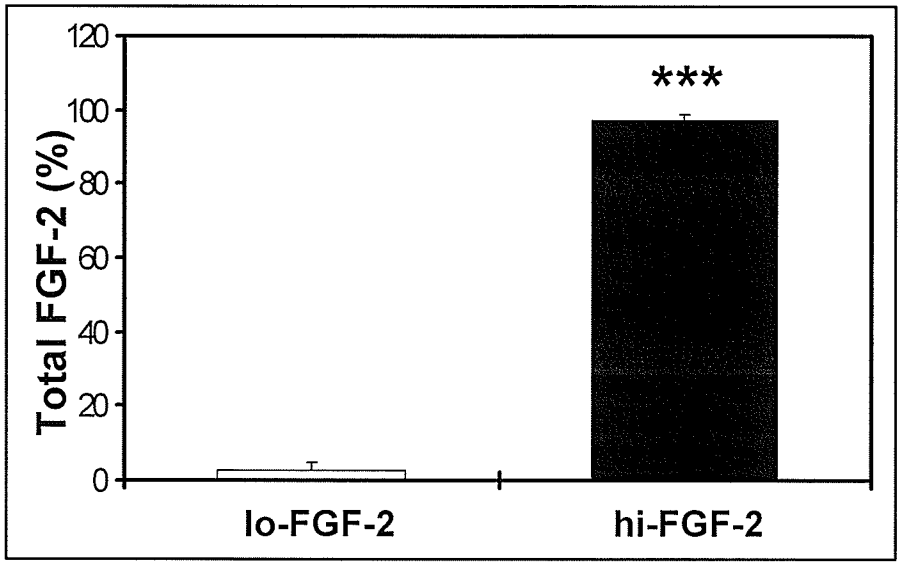

B
Total FGF-2 (%)
120
100
80
60
40
20
0
***
lo-FGF-2
hi-FGF-2

Figure 13: Human cardiac myofibroblasts express mostly hi-FGF-2

Panel A: Lysates from confluent human embryonic myofibroblasts were analyzed for FGF-2 by Western blotting. Representative images are shown in Panel A: human hi-FGF-2 migrates as 22-34 kDa bands, while lo-FGF-2 migrates at 18 kDa. Positive controls of His-tagged rat hi- and lo- FGF-2 are included (1 and 2 ng/lane; respectively). Recombinant FGF-2 appeared higher than the corresponding endogenous FGF-2 due to the histidine-tags used for purification process.

Panel B: Quantitative data showing relative levels of human hi- and lo-FGF-2 (% of total) in human myofibroblasts (n=3 per group). Isoforms of hi-FGF-2 were combined as one band and analyzed separately from lo-FGF-2. The intensity for each sample (hi-FGF-2 and lo-FGF-2) was added together to get the total FGF-2. ***P<0.0001; considered extremely significant.

Figure 14

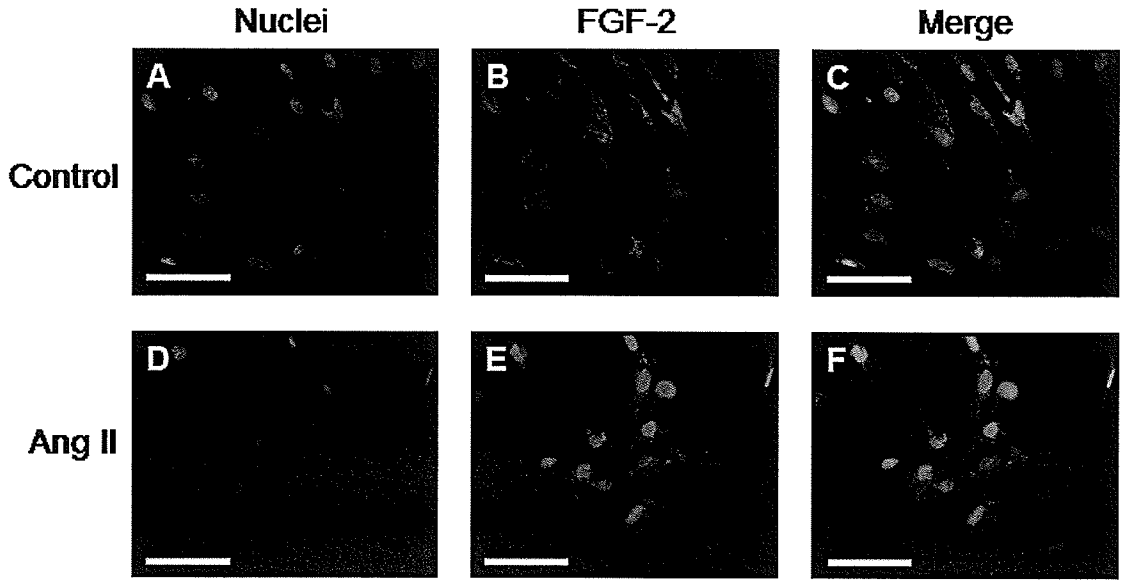


Figure 14: Immunofluorescence staining for total FGF-2 in human cardiac myofibroblasts with or without Angiotensin II

Panels A and D: Nuclear staining of cardiac myofibroblasts with Hoechst shown in blue. Myofibroblasts were starved for 24 hours in low serum before treatment. Cells were treated without (control) or with Ang II (10^{-7} M) for 48 hours.

Panels B and E: Immunofluorescent staining (shown in red) of total FGF-2 (hi- and lo-FGF-2) in cells treated without (control) or with Ang II (10^{-7} M) for 48 hours.

Panels C and F: Merge pictures with double staining of nuclei as shown in Panels A and D and FGF-2 as shown in Panels C and F, respectively. Sizing bars represent 20 μM.

Figure 15

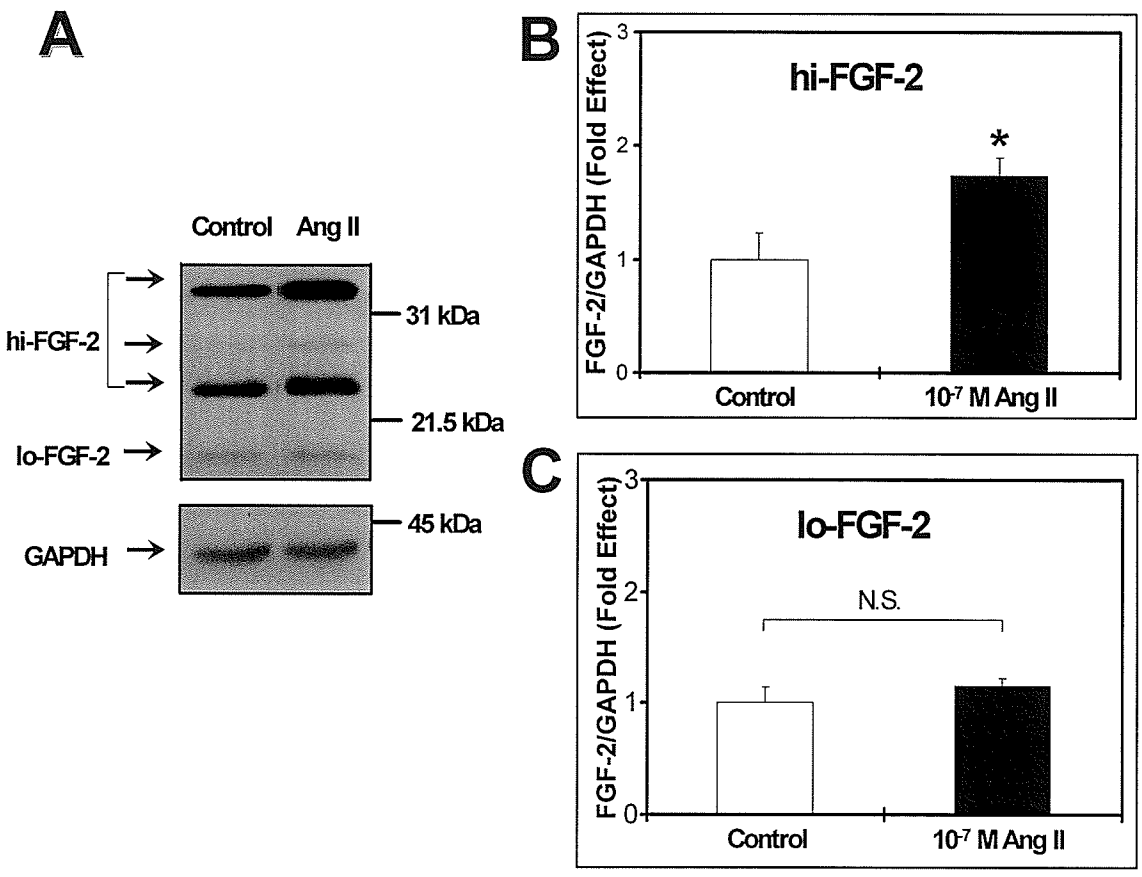

A
Control
Ang II
hi-FGF-2
lo-FGF-2
GAPDH
31 kDa
21.5 kDa
45 kDa
B
hi-FGF-2
FGF-2/GAPDH (Fold Effect)
0
1
2
3
*
Control
10-7 M Ang II
C
lo-FGF-2
FGF-2/GAPDH (Fold Effect)
0
1
2
3
N.S.
Control
10-7 M Ang II

Figure 15: Angiotensin II stimulated accumulation of hi-FGF-2 in human cardiac myofibroblasts

Panel A: Representative Western blot showing expression of hi-FGF-2 in human cardiac myofibroblasts untreated or treated with Ang II. Cells were grown in conditioned medium; and treated or not with 10^{-7} M Ang II for 48 hours after 48 hours of starvation in low serum.

Panels B and C: Quantitation of hi- and lo-FGF-2 isoforms, respectively, in human cardiac myofibroblasts untreated or treated with Ang II (10^{-7} M). Data are expressed as mean ± SEM, where control is set to 1. Statistical comparison shows *$P<0.05$ vs. control using Tukey-Kramer Multiple Comparisons Test (Panel B). The band intensities for FGF-2 were normalized against GAPDH, the loading control (n=3/group). No significant difference was observed with respect to lo-FGF-2 ($P>0.05$) as shown in Panel C.

Figure 16

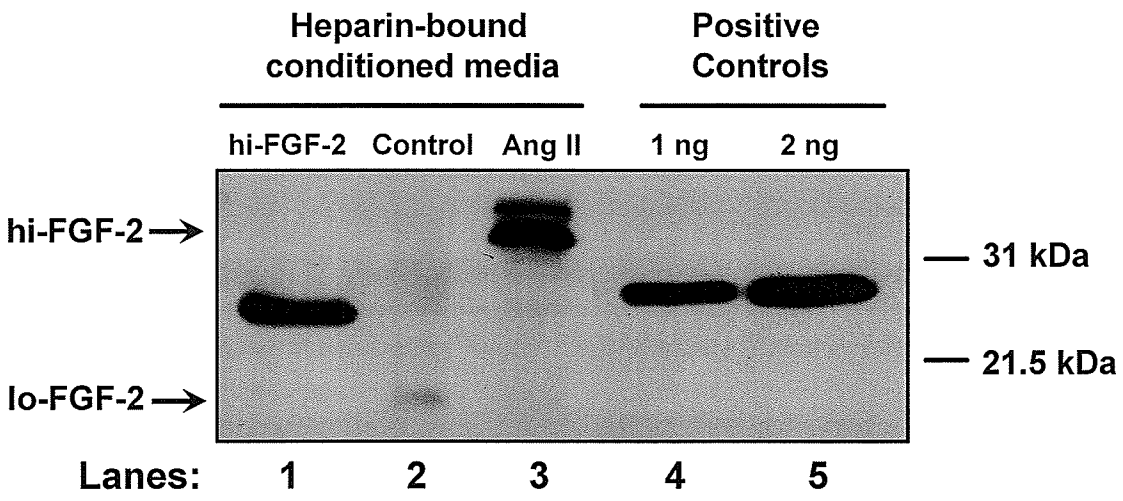


Figure 16: A 33-34 kDa hi-FGF-2 is released into the conditioned media of human cardiac myofibroblasts

Representative Western blot probed for FGF-2. Lane 1 contains the heparin-Sepharose-bound fraction from culture medium supplemented with 1 ng of recombinant hi-FGF-2 (positive control). Lanes 2 and 3 contain the heparin-Sepharose-bound fraction obtained from conditioned media of untreated or 10^{-7} M Ang II-treated confluent human cardiac myofibroblasts (48 hours of starvation time before additional 48 hours of treatment), respectively (using five 100 mm plates of cells in total for each group). Lanes 4 and 5 represent non-heparin enriched samples of recombinant rat hi-FGF-2 at different protein concentrations (1-2 ng of protein), denatured in 1x SDS buffer, and used as additional positive controls.

Figure 17

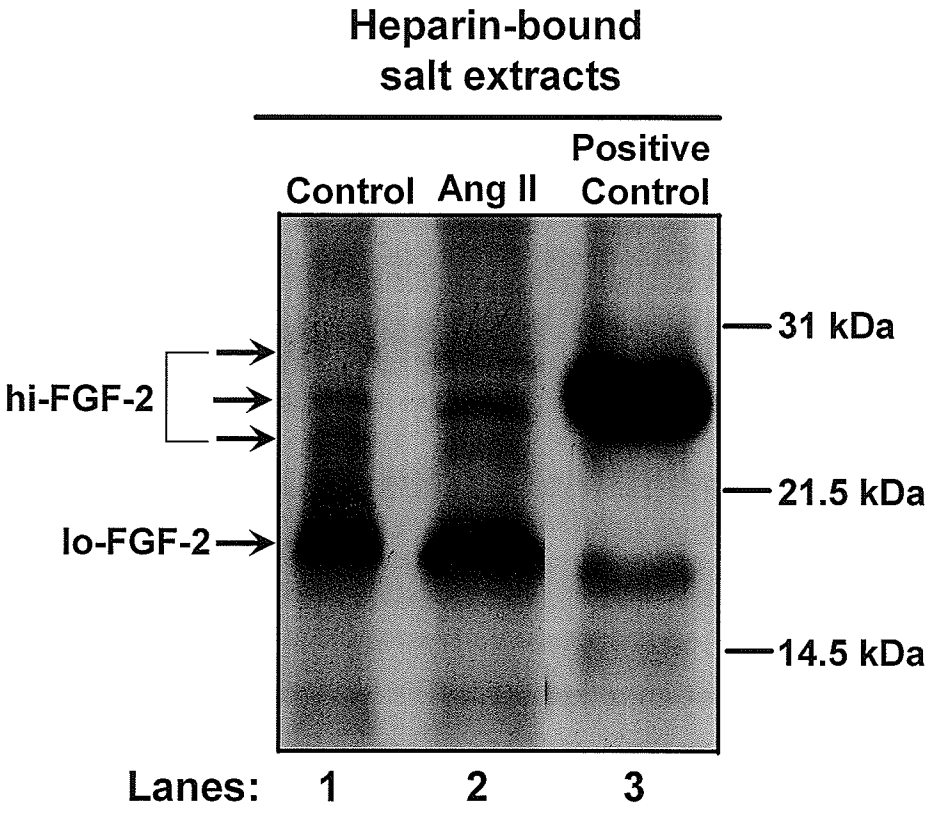
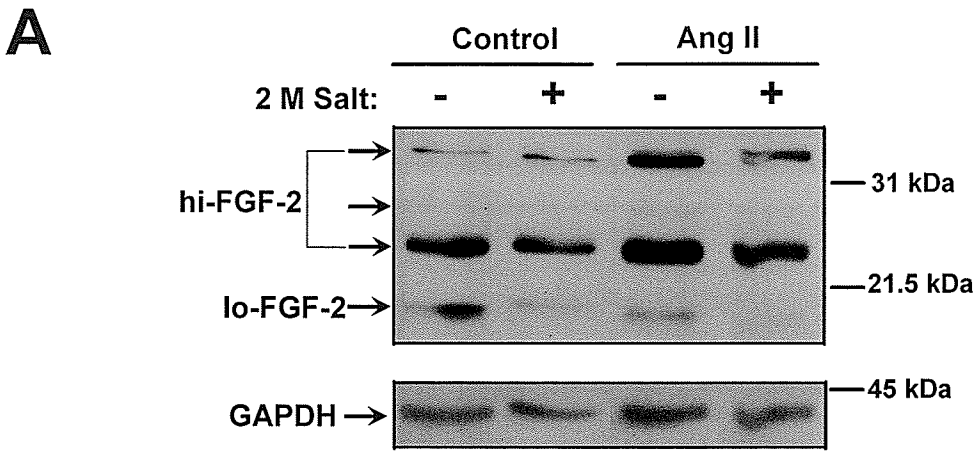
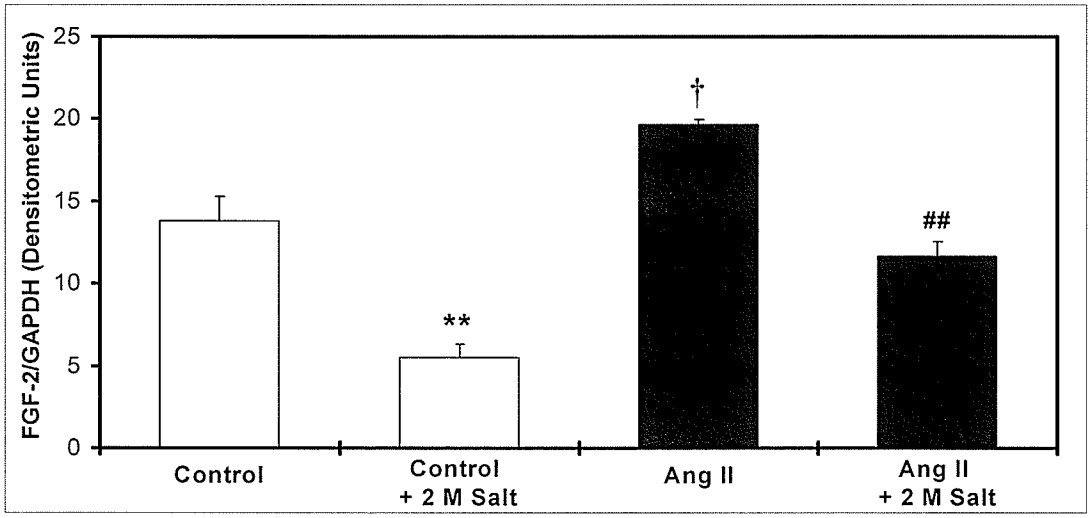


Figure 17: Cell surface and extracellular matrix-associated hi- and lo-FGF-2 in human cardiac myofibroblasts

Secreted FGF-2 proteins, bound to the cell surface and extracellular matrix, were extracted with a 2 M salt wash. The heparin-Sepharose-bound fraction of the 2 M salt wash was analyzed by Western blotting for FGF-2. Lane 1 contains the heparin-bound fraction from the salt wash of untreated cells. Lane 2 contains the heparin-bound fraction from the salt wash of Ang II-treated cells (10^{-7} M for 48 hours after 48 hours of starvation). Lane 3 contains the heparin-bound fraction from the salt extraction buffer supplemented with recombinant hi-FGF-2 (1 ng).

Figure 18


A
Control
Ang II
2 M Salt:
-
+
-
+
hi-FGF-2
lo-FGF-2
GAPDH
31 kDa
21.5 kDa
45 kDa


B
FGF-2/GAPDH (Densitometric Units)
25
20
15
10
5
0
**
†
##
Control
Control
+ 2 M Salt
Ang II
Ang II
+ 2 M Salt

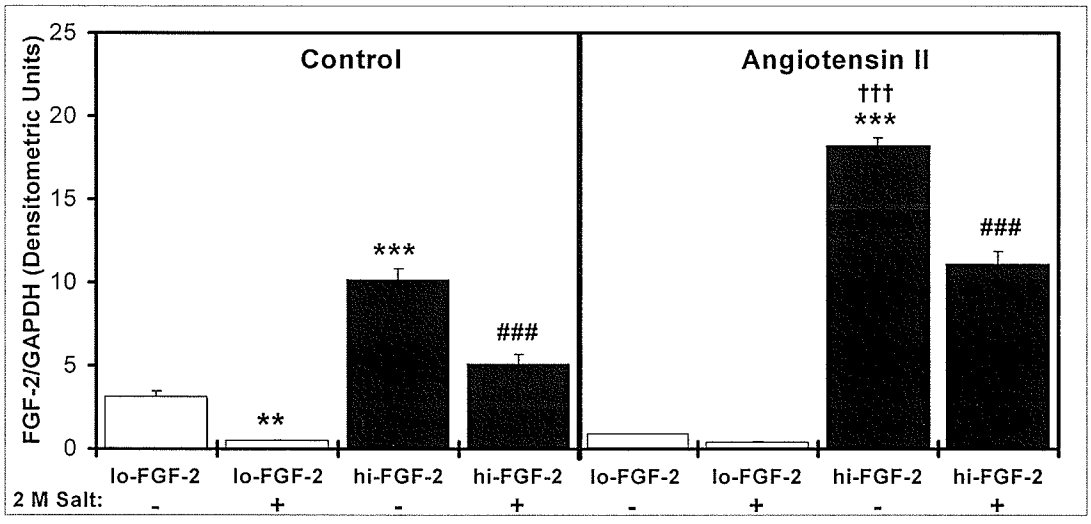

C
FGF-2/GAPDH (Densitometric Units)
25
20
15
10
5
0
Control
Angiotensin II
**
***
###
†††
***
###
lo-FGF-2
lo-FGF-2
hi-FGF-2
hi-FGF-2
lo-FGF-2
lo-FGF-2
hi-FGF-2
hi-FGF-2
2 M Salt:
-
+
-
+
-
+
-
+

Figure 18: Analysis of cell-extracted FGF-2 in human cardiac myofibroblasts before and after 2 M salt wash

Panel A: Representative Western blot for hi- and lo-FGF-2 present in lysates obtained from confluent human myofibroblast cell cultures subjected (+) or not (-) to a 2 M salt wash, and treated or not with Ang II (10^{-7} M) for 48 hours after 48 hours of starvation in low serum, as indicated.

Panel B: Quantitation of total FGF-2 present in lysates obtained from human myofibroblast cell cultures subjected (+) or not (-) to a 2 M salt wash, and treated or not with Ang II (10^{-7} M) for 48 hours after 48 hours of starvation in low serum, as indicated. The band intensities for FGF-2 were normalized against GAPDH, the loading control. The data are expressed as mean ± SEM. Statistical difference (using Tukey-Kramer Multiple Comparisons Test) is shown as $^{**}P<0.01$ vs. control, $^{\#\#}P<0.01$ vs. Ang II, and $^{\dagger}P<0.05$ vs. control (n=3/group).

Panel C: Quantitation of hi- and lo-FGF-2 present in lysates obtained from human myofibroblast cell cultures subjected (+) or not (-) to a 2 M salt wash, and treated or not with Ang II (10^{-7} M) for 48 hours, as indicated. The band intensities for FGF-2 were normalized against GAPDH, the loading control. Data are expressed as mean ± SEM. Statistical difference using Tukey-Kramer Multiple Comparisons Test is shown as $^{**}P<0.01$ vs. control lo-FGF-2 (no salt), $^{***}P<0.001$ vs. control/Ang II-treated lo-FGF-2 (no salt) respectively, $^{\#\#\#}P<0.001$ vs. control/Ang II-treated hi-FGF-2 (no salt) respectively, and $^{\dagger\dagger\dagger}P<0.001$ vs. control hi-FGF-2 (no salt), where n=3 per group.

Chapter 4 Discussion

4.1 Cardiac fibroblasts acquire contractile and synthetic features during differentiation into myofibroblastic phenotype

Our studies used neonatal cardiac non-muscle cells as a model for cardiac fibroblasts or myofibroblasts. We undertook a characterization study in order to ascertain the identity and phenotype of the isolated cells. Studies-to-date have shown that myofibroblasts resemble fibroblast cells by having an extensive rough endoplasmic reticulum and Golgi apparatus, but in contrast to fibroblasts, myofibroblasts display *de novo* expression of α-SMA, supermature focal adhesions, and in several cases, an embryonic isoform of myosin heavy chain or SMemb (Gabbiani et al, 2003). The acquisition of specialized contractile features is essential for the modulation of fibroblastic cells towards the myofibroblastic phenotype (Tomasek et al, 2002). Therefore, in our study, the cells were analyzed for the presence of vimentin and α-smooth muscle actin, representative markers of fibroblasts and myofibroblasts, respectively. Our studies indicated that the isolated cells were indeed fibroblasts (Figure 1), and that after P0 they started expressing α-SMA as shown in Figure 2. In other words, our cells acquired a myofibroblast phenotype after P0, as established previously (Vaughan et al, 2000). Our data also indicated that expression of α-SMA was not affected by Ang II (10^{-8} M) treatment (Figure 1B), in contrast to a report by Tsuruda et al (2005) showing a significant increase in α-SMA in the presence of Ang II. This apparent discrepancy may reflect differences in: (a) experimental model, since these authors employed heart tissue sections, while we used isolated cells, and

the fact that the cells were sub-cultured contributing further to artificial conditions in expressing α-SMA; and (b) method of measurement, since they used an immunohistochemistry-based assessment while we used Western blot detection. At this point, however, one cannot exclude the possibility that the discrepancy may reflect different behavior of cells *in vitro* versus *in vivo*, and needs to be further investigated.

In order to compare results between different preparations of our cells, it was important to examine the effect of passage number on cell phenotype. Cells were thus examined through P0-P3 for markers of myofibroblasts and focal adhesion complexes. Consistent with other studies (Vaughan et al, 2000; Tomasek et al, 2002; Raizman et al, 2007), we found a significant increase in α-SMA between P2-P3 and a significant increase in expression of SMemb at the very first passage until P3 (Figure 2). Interestingly, vimentin also showed a significant increase but only at the latest passage (P3). We speculate that at later passages, i.e. P3 and beyond, cells revert back to the fibroblastic phenotype as is often the case after normal wound healing (Darby et al, 1990). At this point further studies must be undertaken to determine if cells at P3 and beyond are reverting back to fibroblasts or perhaps as proto-myofibroblasts, a subclass of intermediate fibroblasts before fully acquiring a myofibroblastic phenotype (Tomasek et al, 2002). It is important to point out that there is a physiological significance to this phenomenon since myofibroblasts that do not undergo apoptosis or do not revert back to fibroblastic phenotype, for some unknown reason, are the major cause of excessive collagen production or fibrosis during

wound healing. Thus many studies are being conducted to identify factors that may stimulate myofibroblasts to differentiate back to a fibroblastic phenotype.

Analysis of total cell lysates also showed a significant upregulation of focal adhesion proteins, suggesting that these cells become less motile and more anchored into the extracellular matrix. As shown in Figure 3, expression of paxillin, a multi-domain protein that localizes primarily in focal adhesion sites which are the structural links between the extracellular matrix and the actin cytoskeleton of cells (Tuner et al, 2000), was increased at P3, while accumulation of anchor proteins, vinculin and tensin, were also significantly increased between P1-P3 and P2-P3, respectively. Expression of a splice variant of fibronectin (ED-A sequence), which has been shown to be important in further myofibroblastic differentiation (Serini et al, 1998), also increased between P2-P3. Focal adhesion kinase, a non-receptor protein tyrosine kinase and a key element in cell spreading, differentiation, migration and cell cycling, was also found to be upregulated between P2-P3. Altogether, our data suggested that the number of passages influences the phenotypic modulation of fibroblasts into myofibroblasts. Furthermore, we have unpublished data supporting the notion that these cells not only acquire contractile features but also become synthetic in regards to the production of various cytokines (including FGF-2) and collagen.

Thus, because passage number influences the phenotype of fibroblasts in our rat model system, we used cells that are no greater than P3 in order to be consistent throughout the studies. We have also determined that both lo- and hi-

FGF-2 isoforms significantly increase within these passage numbers when compared to P0 (first plating of cells after isolation).

4.2 Cardiac myofibroblasts express predominantly hi-FGF-2 over lo-FGF-2 isoforms

Cardiac fibroblast cells are a major source of FGF-2 (Akimoto et al, 1999), but there is no information as to whether the transition from fibroblast to myofibroblast, and the passage number, affects FGF-2 expression. Relative expression of hi- versus lo-FGF-2 is also not known for these cells. To examine expression of FGF-2 isoforms during the phenotypic modulation of fibroblasts into myofibroblasts, the cells were grown at different stages (P0-P3), and once cells reached confluence, total cell lysates were analyzed. Two major findings were obtained. Firstly, rat cardiac fibroblasts (P0) or myofibroblasts (P1-P3) were found to express predominantly (over 90% of total) hi-FGF-2 (Figure 5). Secondly, expression of both hi- or lo-FGF-2 by cells in P1-P3 increased significantly over expression in P0, reaching maximal levels at P2 (Figure 4). Accumulation of the FGF-2 protein is therefore upregulated during the transition from fibroblast to myofibroblast, and may play an active role in this process, in an autocrine/paracrine manner. This is in agreement with reports that FGF-2 stimulates expression of α-SMA in cardiac myofibroblasts (Cheng et al, 2002). From this point and onwards, all our subsequent studies using rat cardiac myofibroblasts were conducted in confluent P1-P3 cells.

Rat hi- and lo-FGF-2 isoforms were identified based on antibody recognition and electrophoretic mobility; bands migrating at 22-23 kDa were presumed to be hi-FGF-2. Nevertheless, the possibility that the slower electrophoretic motility of immunoreactive bands reflected a post-translational modification of lo-FGF-2, such as glycosylation, needed to be examined; and because several members of the FGF family have been shown to be glycosylated, for example FGF-4, FGF-5, FGF-9, and FGF-16 (Bates et al, 1991; Miyakawa et al, 1999; Revest et al, 2000; Miyakawa et al, 2003), there is a possibility that FGF-2 may also be glycosylated. However, as endoglycosidase treatment did not noticeably affect the motility of the 22-23 kDa immunoreactive bands (Figure 9), we concluded that glycosylation was not a significant parameter in FGF-2 electrophoretic migration and that the slower migrating bands were indeed the hi-FGF-2 isoforms. The apparent slight increase in the 22 kDa and 18 kDa bands at 4 hours of incubation period may be due to the increase in activity of proteases leading to the degradation of 23 kDa hi-FGF-2 into 22 kDa or 18 kDa isoforms at longer time point in a 37°C water bath. However, we think this is not the case since we have incorporated protease inhibitors during protein extraction. Perhaps, endoglycosidase treatment affected the activity of protease inhibitors which may then lead to the increase activity of proteases and degradation of hi-FGF-2. This however requires further examination. At any rate, the apparent increase seems to be minor, so any contribution of glycosylation is not expected to contribute in a substantial manner.

As shown in Figure 5A, hi-FGF-2 isoforms migrated at 22-23 kDa and lo-FGF-2 at 18 kDa in our rat model system. Human embryonic myofibroblasts, on the other hand, presented hi-FGF-2 bands not only at 22-24 kDa but also at 34 kDa (Figure 13). This finding is fully in agreement with previous reports on human hi-FGF-2 (Arnaud et al, 1999), and also demonstrated that the 34 kDa FGF-2 isoform is expressed not just in transformed cell types but in primary cells as well.

Predominant expression of hi-FGF-2 by cardiac myofibroblasts (>90% of the total in both rat and human species) as shown in Figures 5 and 13, is intriguing due to its potential physiological significance. As mentioned in the Introduction section, hi- and lo-FGF-2 display both similar and isoform-specific properties. When acting without exiting the cell, in an intracrine fashion, hi- (but not lo-) FGF-2 can promote apoptosis; when acting from the outside, on plasma membrane receptors, both types of isoforms can exert cytoprotection and stimulate proliferation, but only lo-FGF-2 can promote cell migration (see review Kardami et al, 2007). In addition, previous work in our lab has demonstrated that the hi-FGF-2 (but not lo-FGF-2) isoform(s) stimulated significant cardiomyocyte hypertrophy both *in vitro* and *in vivo* (Jiang et al, 2007). Thus expression of hi-FGF-2 by cardiac myofibroblasts suggests the possibility that these cells contribute to the development of myocardial hypertrophy *in vivo*.

The mechanism underlying predominant accumulation of hi-FGF-2 in cardiac myofibroblasts is currently unknown. We speculate that the interaction of as yet unknown cell-specific factors with the 5'- and/or 3'-untranslated regions of

the FGF-2 mRNA determines whether translation will be initiated in an AUG or CUG site. It is also theoretically possible that protease(s) capable of converting hi- to lo-FGF-2 by limited proteolysis of the N-terminal region, and thus increasing relative levels of lo-FGF-2, are downregulated in these cells.

4.3 Angiotensin II stimulates hi-FGF-2 accumulation in primary cardiac myofibroblasts: requirement for the AT_1 receptor

Ang II mediates development of maladaptive cardiac hypertrophy, fibrosis, and heart failure (Sadoshima et al, 1993, Dostal et al, 1997, Billet et al, 2007); and studies have shown that the effects of Ang II on hypertrophy require FGF-2 expression (Pellieux et al, 2001). Ang II has also been reported to stimulate expression of the FGF-2 gene in cardiac myocytes and microvascular endothelial cells (Fischer et al, 1997), and contribute to the upregulation of hi-FGF-2 in neuronal cell type (Peng et al, 2002; Wang et al, 2005). Thus we hypothesized that Ang II would stimulate accumulation of pro-hypertrophic FGF-2 (i.e. the hi-FGF-2 isoform) in cardiac myofibroblasts. Indeed our data showed upregulation of hi-FGF-2 in Ang II dose- and time-dependent manner. Ang II was effective at concentrations within the physiological and/or pathophysiological range, at 10^{-8} – 10^{-6} M (Figure 7); and hi-FGF-2 increases were evident as early as 24 hours after treatment, lasting for at least 48 hours (Figure 6). While some increase in lo-FGF-2 was also detected in response to Ang II, increases in hi-FGF-2 were far greater than increases in lo-FGF-2 when comparing the same concentration of Ang II at the same time point. In effect, therefore, Ang II could be seen as

preferentially upregulating the pro-hypertrophic hi-FGF-2. In addition, as the stimulatory effect of Ang II on hi-FGF-2 accumulation was also evident in adult rat cardiac myofibroblasts (Figure 8) and human cardiac myofibroblasts (Figures 14 and 15), there is a great possibility that the Ang II effect is non-age or species-specific.

As mentioned, it has been established that Ang II increases the level of FGF-2 mRNA (Fischer et al, 1997), however this does not translate to an increase accumulation of FGF-2 proteins. This is why we investigated the protein content and isoform composition of FGF-2 in cardiac myofibroblasts. Because there is a preferential upregulation of hi-FGF-2 in these cells and both FGF-2 comes from a single mRNA, a possible role of Ang II in the translational regulation of FGF-2 can be inferred, although there are no specific studies on the topic. We also cannot ignore the fact that Ang II may be involved in FGF-2 mRNA and protein stability (pre- and post-translational processing). It has been shown that Ang II can increase stability of monocyte chemoattractant protein-1 mRNA due to the activation of a soluble inhibitor of RNase (Liu B et al, 2006); and Ang II can increase phosphorylation of initiation factors and its binding proteins that regulate the initiation phase of protein translation (Feliers et al, 2005). Ang II may also activate factors that are involved in hi-FGF-2 protein stability so that susceptible sites are no longer accessible to proteases. Nevertheless, oxidative stress has been shown to stimulate preferential translation of hi-FGF-2 (Vagner et al, 1995). Since Ang II has been implicated in the production of oxygen radicals via NAD(P)H oxidase, thus contributing to oxidative stress (Hitomi et al,

2007), it is possible that the effects of Ang II on hi-FGF-2 accumulation are the result of oxidative stress.

Ang II has also been implicated in promoting apoptosis of cardiomyocytes, compounding the effects of progressing hypertrophy towards heart failure (Dimmeler et al, 2000). It is therefore intriguing that upregulation of intracellular nuclear hi-FGF-2 also leads to chromatin compaction and cell death by an intracrine mechanism (Ma et al, 2007). In the present study, we have not assessed the role of Ang II-mediated hi-FGF-2 accumulation in the induction of apoptosis during our treatment. However, we have determined that hi-FGF-2 accumulates in the nucleus of cardiac myofibroblasts in the presence of Ang II (Figure 14). Thus, we can only speculate that perhaps Ang II-induced hypertrophy may be mediated by pro-hypertrophic hi-FGF-2 as an adaptive response; however, prolonged stimulation or presence of increased Ang II may lead to accumulation of hi-FGF-2 in the nucleus, over time leading to cell death and the subsequent heart failure. This however remains to be investigated.

In mammalian cells, Ang II operates via the Ang II Type 1 (AT_1) and Type II (AT_2) plasma membrane receptors (Touyz et al, 2002). The receptor subtypes can be distinguished pharmacologically using specific antagonists: AT_1 receptors can be blocked by biphenylimidazoles including losartan, whereas AT_2 receptors are inhibited by tetrahydroimidazopyridines such as PD123319 (Touyz et al, 2002). We found that the Ang II–induced hi-FGF-2 upregulation was prevented by losartan but not PD123319 (Figure 10), and thus concluded that it is mediated by the G-protein coupled AT_1 receptor. Ang II-induced cardiac hypertrophy is

also mediated by the AT_1 receptor; as this receptor is more abundant in cardiac fibroblasts compared to cardiomyocytes (Gray et al, 1998), it is possible that the pro-hypertrophic effect of Ang II in the heart is exerted, to a large extent, indirectly by binding to the fibroblast AT_1 receptors and promoting expression and release of growth factors such as hi-FGF-2 by these cells (Kim et al, 1995; Gray et al, 1998; Manabe et al, 2002). These factors can then act on cardiomyocytes to promote hypertrophic growth.

4.4 Hi-FGF-2 is released by primary cardiac myofibroblasts in response to Angiotensin II

In order for hi-FGF-2 to have a physiologically relevant role in the induction of myocardial hypertrophy, it must be capable of being released by cardiac myofibroblasts, so that it can then act on myocyte plasma membrane FGF-2 receptors. FGF-2 release to the environment is well documented, despite the absence of a traditional secretory sequence, but the prevailing belief is that only the lo-FGF-2 isoform is released by cells (Florkiewicz et al, 1995; Delrieu, 2000; Ornitz et al, 2001). Nevertheless a few studies have reported that hi-FGF-2 can be released under specific conditions. Some of these reports can be challenged because investigators have employed non-physiological conditions using cell lines overexpressing hi- or lo-FGF-2 (Piotrowicz et al, 1997; Giampietri et al, 2000; Taverna et al, 2003). Because of lack of concrete knowledge concerning its release by primary, non-overexpressing cells, we investigated if conditions (such as Ang II stimulation) that promote release of pro-hypertrophic

factors from cardiac myofibroblasts, would also stimulate release of hi-FGF-2 to the extracellular environment. Our data showed the presence of ~23 kDa hi-FGF-2 in the conditioned medium of rat cardiac myofibroblasts in response to Ang II stimulation (Figure 11), leading us to conclude that hi-FGF-2 can indeed be released by primary cells, possibly by a regulated mechanism. Release of hi-FGF-2 is not likely to be the result of lethal cell damage, since our cultures, examined morphologically and also for release of cytosolic enzymes to the medium (unpublished data) did not show any evidence of injury.

Because cells were incubated in low-serum containing medium for the release studies, one should consider the possibility that the 23 kDa immunoreactive band represented serum-derived FGF-2 that had been retained by cells and remained bound even after repeated washes. We do not think this to be the case because the 23 kDa protein was not detected in conditioned medium from non-Ang II–treated cells.

Human myofibroblasts also released hi-FGF-2 to the conditioned medium in response to Ang II. Surprisingly, it was the 34 kDa isoform but not the 22-24 kDa hi-FGF-2 that was released (Figure 16). This is the first time, to our knowledge, that 34 kDa hi-FGF-2 has been detected in the conditioned medium from primary cells, and its preferential release in response to Ang II suggests that it may have different functions compared to the other isoforms. It remains to be seen what these functions may be; there is no information as to whether the 34 kDa hi-FGF-2 is pro-hypertrophic. However, the 34 kDa human hi-FGF-2, but none of the other isoforms, has been proposed by Arnaud and colleagues (1999)

to act as a survival factor when cells are kept in low serum. Whether this is an intracrine or autocrine/paracrine activity is not known. Teshima-Kondo et al (2004) and Prats et al (2002) have reported that under stress conditions (γ-irradiation, hypoxia, heat shock, and hyperglycemia), cap-dependent translation of FGF-2 producing the 34 kDa species is inhibited and IRES-mediated translation is enhanced producing 18, 22, 22.5, and 24 kDa FGF-2. The increased 34 kDa FGF-2 accumulation found in our cells would suggest that the Ang II concentrations used in our experiments did not represent 'stress conditions' and did not block cap dependent translation.

The mechanism by which hi-FGF-2 is released by myofibroblasts in the presence of Ang II is unknown, but we expect it is similar to the mechanisms proposed for lo-FGF-2 release as reviewed in the Introduction. Taverna et al (2003) have recently reported that secretion of all FGF-2 isoforms (with the exception of the 34 kDa species) occurs in an energy-dependent shed-vesicle-mediated export. Zehe et al (2006) have also reported a role of low affinity heparan sulfate proteoglycans in the export of FGF-2 isoforms via membrane translocation. Warnock et al (1999) have described the identification of p32, a nuclear trafficking protein that selectively interacts with hi-FGF-2 isoforms but not lo-FGF-2. This interaction can target hi-FGF-2 to the nucleus of cells and thus away from the export machinery governing FGF-2 release (Trudel et al, 2000). Preferential upregulation of hi-FGF-2 in the presence of Ang II may saturate this interaction and mask the negative control of p32 permitting the release of hi-

FGF-2 from cells. It is also possible that perhaps Ang II may play a role in the expression or regulation of p32.

4.5 Exported FGF-2 can remain associated with the cells

FGF-2 isoforms are rarely detected in conditioned media unless they are significantly overexpressed (Bikfalvi et al, 1997); or if cells are specifically stimulated to release FGF-2 into the extracellular environment (Yu et al, 2007). Studies have shown that translocation of FGF-2 across the plasma membrane may occur but the proteins remain tethered at the cell surface, bound to heparan sulfate proteoglycans. Thus, proteins may be released by cells but retained at the cell surface and may not reach the conditioned media (Trudel et al, 2000).

We examined the extracellular destination of FGF-2 isoforms released by cardiac rat or human myofibroblasts, in both control and Ang II treated cells. Two possible locations of cell-exported FGF-2 were examined: (a) the extracellular matrix/plasma membrane, where FGF-2 would be expected to be stored and/or exert autocrine/paracrine activities, and (b) the conditioned medium, suggestive of systemic release and action of FGF-2, as discussed in the previous section. Matrix and plasma membrane associated FGF-2 was obtained by a gentle 2 M salt wash of the cell layer after removal of the conditioned medium. Previous studies have shown that FGF-2 generated by fibroblasts can indeed be released by this treatment, without breaking or damaging the cells (Murakami-Mori et al, 1998; Dowd et al, 1999; Molloy et al, 2003; Khalil et al, 2005).

By measuring the FGF-2 content and isoform composition in lysates from cells obtained before or after the 2 M salt wash, we were able to show that a substantial amount of cell-produced FGF-2 was exported/released but remained associated with the cells, in agreement with previous reports. For the first time, however, our data have shown that hi-FGF-2 isoforms predominated in the extracellular, cell-bound FGF-2 fraction. This was the case for both rat and human myofibroblasts. Cell-bound hi-FGF-2 therefore can also be considered a candidate for autocrine-paracrine activity.

An apparent discrepancy in the thrust of our data from both rat and human cells was evident when we used a different approach to analyze cell and matrix-bound FGF-2, namely when we examined the FGF-2 content and composition of the 2 M NaCl cell washes. In the case of rat cells, this approach indicated that Ang II actually decreased the levels of extracellular and cell associated FGF-2 (hi- and lo-FGF-2) as shown in Figure 12. In the case of human cells, this approach indicated that only lo-FGF-2 was released and cell-bound; and that Ang II had no effect (Figure 17). While the quantitative discrepancies can be explained by difficulties in assessing proper loading of gel lanes, and errors introduced during the several steps required for this analysis, it is more difficult to reconcile qualitative differences (compare for example Figure 17, showing lo-FGF-2 as the FGF-2 isoform present in the 2 M salt wash, and Figure 18, showing that hi-FGF-2 levels in cell lysates are decreased by the 2 M salt wash). In the case of human cells, we speculate that any hi-FGF-2 present in the 2 M NaCl wash (Figure 17) may have been subjected to partial degradation during

the elution process by co-extracted extracellular proteases that have escaped the protease inhibitors in the buffer through binding to heparin-Sepharose beads accordingly, resulting in the conversion of hi-FGF-2 to the 18 kDa isoform. This would be in agreement with the report by Doble et al (1990), who showed that heparin-bound hi-FGF-2 was converted to lo-FGF-2 by co-extracted and heparin-binding protease(s). Proteolysis could also explain the differences in data from rat cells; compare Figure 12A with Figure 12C. There is also a possibility that hi-FGF-2 is secreted normally but degraded in the extracellular matrix and stored as lo-FGF-2. However, we think this is not the case since transport across the plasma membrane involves HSPGs (Zehe et al, 2006), which serve as local reservoir for FGF-2 and offer protection from proteolytic degradation (Vlodavsky et al, 1991). Altogether, we think that measuring FGF-2 content and isoform composition in lysates from cells before and after high salt wash is a more reliable method than assessing the content of the salt wash because (a) it requires fewer steps and thus is prone to fewer systematic errors, (b) allows for quantitation controls (through GAPDH), and (c) is less subject to degradation during handling because cells are lysed directly into SDS/PAGE sample buffer.

Overall, a number of similarities were identified between rat and human myofibroblasts. First, both rat and human cardiac myofibroblasts expressed predominantly hi-FGF-2 isoforms. Second, both released hi-FGF-2 into the conditioned medium in response to Ang II. This would indicate that in both species hi-FGF-2 released by cells can find its way into the soluble phase, 'escaping' from the HSPGs at the cell surface and matrix, and by extrapolation to

the *in vivo* situation, is potentially capable of systemic action. A potential for systemic action for FGF-2 has been inferred by its detection in the serum and other body fluids (Olson et al, 1992; Soutter et al, 1993; Leunig et al, 1998). On the other hand, rat cells released a 23 hi-FGF-2 to the medium, while human cells released the 33-34 kDa isoform, and not the human 22-24 kDa hi-FGF-2 species. The significance and/or mechanism underlying this difference are currently unknown; the N-terminal extension in rat hi-FGF-2 has regions which differ in sequence to those of human hi-FGF-2 of similar size. One can speculate that these sequence differences may prevent human 22-24 kDa hi-FGF-2 from 'escaping' the attraction of HSPGs at the cell surface and matrix. Third, in both rat and human myofibroblasts, Ang II upregulated hi-FGF-2; and fourth, in both species a substantial amount of hi-FGF-2 was extracellular (presumably released from cells) while remaining in tight association with the cell surface/extracellular matrix. Cell and matrix-associated hi-FGF-2 would, presumably, be available for interaction with plasma membrane FGF-2 receptors of nearby cells. While there is no information as to the mechanism(s) that determines whether the FGF-2 isoforms remain cell-surface associated or become released to the medium (or circulation), it is possible that any factor(s) preventing or weakening the FGF-2-HSPG interaction, or conditions promoting degradation of HSPGs by heparinases, as might occur during injury, would increase the concentration of free FGF-2 to the medium.

Our data suggested that cardiac myofibroblast-released hi-FGF-2 may have both long-range (systemic) as well as local (cell and matrix-associated)

effects. We cannot comment on the role of lo-FGF-2 because its relative levels were quite low compared to hi-FGF-2 in both rat and human cells, and this made it difficult to assess relative differences accurately. Nevertheless, we failed to detect even traces of lo-FGF-2 in the conditioned medium of cells stimulated with Ang II, and this suggests that perhaps lo-FGF-2 remains preferentially bound to the cell surface and/or that 'liberation' of hi-FGF-2 (versus lo-FGF-2) to the conditioned medium is an isoform-specific event.

4.6 Concluding remarks and future directions

Our most significant findings are that cardiac myofibroblasts, a major cell type in the heart, preferentially upregulate and export/release hi-FGF-2 in response to the pro-hypertrophic peptide Ang II, and that this response is mediated by the AT_1 receptor. This finding implicates hi-FGF-2 as a mediator of Ang II-induced hypertrophy and ensuing heart failure, but remains to be demonstrated by direct studies.

Future studies should also address the particular role of the 33-34 kDa human hi-FGF-2 isoform, the mechanism of hi-FGF-2–induced release, and the mechanism of hi-FGF-2-induced hypertrophy. The possibility that the different hi-FGF-2 isoforms may play different roles needs to be examined as well. Finally, identifying the mechanism governing synthesis and/or accumulation of lo- and hi-FGF-2 represents a central missing component of FGF-2 biology.

In conclusion, our results have confirmed our hypothesis. We have shown, for the first time, that hi-FGF-2 can be released to the extracellular space in a

physiological or pathophysiological condition; until now hi-FGF-2 was presumed to remain nuclear and not play a role as an extracellular cytokine. Furthermore, we have validated our findings in the human cardiac myofibroblast model system, rendering them clinically relevant. By documenting the stimulation of hi-FGF-2 accumulation and release by Ang II, we have provided the foundation of a plausible scenario in which the known deleterious effects of Ang II on the heart can be attributed, in part, to hi-FGF-2 upregulation. Conversely, hi-FGF-2 down-regulation may contribute to the established beneficial effect of Ang II signaling inhibitors. Thus, the regulation of hi-FGF-2 expression and release by cardiac myofibroblasts provides a possible therapeutic target for reversal or management of heart disease.

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