Casein kinase 2 mediates nuclear disruption by high molecular weight Fibroblast Growth Factor 2

BY

XIN MA

A Thesis Submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Human Anatomy and Cell Sciences

Faculty of Medicine

University of Manitoba

Winnipeg , Manitoba

August 2004

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

Casein Kinase 2 Mediates Nuclear Disruption by High Molecular Weight Fibroblast Growth Factor 2

BY

Xin Ma

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

of

MASTER OF SCIENCE

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

There are many people to whom I owe a debt of thanks for their support over the last two years. First of all, I would like to sincerely acknowledge my supervisor, Dr. Elissavet Kardami. I will never forget your rigorous attitude toward science and straightforward and fair-minded character. Thanks for your kindly patience on my progress in the research and language and all guidance throughout the development of this study. Thanks for all the time you spent to share knowledge and expertise with me. Your comments have been of the greatest help at all times.

I would like to extend my sincere thanks to my advisory committee: Dr. Judy Anderson, Dr. Jiming Kong and Dr. Ian Dixon. Without your valuable suggestions and help, this master thesis would not have been possible.

I would also like to thank all colleagues in our lab. Special thanks are due to Robert Fandrich, Dr. Barb Nickel and Dr. Xitong Dang. It is you that teach me techniques and help me figure out every problem I met in the experiments. Without your support, it is hard to imagine what my experiment results would look like. Thanks to the other lab members: Zhisheng Jiang, Sarah Jimenez, Madhu Jeyaraman, Wattamon Srisakuldee, Jon –Jon Santiago. I really enjoy the time we worked together.

Lastly, I would like to thank my family members, my mum and dad, Fang Zhao and Yinglin Ma, my aunts Yong Zhao and Peilun Zhao, my sister Qingyan Zhao, my girlfriend Haiping Wang. Thanks for all of your unconditional love, encouragement and solicitude, to help me overcome countless obstacles.

ABSTRACT

Fibroblast growth factor 2 (FGF2) belongs to a large family of heparin binding growth factors, which consist of at least 23 structurally related proteins. It exists as multiple isoforms, resulting from alternative translation from different initiation codons within a single –copy gene. AUG codon generates 18 kDa (lo) FGF2 and CUG codons initiate 22, 22.5, 24 and 34 kDa (hi) FGF2. FGF2 affects many cell processes, inclusive of cell proliferation, differentiation, migration, apoptosis, angiogenesis and cardiomyocyte hypertrophy. Hi and lo FGF2 exhibit distinct and tissue-specific patterns of accumulation, intracellular distribution and biological activities. Stress treatments (heat shock or hypoxia) induce selective translation of the predominantly nuclear hi FGF2, as well as cell injury and apoptosis, suggesting a cause and effect relationship. Overexpression of hi FGF2 in cardiomyocytes elicited a distinct nuclear phenotype characterized by non-mitotic chromatin condensation ('clumping') and eventually leading to mitotic arrest and cell death seems to occur with apoptotic features, via an intracrine mechanism. The intracrine effects of hi FGF2 were mediated by intracellular FGF2 receptor 1 (FGF R1) and the extracellular signal kinase (ERK) 1/2. We have now used a cell line, human embryonic kidney cells (HEK 293) to explore further the mechanism of hi FGF2-induced chromatin clumping. Firstly, we established that HEK293 cells responded to ectopic hi FGF2 overexpression in a manner similar to cardiomyocytes, i.e. by chromatin clumping and eventually apoptosis-like cell death, and that the effect was decreased significantly by pharmacological inhibition of ERK1 with PD 98059. Similar results were obtained using either human hi FGF2 or rat (Ds-Red labeled) hi FGF2. We then tested the hypothesis that casein kinase 2 (CK 2), a ubiquitous Ser/ Thr kinase

interacting with intracellular FGF2 via its beta subunits, was mediating chromatin clumping. Pharmacological inhibition of the catalytic subunits of CK2 (alpha subunits) with emodin or overexpression of a hi FGF2 mutant unable to bind the beta subunits of CK2 (hi-S117A-FGF2) significantly decreased DNA clumping. Overexpression of lo-FGF2 (localizes to cytosol and nucleus), the truncated N-terminal of hi FGF2 (localizes to the nucleus) had no effect on chromatin. Overexpression of a double hi FGF2 mutant (R149G-R151G) that does not localize to the nucleus also failed to elicit chromatin clumping.

In conclusion: We have shown that the intracrine effects of hi FGF2 on chromatin are not cell-type specific, as they occur in both primary cardiomyocytes and the HEK293 cell line. The signal transduction pathway leading to hi-FGF2-induced chromatin clumping requires the activity of ERK1/2 and of CK2, as well as the ability of the beta subunit of CK2 to interact with FGF2. Finally, nuclear localization of hi FGF2 is necessary for the chromatin effect, requiring both the N-terminal extension as well as the 'core' FGF2 sequence.

LIST OF FIGURES

ABBREVIATIONS

Ad..... adenovirus

AIP..... apoptosis-inducing factor

AUG..... methionine

BAMC..... bovine adrenal medullary cells

BCA..... bicinchoninic acid

Bcl-2.....B cell leukemia/lymphoma-2

bFGF..... basic fibroblast growth factor

BSA..... bovine serum albumin

°C.....Degrees Celcius

Ca^{2+}..... calcium

cDNA..... complementary deoxyribonucleic acid

CUG..... leucine

DMEM..... Dulbecco's Modified Eagle's medium

DN..... dominant negative

DNA..... deoxyribonucleic acid

DNase..... deoxynuclease

EDTA..... ethlenediaminetetraacetic acid

Erk..... extracellular signal regulated protein kinase

FBS..... fetal bovine serum

FGF2..... fibroblast growth factor 2

FGFR 1..... fibroblast growth factor receptor 1

g..... gram

Hi FGF2.....high molecular weight fibroblast growth factor 2

hr .....hour

HSPG..... heparin sulfate proteoglycan

Ig G.....immunoglobulin

IL – 6.....interleukin 6

IP 3..... 1,4,5 triphosphate

IRES..... internal ribosome entry site

kDa..... kilodalton

L..... litre

Lo FGF2.....low molecular weight fibroblast growth factor 2

M..... molar

MAPK..... mitogen-activated protein kinase

MEK 1..... MAP kinase kinase

m.o.i. .....multiplicity of infection

µg..... microgram

µl.....microliter

µM..... micromolar

mL..... milliliter

mM.....millimolar

mRNA..... messenge ribonucleic acid

NLS..... nuclear localization sequence

N.S. ..... not significant

PBS..... phosphate bufferes saline

PKC..... protein kinase C

PLC..... phospholipase C

PVDF..... polyvinylidene difluoride

RNA..... ribonucleic acid

RPM..... rotation per minute

SDS..... sodium dodecyl sulfate

SEM..... standard error of the mean

TBST..... Tris buffered saline with Tween

TUNEL.....TdT-mediated dUTP nick end labeling

UPA..... urokinase type plasminogen activator

VEGF.....vascular endothelial growth factor

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Introduction

Fibroblast growth factor 2 (FGF2), also known as basic FGF (or bFGF) is a prototypical member of a large FGF family which consists of at least 23 members (Kardami et al., 2004). This heparin-binding protein exists as multiple isoforms due to translation from alternative initiation codons within a single-copy gene. Translation from the traditional AUG codon produces low molecular weight (lo) FGF2 (18 kDa), while upstream CUG codons generate high molecular weight (hi) FGF2 (22-34 kDa). Most studies have focused on lo FGF2 since it can be released from cells and activate multiple signal transduction pathways through its cell surface receptor. Lo FGF2 modulates a variety of cell behaviors including proliferation, migration, differentiation and apoptosis. Hi FGF2 is presumed to remain intracellular/nuclear after biosynthesis, i.e. to exert effects in an intracrine mode, although there is increasing evidence that hi FGF2 can also be exported from the cells and therefore is capable of autocrine and paracrine effects as well. This review summarizes major findings on hi FGF2 in the last few decades, from our lab and from other groups.

1.2 Hi FGF2 Gene Expression: From gene to protein

Accumulation of hi FGF2 is developmentally regulated and shows tissue specificity. Hi FGF2 is the predominant FGF2 species of neonatal hearts, while lo FGF2 predominates in the adult myocardium (Liu et al., 1993). On the other hand, hi FGF2 in the rat central nervous system follows the opposite trend, which displays basal level at

early developmental stages and progressively approaches to a maximum in the adult (Delrieu, 2000). Tissue-specific accumulation (translation) of the hi FGF2 was also evident in transgenic mice overexpressing all isoforms of human FGF2. Brain, lung, liver, heart and spleen in these mice expressed both hi and lo FGF2 isoforms (human as well as the endogenous mouse isoforms) while kidney and skeletal muscles only expressed lo FGF2 (Coffin et al., 1995).

A single gene and mRNA produce all FGF2 isoforms. FGF2 transcription is regulated by various stimuli. Many bioactive peptides and hormones (angiotensin II, endothelin-1 and interleukin-1, parathyroid hormone, β adrenergic receptor stimulation, nicotinic acetylcholine receptors stimulation), as well as stress stimuli (angiotensin, oxidative stress) activate transcription of the FGF2 gene (Peng et al., 2001; Stachowiak et al., 1994). Protein kinase C (PKC) and cyclic adenosine 5'-monophosphate (cAMP) signaling pathways were implicated in up-regulation of FGF2 gene transcription induced by stimulation of angiotensin II receptor and acetylcholine receptor (Stachowiak et al., 1994). In addition FGF2 itself is capable of activating its own gene expression (Jimenez et al., 2004). There is evidence suggesting that activation of the FGF2 gene occurs by binding of nuclear FGF receptor 1 directly onto the FGF2 promoter (Peng et al., 2001; Stachowiak et al., 1994).

As all isoforms of FGF2 are derived from a single mRNA, whether the final product is hi or lo FGF2 is determined by regulation at the translational level. While most eucaryotic mRNAs (>90%) require cap-dependent translation initiation at their 5' end, the FGF2 mRNA is translated by both cap-dependent and IRES-dependent (internal ribosome entry) mechanisms: the 34 kDa CUG-initiated FGF2 is made by cap-dependent

translation, while a single IRES determines translation initiation of the remaining three CUG- and one AUG-initiated FGF2 isoforms (Arnaud et al., 1999).

Transformed cells have been reported to produce a higher level of CUG-initiated hi FGF2 compared to normal cells (Vagner et al., 1996). Heat shock or oxidative stress promotes preferential translation of hi FGF2 in normal cells such as primary human skin fibroblasts (Vagner et al., 1996). Hi FGF2 (but no lo) expression was also specifically affected by certain cytokines and hormonal status. Rat hippocampal astrocytes exhibit selectively elevated hi FGF2 as a consequence of epidermal growth factor (EGF), interleukin 1-beta or tumor necrosis factor alpha treatment (Kamiguchi et al., 1996). Thyroid status regulates hi FGF2 level in the rat in a tissue specific manner, for example, hypothyroidism promoted hi FGF2 accumulation in rat hearts but not in brain tissues (Liu et al., 1993).

Hi FGF2 is post-translationally methylated on arginines at different locations within the protein, which results in 1 or 2 kDa increase in molecular mass. Inhibition of methylation significantly decreased the nuclear localization of hi FGF2 in NIH 3T3 cells, indicating its role in regulating the intracellular distribution of hi FGF2 (Burgess et al., 1991; Klein et al., 2000; Pintucci et al., 1996).

1.3 Hi FGF2 intracellular localization

Hi FGF2 and lo FGF2 exhibit different subcellular distributions, which can result in distinct effects on cell phenotypes (Delrieu, 2000). Hi FGF2 was found predominantly in nuclear fractions in transfected NIH 3T3, SK-Hep-1 cells (Quarto et al., 1991a; Zagzag et al., 1990), Cos1 GM 7373 (Gualandris et al., 1994), OB Ros 17/2.8 cells (Xiao et al.,

2003) and Cos 7 cells (Bugler et al., 1991). Lo FGF2 was found mostly in the cytosol in transfected OB Ros 17/2.8 cells (Xiao et al., 2003), SK –Hep –1 cells, NIH 3T3 cells (Zagzag et al., 1990) and Cos 7 cells (Bugler et al., 1991). On the other hand, both lo and hi FGF2 have been localized to the nucleus of cardiac and neuronal cell types (Claus et al., 2003; Pasumarthi et al., 1994).

The mechanisms of hi FGF2 nuclear localization have been investigated by several groups. Hi and lo FGF2 differ only in the length of amino terminal extension, within which a 37 amino acid sequence between the second CUG and AUG codon has been identified as the nuclear localization signal (NLS) for human hi FGF2 (Patry et al., 1994; Quarto et al., 1991a). This NLS is responsible for the nuclear localization of human hi FGF2s. For the rat FGF2 an additional non-canonical NLS located within the AUG-initiated " core" sequence (common in both hi and lo FGF2) has been identified, requiring arginines 149 and 151(Claus et al., 2003; Dormond et al., 2004). Hi or lo FGF2 carrying a double mutation (R149G /R151G) fail to localize to the nucleus, resulting in cytoplasmic redistribution. These findings indicate that nuclear localization of rat hi FGF2 is governed by both amino terminal extension and specific sequences in the C-terminus (Claus et al., 2003; Dormond et al., 2004). They also imply that there may be differences in the mechanism of nuclear localization between human and rat hi FGF2. In addition to amino-acid sequence, Quarto *et al.* also found that post-translational methylation on arginine plays a key role for hi FGF2 nuclear accumulation (Pintucci et al., 1996).

1.4 Hi FGF2 release

Sequencing of the FGF2 gene does not reveal any secretory signal sequence. Nevertheless, lo FGF 2 is released into the extracellular matrix, biological fluid and plasma in vivo, through active exocytosis via an energy-dependent and ER-Golgi - independent mechanism (Kardami et al., 2004; Liu et al., 1993; Mignatti et al., 1992). This process involves the α-subunit of Na^+-K^+ ATPase on the cell plasma membrane (Dahl et al., 2000).

Increasing evidence shows that hi FGF2 can also be released actively in response to other stimuli, in addition to being passively released from dead or injured cells. Beta-estradiol induced hi FGF2 release from bovine arterial endothelial cells and HCAEC cells into medium, a process facilitated by overexpression of human 27 kDa heat shock protein (Albuquerque et al., 1998; Piotrowicz et al., 2001; Piotrowicz et al., 1997) and requiring the PKC signaling pathway and the cell surface estrogen receptor (Albuquerque et al., 1998). Furthermore, hi FGF2 can also be released through plasma membrane vesicle shedding. Addition of serum to serum-starved SK Hep 1 cells and NIH 3T3 cells induces extracellular vesicle shedding which is found to be carrying both lo and hi FGF2 (Taverna et al., 2003).

1.5 Biological activities

1.5.1 Proliferation and survival

Lo FGF2 is widely accepted as a mitogen and exerts its effect through activating its cell surface FGF receptor, as well as through internalization into cells and subsequent translocation into the nuclei (Bossard et al., 2003). One of its nuclear targets has been

identified to be Casein Kinase 2 (CK2) (Delrieu, 2000). Hi FGF2 stimulates MCF 7 cell proliferation in a manner similar to lo FGF2, whereas it has no effect on bovine arterial endothelial cells (Piotrowicz et al., 1999). Lan Ding *et al.* report that the truncated 18 kDa "core" sequence possesses as much mitogenic activity as hi FGF2 on MCF –7 cells and that the amino-terminal extension of hi FGF 2 has little effect on cell proliferation (Ding et al., 2002). Constitutive overexpression of hi FGF2 stimulates the proliferation of several cell lines including fibroblasts, ABAE and osteoblastic cells (Bikfalvi et al., 1995; Gualandris et al., 1999; Seghezzi et al., 1998; Xiao et al., 2003). Moreover, hi FGF2 confers upon transfected cells (from cell lines) tumorigenic properties characterized by the ability to grow in low-serum or serum-free medium, as well as anchorage – independent growth on soft agar (Bikfalvi et al., 1995; Estival et al., 1993; Estival et al., 1996; Gualandris et al., 1999; Xiao et al., 2003). Hi FGF2 exerts its effect through an intracrine mode of action (Bikfalvi et al., 1995; Stachowiak et al., 1997; Xiao et al., 2003) that is mediated by intracellular FGF receptor (Stachowiak et al., 1997). In vascular endothelial cells, hi FGF2 stimulates cell growth by up-regulating VEGF gene expression (Seghezzi et al., 1998).

1.5.2 Cell migration

Lo FGF2 is widely recognized as a potent stimulator for cell migration (e.g. vascular endothelial cells) and a major angiogenic factor for new capillary development (Detillieux et al., 2003). The effect of hi FGF2 on cell migration is cell type-dependent. Exogenously added hi FGF2 stimulates the migration of bovine coronary venular endothelial cells and NIH 3T3 fibroblasts at the same degree as lo FGF2 (Bikfalvi et al., 1995); (Gualandris et al., 1994). However, hi FGF2 displayed inhibitory activity on cell

migration of MCF –7 cells and bovine aortic endothelial cells (Piotrowicz et al., 1999). Antibodies against the 55 amino terminal extension of 24 kDa FGF2 abolish its inhibitory activity for cell migration (Piotrowicz et al., 1999).Furthermore, a truncated mutant of hi FGF2 (amino terminal extension plus downstream 31 amino acid sequence) demonstrates a similar negative effect on cell migration as wild type hi FGF2 (Ding et al., 2002). The inhibition of cell migration by hi FGF2 follows an estrogen receptor (ER)-dependent mechanism since hi FGF2 treatment is found to activate ER and depletion of ER abrogated its inhibitory effect on cell migration. Overexpression of a dominant negative form of FGFR 1 prevented hi FGF2-induced ER activation in NIH 3T3 cells, indicating FGFR 1's involvement in the signaling pathway. Even though the ERK1/2 kinase was activated in response to hi FGF2 treatment, this signaling pathway is not relevant to the inhibition of cell migration by hi FGF2 (Piotrowicz et al., 2001; Piotrowicz et al., 1999).

1.5.3 Additional Properties of Hi FGF2

The nuclear CUG –initiated FGF2 isoforms can regulate the expression of other cytokines such as interleukin 6 (IL-6), in a cell-specific manner. NIH 3T3 cells displayed up-regulated level of IL-6 in response to hi FGF2 overexpression and HeLa cells showed the opposite response (Delrieu et al., 1998; Delrieu et al., 1999). Effect on cell morphology by hi FGF2 have also been reported. Multinucleated or binucleated phenotypes were displayed in the NIH 3T3 cells, PC cells (sympathetic post-ganglionic neuronal cells) and cardiomyocytes overexpressing hi FGF2 (Claus et al., 2004; Pasumarthi et al., 1996; Quarto et al., 1991b).

1.6 FGFR 1 mediated nuclear signaling pathway

FGF2 interacts with two classes of receptors: low affinity FGF2 receptors (heparin sulfate proteoglycan, HSPG) and high affinity tyrosine kinase receptors (FGFR 1-4). HSPG is found in the extracellular matrix and as a component of the plasma membrane, can protect FGF2 from degradation and promotes the interaction of FGF2 with FGFR on the cell surface (Tumova et al., 2000). FGFR 1-4 share a highly homologous structure consisting of an extracellular ligand –binding domain, a transmembrane domain and an intracellular tyrosine kinase domain(Johnson and Williams, 1993).

FGFRs on the cell surface mediate most cellular responses to extracellular FGF2. Activation of these receptors initiates an intracellular signal cascade toward the nucleus, ultimately modulating cell behavior by regulating gene expression (Reilly and Maher, 2001).

In addition to traditional, plasma membrane-initiated signaling, accumulating evidence indicates that functional FGFR 1 exists in the nucleus of many cell types (e.g. BAMC, astrocyte and glioma cells) and accounts for high affinity binding sites for nuclear FGF2 (Stachowiak et al., 2003b). A distinct integrative nuclear FGFR1 signaling (INFS) pathway has been characterized by Stachowiak *et al.* Several stimuli elicit nuclear accumulation of FGFR 1 such as lo FGF2, angiotensin II, forskolin and phorbol esters (Stachowiak et al., 2003b). Lo FGF2 treatment induces FGFR 1 to translocate from cell surface to the nucleus in NIT 3T3 cells (Maher, 1996). Stimulation by angiotensin, cAMP and PKC induce FGFR that is localized at the endoplasmic reticulum to be released to the cytosol by 'retrograde transport', followed by its nuclear transportation (Myers et al., 2003). The mechanism of FGFR 1 nuclear entry has also been investigated. Gene

sequencing does not identify a nuclear localization signal. It is proposed that FGFR 1 binds to intracellular hi FGF2 which does have an NLS that can be recognized by beta-importin (Stachowiak et al., 2003b). Beta-importin is a major component of cellular nuclear import mechanism, shuttling between the cytosol and the nucleus (Stachowiak et al., 2003b).

Nuclear FGFR 1 signaling is associated with many cellular processes, including cell proliferation, differentiation and gene expression. Transfection of FGFR 1 into glioma cells results in its nuclear accumulation, which is associated with an increased proliferative phenotype (Joy et al., 1997). In addition, nuclear FGFR 1 mediates cAMP / bone morphogenetic protein (BMP)-induced differentiation in human neuronal progenitor cells as well (Stachowiak et al., 2003a). Furthermore, Peng *et al.* reported that AII, veratridine, or PMA induced upregulation of tyrosine hydroxylase and that FGF2 promoter activity was mediated by FGFR 1 accumulating in the nuclei in BAMC cells. In both cases, the nuclear FGFR 1 acts as a transcription factor, directly interacting with the promoter sequences and regulating gene expression (Peng et al., 2001; Peng et al., 2002).

1.7 FGF2 and CK2

Casein kinase 2 (CK2), also known as protein kinase 2, is a ubiquitous Ser/Thr kinase found in all eukaryotic organisms. Existing as a hetero-tetrameric complex, CK2 consists of two catalytic subunits (α and/or α') assembled with a dimeric "regulatory" ß subunit, which serves to stabilize the CK2 tetrameric conformation, dock potential kinase regulators (e.g. FGF2) and modulate substrate selectivity. The presence of individual

CK2 subunits (designated as CK2 α_2, CK2 α_2' and CK2 $ß_2$) independently of tetramer structure has been detected in many organisms (Litchfield, 2003).

CK2 is involved in a variety of cellular processes, including cell proliferation, cell survival(Litchfield, 2003) and chromatin remodeling (Barz et al., 2003) etc.. Over three hundreds CK2 substrates have been identified to date, most of which are involved in gene expression, protein synthesis and signaling pathway essential for cell viability (Meggio and Pinna, 2003). An essential role of CK2 in supporting cell viability has been characterized, which might result from its anti-apoptotic function (Litchfield, 2003). Inhibition of CK2 activity elicits apoptosis in cancer cells and overexpression of the protein kinase protects cell from various reagents-induced apoptosis (Unger et al., 2004). CK2 exerts its anti-apoptosis effect either through protecting specific proteins (e.g. connexin, Max etc) from caspase-mediated degradation or activating apoptosis repressor with caspase recruitment domain (ARC) to inhibit caspase 8 activity (Litchfield, 2003). CK2 regulates cell proliferation by phosphorylating physiological substrates involved in cell cycle progression, such as topoisomerase II, cdc 34 etc (Litchfield, 2003). Bouche *et al.* have established that CK2 is a key mediator for lo FGF2- induced cell proliferation. A lo FGF2 mutant S117A (carrying a serine 117 substitution to alanine), which is unable to interact with the CK2 ß subunits, exhibits compromised mitogenic activity for NIH 3T3 cells and cardiomyocytes (Bailly et al., 2000; Jiang et al., 2004). CK2 also mediates lo FGF2-induced ribosome synthesis by phosphorylating nucleolin, a major protein in the nucleolus (Bouche et al., 1994). Jiang *et al.* have demonstrated that CK2 mediates long-term cardioprotection and angiogenesis by lo FGF2 (Jiang et al., 2004).

1.8 Hi FGF2 in the heart / Previous work in our lab

Our lab is particularly interested in the effects of both hi and lo FGF2 on immature as well as adult cardiomyocytes. Lo FGF2 exerts both acute (Jiang et al., 2002; Padua et al., 1998; Padua et al., 1995) and sustained cardioprotection after myocardial infarction (Jiang et al., 2002; Jiang et al., 2004). Cardioprotection is mediated by FGFR and PKC epsilon (Jiang et al., 2002; Padua et al., 1995). Hi FGF2 shares the acute protective effect of lo FGF2 when acting via a paracrine or autocrine mechanism (Dr. Kardami's lab, unpublished data). In contrast, their intracrine effects are quite distinct: hi FGF2, but not lo FGF2, induced chromatin disruption (an irregular pattern of non-mitotic chromatin condensation or 'clumping') and apoptosis-like cell death when constitutively overexpressed in cardiomyocytes (Hirst et al., 2003). This part of the review focuses on the major findings of hi FGF2 on cardiomyocytes in our lab from the last 10 years.

1.8.1 Regulation of Hi FGF2 expression

Hi and lo FGF2 display different patterns of relative accumulation in rat hearts. Hi FGF2 is the predominant isoform in neonatal hearts, while adult hearts synthesize primarily lo FGF2. Thyroid hormone down-regulates hi FGF2 accumulation in the heart (Liu et al., 1993). Thyroid hormone levels in the blood increase postnatally in a manner coinciding with differentiation of cardiomyocytes. In fact thyroid hormone regulates expression of several myofibrillar genes (Liu et al., 1993). Hyperthyroidism causes myocardial hypertrophy and also promotes increases in lo FGF2, while hypothyroidism increases the relative levels of hi FGF2 in the heart (Liu et al., 1993). The effects of

thyroid hormone on hi FGF2 accumulation are tissue specific, as hypothyroidism had no effect on brain hi FGF2 (Liu et al., 1993).

Hi FGF2 was also shown to increase in the heart transiently one day after isoproterenol-induced injury, to be replaced by lo FGF2 at later time points (Padua and Kardami, 1993). It is not known which cells produced hi FGF2 in response to injury in the heart. Immunofluorescent labeling with antibodies recognizing all FGF2 species showed increased FGF2 staining of necrotic cardiomyocytes one day after isoproterenol injection (Padua and Kardami, 1993), suggesting an association between relative increases in hi FGF2 and myocyte cell death.

Overexpression of lo FGF2 in the hearts of transgenic mice resulted in the accumulation of the endogenous hi FGF2 species, by auto-induction (Detillieux et al., 2003).

1.8.2 The effects of ectopically expressed Hi FGF2 on cardiomyocytes

To investigate the effect of hi versus lo FGF2 on cardiomyocytes, Pasumarthi *et al.* transfected rat hi or lo FGF2 cDNAs into chicken or rat neonatal ventricular cardiomyocytes using calcium phosphate/DNA precipitation-mediated gene transfer (Pasumarthi et al., 1994; Pasumarthi et al., 1996). There was no difference in the response of chicken versus rat cardiomyocytes. Hi FGF2 was found to localize predominantly to the nucleus while lo FGF2 was found in both nuclei and cytoplasm. Hi FGF2 stimulated DNA synthesis, protein synthesis and cell proliferation to the same extent as lo FGF2, and this effect was paracrine or autocrine in nature since it was blocked by anti-FGF2 neutralizing antibodies. In addition, hi FGF2 (but not lo FGF2)

overexpressing cells exclusively exhibited a unique nuclear phenotype characterized by DNA "clumping", i.e. appearance of several condensed chromatin 'clumps' within an apparently intact nucleus. In addition, a significant proportion of hi FGF2 overexpressing myocytes were binucleated. Neither chromatin clumping nor binucleation were prevented by anti-FGF2 neutralizing antibodies, pointing to an intracrine mode of action for hi FGF2 (Pasumarthi et al., 1994; Pasumarthi et al., 1996).

The above studies used rat hi and lo FGF2. Sun *et al.* demonstrated that human hi FGF2, introduced into rat cardiomyocytes by gene transfer, elicited identical effects on the nuclear phenotype (Sun et al., 2001). Sun *et al.* then examined whether chromatin condensation induced by hi FGF2 was related to apoptosis or mitosis. They concluded that chromatin clumping was not the consequence of apoptosis since the phenotype was not prevented by general caspase inhibitors, there was absence of TdT-mediated dUTP nick end labeling (TUNEL) and activated caspase 3, while anti-lamin B staining showed intact nuclear lamina without interruption. Moreover, co-transfection of B cell leukemia /lymphoma-2 (Bcl 2) did not prevent DNA clumping induced by hi FGF2. These data indicated the condensed nuclear phenotype was not initiated by an apoptosis-like mechanism. To determine whether DNA clumping was linked to mitotic stimulation, mitotic markers were utilized for phosphorylated H1 and H3. No increase in phosphorylation of H1 and H3 were observed in overexpressing cells, indicating a mitosis-independent mechanism. Furthermore, purified hi FGF2 bound to and elicited chromatin condensation in the test tube, suggesting that the observed nuclear phenotypes might be due to the direct interaction of nuclear hi FGF2 to chromatin(Sun et al., 2001).

The gene transfer methods used in the above studies had relatively low efficiency (~ 15%) and thus allowed mostly qualitative observations; in addition, they did not explore cell phenotype in response to hi FGF2 overexpression at later time points. Hirst *et al.* therefore used infection with adenoviral vector carrying human hi FGF2 to explore further mechanisms of DNA clumping by hi FGF2 in cardiomyocytes. The adenoviral vector allowed significantly increased transfection efficiency (over 90%) so that dose- (50 and 200 m.o.i.) and time- (2-4 days post-infection) dependent studies of hi FGF2's effect on cardiomyocytes became feasible. Hirst *et al.* showed that the hi FGF2-induced chromatin clumping was both dose- and time- dependent, being maximal at 200 m.o.i. and at 3 days post-infection. Most importantly, she showed the appearance of DNA ladder, as well as cell loss, at three days post-infection. These findings established that overexpression of hi FGF2 initiates events that include disruption of chromatin and eventually (after three days) lead to cell death presenting apoptosis-like features (Hirst et al., 2003).

The mechanisms involved in chromatin disruption were also investigated. Dominant-negative forms of FGFR 1 (the main FGF2 receptor in myocytes) and / or of MEK 1 (an enzyme activated by FGF2/ FGFR1 leading to ERK1/2 activation) were used and found to prevent chromatin disruption by hi FGF2. In contrast, the dominant negative forms of PKC epsilon did not prevent the clumped nuclear phenotype (Dr. Kardami's lab, unpublished data). Overall, data by Hirst *et al.* established that hi FGF2 exerts its effects on chromatin (and eventually cell death) by activating an intracellular FGFR1-MEK1-pathway (Hirst et al., 2003).

1.9 Rationale for Project

In contrast to the cardioprotective role of lo FGF2 acting on cell surface FGFR1, hi FGF2 caused cell death by activating intracellular/nuclear pathways. Very little is currently known about the hi FGF2 activated intracellular signals leading to chromatin disruption / cell death. CK2 is an important multifunctional kinase that associates with FGF2 (both hi and lo isoforms) in the cell nucleus (Bonnet et al., 1996), and plays a role in chromatin remodeling (Barz et al., 2003); its role on chromatin clumping by hi FGF2 is not known. It is also not known whether hi FGF2 needs to be in the nucleus to trigger DNA clumping.

To further explore the intracellular mechanism of hi FGF2-induced chromatin disruption, we have used HEK 293 cells (a human embryonic kidney cell line) as an *in vitro* model. First we examined whether HEK 293 cells responded to (human and/or rat) hi FGF2 overexpression in manner similar to cardiomyocytes, i.e. by developing chromatin clumping followed by cell death with apoptosis-related characteristics in a MEK 1-dependent manner. We were then able to use HEK 293 cells to examine the following hypothesis:

Chromatin disruption by hi FGF2 (a) is dependent on its ability to bind the beta subunit of CK2; (b) is dependent on CK2 activity, (c) requires hi FGF2 presence in the nucleus, and (d) is regulated by arginines 149/151 in hi FGF2.

Cell lines exhibit certain advantages over primary cells. A cell line can be passaged several times and thus is more readily available, in a relatively unlimited supply, compared to cardiomyocytes. Furthermore, HEK 293 cells can be transfected efficiently and reproducibly with TransIT®-293 Transfection Reagent (Mirus), and thus do not

require adenoviral vector creation (a lengthy process with uncertain outcome) necessary for gene transfer in cardiomyocytes. HEK 293 cells enabled us to use many different plasmids, coding for human or (Ds-red labeled) rat hi FGF2, human hi FGF2 mutants, and Ds-Red rat-hi FGF2 truncations. The findings from HEK 293 cells will narrow down the range of reagents to be tested in the cardiac system to investigate the mechanisms of hi FGF2-induced chromatin disruption/ cell death.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

MEK 1 inhibitor, PD 98059, was purchased from Cell Signaling Technology. The inhibitor that blocks binding of FGF2 to cell surface FGF receptor, inositolhexakisphosphate 6 (IP 6) and casein kinase II inhibitor, Emodin, were both from Sigma (St. Louis, MO). Mitochondria-mediated apoptosis pathway inhibitor, Bax inhibiting peptide (Bip V5), and its inert control peptide Bip–NC were from Calbiochem. All other reagents used in the experiments, if not specified, were all from Invitrogen (Ontario, Canada).

2.2 Cell Culture

HEK 293 cells were grown in 5% Dulbecco's Modified Eagle's Medium supplemented with 0.35 % glucose and 100U / ml penicillin, 100ug/ ml streptomycin in 100mm dishes. The cells were incubated in 37 °C incubator with 5% CO_2. At 70% confluence, the cells were passaged to 6-wells 35mm plates with or without coverslips. After incubation for another 24hr, the cells are ready for transfection.

2.3 Plasmids

Human hi FGF2 cDNA (pcDNA3-hi FGF2), encoding all three isoforms of hi FGF2, was a kindly gift of Dr. RZ Florkiewicz (CIBLEX Corporation, San Diego,

California), Human hi FGF2 adenovirus was a generous gift from Dr. Meenhard Herlyn (Wistar Institute in Philadelphia, Pennsylvania) and was described previously (Sauter et al., 2001). Plasmids coding for mutant hi FGF2 (S117A-hi-FGF2, S105A-hi-FGF2, and a dominant-negative form of FGFR 1) were all produced by Dr. Yan Jin in collaboration with Dr. P. Cattini (Department of Physiology, University of Manitoba, Canada). Plasmids coding for rat Ds-red hi FGF2, Ds-red Tag, Ds-red hi FGF2 amino terminal fragment, rat Ds-red lo FGF2 and rat hi FGF2 R149G and R151G were generous gifts from Drs Claudia Grothe and Peter Claus (Neuroanatomy, Hannover Medical School, Germany), and have been described in detail in the literature (Claus et al., 2003).

2.4 Transient gene transfer

One day before transfection, HEK 293 cells were seeded in 6-well 35mm plates or a 100 mm dish at 0.5-1.0 x10^5 cells/ ml. The transfection procedure followed manufacturer's instruction. In brief, 3 µl of TransIT®-293 Transfection Reagent (Mirus) was added into 200 µL of serum-free DMEM Hi-Glucose, vortexed for a few seconds and incubated for 15 minutes at room temperature. Then 10 µl of plasmid (1µg/10 µl) of various kinds was added to the diluted TransIT®-293 Transfection Reagent, mixed by pipetting up and down 20 times and incubated for another 15 minutes at room temperature. Finally, the above mixture was added dropwise to the cells in 35 mm wells and the plates were shaken gently. For 100 mm dishes, 6 times the above DNA and TransIT 293 reagent mixture was added in the same fashion as in 6-well plates.

2.5 Immunofluorescence

The cells, plated on glass coverslips contained within 35 mm dishes, were allowed to transiently express the transfected genes for 24, 48 or 72 hr. Coverslips were washed with phosphate buffered saline, fixed for 15 minutes at 4 °C in 4 % paraformaldehyde and then permeabilized with 0.1 % Triton X –100 (Roche) in PBS at 4 °C for another 15 min. After permeabilization, cells were incubated with primary antibodies diluted in 1 % BSA/PBS at room temperature for 1 hr. Primary antibodies were used were: 1) Mouse monoclonal anti- FGF2 antibody (Upstate Biotechnology) (1:1,000); 2) Mouse monoclonal anti-FGFR 1 antibody (QED Bioscience Inc.) (1: 500); 3) rabbit polyclonal anti-hi FGF2 antibodies (from Dr. Levin EG, La Jolla Institute for Molecular Medicine, San Diego, California) (1: 1,500). Secondary antibodies were: 1) anti-mouse biotinylated immunoglobulins (Amersham, Arlington Heights, IL) (1:100), and 2) anti-rabbit biotinylated immunoglobulins (Amersham, Arlington Heights, IL) (1:20). The third reagent, fluorescein-conjugated streptavidin (Amersham, Arlington Heights, IL), was added at 1:20 dilution. Thereafter, nuclear DNA was stained with 2.5 μM Hoechst 33342 (Calbiochem-Novabiochem, San Diego, CA) for 3 minutes and then the coverslips were mounted with ProLong antifade mounting medium (Molecular Probe, Eugene, Oregon). Between each step, the cells were rinsed 4 times with PBS. Zeiss Axiovert 200 microscope was used to view the results.

For cells transfected with Ds-red labeled cDNAs, Hoechst staining was performed directly after fixation and permeabilization. Then the coverslips were mounted and viewed as described above.

2.6 Western blot

Transfected or control cells were first washed 3 times with ice-cold PBS and the cells were lysed in 2 % SDS lysis buffer (50 mM Tris- HCl pH=8.0, 1mM EDTA, 2 μg/ml leupeptin, pepstatin A, aprotinin, E 64, 1mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 25 mM beta-glycerophosphate) for 60 seconds. Thereafter, the cells suspensions were scraped with a rubber policeman and transferred to an Eppendorf tube. Cell lysates were subjected to sonication and centrifugation to remove cell debris. The protein content was determined using BCA analysis (Pierce). 10-20 μg protein was resolved on 15 % polyacrylamide gel and then transferred to PVDF membrane (Roche). The membrane was first blocked with 3 % BSA in Tris-buffered saline containing 0.1 %Tween-20 (Sigma) (TBST) at room temperature for 1hr. After a quick rinse with TBST, the membrane was incubated with the primary antiboby either against FGF2 (mouse monoclonal anti- FGF2 antibody from Upstate Biotechnology, 1:1,000) or against activated caspase 3, (Cell Signaling Technology 1:1000) for 1 hour. After 4 rinses with 0.1 % BSA / TBST, the membrane was then incubated with the secondary antibodies, anti-mouse horseradish peroxidase (1: 10,000 dilution in 1 % BSA/TBST, Biorad) or anti-rabbit horseradish peroxidase (1: 10,000 dilution in 1 % BSA/TBST, Biorad) for another 1 hour. All antibodies incubation was performed at room temperature and with gentle agitation on a shaker. Finally, the membrane was washed 4 times with TBST and the bands were visualized by chemiluminescent reaction (ECL, Amersham Life Science).

2.7 Data collection and statistical analysis

We determined the 'nuclear clumping index' or 'percent clumping', i.e. the proportion of overexpressing cells presenting nuclear disruption over the total number of overexpressing cells per visual field. Each coverslip (22 x 22 mm) was divided into 8 equal triangles, one field was randomly selected in each triangle for observation under low magnification (10 x lens) and scoring; a total of 24 fields (distributed in three coverslips amounting to approximately 1200 cells) were counted per experimental group. Percent clumping between groups was statistically compared with Student one-tailed paired test and one-way ANOVA, using GraphPad InStat 3.0 program. Differences were considered significant when $P < 0.05$.

CHAPTER 3

RESULTS

3.1 Effect of human hi FGF2 overexpression on the nuclear phenotype of HEK 293 cells

The cDNA coding for human hi FGF2 was introduced into HEK 293 cells using transient gene transfer and the cells were allowed to express the gene for 24, 48 or 72hr after transfection. Transfection with TransIT®-293 Transfection Reagent (Mirus) resulted in 60-70% efficiency, as confirmed by immunofluorescence (not shown). Expression and intracellular localization of the cDNA products were examined by Western blot and immunostaining techniques, using antibodies that were either hi FGF 2 specific (immunofluorescence) or capable of recognizing all FGF2 species (Western blot). Human hi FGF2 cDNA expressing cultures generated 22-24 kDa bands (corresponding to CUG –initiated hi FGF2) as shown in Figure 1. Endogenous FGF2 migrated at 18 Kda and 34 Kda species. Hi FGF2 localized to the nucleus, as shown in Figures 2a and 2c. Disrupted nuclear phenotype, characterized by several distinct condensed chromatin 'clumps' surrounded by an apparently intact nuclear membrane, was detected in cultures expressing hi FGF2 (Figures 2b and 2d), but not in vector-only transfected cultures (Figures 2e and 2f), nor in non transfected cells (data not shown). Vector-only transfected cells were also showing some extent of immunoreactivity (Figure 2e), representing endogenous FGF2, but the nuclear phenotypes were indistinguishable from those of non-transfected cell, which is characterized by homogenous DNA staining.

3.2 Effect of rat hi FGF2 overexpression on the nuclear phenotype of HEK 293 cells

Through collaboration with a group in Germany we obtained a cDNA coding for hi FGF2, fused with a fluorescent marker Ds-red protein (Claus et al., 2003). Total cell lysates from HEK 293 cells transfected with rat Ds-red hi FGF2 cDNA was analyzed by Western blot with monoclonal anti-FGF2-antibody. As shown in Figure 3, expressing cultures elicited anti-FGF2 bands at 50 Kda reflecting the added size contributed by the fusion protein. Cells expressing Ds-red hi FGF2 showed nuclear localization associated with a disrupted ('clumped') nuclear phenotype, at 24 h and 72h. Ds-red hi FGF2 seemed to co-distribute extensively with condensed chromatin at both 24 and 72h. The Ds-red protein alone did not have any effect on nuclear phenotype (Figures 4A e and 4A f and Figure 4B). DNA clumping was not encountered in non-transfected cells (data not shown). HEK 293 cells overexpressing human or rat hi FGF2 exhibited apoptotic looking nuclei at 72 hr post transfection, characterized by a small number of large condensed DNA clumps (Figure 2d and Figure 3A d). Figure 4B also shows the proportion of nuclei presenting the clumped phenotype of HEK 293 cells overexpressing rat Ds-red hi FGF2 at 24 hr.

3.3 Does hi FGF2 expression cause apoptotic cell death in HEK 293 cells?

We next examined if the hi FGF2-induced DNA clumping in HEK 293 cells was linked to apoptotic cell death. First we examined the activation of caspase 3 (an indicator of apoptotic cell death) as a function of rat hi FGF2 expression. As shown in Figure 5,

elevated level of the P17 caspase fragment (activated caspase 3) was detected in overexpressing cells at 72 hr. In comparison, non-transfected cells showed negligible P17.

We next examined whether an apoptosis inhibitor Bip V5 (Sawada et al., 2003) would block the hi FGF2-induced nuclear phenotype. HEK 293 cells were treated with Bip V5 or its inert control (Bip NC) 1 hour prior to gene transfer. After 24 hr incubation, the degree of nuclear disruption (clumping index) was determined, subsequent to immunofluorescence staining of the cells. As shown in Figure 6, Bip V5 significantly prevented the DNA disruption by rat Ds-red hi FGF2 in HEK 293 cells, while Bip NC had no effect.

3.4 Role of autocrine/paracrine signaling and ERK 1/2 in hi FGF 2- induced nuclear disruption

To examine potential contribution of released FGF2 to the observed phenotype we pretreated HEK 293 cultures with an extracellularly acting FGFR antagonist, inositol hexakis phosphate (IP 6), 1 hour before the cells were transfected with rat Ds-red hi FGF2 (Peng et al., 2001). The assessment of chromatin clumping (clumping index) was performed 24 hour later. IP 6 did not prevent the appearance of DNA clumping by hi FGF2 in 293 cells (Figure 7).

Human hi FGF2-induced chromatin clumping has been shown to be prevented by blocking ERK 1/2 signaling pathway in cardiomyocytes (Dr. Kardami's lab, unpublished data). We investigated the involvement of ERK1/2 in hi FGF2-elicited chromatin clumping in HEK 293 cells. Cells were pre-treated with a specific ERK 1/2 inhibitor, PD

98059 (20 or 50 μM), 1 hr prior to transfection with either human or rat Ds-red hi FGF2 cDNAs. The cultures were incubated for additional 72 hr (for human hi FGF2) or 24 / 72 hr (for rat hi FGF2) and then subjected to immunofluorescence and quantitative analysis. As shown in Figure 8 and Figure 9, PD 98059 significantly decreased both human and rat hi FGF2-induced DNA clumping in HEK 293 cells.

3.5 Role of FGFR 1 on hi FGF2- induced chromatin clumping on HEK 293 cells

Unpublished data in our laboratory had suggested that FGFR 1 was mediating the hi FGF2-induced nuclear clumping (MSc thesis by Cheryl Hirst, 2003). These data were confirmed, as shown in Figure 10. Primary cultures of neonatal cardiomyocytes were infected with an adenoviral vector expressing a kinase-deficient FGFR 1, acting in a dominant-negative manner (FGFR 1-dn) (Sheikh F. et al 2004, in Press), at 50 m.o.i. . Next day, myocytes were infected with an adenovirus expressing human hi FGF2, at 150 m.o.i.. Nuclear clumping index determined 72hr later, showed that expression of FGFR 1-dn prevented hi FGF2-induced chromatin clumping (Figure 10). We then examined if FGFR 1 was involved in the hi FGF2-induced nuclear disruption of HEK 293 cells. Two different approaches were used for human or rat hi FGF2 respectively. In the first approach, HEK 293 cells were simultaneously transfected with rat Ds-red hi FGF2 and FGFR 1-dn. The expression of the corresponding gene products and the effect of FGFR 1-dn on hi FGF2-elicited DNA clumping, assessed by immunostaining, are shown in Figure 11. FGFR 1-dn was expressed in HEK 293 cells and localized appropriately at

cell membrane and peri-nuclear sites (Figure 11 c, Figure 11 e). Expression of FGFR 1-dn did not seem to prevent the hi FGF2-induced nuclear phenotype.

In the second approach, we used a mutant form of human hi FGF2, S105A-hi FGF2, which carries a substitution of glutamine –105 with alamine residue and exhibits dramatically diminished affinity for FGFR 1 (Jiang et al., 2002; Zhu et al., 1995). HEK 293 cells were transfected with the mutant and its effects on DNA clumping were compared to those of wild type human hi FGF2. As shown in Figure 12, the percentage of clumped nuclei remained unchanged in HEK 293 cells overexpressing hi FGF2 S105A.

3.6 Role of CK2 on hi FGF2 -induced chromatin clumping on HEK 293 cells

CK2 is composed of both α and β subunits (Litchfield, 2003). We examined whether hi FGF2-induced DNA clumping in HEK 293 cells is mediated by its binding to the β subunits of CK2. A human hi FGF2 mutant S117A was used which carries a substitution of glutamine 117 with alanine residue and exhibited compromised ability to interact with the CK2 β subunits (Bailly et al., 2000). As shown in Figure 13, the S117A mutant localized to the nucleus but displayed significantly decreased ability to cause nuclear clumping compared to wild type human hi FGF2. To also examine the participation of CK2 α subunit in mediating the DNA clumping by hi FGF2 in HEK 293 cells, the cells were treated with a specific α subunit inhibitor, emodin, 1 hr prior to or 10 hr after transfection with rat Ds-red hi FGF2 cDNA. The nuclear clumping index was determined 24 hr post transfection, subsequent to immunofluorescence. The chromatin disruption was significantly prevented by emodin treatment under both conditions. Emodin itself had no effect on nuclear morphology (Figure 14A and Figure 14B).

3.7 Role of nuclear localization on hi FGF2-induced chromatin disruption on HEK 293 cells

Rat Ds-red hi FGF2 mutant R149G / R151G, carrying a substitution of arginines 149 &151 with glycine residues (Claus et al., 2003), was introduced into HEK 293 cells by gene transfer and its intracellular localization and effect on chromatin disruption were analyzed with immunofluorescence 24 hr later. As shown in Figure 15A a and Figure 15A b, rat Ds-red hi FGF2 R149G / R151G (designated as P56) displayed a cytoplasmic re-distribution in HEK 293 cells, which resulted in complete prevention of DNA clumping induced by wild type nuclear hi FGF2 (Figure 15B).

Expression of amino terminal fragment of rat hi FGF2, designated as P1-34, had no effect on nuclear morphology, although it localized to the nucleus (Figure 15A c, Figure 15A d and Figure 15B). Expression of Ds-red lo FGF2 (termed as P18) had no effect on nuclear morphology either and it also localized to the nucleus (Figure 15A e, Figure 15A f and Figure 15B).

CHAPTER 4

DISCUSSION

4.1 Hi FGF2 induces chromatin clumping in HEK 293 cells

Several previous studies in our laboratory have demonstrated the ability of hi FGF2 to induce chromatin clumping (compaction, non-mitotic condensation) leading to cell death in cardiomyocytes. To examine if hi FGF2 could exert similar effects in another cell type, we used the cell line HEK293. Because we observed a very similar pattern of response on HEK293 cells, we can conclude that, as in cardiomyocytes, ectopic expression of hi FGF2 induces a characteristic type of nuclear disruption. This phenotype becomes visible by immunofluorescence at 24-48 h, and is characterized by the appearance of numerous (10-30) small condensed chromatin clumps within an apparently intact nuclear membrane, developing into a more typically apoptotic looking morphology (fewer and larger chromatin clumps) at 72 h. As in cardiomyocytes, the nuclear effects reflected an intracrine mode of action, because they were not prevented by IP6, a compound that blocks the interaction of extracellular FGF2 with the cell surface (Peng et al., 2001).

Both human hi FGF-2 or Ds-red-labeled rat hi FGF2 induced qualitatively similar patterns of nuclear disruption, at both early and late time points, indicating that the effect is not species-specific, and that Ds-red had no effect in and of itself on the phenotype, in agreement with previous reports (Claus et al., 2003; Hirst et al., 2003; Pasumarthi et al., 1994; Pasumarthi et al., 1996; Sun et al., 2001). Nuclear disruption in rat Ds-red-labeled- versus human hi FGF2 expressing cells was clearly detectable at 24 or 48 h, respectively.

In addition, rat hi FGF2 overexpressing cells tended to have higher nuclear clumping index and more apparent "apoptotic" nuclei than human hi FGF2 at 72hr. One possibility for more overt expression of the phenotype in the Ds-red-labeled FGF2 transfected cells is that the rat isoform is more 'active'. The increased 'activity' may have been conferred by the Ds-red label, or may reflect a real difference between human and rat hi FGF2. We think that the first possibility is unlikely, because Ds-red protein by itself, or in conjunction with lo-FGF2 had no effect on chromatin. The second possibility merits further investigation, since the rat hi FGF2 N-extension shares both areas of similarity as well as difference with its human counterpart, and thus theoretically may have somewhat different nuclear properties. Even though known as a highly conserved protein, human and rat hi FGF2 demonstrate significant divergence in the amino terminal extensions. Human hi FGF2 consists of 41, 46 and 55 amino acid residues, compared to 26, 34 residues for rat hi FGF2 in the N-terminal extension (Sun et al., 2001). Another suggestion that this may be the case comes from their respective nuclear localization patterns: human hi FGF2 localizes to the nucleus but shows only partial co-localization with the clumped chromatin (Hirst et al., 2003). On the other hand, we found that Ds-red-labeled rat hi FGF2 co-localized extensively with the clumped chromatin. Irrespectively however of whether human and rat hi FGF2 have somewhat different sub-nuclear localization, their end effects, chromatin clumping and cell death, are the same. Complete co-localization with condensed chromatin is therefore not essential for the induction of the 'clumped' phenotype.

It is also possible that the method of detection used affected the sensitivity of detection, and thus created the appearance of differences between rat and human FGF2.

Human hi FGF2 detection was achieved indirectly, by immunodetection with specific antibodies; antibody-based detection is subject to interference from epitope masking. Hi-FGF2 epitopes may have become masked upon interaction with chromatin, creating an appearance of non-codistribution. This would not happen in the tagged-hi-FGF2, where detection of the fluorescent tag (Ds-red) is achieved directly. This question should therefore be addressed by using the same method of detection for both human and rat hi FGF2.

4.2 Hi FGF2 induces cell death with apoptotic features

We previously reported that the human hi FGF2-induced chromatin disruption in cardiomyocytes presented some apoptotic features, i.e. development of DNA ladder and nuclear morphology (Hirst et al., 2003), becoming evident at 72 h after transfection. We undertook similar studies to examine if hi FGF2-induced apoptotic cell death in HEK293 cells. HEK 293 cells were transfected with rat Ds-red hi FGF2 and two apoptotic indices were examined: activation of caspase 3 and the involvement of a pro-apoptotic protein Bax in the process.

Apoptosis (also known as programmed cell death) is a highly conserved cellular process and essentially required for embryonic development and normal tissue homeostasis (Danial and Korsmeyer, 2004). Apoptotic cells present distinct morphological features including cell shrinking, plasma membrane blebbing, DNA condensation and fragmentation, and eventually formation of apoptotic bodies (Van Cruchten and Van Den Broeck, 2002). Caspase-dependent apoptosis can occur by a death receptor mechanism or by a mitochondrial-mediated mechanism (Fesik, 2000). In the

first pathway, extracellular effectors (e.g. TNF) interact with cell surface death receptor, initiating an intracellular signaling cascade and eventually activating caspase 8. In the mitochondrial pathway, intrinsic apoptotic stimuli induce release of cytochrome C from mitochondria to cytosol. Cytochrome C can bind to the protein Apaf-1 in the cytosol to recruit and activate procaspase 9. Caspase 3 is a downstream molecule to caspase 8 and caspase 9, being a key mediator in both of the mechanisms (Fesik, 2000). Recently, a newly- characterized apoptosis, which is mitochondrion-mediated but caspase-independent pathway, was reported (Lockshin and Zakeri, 2004). Not too much has been defined regarding to this novel signaling pathway, which involves translocation of apoptosis-inducing factor (AIP) and/or endonuclease G, from mitochondria to nuclei and most likely trigger cell death through their effects on nuclei (Kaufmann and Hengartner, 2001). DNA degradation as indicated by DNA condensation and fragmentation is a morphological hallmark of apoptosis, whichever mechanisms are involved (Danial and Korsmeyer, 2004).

In our systems, elevated level of activated caspase 3 was detected in hi FGF2 overexpressing cells, pointing to a caspase-dependent signaling pathway. We also examined the role of the protein Bax in the signaling pathway. Bcl-2 family of proteins regulate mitochondrially-mediated apoptosis in either positive or negative manner (Sorenson, 2004). Bax, as a pro-apoptotic effector of the family, translocates from the cytosols to the mitochondria upon apoptotic stimuli. Translocating Bax induces the release of cytochrome C, AIF and endonuclease G from mitochondria, resulting in either caspase-dependent and/or caspase-independent cell death (Green, 2000). It was reported that the protein Ku70 could prevent such translocation by binding to Bax in the cytosol

and forming a multimeric complex (Sawada et al., 2003). This property has been exploited to create pentapeptides containing the putative Bax-binding sequence of Ku70 and capable of suppressing Bax-mediated cell death (Sawada et al., 2003; Yoshida et al., 2004). We used such a peptide, BIP V5, as well as its scrambled negative control (BIP NC) to pretreat HEK293 cells. Because the BIP V5 peptide prevented hi FGF2-induced nuclear clumping, while BIP NC did not, we conclude that hi FGF2 expression may trigger a Bax-dependent pathway towards cell death. Given the exclusive nuclear localization of hi FGF2 and its effect on chromatin remodeling, how mitochondria get involved in the signaling pathway should be further examined. Some groups worked on DNA damage-induced apoptosis (DDIA), in which the DNA damage signal is transduced from nuclei to mitochondria in a P53-dependent or Caspase2/Nur77-dependent manner. It remains unknown if hi FGF2 could cause DNA damage within nucleus, independent of mitochondria. Another possibility would be that hi FGF2 up-regulates certain genes, triggering Bax-dependent cell death program leading to apoptotic nuclear events. This needs to be addressed in further studies.

4.3 The MEK1-ERK1/2 pathway but not FGFR1 mediates hi FGF2-induced chromatin clumping in HEK 293 cells

Previous work in our laboratory indicated that inactivation of the MEK1-ERK1/2 pathway, either using a dominant negative inhibitor of MEK1, or pharmacological inhibition (PD 98059), prevented the effects of hi FGF2 on cardiomyocyte chromatin. Because pre-treatment of HEK293 cells with PD 98059 suppressed the effects of either human hi or rat Ds-red FGF2 on chromatin, we conclude that, as in cardiomyocytes,

activation of the MEK1-ERK1/2 pathway is required for hi FGF2-induced nuclear disruption. Our data are in apparent disagreement with a majority of studies that have implicated the MEK1-ERK1/2 pathway in prevention of apoptosis and cell death (Jacobs et al., 2004; Saxena et al., 2004; Wu et al., 2004). Nevertheless there are studies that indicate that in certain instances this pathway is actually engaged towards the induction of cell death: Yang *et al.* recently showed that in human aorta smooth muscle cells, hydrogen sulfide induced apoptosis by a mechanism requiring the activation of ERK1/2 and caspase 3 (Yang et al., 2004). Involvement of ERK1/2 in chromatin remodeling or compaction has been suggested by Zhou *et al.* (Zhou et al., 2000). These investigators reported that ERK1/2 associate with histone deacetylase 4. Deacetylases remove acetyl groups from core histones and thus promote chromatin compaction and transcriptional repression (Waterborg, 2002). Activation of ERK1/2 by intracellular hi FGF2 may contribute towards chromatin deacetylation and condensation.

Our previous data showed hi FGF2-induced chromatin disruption/ cell death in cardiomyocytes could not be reversed by exogenous treatment of lo FGF2, indicating hi FGF2's intracrine mode of action (Dr. Kardami's lab, unpublished data). We investigated if this is the case in HEK 293 cells with a FGFR 1 antagonist (IP6), which block FGF2 binding to the cell surface receptors (Peng et al., 2001). Because IP6 did not prevent hi FGF2-induced phenotype, we conclude that hi FGF2 's effects on HEK 293 cells follow an intracrine mode of action, as in cardiomyocytes.

The effects of hi FGF2 on cardiac myocytes were mediated by FGFR1, as shown by Hirst, C (MSc thesis) and as confirmed by us (Fig.11). This was not the case in HEK293 cells. Neither expression of FGFR1-dn, nor of a hi-FGF2 mutant with

diminished affinity for FGFR1 had any effect on the nuclear phenotype. The reason for this difference is not known at present. One possibility could be that HEK293 cells do not express FGFR1. Preliminary data have indicated that this is not the case, since we have found that HEK293 cells express immunoreactive FGFR1 protein, by Western blotting (Dr. Kardami's lab, unpublished data). Another possibility is that, unlike cardiomyocytes expressing predominantly FGFR1, HEK293 cells express other FGFR members that would not be inhibited by the methods used, and would mediate the effects of hi FGF2. There are four FGFR members, FGFR1-4, all tyrosine kinases triggering similar signal transduction pathways. These receptors display ligand as well as cell type specificity (Jaye et al., 1992). Preliminary studies have suggested that HEK293 cells do indeed express other FGFR (FGFR 2- 4). It remains to be seen if selective inhibition of these receptors will prevent the effects of hi FGF2 in HEK293 cells. Finally, a third possibility is that the intracrine effects of hi FGF2 are wholly FGFR-independent in HEK293 cells.

Overall our studies have shown that the mechanism of hi FGF2-induced nuclear clumping in HEK293 cells has certain mechanistic aspects in common with cardiomyocytes, such as requiring the MEK1-ERK1/2 pathway and following an intracirne mode of action. On the other hand, there may exist cell-type specific signaling steps, such as the involvement of FGFR1 for cardiomyocytes but not HEK 293 cells.

4.4 CK2 mediates the nuclear effects of hi FGF2

The deacetylated histone state contributes to chromatin condensation (Mahlknecht et al., 2000). Factors and conditions therefore that stimulate the activity of histone deacetylating enzymes would be expected to promote chromatin remodeling and compaction. One such protein is CK2, a known interacting partner of hi and lo FGF2

(Bonnet et al., 1996). CK2 is a ubiquitous serine/threonine kinase, implicated in transcription control, cell proliferation, and survival. Genome-wide screens have shown that many CK2-affected genes encode proteins involved in chromatin remodeling and modification including histone deacetylation (Barz et al., 2003). In yeast, all CK2 subunits interact with histones and general chromosomal remodeling proteins; architectural non-histone chromatin proteins such as the heterochromatin-associated protein and others are phosphorylated by CK2, resulting in changes in their DNA binding and gene silencing activities. CK2 furthermore binds and phosphorylates human histone deacetylases 1 and 2 (HDAC1, 2), promoting their activity (Sun et al., 2001). CK2 has two alpha and two beta subunits forming the tetrameric holoenzyme; the alpha and beta subunits are reported to act independently as well as in complex with each other. CK2 interacts with numerous cellular targets at many subcellular sites including the nucleus (Litchfield, 2003). To test if either of the CK2 subunits were involved in the effects of hi FGF2 in the nucleus, we used two different strategies, affecting the beta or alpha subunits. One strategy exploits the finding by Bouche and co-workers (Bailly et al., 2000) that a single mutation on (lo) FGF2, S117A, prevents: (i) interaction with the beta subunit of CK2, (ii) the FGF2-induced activation of CK2, and (iii) the lo FGF2-induced stimulation of proliferation. We therefore created the same mutation on hi FGF2, and tested it for ability to cause nuclear disruption. Because hi (S117A) FGF2 has diminished ability to cause nuclear disruption, we conclude that interaction with the beta subunit of CK2, and, presumably, the subsequent activation of CK2 in the nucleus, mediate the effects of hi FGF2 on chromatin.

Because beta subunits can act independently of alpha CK2 subunits in some instances, we examined if the alpha subunit activity was required for the effects of hi FGF2. The strategy involved use of the highly specific alpha (catalytic) subunit inhibitor emodin. Because emodin, added to the cells either prior to or 10 h after transfection with the cDNA for hi FGF2, decreased the occurrence of the hi FGF2 nuclear phenotype, we suggest that alpha CK2 subunit activity is needed for the effect. Overall our data suggest that the CK2 holoenzyme (both alpha and beta subunits) is required for the nuclear effects of hi FGF2. Filol *et al.* have demonstrated that the CK2 holoenzyme remains cytosolic unless bound to FGF2 that carries it to the nucleus. Prevention of interaction between the beta subunit and (lo) FGF2 prevented the nuclear translocation of the enzyme (Filhol et al., 2004). We thus hypothesize that hi FGF2 also binds to the beta subunit of CK2 in the cytosol, and promotes translocation and activation of the holoenzyme in the nucleus. In agreement, our non-CK2 binding hi FGF2 mutant may have been unable to cause nuclear compaction despite its nuclear localization because it would have been unable to bring the CK2 holoenzyme into the appropriate nuclear domains. In other words, we hypothesize that CK2 is the downstream effector of hi FGF2 as far as chromatin condensation is concerned. As mentioned already, hi and lo FGF2 display distinct distributions within the nucleus, an thus they are likely to carry their binding partners (such as CK2) to different nuclear sites (Claus et al., 2003). Lo FGF2-associated nuclear sites are presumably important for the proliferative response of cells while hi FGF2- associated nuclear/chromatin sites can (via CK2) lead to gene silencing, chromatin compaction and cell death.

The above model is dependent on the nuclear translocation of hi FGF2. In agreement, non-nuclear hi FGF2 (the double R149/R151 mutant) had no effect on nuclear morphology. It also requires a hi FGF2-specific sub-nuclear localization. In agreement, lo FGF2, localizing to different domains (Claus et al., 2003) does not affect nuclear morphology and chromatin compaction. Finally, the N-terminal extension of hi FGF2, although capable of nuclear translocation, was, by itself, unable to cause nuclear disruption, due perhaps to an inability to bind and translocate CK2.

4.5 Concluding remarks and future directions

We have shown that hi FGF2, ectopically expressed in HEK 293 cells, promotes chromatin compaction leading to an apoptotic cell death that likely follows a mitochondrion-dependent as well as caspase 3-dependent pathway. The effects of hi FGF2 on chromatin require the activity of ERK1/2, as well as CK2 and are dependent on both the interaction of hi FGF2 with the beta subunit of CK2 and its ability for nuclear translocation. A likely mechanism by which ERK1/2 and CK2 mediate the effect of hi FGF2 on chromatin is via their known property to stimulate deacetylase activity and chromatin compaction. This, however, remains to be shown.

Future studies should address further the mechanism of hi FGF2-induced cell death. The role of mitochondrial pathways including cytochrome c release, the role of apoptosis-inducing factor, and the role of various caspases should be examined. Identification of the enzyme responsible for DNA cleavage in response to hi FGF2 is also an important question. Endonuclease G and DNA fragmentation factor are involved in caspase-independent or caspase-dependent pathways, and are candidates for future

research. The role of CK2 should also be investigated further, by examining the effect of hi FGF2, and its non-CK2 binding mutant, on the translocation of its alpha and beta subunits to the nucleus. RNA interference could be used to deplete cells of CK2, to further establish its role on the effect of hi FGF2 on chromatin. Manipulations aimed at suppressing the activity of deacetylases should also be examined, to investigate if the hi FGF2-induced phenotype depends on histone deacetylation. Last but not least, the above studies should also be done in primary cell cultures such as cardiomyocytes, to establish universality (or not) of the mechanism.

Cell death and chromatin disruption induced by hi FGF2 are processes highly relevant to heart disease and carcinogenesis. Understanding the mechanism of hi FGF2 action may allow us to identify new therapeutic targets to either prevent cell death (cardiomyocytes) or induce cell death (cancer).

Figure 1. **Overexpression of human hi FGF2 in HEK 293 cells.** HEK 293 cells were transfected with human hi FGF2 or vector for 72 hr. Cells were extracted, and lysates analyzed by Western blotting (50 ug/lane) and monoclonal antibodies against FGF2. Arrows point to the bands corresponding to the overexpression of three isoforms of hi FGF2 (22, 22.5 and 24 kDa) in transfected cells. These bands are not detected in samples from vector-only transfected cultures.

Figure 1

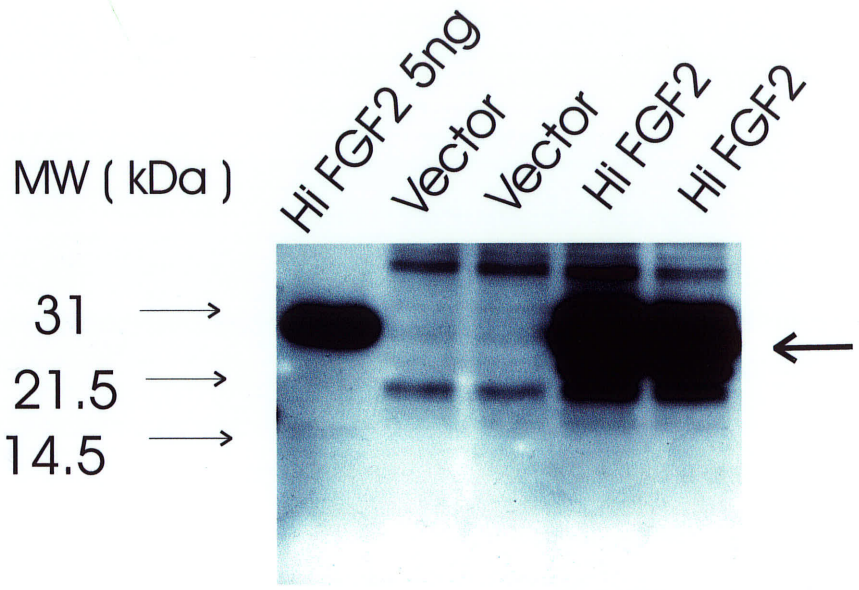


Figure 2. **Effect of human hi FGF2 overexpression on chromatin in HEK 293 cells.** **(a, c, e)** and **(b, d, f)**, double fluorescence labeling for hi FGF2 and DNA, respectively. Cells shown in **a, b** and **c, d** were examined 48 and 72 hr after transfection with the cDNA for human hi FGF2, as indicated. Vector-transfected cells are also shown **(e, f)**. The bars shown in **a, c, e** represent 20 μM.

Figure 2

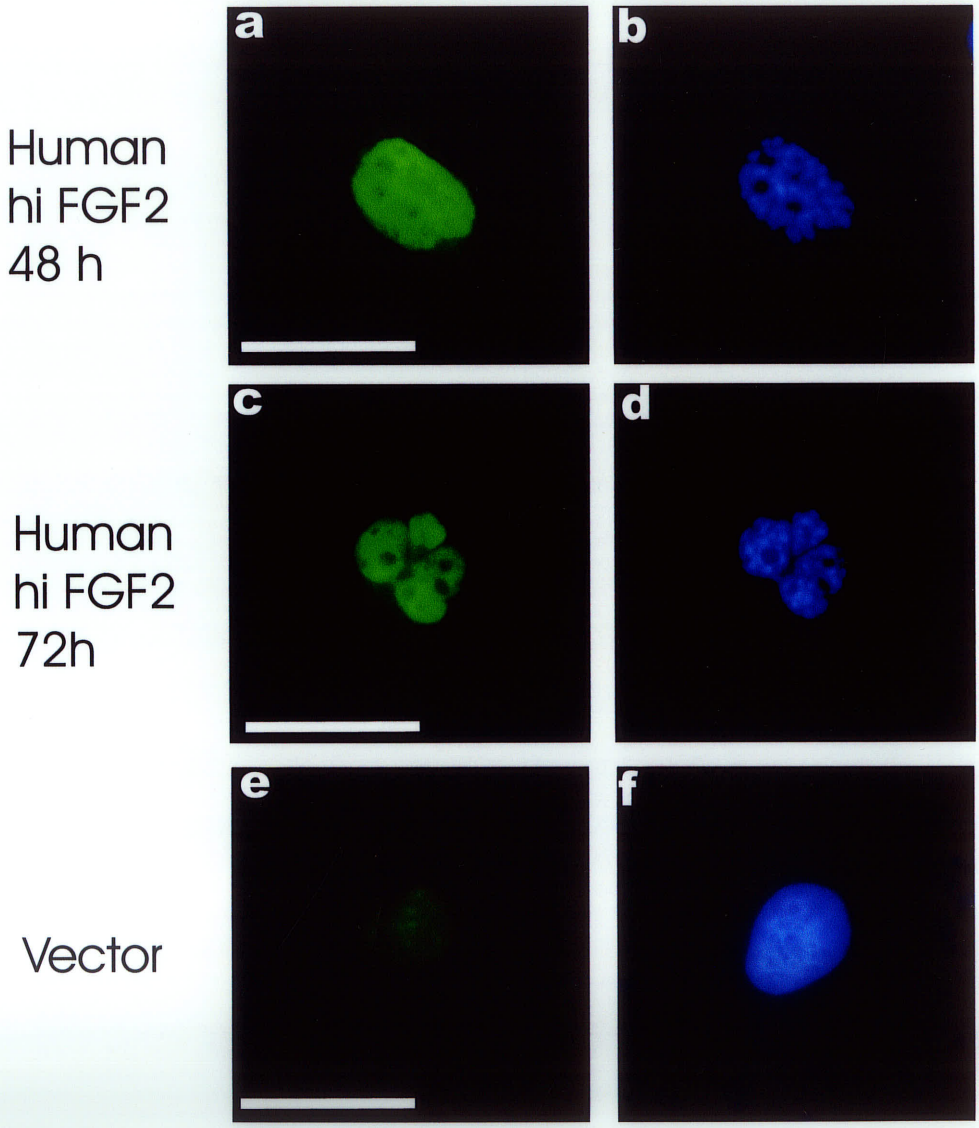
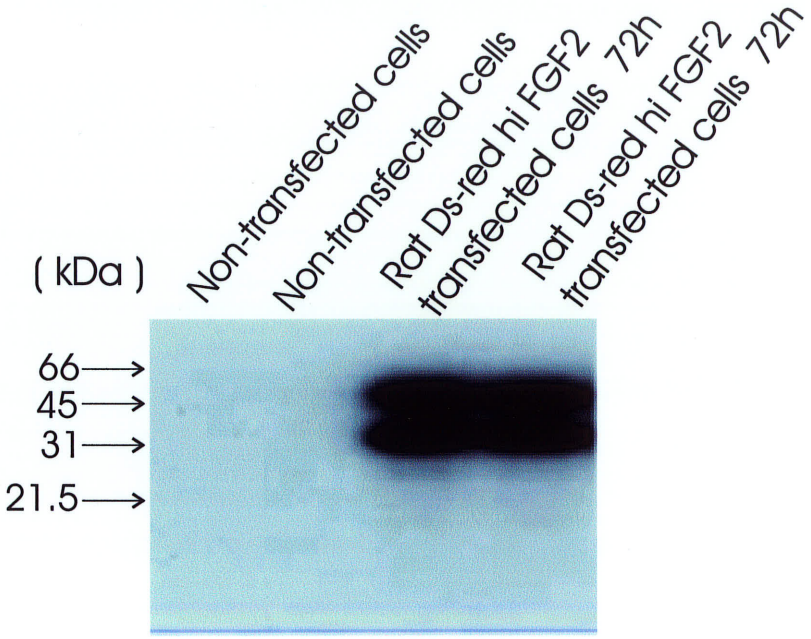


Figure 3. **Overexpression of rat Ds-red hi FGF2 in HEK 293 cells.** HEK 293 cells were transfected with the cDNA for rat Ds-red hi FGF2. Cell lysates were obtained one day later and analyzed by Western blotting and monoclonal antibodies against FGF2. Arrows point to the bands corresponding to the overexpression of Ds-red hi FGF2 in transfected cells.

Figure 3



Anti FGF2

Figure 4 **Effect of rat Ds-red hi FGF2 overexpression on chromatin in HEK 293 cells.**

Figure 4A. Double fluorescence labeling for rat Ds-red hi FGF2 **(a, c, e)** and DNA **(b, d, f)**, respectively. **(a,b)** and **(c,d)** show cells examined at 24 hr and 72 hr post-transfection, as indicated. **(e, f)** show cells transfected with the cDNA for Ds-red protein. The bars shown in **a, c, e** represent 20 μM.

Figure 4B: **Effect of Ds-red protein overexpression on chromatin in HEK 293 cells.** HEK 293 cells were transfected with either rat Ds-red hi FGF2 or Ds-red tag protein. Cells were subjected to DNA staining and quantitatively analyzed for nuclear clumping index 24 hr post-transfection. Data are shown as the mean ± S.E.M., n= 24.

Figure 4A

Rat hi FGF2 24 h

Rat hi FGF2 72h

Ds-red tag protein

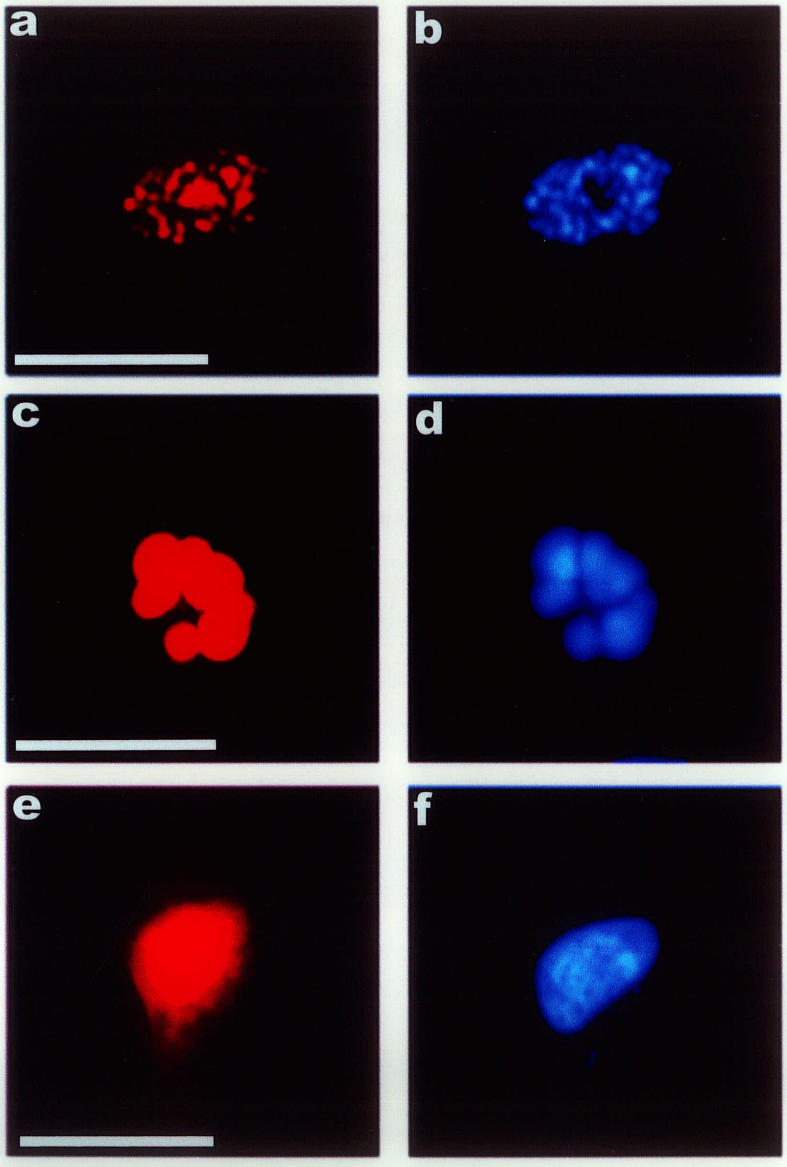


Figure 5. **Effect of rat Ds-red hi FGF2 overexpression on activation of caspase 3 in HEK 293 cells.** HEK 293 cells were transfected with rat Ds-red hi FGF2 for 72 hr. Lysates from transfected or non-transfected cells were analyzed by Western blotting and antibodies to activated caspase 3 (P17). The arrow point to elevated levels of activated caspase 3 in overexpressing cells.

Figure 4B

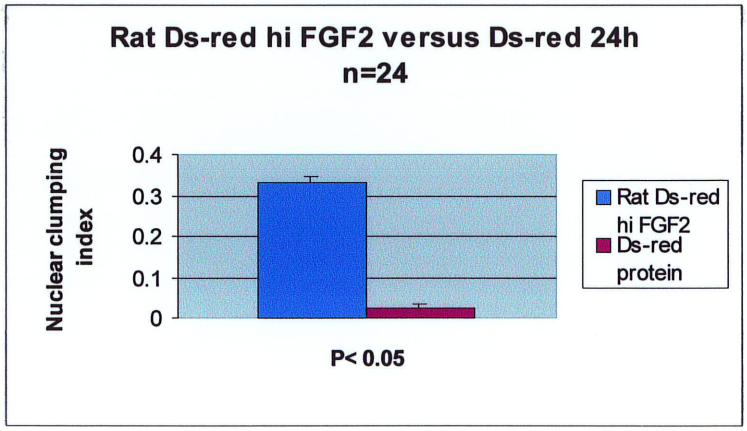
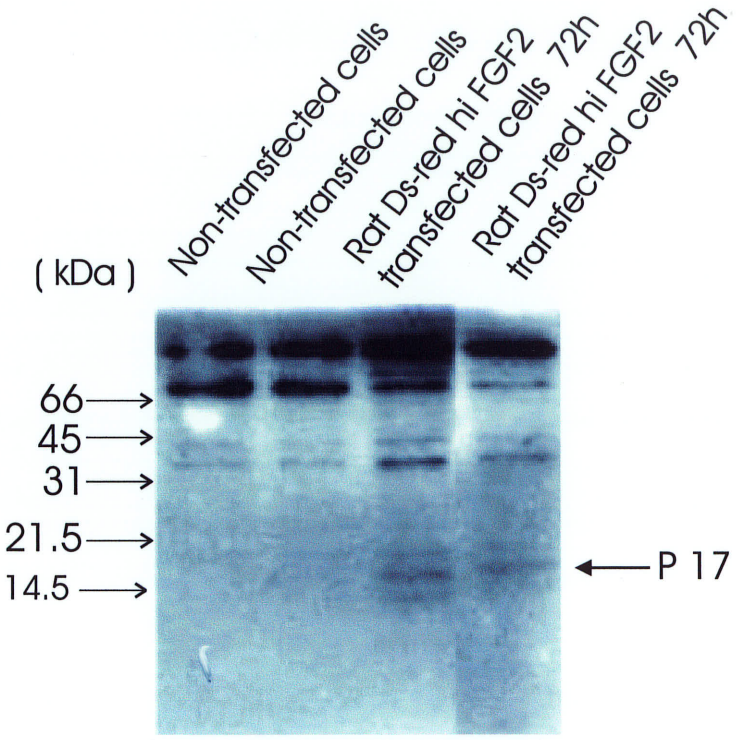

Rat Ds-red hi FGF2 versus Ds-red 24h
n=24
Nuclear clumping index
0.4
0.3
0.2
0.1
0
Rat Ds-red hi FGF2
Ds-red protein
P< 0.05

Figure 6. **Effect of Bax inhibiting peptide (Bip V5) on rat Ds-red hi FGF2-induced chromatin disruption in HEK 293 cells**

Figure 6A: Double fluorescence labeling for rat Ds-red hi FGF2 **(a, c, e)** and DNA **(b, d, f)**, respectively. **a, b:** HEK 293 cells overexpressing rat Ds-red hi FGF2 for 24 hr. **c, d:** HEK 293 cells overexpressing rat Ds-red hi FGF2 for 24 hr but treated with BIP V5 (200 μM) 1hr before transfection. **e, f:** HEK 293 cells overexpressing rat Ds-red hi FGF2 for 24hr and treated with BIP NC peptide (200 μM) 1hr before transfection (NC= negative control). The bars shown in **a, c, e** represent 20 μM.

Figure 6B: HEK 293 cell cultures were pretreated with or without BIP V5 or BIP NC 1hr before transfection with rat Ds-red hi FGF2. The cells were subjected to quantitative analysis of nuclear clumping index 24 hr post transfection. Data are shown as the mean ± S.E.M., n= 24.

Figure 5



Anti activated caspase 3 (P17)

Figure 6A

Rat Ds-red
hi FGF2

Rat Ds-red
hi FGF2 &
Bip V5
200uM

Rat Ds-red
hi FGF2 &
Bip NC
200uM

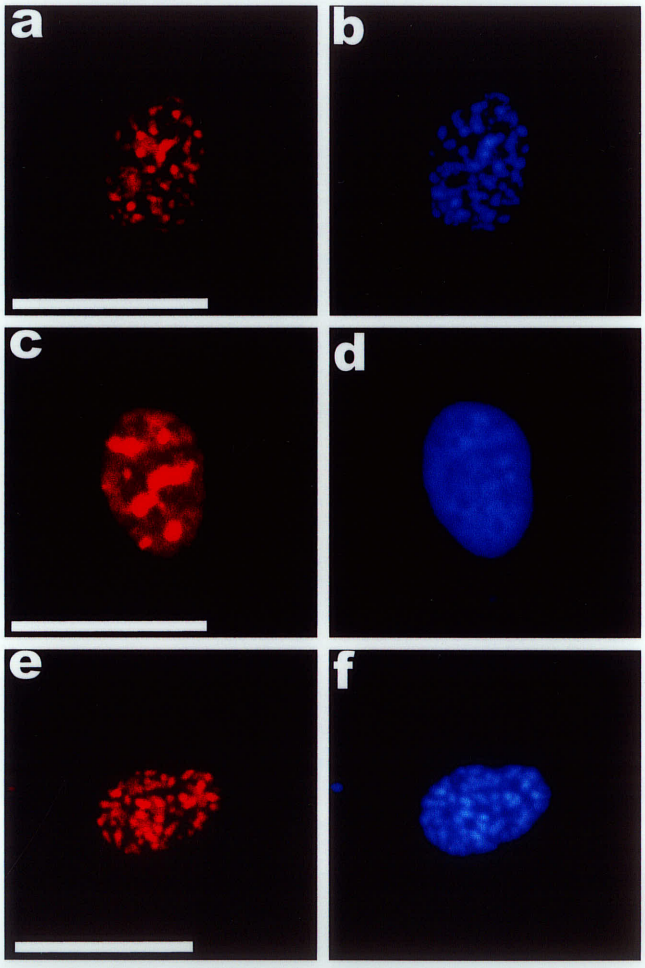


Figure 6B

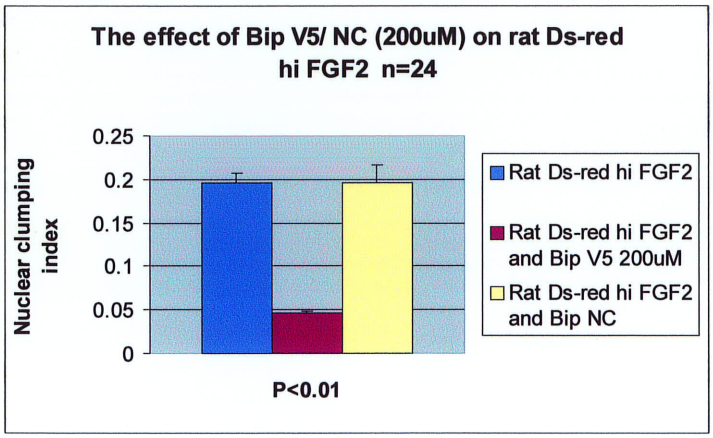

The effect of Bip V5/ NC (200uM) on rat Ds-red hi FGF2 n=24
Nuclear clumping index
0.25
0.2
0.15
0.1
0.05
0
Rat Ds-red hi FGF2
Rat Ds-red hi FGF2 and Bip V5 200uM
Rat Ds-red hi FGF2 and Bip NC
P<0.01

Figure 7. **Effect of inositol hexakis phosphate (IP 6) on rat Ds-red hi FGF2-induced chromatin disruption in HEK 293 cells. a, b :** Double fluorescent labeling for rat Ds-red hi FGF2 and DNA in HEK 293 cells transfected with rat Ds-red hi FGF2. **c, d :** Double fluorescent labeling for rat Ds-red hi FGF2 and DNA in HEK 293 cells pretreated with IP 6 (400 μM) 1hr before transfection with the cDNA for rat Ds-red hi FGF2. The arrows point to overexpressing cells and corresponding nuclear phenotypes. The bars shown in **a, c** represent 50 μM.

Figure 7

at Ds-red
i FGF2

at Ds-red
i FGF2 and
? 6 400uM

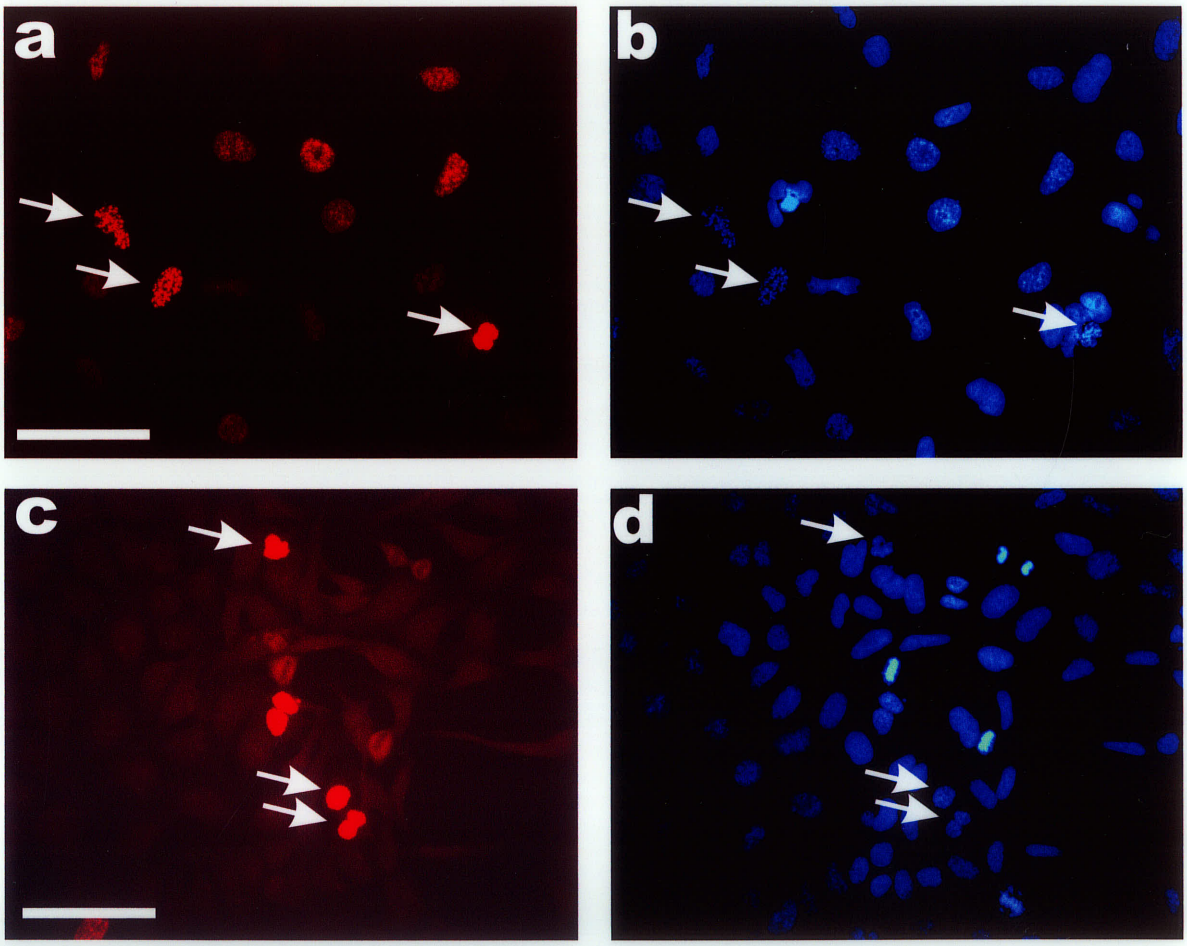


Figure 8. **Effect of PD 98059 on human hi FGF2-induced chromatin disruption in HEK 293 cells**

Figure 8A: Double fluorescence labeling for human hi FGF2 (**a, c**) and DNA (**b, d**). **a, b:** HEK 293 cells overexpressing human hi FGF2 for 72 hr. **c, d :** HEK 293 cells pretreated with PD 98059 (20 μM) 1 hr before transfection with the cDNA for human hi FGF2 for 72 hr .The arrows point to the overexpressing cells and corresponding nuclear phenotypes. The bars shown in **a, c** represent 50 μM.

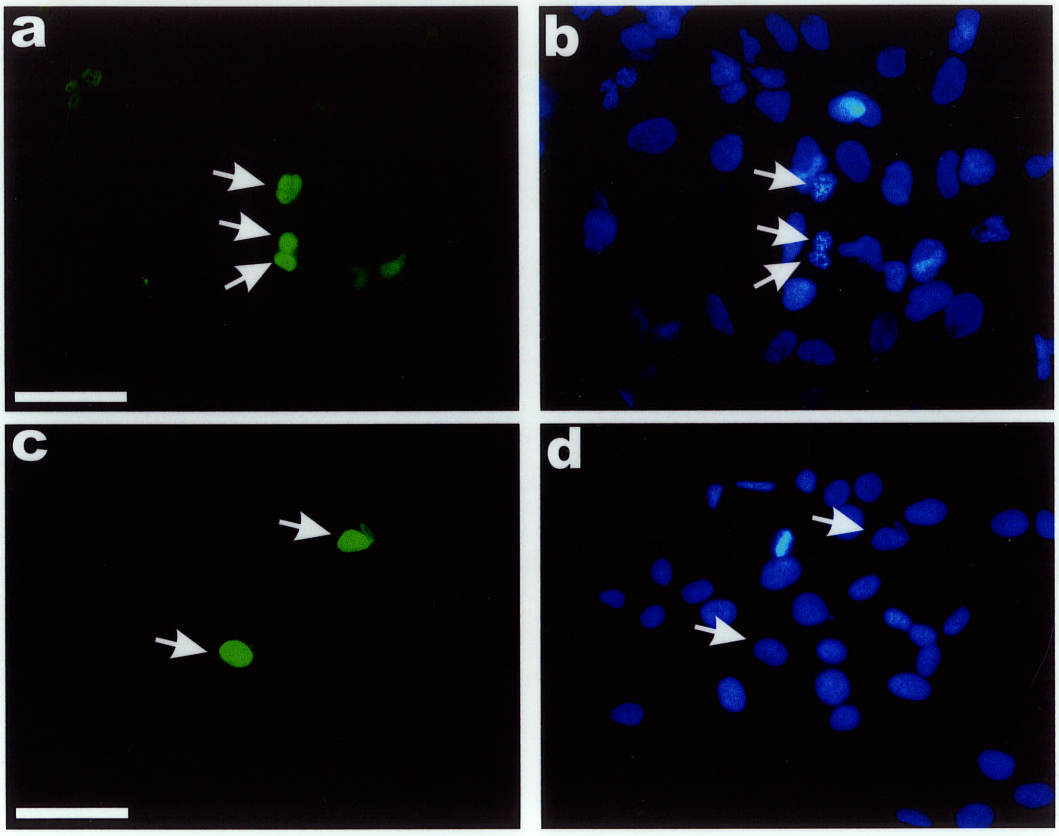
Figure 8B: HEK 293 cell cultures were pretreated with / without PD 98059 (20 μM) 1hr before transfected with human hi FGF2. The cells were subjected to double immunostaining for hi FGF2 and DNA and quantitative analysis of nuclear clumping index. Data are shown as the mean ± S.E.M., n= 16.

Figure 8

A

Human hi FGF2 72h

Human hi FGF2 72h and PD 98059 20uM



B

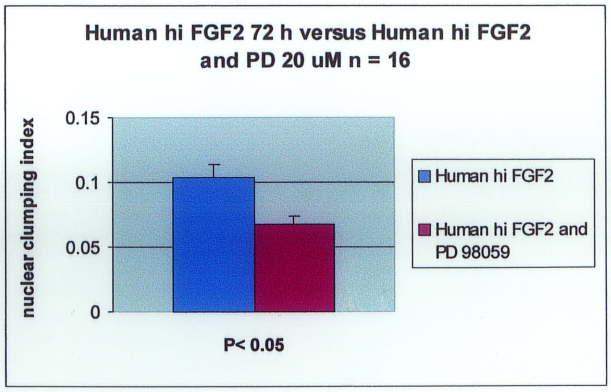


Figure 9. **Effect of PD 98059 on rat Ds-red hi FGF2-induced chromatin disruption in HEK 293 cells**

Figure 9A: Double fluorescent labeling for rat Ds-red hi FGF2 (**a, c, e**) and DNA (**b, d, f**). **a, b:** HEK 293 cells overexpressing rat Ds-red hi FGF2 for 24 hr. **c, d:** HEK 293 cells pretreated with PD 98059 (50 μM) 1hr before transfection and overexpressing rat Ds-red hi FGF2 for 24 hr. **e, f:** HEK 293 cells pretreated with PD 98059 (20 μM) 1 hr before transfection and overexpressing rat Ds-red hi FGF2 for 72 hr. The bars shown in **a, c, e** represent 20 μM.

Figure 9B: HEK 293 cell cultures were pretreated with / without PD 98059 (20/50 μM) 1 hr before transfected with rat Ds-red hi FGF2. The cells were subjected to immunostaining for DNA and quantitative analysis of nuclear clumping index 24 / 72 hr later. Data are shown as the mean ± S.E.M., n= 16.

Figure 9A

Rat Ds-red
hi FGF2

Rat Ds-red
hi FGF2 and
PD 50uM 24 h

Rat Ds-red
hi FGF2 and
PD 20uM 72 h

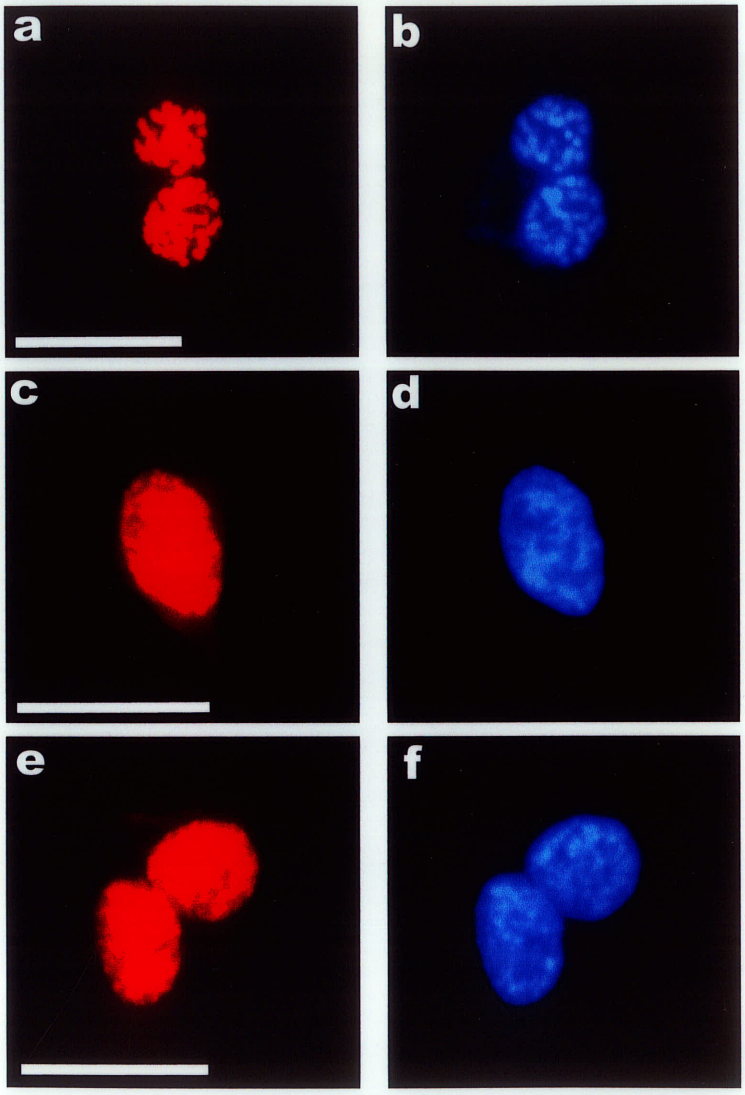
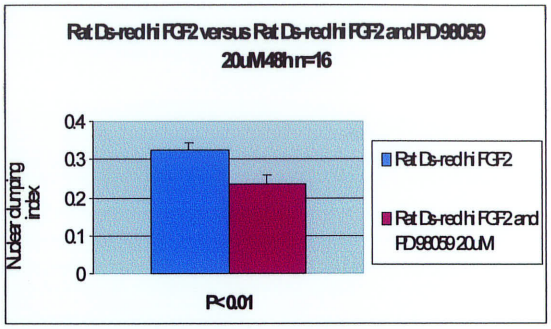


Figure 9B


Rat Ds-red hi FGF2 versus Rat Ds-red hi FGF2 and PD98059 20uM 48h n=16
Nuclear clumping index
0.4
0.3
0.2
0.1
0
Rat Ds-red hi FGF2
Rat Ds-red hi FGF2 and PD98059 20uM
P<0.01

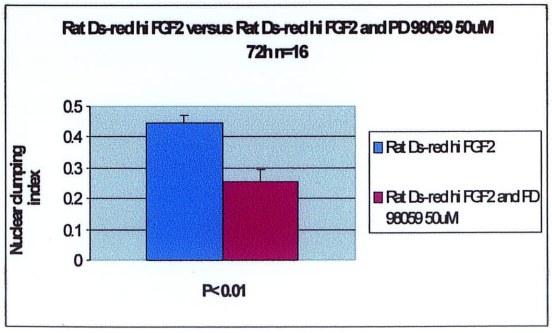

Rat Ds-red hi FGF2 versus Rat Ds-red hi FGF2 and PD 98059 50uM 72h n=16
Nuclear clumping index
0.5
0.4
0.3
0.2
0.1
0
Rat Ds-red hi FGF2
Rat Ds-red hi FGF2 and PD 98059 50uM
P<0.01

Figure 10. **Effect of preventing FGF2 receptor 1 activation on hi FGF2-induced chromatin disruption in rat cardiomyocytes.** Rat neonatal cardiomyocytes were infected with an adenoviral vector for human hi FGF2 in the presence or absence of pre-infection with an adenoviral vector for a dominant-negative form of FGFR 1. Myocytes were examined 72 hr post-infection. Data for quantitative analysis of nuclear clumping index are shown as the mean ± S.E.M., n= 16.

Figure 10

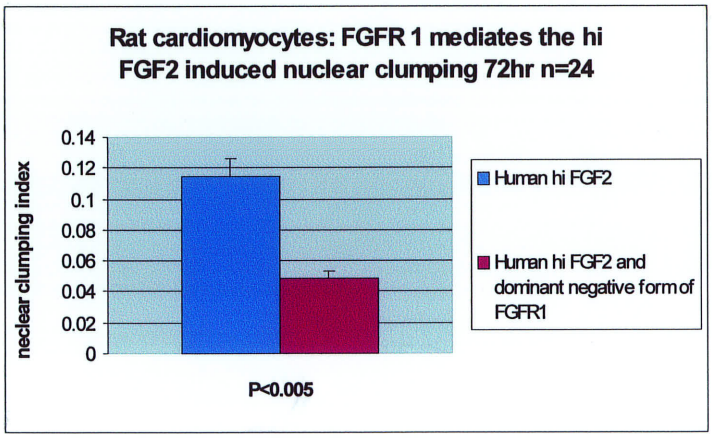

Rat cardiomyocytes: FGFR 1 mediates the hi FGF2 induced nuclear clumping 72hr n=24
neclear clumping index
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
P<0.005
Human hi FGF2
Human hi FGF2 and dominant negative form of FGFR1

Figure 11. **Effect of preventing FGF2 receptor 1 activation on hi FGF2-induced chromatin disruption in HEK 293 cells. a, b :** Double fluorescence labeling for FGF2 and DNA in HEK 293 cells overexpressing rat Ds-red hi FGF2 for 48 hr. **c, d :** Double fluorescence labeling for FGFR 1 (green) and DNA in HEK 293 cells overexpressing dominant and negative form of FGFR 1 for 48 hr. **e, f, g :** triple fluorescence labeling for rat Ds-red hi FGF2, FGFR 1 (green) and DNA in HEK 293 cells co-transfected with rat Ds-red hi FGF2 and a dominant negative form of FGFR 1 at 48 hr. **h, i:** double fluorescence labeling for FGFR 1 and DNA in non-transfected HEK 293 cells. The bars shown in **a, c, e and h** represent 20 μM.

Figure 11

Rat Ds-red hi FGF2 expressing HEK 293 cellls

DN-FGFR 1 expressing HEK 293 cells

Rat Ds-red hi FGF2 and DN-FGFR 1 expressing HEK 293 cells

Non-transfected HEK 293 cells

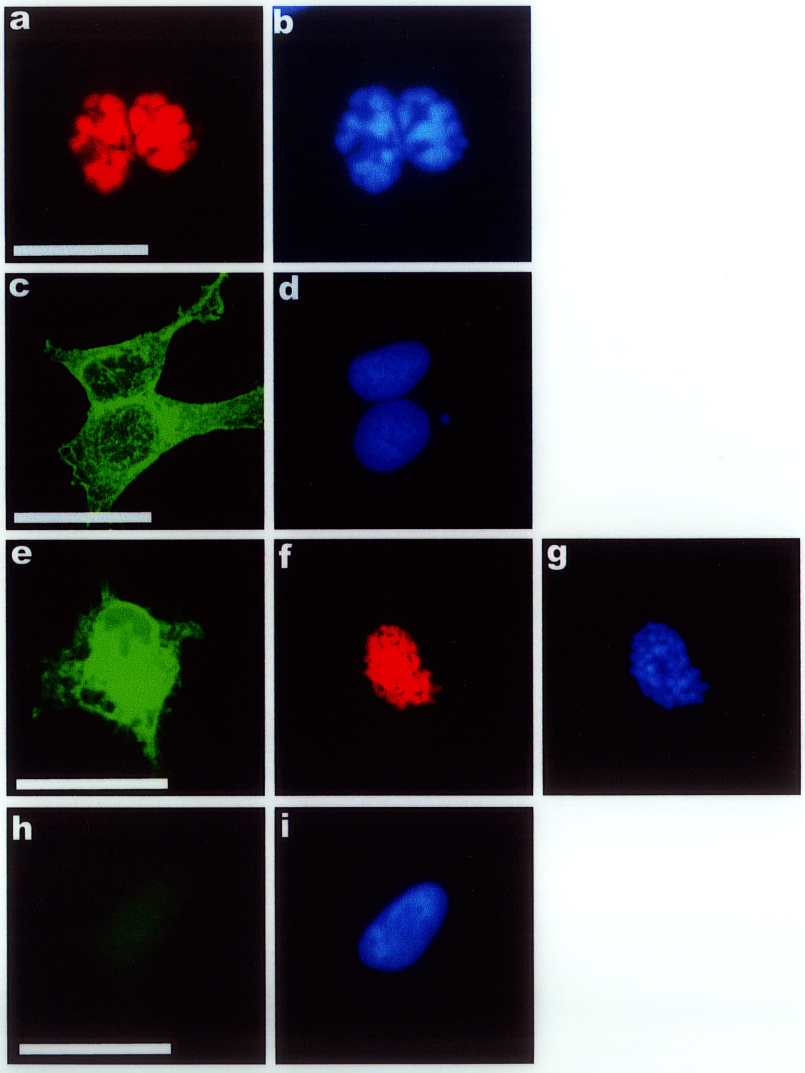


Figure 12. **Effect of decreasing the affinity of FGF2 for FGFR 1 on hi FGF2-induced chromatin clumping in HEK 293 cells**

Figure 12A **a, b:** Double fluorescence labeling for FGF2 and DNA in HEK 293 cells overexpressing human hi FGF2 for 72 hr. **c, d:** Double fluorescence labeling for FGF2 and DNA in HEK 293 cells overexpressing human hi FGF2 S105A for 72 hr. The bars shown in **a, c** represent 20μM.

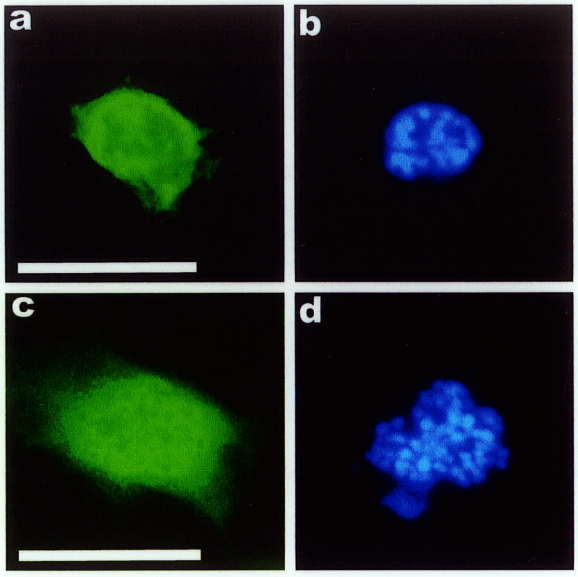
Figure 12B HEK 293 cell cultures were transfected with human hi FGF2 or human hi FGF2 mutant S105A. The cells were subjected to double immunostaining for FGF2 and DNA and quantitative analysis of nuclear clumping index 72 hr later. Data are shown as the mean ± S.E.M., n= 32.

Figure 12

A

Human hi FGF2 72 h

Human hi FGF2 S105A 72h



B

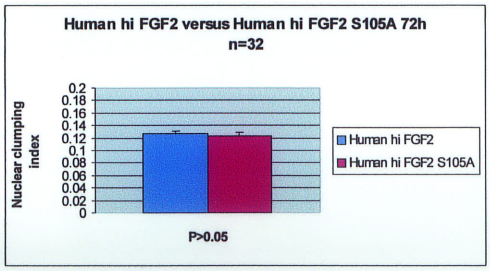


Figure 13. **Role of the CK2 beta subunits in rat Ds-red hi FGF2-induced chromatin disruption in HEK 293 cells**

Figure 13A **a, b:** Double fluorescence labeling for FGF2 and DNA in HEK 293 cells overexpressing human hi FGF2 for 72 hr. **c, d :** Double fluorescence labeling for FGF 2 and DNA in HEK 293 cells overexpressing human hi FGF2 S117A for 72 hr. Arrows point to the overexpressing cells and corresponding nuclear phenotypes. The bars shown in **a, c** represent 50 μM.

Figure 13B HEK 293 cell cultures were transfected with human hi FGF2 or human hi FGF2 mutant S117A. The cells were subjected to double immunostaining for FGF2 and DNA and quantitative analysis of nuclear clumping index 72 hr later. Data are shown as the mean ± S.E.M. and n= 24 or 32, as indicated.

Figure 13A

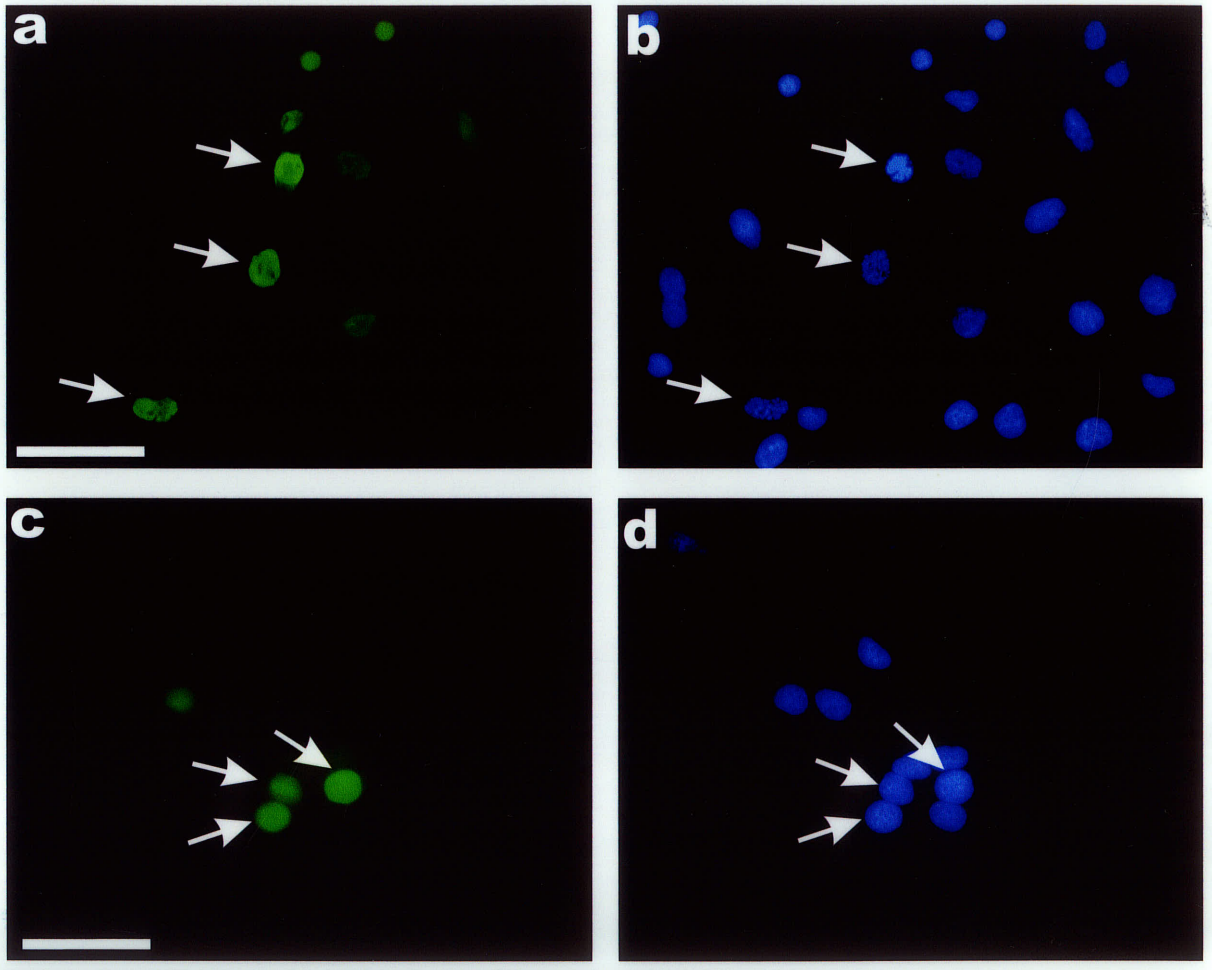
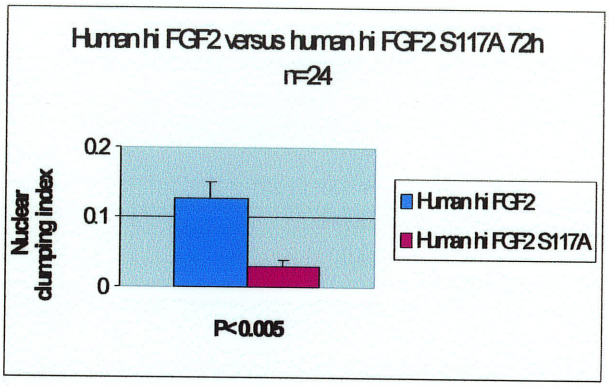


Figure 13B


Human hi FGF2 versus human hi FGF2 S117A 72h
n=24
Nuclear clumping index
0.2
0.1
0
Human hi FGF2
Human hi FGF2 S117A
P<0.005

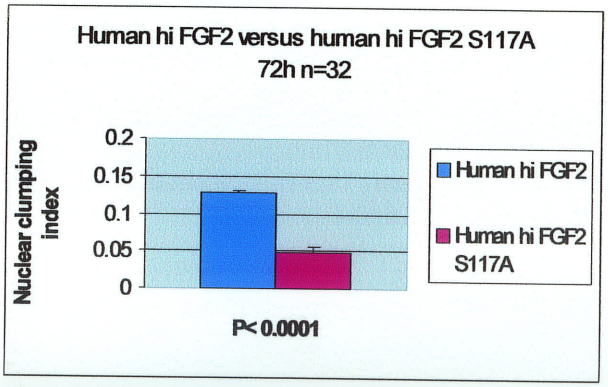

Human hi FGF2 versus human hi FGF2 S117A
72h n=32
Nuclear clumping index
0.2
0.15
0.1
0.05
0
Human hi FGF2
Human hi FGF2 S117A
P< 0.0001

Figure 14. **Role of the alpha subunits of CK2 in rat Ds-red hi FGF2-induced chromatin disruption in HEK 293 cells.**

Figure 14A **a, b:** Double fluorescent labeling for rat Ds-red hi FGF2 and DNA in HEK 293 cells overexpressing rat Ds-red hi FGF2 for 24 hr. **c, d:** Double fluorescent labeling for rat Ds-red hi FGF2 and DNA in HEK 293 cells pretreated with emodin (10 μM) 1hr before transfection with the cDNA for rat Ds-red hi FGF2 for 24 hr. The bars shown in **a, c** represent 20 μM.

Figure 14B **a:** HEK 293 cells were pretreated with or without emodin (10 μM) 1 hr before transfection with the cDNA coding for rat Ds-red hi FGF2 for 24 hr. The cells were subjected to fluorescent labeling for DNA and quantitative analysis of nuclear clumping index. Data are shown as the mean ± S.E.M., n= 24. The effect of emodin itself on chromatin disruption is also shown. **b:** HEK 293 cells were treated with or without emodin (20 μM) 1 hr prior to transfection with the cDNA coding for rat Ds-red hi FGF2 for 24 hr. The cells were subjected to fluorescent labeling for DNA and quantitative analysis of nuclear clumping index. Data are shown as the mean ± S.E.M., n= 24. **c:** HEK 293 cells were treated with or without emodin (20 μM) 10 hr after transfection with the cDNA coding for rat Ds-red hi FGF2 for 24 hr. The cells were subjected to fluorescent labeling for DNA and quantitative analysis of nuclear clumping index. Data are shown as the mean ± S.E.M., n= 24.

Figure 14A

Rat Ds-red hi FGF2 versus rat Ds-red hi FGF2 and emodin

Rat Ds-red hi FGF2

Rat Ds-red hi FGF2 and emodin 10uM 24 h

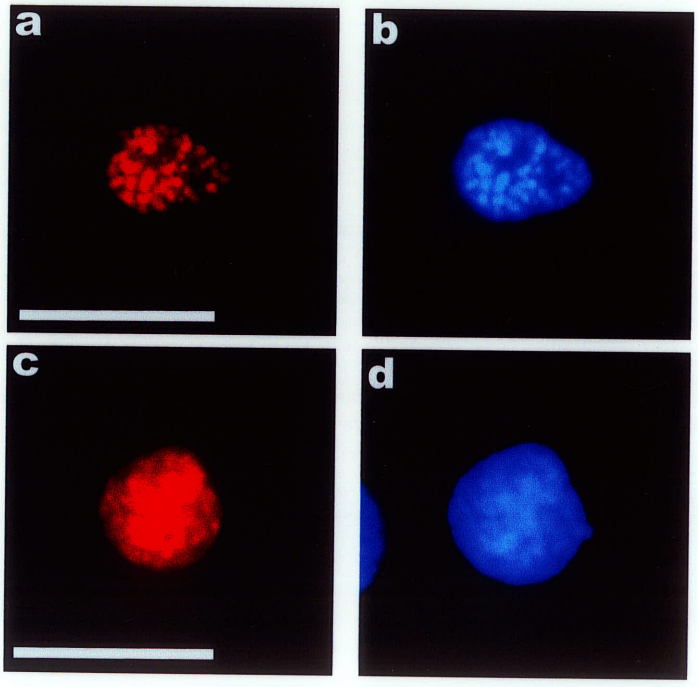
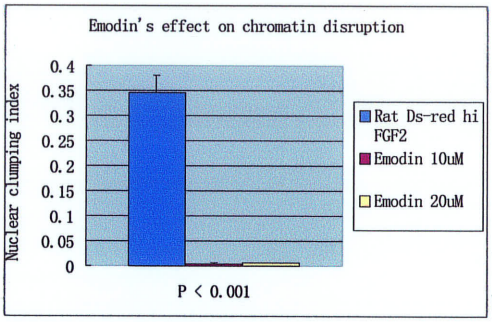
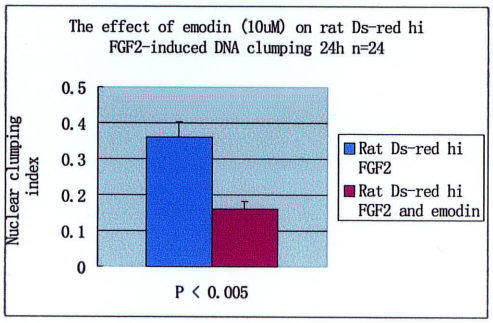


Figure 14B

a



b



c

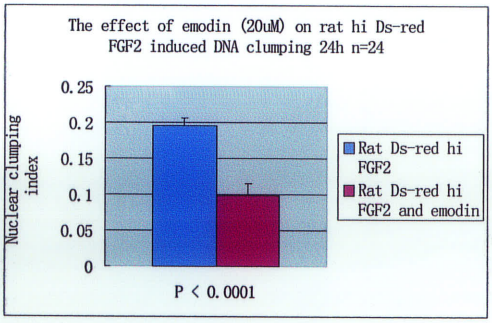


Figure 15. **Effect of hi FGF2 mutants on chromatin disruption in HEK 293 cells.**

Figure 15A **a, b:** Double fluorescent labeling for non-nuclear rat Ds-red hi FGF2 mutant R149G/ R151G (P56) and DNA in overexpressing HEK 293 cells. **c, d:** Double fluorescent labeling for amino-terminal extension of rat Ds-red hi FGF2 (P1-34) and DNA in overexpressing HEK 293 cells. **e, f:** Double fluorescent labeling for rat Ds-red lo FGF2 (P18) and DNA in overexpressing HEK 293 cells. The bars shown in **a, c, e** represent 20 μM.

Figure 15B HEK 293 cells were transfected with rat Ds-red hi FGF2, rat Ds-red hi FGF2 mutant R149G/ R151G (P56), amino-terminal extension of rat Ds-red hi FGF2 (P1-34) and rat Ds-red lo FGF2 (P18). After 24 hr incubation, the cells were subjected to fluorescent labeling for DNA and quantitative analysis of nuclear clumping index. Data are shown as the mean ± S.E.M., n= 24.

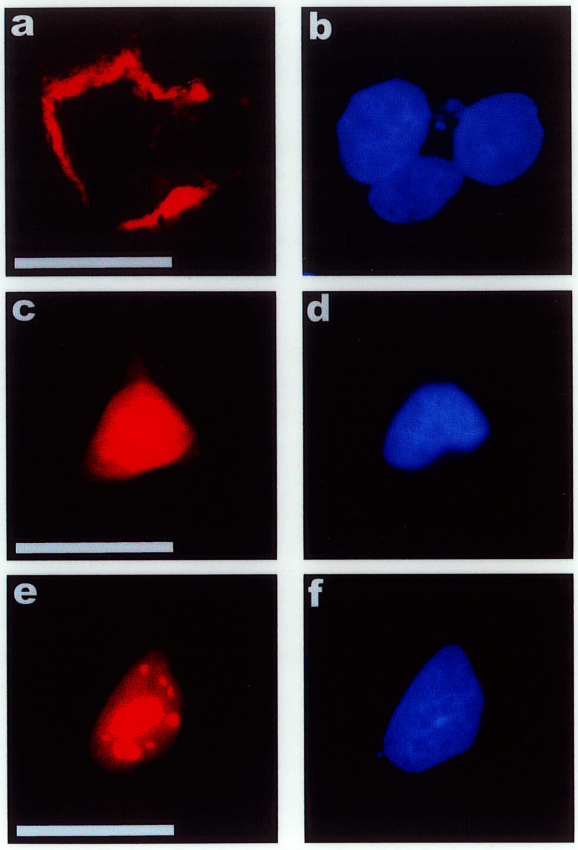
Figure 15

A

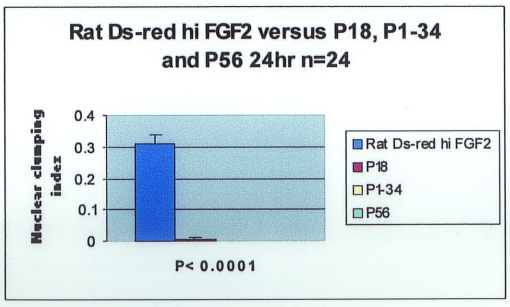
Rat Ds-red hi FGF2 R149G/ R151G (P56)

Amino terminal extension of rat Ds-red hi FGF2 (P1-34)

Rat Ds-red lo FGF2 (P18)



B



CHAPTER 5

REFERENCES

Albuquerque, M.L., S.K. Akiyama, and H.W. Schnaper. 1998. Basic fibroblast growth factor release by human coronary artery endothelial cells is enhanced by matrix proteins, 17beta-estradiol, and a PKC signaling pathway. *Exp Cell Res.* 245:163-9.

Arnaud, E., C. Touriol, C. Boutonnet, M.C. Gensac, S. Vagner, H. Prats, and A.C. Prats. 1999. A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol Cell Biol.* 19:505-14.

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

Barz, T., K. Ackermann, G. Dubois, R. Eils, and W. Pyerin. 2003. Genome-wide expression screens indicate a global role for protein kinase CK2 in chromatin remodeling. *J Cell Sci.* 116:1563-77.

Bikfalvi, A., S. Klein, G. Pintucci, N. Quarto, P. Mignatti, and D.B. Rifkin. 1995. Differential modulation of cell phenotype by different molecular weight forms of basic fibroblast growth factor: possible intracellular signaling by the high molecular weight forms. *J Cell Biol.* 129:233-43.

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

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

Bouche, G., V. Baldin, P. Belenguer, H. Prats, and F. Amalric. 1994. Activation of rDNA transcription by FGF-2: key role of protein kinase CKII. *Cell Mol Biol Res.* 40:547-54.

Bugler, B., F. Amalric, and H. Prats. 1991. Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol Cell Biol.* 11:573-7.

Burgess, W.H., J. Bizik, T. Mehlman, N. Quarto, and D.B. Rifkin. 1991. Direct evidence for methylation of arginine residues in high molecular weight forms of basic fibroblast growth factor. *Cell Regul.* 2:87-93.

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

Claus, P., S. Werner, M. Timmer, and C. Grothe. 2004. Expression of the fibroblast growth factor-2 isoforms and the FGF receptor 1-4 transcripts in the rat model system of Parkinson's disease. *Neurosci Lett.* 360:117-20.

Coffin, J.D., R.Z. Florkiewicz, J. Neumann, T. Mort-Hopkins, G.W. Dorn, 2nd, P. Lightfoot, R. German, P.N. Howles, A. Kier, B.A. O'Toole, and et al. 1995. Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol Biol Cell.* 6:1861-73.

Dahl, J.P., A. Binda, V.A. Canfield, and R. Levenson. 2000. Participation of Na,K-ATPase in FGF-2 secretion: rescue of ouabain-inhibitable FGF-2 secretion by ouabain-resistant Na,K-ATPase alpha subunits. *Biochemistry.* 39:14877-83.

Danial, N.N., and S.J. Korsmeyer. 2004. Cell death: critical control points. *Cell.* 116:205-19.

Delrieu, I. 2000. The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. *FEBS Lett.* 468:6-10.

Delrieu, I., E. Arnaud, G. Ferjoux, F. Bayard, and J.C. Faye. 1998. Overexpression of the FGF-2 24-kDa isoform up-regulates IL-6 transcription in NIH-3T3 cells. *FEBS Lett.* 436:17-22.

Delrieu, I., J.C. Faye, F. Bayard, and A. Maret. 1999. Inhibition of interleukin-6 promoter activity by the 24 kDa isoform of fibroblast growth factor-2 in HeLa cells. *Biochem J.* 340 (Pt 1):201-6.

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

Ding, L., F. Donate, G.C. Parry, X. Guan, P. Maher, and E.G. Levin. 2002. Inhibition of cell migration and angiogenesis by the amino-terminal fragment of 24kD basic fibroblast growth factor. *J Biol Chem.* 277:31056-61.

Dormond, O., L. Ponsonnet, M. Hasmim, A. Foletti, and C. Ruegg. 2004. Manganese-induced integrin affinity maturation promotes recruitment of alpha V beta 3 integrin to focal adhesions in endothelial cells: evidence for a role of phosphatidylinositol 3-kinase and Src. *Thromb Haemost.* 92:151-61.

Estival, A., D. Louvel, B. Couderc, H. Prats, E. Hollande, N. Vaysse, and F. Clemente. 1993. Morphological and biological modifications induced in a rat pancreatic acinar cancer cell line (AR4-2J) by unscheduled expression of basic fibroblast growth factors. *Cancer Res.* 53:1182-7.

Estival, A., V. Monzat, K. Miquel, F. Gaubert, E. Hollande, M. Korc, N. Vaysse, and F. Clemente. 1996. Differential regulation of fibroblast growth factor (FGF) receptor-1 mRNA and protein by two molecular forms of basic FGF. Modulation of FGFR-1 mRNA stability. *J Biol Chem.* 271:5663-70.

Fesik, S.W. 2000. Insights into programmed cell death through structural biology. *Cell.* 103:273-82.

Filhol, O., J.L. Martiel, and C. Cochet. 2004. Protein kinase CK2: a new view of an old molecular complex. *EMBO Rep.* 5:351-5.

Green, D.R. 2000. Apoptotic pathways: paper wraps stone blunts scissors. *Cell.* 102:1-4.

Gualandris, A., M. Arese, B. Shen, and D.B. Rifkin. 1999. Modulation of cell growth and transformation by doxycycline-regulated FGF-2 expression in NIH-3T3 cells. *J Cell Physiol.* 181:273-84.

Gualandris, A., C. Urbinati, M. Rusnati, M. Ziche, and M. Presta. 1994. Interaction of high-molecular-weight basic fibroblast growth factor with endothelium:

biological activity and intracellular fate of human recombinant M(r) 24,000 bFGF. *J Cell Physiol.* 161:149-59.

Hirst, C.J., M. Herlyn, P.A. Cattini, and E. Kardami. 2003. High levels of CUG-initiated FGF-2 expression cause chromatin compaction, decreased cardiomyocyte mitosis, and cell death. *Mol Cell Biochem.* 246:111-6.

Jacobs, C.M., K.A. Boldingh, H.H. Slagsvold, G.H. Thoresen, and R.E. Paulsen. 2004. ERK2 prohibits apoptosis induced subcellular translocation of orphan nuclear receptor NGFI-B/TR3. *J Biol Chem.*

Jaye, M., J. Schlessinger, and C.A. Dionne. 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochim Biophys Acta.* 1135:185-99.

Jiang, Z.S., R.R. Padua, H. Ju, B.W. Doble, Y. Jin, J. Hao, P.A. Cattini, I.M. Dixon, and E. Kardami. 2002. Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C. *Am J Physiol Heart Circ Physiol.* 282:H1071-80.

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

Jimenez, S.K., F. Sheikh, Y. Jin, K.A. Detillieux, J. Dhaliwal, E. Kardami, and P.A. Cattini. 2004. Transcriptional regulation of FGF-2 gene expression in cardiac myocytes. *Cardiovasc Res.* 62:548-57.

Johnson, D.E., and L.T. Williams. 1993. Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res.* 60:1-41.

Joy, A., J. Moffett, K. Neary, E. Mordechai, E.K. Stachowiak, S. Coons, J. Rankin-Shapiro, R.Z. Florkiewicz, and M.K. Stachowiak. 1997. Nuclear accumulation of FGF-2 is associated with proliferation of human astrocytes and glioma cells. *Oncogene*. 14:171-83.

Kamiguchi, H., K. Yoshida, H. Wakamoto, M. Inaba, H. Sasaki, M. Otani, and S. Toya. 1996. Cytokine-induced selective increase of high-molecular-weight bFGF isoforms and their subcellular kinetics in cultured rat hippocampal astrocytes. *Neurochem Res*. 21:701-6.

Kardami, E., Z.S. Jiang, S.K. Jimenez, C.J. Hirst, F. Sheikh, P. Zahradka, and P.A. Cattini. 2004. Fibroblast growth factor 2 isoforms and cardiac hypertrophy. *Cardiovasc Res*. 63:458-66.

Kaufmann, S.H., and M.O. Hengartner. 2001. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol*. 11:526-34.

Klein, S., J.A. Carroll, Y. Chen, M.F. Henry, P.A. Henry, I.E. Ortonowski, G. Pintucci, R.C. Beavis, W.H. Burgess, and D.B. Rifkin. 2000. Biochemical analysis of the arginine methylation of high molecular weight fibroblast growth factor-2. *J Biol Chem*. 275:3150-7.

Litchfield, D.W. 2003. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J*. 369:1-15.

Liu, L., B.W. Doble, and E. Kardami. 1993. Perinatal phenotype and hypothyroidism are associated with elevated levels of 21.5- to 22-kDa basic fibroblast growth factor in cardiac ventricles. *Dev Biol*. 157:507-16.

Lockshin, R.A., and Z. Zakeri. 2004. Caspase-independent cell death? *Oncogene*. 23:2766-73.

Maher, P.A. 1996. Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. *J Cell Biol.* 134:529-36.

Mahlknecht, U., S. Schnittger, O.G. Ottmann, C. Schoch, M. Mosebach, W. Hiddemann, and D. Hoelzer. 2000. Chromosomal organization and localization of the human histone deacetylase 5 gene (HDAC5). *Biochim Biophys Acta.* 1493:342-8.

Meggio, F., and L.A. Pinna. 2003. One-thousand-and-one substrates of protein kinase CK2? *Faseb J.* 17:349-68.

Mignatti, P., T. Morimoto, and D.B. Rifkin. 1992. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J Cell Physiol.* 151:81-93.

Myers, J.M., G.G. Martins, J. Ostrowski, and M.K. Stachowiak. 2003. Nuclear trafficking of FGFR1: a role for the transmembrane domain. *J Cell Biochem.* 88:1273-91.

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

Padua, R.R., P.L. Merle, B.W. Doble, C.H. Yu, P. Zahradka, G.N. Pierce, V. Panagia, and E. Kardami. 1998. FGF-2-induced negative inotropism and cardioprotection are inhibited by chelerythrine: involvement of sarcolemmal calcium-independent protein kinase C. *J Mol Cell Cardiol.* 30:2695-709.

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

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

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

Patry, V., E. Arnaud, F. Amalric, and H. Prats. 1994. Involvement of basic fibroblast growth factor NH2 terminus in nuclear accumulation. *Growth Factors.* 11:163-74.

Peng, H., J. Moffett, J. Myers, X. Fang, E.K. Stachowiak, P. Maher, E. Kratz, J. Hines, S.J. Fluharty, E. Mizukoshi, D.C. Bloom, and M.K. Stachowiak. 2001. Novel nuclear signaling pathway mediates activation of fibroblast growth factor-2 gene by type 1 and type 2 angiotensin II receptors. *Mol Biol Cell.* 12:449-62.

Peng, H., J. Myers, X. Fang, E.K. Stachowiak, P.A. Maher, G.G. Martins, G. Popescu, R. Berezney, and M.K. Stachowiak. 2002. Integrative nuclear FGFR1 signaling (INFS) pathway mediates activation of the tyrosine hydroxylase gene by angiotensin II, depolarization and protein kinase C. *J Neurochem.* 81:506-24.

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

Piotrowicz, R.S., L. Ding, P. Maher, and E.G. Levin. 2001. Inhibition of cell migration by 24-kDa fibroblast growth factor-2 is dependent upon the estrogen receptor. *J Biol Chem.* 276:3963-70.

Piotrowicz, R.S., P.A. Maher, and E.G. Levin. 1999. Dual activities of 22-24 kDA basic fibroblast growth factor: inhibition of migration and stimulation of proliferation. *J Cell Physiol.* 178:144-53.

Piotrowicz, R.S., J.L. Martin, W.H. Dillman, and E.G. Levin. 1997. The 27-kDa heat shock protein facilitates basic fibroblast growth factor release from endothelial cells. *J Biol Chem.* 272:7042-7.

Quarto, N., F.P. Finger, and D.B. Rifkin. 1991a. The NH2-terminal extension of high molecular weight bFGF is a nuclear targeting signal. *J Cell Physiol.* 147:311-8.

Quarto, N., D. Talarico, R. Florkiewicz, and D.B. Rifkin. 1991b. Selective expression of high molecular weight basic fibroblast growth factor confers a unique phenotype to NIH 3T3 cells. *Cell Regul.* 2:699-708.

Reilly, J.F., and P.A. Maher. 2001. Importin beta-mediated nuclear import of fibroblast growth factor receptor: role in cell proliferation. *J Cell Biol.* 152:1307-12.

Sauter, E.R., M. Nesbit, D. Tichansky, Z.J. Liu, T. Shirakawa, J. Palazzo, and M. Herlyn. 2001. Fibroblast growth factor-binding protein expression changes with disease progression in clinical and experimental human squamous epithelium. *Int J Cancer.* 92:374-81.

Sawada, M., P. Hayes, and S. Matsuyama. 2003. Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70. *Nat Cell Biol.* 5:352-7.

Saxena, N.K., M.A. Titus, X. Ding, J. Floyd, S. Srinivasan, S.V. Sitaraman, and F.A. Anania. 2004. Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk) and Akt phosphorylation. *Faseb J.*

Seghezzi, G., S. Patel, C.J. Ren, A. Gualandris, G. Pintucci, E.S. Robbins, R.L. Shapiro, A.C. Galloway, D.B. Rifkin, and P. Mignatti. 1998. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol.* 141:1659-73.

Sorenson, C.M. 2004. Bcl-2 family members and disease. *Biochim Biophys Acta.* 1644:169-77.

Stachowiak, E.K., X. Fang, J. Myers, S. Dunham, and M.K. Stachowiak. 2003a. cAMP-induced differentiation of human neuronal progenitor cells is mediated by nuclear fibroblast growth factor receptor-1 (FGFR1). *J Neurochem.* 84:1296-312.

Stachowiak, E.K., P.A. Maher, J. Tucholski, E. Mordechai, A. Joy, J. Moffett, S. Coons, and M.K. Stachowiak. 1997. Nuclear accumulation of fibroblast growth factor receptors in human glial cells--association with cell proliferation. *Oncogene.* 14:2201-11.

Stachowiak, M.K., X. Fang, J.M. Myers, S.M. Dunham, R. Berezney, P.A. Maher, and E.K. Stachowiak. 2003b. Integrative nuclear FGFR1 signaling (INFS) as a part of

a universal "feed-forward-and-gate" signaling module that controls cell growth and differentiation. *J Cell Biochem.* 90:662-91.

Stachowiak, M.K., J. Moffett, A. Joy, E. Puchacz, R. Florkiewicz, and E.K. Stachowiak. 1994. Regulation of bFGF gene expression and subcellular distribution of bFGF protein in adrenal medullary cells. *J Cell Biol.* 127:203-23.

Sun, G., B.W. Doble, J.M. Sun, R.R. Fandrich, R. Florkiewicz, L. Kirshenbaum, J.R. Davie, P.A. Cattini, and E. Kardami. 2001. CUG-initiated FGF-2 induces chromatin compaction in cultured cardiac myocytes and in vitro. *J Cell Physiol.* 186:457-67.

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

Tumova, S., A. Woods, and J.R. Couchman. 2000. Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. *Int J Biochem Cell Biol.* 32:269-88.

Unger, G.M., A.T. Davis, J.W. Slaton, and K. Ahmed. 2004. Protein kinase CK2 as regulator of cell survival: implications for cancer therapy. *Curr Cancer Drug Targets.* 4:77-84.

Vagner, S., C. Touriol, B. Galy, S. Audigier, M.C. Gensac, F. Amalric, F. Bayard, H. Prats, and A.C. Prats. 1996. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. *J Cell Biol.* 135:1391-402.

Van Cruchten, S., and W. Van Den Broeck. 2002. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat Histol Embryol.* 31:214-23.

Waterborg, J.H. 2002. Dynamics of histone acetylation in vivo. A function for acetylation turnover? *Biochem Cell Biol.* 80:363-78.

Wu, J., W.W. Wong, F. Khosravi, M.D. Minden, and L.Z. Penn. 2004. Blocking the Raf/MEK/ERK pathway sensitizes acute myelogenous leukemia cells to lovastatin-induced apoptosis. *Cancer Res.* 64:6461-8.

Xiao, L., P. Liu, T. Sobue, A. Lichtler, J.D. Coffin, and M.M. Hurley. 2003. Effect of overexpressing fibroblast growth factor 2 protein isoforms in osteoblastic ROS 17/2.8 cells. *J Cell Biochem.* 89:1291-301.

Yang, G., X. Sun, and R. Wang. 2004. Hydrogen sulfide-induced apoptosis of human aorta smooth muscle cells via the activation of mitogen-activated protein kinases and caspase-3. *Faseb J.*

Yoshida, T., I. Tomioka, T. Nagahara, T. Holyst, M. Sawada, P. Hayes, V. Gama, M. Okuno, Y. Chen, Y. Abe, T. Kanouchi, H. Sasada, D. Wang, T. Yokota, E. Sato, and S. Matsuyama. 2004. Bax-inhibiting peptide derived from mouse and rat Ku70. *Biochem Biophys Res Commun.* 321:961-6.

Zagzag, D., D.C. Miller, Y. Sato, D.B. Rifkin, and D.E. Burstein. 1990. Immunohistochemical localization of basic fibroblast growth factor in astrocytomas. *Cancer Res.* 50:7393-8.

Zhou, X., V.M. Richon, A.H. Wang, X.J. Yang, R.A. Rifkind, and P.A. Marks. 2000. Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and

2, and its cellular localization is regulated by oncogenic Ras. *Proc Natl Acad Sci U S A*. 97:14329-33.

Zhu, H., K. Ramnarayan, J. Anchin, W.Y. Miao, A. Sereno, L. Millman, J. Zheng, V.N. Balaji, and M.E. Wolff. 1995. Glu-96 of basic fibroblast growth factor is essential for high affinity receptor binding. Identification by structure-based site-directed mutagenesis. *J Biol Chem*. 270:21869-74.