MECHANISM OF CUG-FGF-2-INDUCED CHROMATIN COMPACTION OF CARDIOMYOCYTES

BY

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MECHANISM OF CUG-FGF-2-INDUCED CHROMATIN COMPACTION OF CARDIOMYOCYTES

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

Of

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For

Andy

and

Isaiah

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ABSTRACT

Fibroblast growth factor 2 (FGF-2) is a member of a large family of heparin-binding proteins. This multifunctional protein is released from cells after cell death, wounding, chemical injury or irradiation. FGF-2 is found in all developmental stages and tissues and has been implicated in several events in the heart such as embryonic growth, myocyte hypertrophy, angiogenesis and protection from ischemia reperfusion injury. It exists as four different molecular weight isoforms that originate from alternative initiation codons within the same RNA molecule. CUG or leucine-initiated forms of 22, 22.5, 24 and 34 kDa (hi FGF-2) contain nuclear localization signal-like sequences at their N-termini that targets them to the nucleus. An AUG or methionine-initiated form of 18 kDa (lo FGF-2) is found in the nucleus, cytosol and in association with the cell surface. Stress or cell transformation has been shown to induce preferential translation of hi FGF-2 nevertheless its function is not characterized as yet. Previous studies in our laboratory showed that overexpression of hi FGF-2 caused a distinct nuclear phenotype in cardiomyocytes.

The objective of our studies was to explore further the relationship between hi FGF-2 expression levels, mitotic entry, cell number, chromatin compaction and the development of apoptotic DNA laddering in neonatal (1 day old) rat ventricular cardiac myocytes. A second objective was to establish the signal transduction pathway leading to hi FGF-2-induced

chromatin compaction. Primary cultures of cardiac myocytes were transfected with different multiplicity of infections (m.o.i. 50-200) of adenoviral vectors expressing hi FGF-2. Relative levels of hi FGF-2 protein (Western blotting), mitotic index (labeling for phosphorylated histone H3), appearance of compacted chromatin (Hoechst staining) and appearance of a DNA ladder (gel analysis) were examined at 1-3 days post-transfection. At an m.o.i. of 50, levels of hi FGF-2 (assessed by Western blotting) and mitotic index (fraction of myocyte nuclei staining positive for phosphorylated histone H3) increased in parallel, becoming maximal at two days. At 200 m.o.i., maximal expression of hi FGF-2 (approximately double that at 50 m.o.i) was achieved at two days and coincided with decreased mitotic index and increased chromatin compaction. At three days, compaction was maximal, mitotic index was minimal, and cell numbers decreased, accompanied by the appearance of DNA laddering, an indicator of apoptosis.

The second objective was achieved by examining the role of the plasma membrane or intracellular FGF-2 receptor-1 (FGFR1) in this process. Neutralizing anti-FGF-2 antibodies decreased FGF-2-stimulated DNA synthesis but not the extent of chromatin clumping. Expression of a kinase-deficient, dominant negative FGFR1(dn), by adenovirally-mediated transfection, decreased DNA synthesis as well as completely prevented the 'clumped' chromatin phenotype. Downstream of the FGFR1 is the ras-raf-MEK1-erk1/2 pathway and expression of a dominant negative (dn)

version of mitogen kinase kinase 1 decreased the activation of erk1/2 (implicated in nuclear transport) and also prevented hi FGF-2-induced chromatin disruption. Neither Ad.FGFR1(dn), nor Ad.MKK1(dn) prevented nuclear accumulation of CUG-FGF-2, indicating that this pathway did not affect nuclear compaction by altering nuclear accumulation of hi FGF-2. Overexpression of a dominant negative PKCε did not prevent the nuclear effects of hi FGF-2, thus we conclude that it has no role in hi FGF-2-induced chromatin compaction under the assay conditions used.

Our data indicate that relatively low m.o.i. delivery of hi FGF-2 and early time points favored a proliferative phenotype, while the higher m.o.i. regimen, and later time points, promoted chromatin compaction, with inhibition of proliferation and myocyte apoptosis. In addition, the effects of hi FGF-2 on chromatin require activation of intracellular, possibly nuclear, FGFR1, and the erk1/2 pathway, but do not reflect changes on its nuclear accumulation. While plasma membrane FGFR1 mediates cardioprotection and stimulation of DNA synthesis, the interaction of intracellular/nuclear hi FGF-2 and FGFR1 can lead to chromatin disruption and cell death.

LIST OF FIGURES

ABBREVIATIONS

Ad.....adenovirus

anti-P-H3.....anti-phospho histone 3

AUG.....methionine

BAMCs.....bovine adrenal medullary cells

BCA.....bicinchonic acid

bFGF.....basic fibroblast growth factor

BSA.....bovine serum albumin

°C.....degrees centigrade

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

cDNA.....complimentary deoxyribonucleic acid

ChIP.....chromatin immunoprecipitation

CO_2.....carbon dioxide

CUG.....leucine

DAG.....diacylglycerol

DMEM.....Dulbecco's modified Eagle's medium

dn.....dominant negative

DNA.....deoxyribonucleic acid

DNase.....deoxyribonuclease

EDTA.....ethylenediaminetetraacetic acid

Egr-1.....early growth response-1 protein

Erk.....extracellular-signal-regulated protein kinase

FBS.....fetal bovine serum

FGF-2.....fibroblast growth factor 2

FGFR1.....fibroblast growth factor receptor 1

g.....grams

GDP.....guanosine 5'-diphosphate

GTP.....guanosine 5'-triphosphate

h.....hour

^{3}H.....tritiated thymidine

H1.....histone 1

H3.....histone 3

hi.....high molecular weight

HSPG.....heparin sulfate proteoglycan

IgG.....immunoglobulin

IGF-1.....insulin growth factor 1

IL-6.....interleukin 6

IP_3.....1,4,5-triphosphate

IRES.....internal ribosomal entry site

kDa.....kilodalton

KOAc.....potassium acetate

L.....litres

lo.....low molecular weight

M.....molar

MAPK.....mitogen activated protein kinase

MEK-1.....MAP kinase kinase

MKK1.....mitogen kinase kinase 1

μCi.....microcurie

μg.....microgram

μL.....microliter

μM.....micromolar

mL.....milliliter

mM.....millimolar

MI.....mitotic index

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

mRNA.....messenger ribonucleic acid

MSK 1.....mitogen and stress activated protein kinase 1

NaCl.....sodium chloride

NLS.....nuclear localization signal / sequence

N.S.....not significant

O.D.....optical density

p42/44.....MAPK

PAI-1.....plasminogen activator inhibitor-1

PBS.....phosphate buffered saline

phospho p42/44.....phosphorylated MAPK

PIP_2.....phosphotidylinositol bisphosphate

PKC.....protein kinase C

PLC.....phospholipase C

PMSF.....phenylmethylsulfonylfluoride

PVDF.....polyvinylidene difluoride

RNA.....ribonucleic acid

RNase.....ribonuclease

RPM.....rotations per minute

SDS.....sodium dodecyl sulfate

SEM.....standard error of the mean

ser.....serine

SH2.....src homology 2

TBST.....Tris buffered saline with TWEEN

TCA.....trichloroacetic acid

t-PA.....tissue-type plasminogen activator

Tris-HCl.....tris (hydroxymethyl) aminomethane – hydrochloric acid

U.....units

uPA.....urokinase type plasminogen activator

UV.....ultraviolet

vec.....vector

VEGF.....vascular endothelial growth factor

CHAPTER 1

REVIEW OF THE LITERATURE

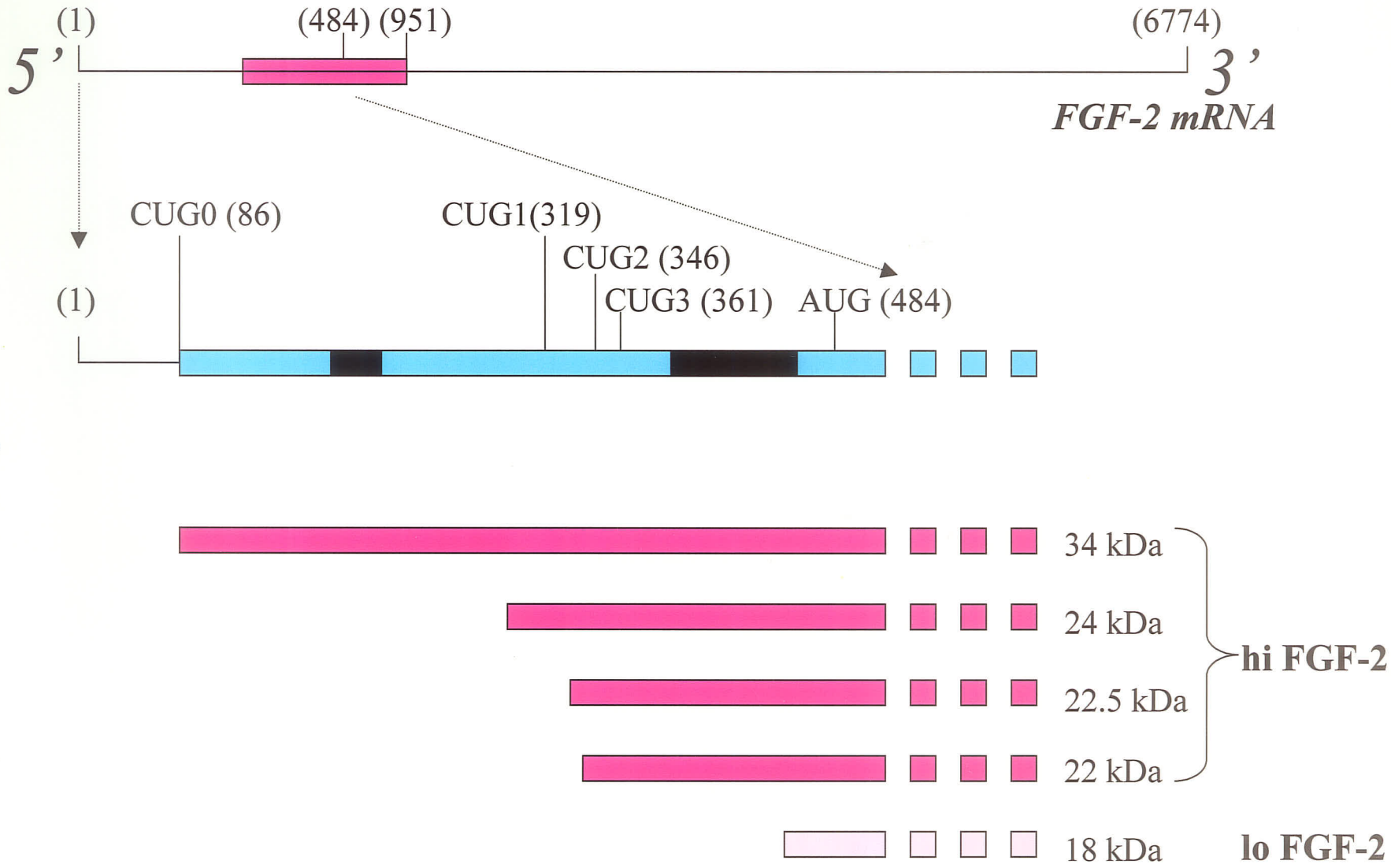
1.1 General

Basic fibroblast growth factor 2 or FGF-2 is a member of a large family of heparin-binding proteins (Bikfalvi et al., 1997), numbering 23 members so far (Yamashita et al., 2000). FGF-2 is one of the best studied and characterized members of this family. A single copy of the human FGF-2 gene encodes five FGF-2 isoforms of varied length (18-35 kDa) (Arnaud et al., 1999; Delrieu 2000). Figure 1 illustrates how translation from an AUG start site produces the 18 kDa isoform (referred to by us as lo FGF-2), while translation from several CUG start sites, found upstream of the AUG site produces 22, 22.5, 24 and 34 kDa FGF-2 (Bugler et al., 1991; Florkiewicz and Sommer 1989; Prats et al., 1989) (referred to collectively by us as hi FGF-2). Rat and chick FGF-2 also have AUG and CUG initiated isoforms. Alternative translation of FGF-2 mRNA occurs by internal ribosomal entry sites (Vagner et al., 1995). The 22-34 kDa forms represent N-terminal extensions of the 18 kDa FGF-2 (Delrieu 2000).

The first FGF-2 form to be isolated and the most widely studied is lo FGF-2 (Maciag et al., 1984). CUG-initiated or hi FGF-2 was discovered in 1989 (Florkiewicz and Sommer 1989). Both hi and lo FGF-2 are capable of stimulating cell proliferation and angiogenesis, however, while lo FGF2 stimulates, hi FGF-2 is reported to inhibit cell migration (Piotrowicz et al., 1997). Furthermore, hi FGF-2 seems to be more effective in conferring

Figure 1. ***Alternative translation of human FGF-2 messenger RNA results in various hi and lo FGF-2 isoforms***

Nucleotide positions relative to the transcription start site are indicated in brackets. The black boxes represent NLS-like sequences. Translation from an AUG start site at the amino-terminal end produces the 18 kDa or lo FGF-2 which can be found in the cytosol and nucleus. CUG start sites produce 22, 22.5, 24 and 34 kDa proteins or hi FGF2 which are exclusively found in the nucleus.



Adapted from FEBS Letters, Delrieu, 2000

aspects of a transformed phenotype (Vagner et al., 1996). Differential effects on gene expression have been reported for hi and lo FGF-2 (Delrieu 2000). Differences in activity between hi and lo FGF-2 have been linked to differences in subcellular localization. While lo FGF-2 can be found in the extracellular matrix as well as the cytosol and nucleus, hi FGF-2 is found exclusively in the nucleus (Bugler et al., 1991; Pasumarthi et al., 1994; Pasumarthi et al., 1996). The N-terminal extension of hi FGF-2 functions as a nuclear localization signal (Bugler et al., 1991; Quarto et al., 1991). However, a sequence within lo FGF-2 is also reported to be important for nuclear localization (Claus et al., 2003).

FGF-2 is known to act in an autocrine/paracrine as well as intracrine fashion (Delrieu 2000; Pasumarthi et al., 1994; Pasumarthi et al., 1996). FGF-2 is exported by cells and is retained at the extracellular space due to its affinity for heparin. Extracellular FGF-2 can then interact and activate tyrosine kinase receptors (FGFR), a step mediating most FGF-2 biological activities. An intracrine mode of action is also described for both lo and hi FGF-2 and involves nuclear localization (Pasumarthi et al., 1994; Pasumarthi et al., 1996). Increasing evidence indicates that intracellular FGFR1 is also participating in FGF-2 nuclear signaling (Maher 1996; Stachowiak et al., 1996; Stachowiak et al., 1996; Stachowiak et al., 1997). Very little is currently known about the intracellular signaling cascades that mediate the effects of hi FGF-2.

FGF-2 is an important growth factor in the heart. Hi FGF-2 predominates in immature or hypothyroid myocardium (Liu et al., 1993); its levels are transiently elevated following cardiac injury (Padua and Kardami 1993). In cultured cardiomyocytes, hi FGF-2, acting in an autocrine/intracrine manner, stimulates cell proliferation, but when it follows an intracrine/nuclear route it causes chromatin compaction (Pasumarthi et al., 1994; Pasumarthi et al., 1996; Sun et al., 2001). Specific questions relating to the relationship between hi FGF-2 expression, chromatin compaction and cell death, and the involvement of FGFR in mediating the hi-FGF-2 effects on myocyte nuclei are the subject of this thesis.

In this introduction, we will focus on reviewing the literature on: (a) expression and distribution of hi FGF-2; (b) the effects of nuclear hi FGF-2 on cell proliferation, transformation, gene expression and survival; (c) the role of nuclear FGFR on intracrine FGF-2 signaling; (d) effects of hi FGF-2 on cell migration; and (e) hi FGF-2 specifically in the heart and the cardiomyocyte (previous work in the lab). We will conclude by providing a rationale for the conducted studies.

1.2 Expression / Distribution of hi FGF-2

A number of studies reported that hi FGF-2 is predominantly associated with transformed cells lines (Vagner et al., 1996) including uterus carcinoma HeLa cells, liver adenocarcinoma Sk-Hep1, epidermoid

carcinoma A-431 cells and pancreas carcinoma MIA PaCa-2 cells, whereas "normal" cells such as skin fibroblasts or aortic endothelial cells, produce predominantly lo FGF-2 (Vagner et al., 1996). Hi FGF-2 was also produced in primary skin fibroblasts in response to heat shock and oxidative stress and UV-crosslinking experiment showed that its expression could be correlated to the binding of several proteins to the 5' region of the FGF-2 mRNA, possibly indicating regulation at the translational level (Vagner et al., 1996). Because hi FGF-2 is rapidly synthesized in response to stress, Vagner et al proposed that hi FGF-2 may be a survival factor (Vagner et al., 1996). In another system, bovine adrenal medullary cells, expression and nuclear localization of both hi and lo FGF-2 was stimulated by angiotensin II, depolarization, activation of protein kinase C or adenylate cyclase (Peng et al., 2002).

In contrast to the findings from cell lines, hi FGF-2 is constitutively accumulated and is the predominant FGF-2 species in adult brain (Delrieu 2000; Giordano et al., 1991; Liu et al., 1993), a differentiated tissue. In fact, hi FGF-2 can be detected to a variable extent in extracts from several adult organs (Liu et al., 1993). A consistent finding is that the relative abundance of hi and lo FGF-2 shows tissue and stage-specificity (Coffin et al., 1995; Liu et al., 1993). Relative levels of hi FGF-2 reach maximum levels in the adult brain, but minimal levels in the adult heart (Liu et al., 1993). Hypothyroidism stimulates accumulation of hi FGF-2 in the heart but not other tissues (Liu et al., 1993).

Expression of FGF-2 is regulated at the translational level by several cis-acting elements of the RNA leader sequence (Prats et al., 1992). The mechanism of translation-initiation of the FGF-2 mRNA differs from the classical model (Vagner et al., 1995). The classical 'cap' dependent model dictates that translation occurs when the ribosome binds to the capped mRNA 5' end and scans the RNA until it finds a 'start' codon (Kozak 1978). In the case of FGF-2, translation occurs independently of the 'cap' following an internal ribosome entry process, due to the presence of an IRES (internal ribosomal entry site) upstream of the CUG start codon (Vagner et al., 1995). This mechanism is uncommon, and, in the case of viral IRES, requires cellular trans-acting factors (Vagner et al., 1996). Cell specific trans-acting factors could account for the cell-specificity of hi FGF-2 accumulation. It was later discovered that while IRES-dependent translation regulated expression of AUG-initiated (lo) or CUG-initiated (hi, 21-25 kDa) FGF-2, a cap-dependent translation regulated expression of the CUG-initiated 34 kDa FGF-2 (Arnaud et al., 1999). Cap-dependent translation is inhibited under stress conditions. It is proposed that IRES-mediated translation is activated in stressed cells resulting in FGF-2 accumulation (Prats and Prats 2002).

An important feature of hi FGF-2 is its nuclear localization. Its N-terminal extension is widely believed to provide the necessary signal for nuclear localization, since it alone can confer nuclear localization to non-nuclear proteins (Claus et al., 2003; Quarto et al., 1991). Nevertheless,

additional elements of the FGF-2 structure that are common between hi and lo FGF-2 may also participate in this phenomenon. Claus and coworkers recently reported that mutations in arginine residues 149 and 151 in the C terminal prevent nuclear localization of hi as well as lo FGF-2 (Claus et al., 2003). These investigators also reported a distinct subnuclear localization pattern for hi FGF-2 and association with the nuclear matrix (Claus et al., 2003).

1.3 Proliferation / Survival

Overexpresson of either lo or hi FGF-2 has been reported to stimulate proliferation in cells grown in complete medium, although only hi FGF-2 was able to stimulate growth in cells grown in 1% serum (Arese et al., 1999; Bikfalvi et al., 1995). Nevertheless, Arese et al. have provided evidence that nuclear targeting of either hi or lo FGF results in a dose-dependent stimulation of DNA synthesis and cell proliferation. All FGF forms, hi and lo, were also capable of stimulating anchorage–independent colony growth, a hallmark of cell transformation (Arese et al., 1999).

The largest hi FGF-2 isoform (34 kDa) was found to behave as a survival factor in NIH-3T3 cells (Arnaud et al., 1999). Increased radioresistance was observed in HeLa cells overexpressing hi FGF-2 (Cohen-Jonathan et al., 1997). This radioprotective effect is associated with an increase in G2 delay and a hyperphosphorylation of the cyclin-dependent kinase $p34^{cdc2}$ (Cohen-Jonathan et al., 1997). Dini et al.

investigated the role of lo and hi isoforms in drug sensitivity modulation and gene amplification potential in NIH-3T3 and A31 cells. Cells overexpressing hi FGF-2 grew in 1% serum, their invasive potential was lower and they were more drug resistant compared to lo FGF-2 overexpressing cells (Dini et al., 2002).

1.4 Cell Migration

Hi FGF-2 is not normally exported by the cell (Delrieu 2000). Nevertheless, there are reports that under certain conditions it is found in the conditioned medium of endothelial cells (Piotrowicz et al., 1997). These conditions require activation of heat shock protein 27 (Piotrowicz et al., 1997) and the estrogen receptor (Piotrowicz et al., 2001). Under those or equivalent conditions of stress, hi FGF-2 could be found at the extracellular space, acting on cell membrane receptors in an intracrine-autocrine fashion and affecting the cell phenotype (Piotrowicz et al., 2001; Piotrowicz et al., 1997). A recent manuscript in fact, reports that serum-stimulated cells shed membrane-encased vesicles containing both hi and lo FGF-2 into the extracellular environment (Taverna et al., 2003). Unless hi FGF-2 is exported, it cannot act on the receptors to affect migration. The effect on migration is not an intracrine effect, it is an autocrine or paracrine effect and is stimulated by adding exogenous hi FGF-2.

Cell migration is important in processes such as angiogenesis, development, wound healing and tumor growth. As a consequence,

several studies have been conducted regarding FGF-2 and its involvement in cell migration. Recombinant hi FGF-2, in contrast to lo FGF-2, inhibited migration of endothelial or breast carcinoma cells (MCF7) (Piotrowicz et al., 1999). This property is attributed to a fragment of the molecule that includes the 55 N-terminal amino-acids (present only in hi FGF-2) and the neighboring 31 amino-acids (common to both hi and lo FGF-2) (Ding et al., 2002). Ding et al. have demonstrated that this fragment retains most of the ability of hi FGF-2 to inhibit cell migration in culture and angiogenesis in vivo, while having lost the ability to bind to the FGFR (Ding et al., 2002). Studies by (Piotrowicz et al., 1999) offered further support to previous studies that used antibodies specific for the N-terminal extension of hi FGF-2 to specifically block its effects and proposed that this region of the molecule was critical for inhibition of migration.

Further studies revealed that inhibition of migration by hi FGF-2 is mediated by the estrogen receptor in both endothelial cells and MCF-7 cells: upon removal of the estrogen receptor, the inhibitory activity of hi FGF-2 was abolished while re-introduction of the estrogen receptor into deficient cells promoted the inhibitory response (Piotrowicz et al., 2001). Because hi FGF-2 inhibited cell migration even in the presence of mitogens such as IGF-1, VEGF and lo FGF-2, it has been proposed that hi FGF-2 can override the signals generated by these growth factors using a novel intracellular pathway that involves the estrogen receptor. This

receptor is activated upon synthesis of the amino-terminal end of FGF-2 (Piotrowicz et al., 2001).

An additional conclusion from the above mentioned studies is that the ability to induce proliferation can be dissociated from the ability to stimulate migration. The hi FGF-2–derived, N terminal fragment retained its ability to inhibit migration while having lost the proliferative activity that resides in the region of the molecule shared with lo FGF-2 (Ding et al., 2002).

Inhibition of migration by hi FGF-2 has also been detected after gene transfer in other cell types as well. Pancreatic tumour cells transfected with the hi FGF-2 migrated less than control cells or cells expressing lo FGF-2, demonstrating the potential of the upregulation of hi FGF-2 levels to reduce spreading of pancreatic cancer cells (Escaffit et al., 2000)

1.5 Effects on Gene Expression

There is evidence that hi FGF-2 can influence the expression of a number of genes, although there is as yet no information as to the detailed mechanism involved. One study used stable transfections of NIH-3T3 cells with hi FGF-2 to show an increase in mRNA expression for the cytokine IL-6, resulting from upregulation of its promoter activity (Delrieu et al., 1998); in another cell type (HeLa cells), hi FGF-2 caused the opposite effect, a down regulation of IL-6, suggesting cell-type specific effects

(Delrieu et al., 1999). Neither of the above effects could be induced by added exogenous lo FGF-2, so the authors suggested that they are not caused by an autocrine/paracrine mechanism (Delrieu et al., 1998; Delrieu et al., 1999). In view, however, of studies showing distinct effects of extracellular hi and lo FGF-2 on cell migration, the above conclusion has not been adequately supported.

Pancreatic tumors overexpress FGF-2 and tissue-type plasminogen activator (t-PA) (Escaffit et al., 2000). T-PA is involved in extracellular matrix degradation by activating plasminogen and metalloproteases while plasminogen activator inhibitor-1 (PAI-1) inhibits t-PA (Escaffit et al., 2000). To investigate the role of FGF-2 isoforms on t-PA and PAI-1 expression, pancreatic carcinoma AR4-2J cells were retrovirally transfected with mutated FGF-2 cDNAs expressing either lo or hi FGF-2. Hi FGF-2 reduced t-PA and PAI-1 synthesis 2-fold while lo FGF-2 had the opposite effect (Escaffit et al., 2000).

Gaubert et al (2001) studied the effect of hi or lo FGF-2 expression (stably or transiently in pancreatic AR4-2J cells) on signal transduction enzymes. The two FGF-2 isoforms differentially modulated PKC levels: hi FGF-2 overexpression down-regulated PKCε and upregulated PKCδ protein and mRNA levels, while lo FGF-2 did not. Furthermore, expression of hi FGF-2 increased erk 1 and 2 activation. Erk activation was dependent on PKCδ indicating that this PKC isotype must be in the active state in hi FGF-2 expressing cells (Gaubert et al., 2001).

Overexpression of lo but not hi FGF-2 increased expression of alpha 5 beta 1 integrin (Klein et al., 1996). In addition and as will be discussed below, nuclear hi FGF-2 can direct the expression of the tyrosine hydroxylase gene in bovine adrenal medullary cells (Peng et al., 2002).

1.6 Nuclear FGFR1

The effects of exported FGF-2 are widely believed to be mediated by an interaction with tyrosine kinase plasma membrane receptors (FGFR1-4) (Klint and Claesson-Welsh 1999; Powers et al., 2000). The traditional view of signaling is that the ligand binds to the receptor at the extracellular space. This event at the plasma membrane is followed by activation of a number of downstream signal transduction pathways in the cytosol and then the nucleus, resulting in a number of end-points. It is, however, becoming increasingly evident that this scenario cannot explain several reports of nuclear localization and intracellular signaling for FGFR1. Studies to date have linked the subcellular and nuclear localization of FGFR1 mostly with that of lo FGF-2. Hi FGF-2 being predominantly nuclear and having similar affinity for FGFR1, as does the lo FGF-2 isoform, is even more likely to be involved in these pathways.

Maher provided strong evidence for nuclear accumulation of plasma membrane FGFR1 in response to FGF-2 stimulation of Swiss 3T3 fibroblasts, and suggested that FGFR may have a direct effect on gene

transcription (Maher 1996). Stachowiak et al., using confocal microscopy as well as subcellular fractionation, showed that FGFR1 was associated with the nuclear matrix-lamina in human astrocytes (Stachowiak et al., 1996). Nuclear accumulation of FGFR1 was found to be associated with cell proliferation in U251MG glioma cells: quiescent cells had little or no nuclear FGFR1 in high-density cultures (Stachowiak et al., 1997). Stachowiak et al., have suggested that nuclear FGFR1 stimulates the transition from the G0/G1 to the S phase of the cell cycle (Stachowiak et al., 1997). Nuclear accumulation of full-length and functional FGFR1, both transfected and endogenous has been shown in BAMCs (Peng et al., 2001; Stachowiak et al., 1996; Stachowiak et al., 1994), astrocytes (Stachowiak et al., 1996; Stachowiak et al., 1997), glioma cells (Stachowiak et al., 1997; Stachowiak et al., 1997) and in sympathetic neurons (Stachowiak et al., 1997) via Western blotting and antibodies specific for FGFR1 epitopes.

Peng et al. (2002) generated an FGFR1 mutant that does not insert to the plasma membrane by deleting the hydrophobic signal peptide required for membrane insertion. A second mutant, in which the deleted signal peptide was replaced by addition of a nuclear localization signal (NLS), localized exclusively to the nucleus. The second mutant proved to be more effective at affecting tyrosine hydroxylase gene expression compared to the first mutant, which was predominantly cytoplasmic. Both mutants showed that nuclear localization (but not plasma membrane

association) were essential for its ability to affect expression of the tyrosine hydroxylase gene. These authors proposed a novel signal transducing mechanism involving nuclear FGFR1 and hi FGF-2 to activate the tyrosine hydroxylase gene, and concluded that intracrine signaling is caused by a separate pool of FGFR1, different from the plasma membrane receptors and has a direct gene transactivating function (Peng et al., 2002).

Nuclear localization and action of FGFR1 may however not occur in all systems. Prudovsky et al. argue that FGFR1 is translocated near the nucleus during G1 of the cell cycle and suggest that it may play an intracellular role near the nucleus as an enzymatic modifier of proteins involved in DNA synthesis in NIH 3T3 cells (Prudovsky et al., 1994).

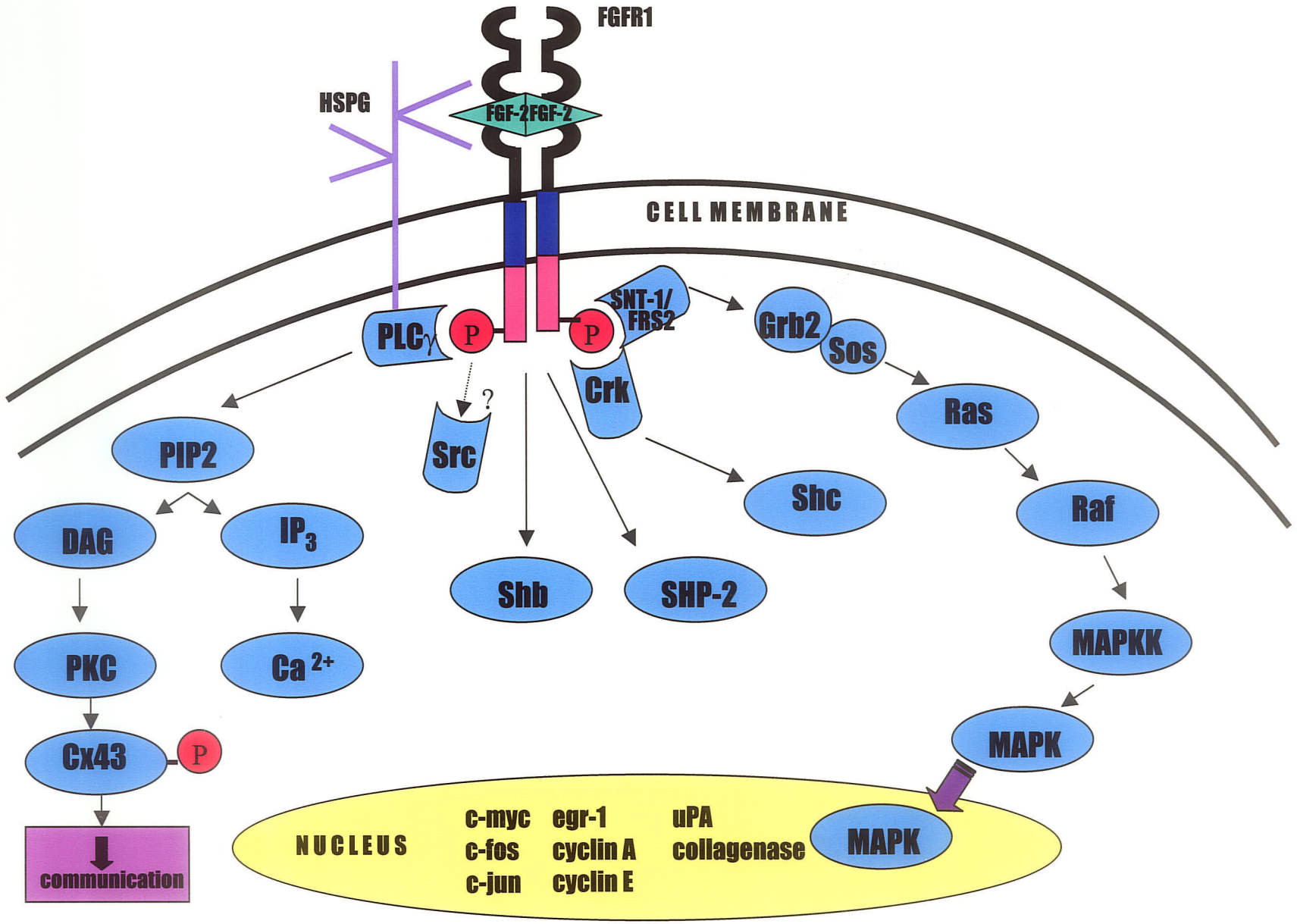
It is not as yet known how FGFR1 is translocated into the nucleus. FGFR1 is a macromolecule of 103-145 kDa with an apparent size of 150 kDa due to glycosylation. It would require active transportation across the nuclear pore membrane (Stachowiak et al., 1996). Transportation across the nuclear pore complex requires that the protein have a nuclear localization signal (NLS) sequence, or, that the protein be bound to another protein that has an NLS (Stachowiak et al., 1996). Reilly and Maher reported that importin-β, a component of multiple nuclear import pathways, interacts with FGFR1 and mediates its nuclear translocation by a mechanism distinct from classical nuclear import (Reilly and Maher 2001).

1.7 FGF-2 Signal Transduction

The FGFR has 4 major members (FGFR1-4) and is expressed in most cells and tissues (Klint and Claesson-Welsh 1999). It is composed of an extracellular domain containing 2 or 3 immunoglobulin (Ig) loops, one acid box consisting of a cluster of acidic amino acids, one transmembrane domain, and a carboxy terminal tail containing a cytosolic tyrosine kinase domain (Jaye et al., 1992; Klint and Claesson-Welsh 1999; Powers et al., 2000). The acid box is situated between the first and second immunogloblin-like folds in all FGFR types and is composed of a row of 8 consecutive acidic residues; it is a unique feature of the FGFR and important for FGFR function (Klint and Claesson-Welsh 1999). Figure 2 presents a schematic diagram of the signal transduction pathway for FGF-2 in cardiomyocytes. FGF-2 ligand binds to the extracellular domain and induces oligomerization of receptors (Jaye et al., 1992). Heparan sulfate proteoglycans (HSPG) promote the interaction of FGF-2 with the FGFR. Proteoglycans are integral components of the basement membranes which contain carbohydrate side chains and are essential for FGF function (Mason 1994; Yayon et al., 1991). The complex formed between FGF-2, FGFR and HSPG promotes FGF-2 binding and signaling. Interaction of FGF-2 with the plasma membrane FGFRs induces dimerization leading to phosphorylation of the tyrosine kinase domain (Klint and Claesson-Welsh 1999). Dimerization of receptor tyrosine kinases appears to be a prerequisite for activation of the tyrosine kinase:

Figure 2. ***Schematic of the signal transduction pathway for FGF-2 in cardiomyocytes***

HSPG promotes the interaction and binding of FGF-2 with the extracellular domain of the FGFR1. This induces receptor dimerization, leading to phosphorylation of the tyrosine kinase domain and initiation of downstream signaling events.


FGFR1
HSPG
FGF-2FGF-2
CELL MEMBRANE
PLCγ
P
P
SNT-1/
FRS2
Grb2
Sos
Ras
Raf
MAPKK
MAPK
Crk
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Src
PIP2
DAG
IP3
Shc
Shb
SHP-2
PKC
Ca 2+
Cx43
P
communication
NUCLEUS
c-myc
c-fos
c-jun
egr-1
cyclin A
cyclin E
uPA
collagenase
MAPK

the close proximity allows trans phosphorylation and is accompanied by conformational changes in the intracellular domain, a critical step in receptor activation (Klint and Claesson-Welsh 1999). The phosphorylated tyrosines are recognized by src homology 2 (SH2) domain-containing molecules and phospholipase C (PLC γ) initiating downstream signaling events in chains or cascades often resulting in changes in gene expression (Boilly et al., 2000; Klint and Claesson-Welsh 1999).

A major pathway involves the receptor tyrosine activation of SNT-1/FGFR substrate (FRS2): phosphorylation of SNT-1/FRS2 recruits the adaptor proteins Grb2/Sos complex and facilitates binding (Grb2 is a small adaptor molecule that binds to Sos, a nucleotide exchange factor, through its SH3 domain (Boilly et al., 2000)). Sos catalyzes the exchange of GDP for GTP on Ras, a GTP-binding proto-oncogene. Ras activates Raf, a serine-threonine kinase, by phosphorylating it. Raf phosphorylates mitogen activated protein kinase kinase (MAPKK or Mek-1), which in turn phosphorylates MAPK or erk1/2. MAPK translocates to the nucleus where it phosphorylates transcription factors that affect gene expression (Powers et al., 2000). Some targets in the nucleus are immediate early genes such as *c-myc*, *c-fos*, *c-jun* and *egr-1*, cell cycle proteins such as cyclins A and E, as well as matrix proteases such as uPA and collagenase (Szebenyi and Fallon 1999; Tomono et al., 1998).

Another major pathway involves the receptor tyrosine phosphorylation of phospholipase C gamma (PLCγ). PLCγ hydrolyzes

phosphatidylinositol 4,5 bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 goes on to release Ca^{2+} from intracellular stores while DAG accumulation activates members of the PKC family (Klint and Claesson-Welsh 1999; Powers et al., 2000). PKC can go on to phosphorylate targets like the gap junction protein connexin 43 (Powers et al., 2000). The FGFR can also indirectly act on src via PLCγ (Powers et al., 2000).

The SH2 domain-containing phosphotyrosine phosphatase SHP-2 is involved in regulation of signal transduction downstream of tyrosine kinases (Klint and Claesson-Welsh 1999).

1.8 Hi FGF-2 in the heart

Relative levels of hi FGF-2 are developmentally regulated in the heart: hi FGF-2 is the predominant FGF-2 species in extracts from immature cardiomyocytes while lo FGF-2 predominates in adult heart extracts (Liu et al., 1993). Since blood levels of thyroid hormone during early neonatal period are low compared to those in the adult animals, and in view of the importance of this hormone in cardiac growth and differentiation, Liu et al. examined the effect of thyroid status on the relative expression of hi FGF-2. Hypothyroidism was accompanied by significant increases in the relative levels of ventricular hi FGF-2, while hyperthyroidism resulted in increased accumulation of lo FGF-2 (Liu et al., 1993). These early data suggested a link between the immature cardiac

phenotype (in neonatal or hypothyroid rats) and expression of hi FGF-2. One could also argue that the 'stress' of induced hypothyroidism, caused the increase in 'hi' FGF-2. Preferential translation of hi FGF-2 in response to stress has been reported (Piotrowicz et al., 1997; Vagner et al., 1996).

Increased and transient accumulation of hi FGF-2 in response to cardiac injury induced by isoproterenol injection was observed by Padua and Kardami (Padua and Kardami 1993). One day after isoproterenol injection, they found increased accumulation of FGF-2 in necrotic cardiomyocytes, assessed by immunofluorescence microscopy and antibodies recognizing all FGF-2 species. This correlated with increased levels of hi FGF-2, detected by western blotting of total cardiac lysates, suggesting the possibility that hi FGF-2 was involved in the injury process (Padua and Kardami 1993). Again, preferential translation of hi FGF-2 during (isoproterenol-induced) stress provides a possible scenario for the detected increases. An additional or parallel possibility is that the increases in hi FGF-2 were a consequence of the intense cellular infiltration and proliferation that occurs at the injury sites at this time point (Padua and Kardami 1993). This latter possibility is in apparent contrast to reports that hi FGF-2 inhibits cell migration (Ding et al., 2002; Piotrowicz et al., 2001; Piotrowicz et al., 1999). It should be noted that these studies have not examined the effects of hi FGF-2 on all the different cell types involved in the injury-repair response. Levels of hi FGF-2 returned to pre-injury levels at one week, post isoproterenol

injection, while those of lo FGF-2 increased (Padua and Kardami 1993). This was associated with full 'healing' of the focal isoproterenol lesions, and absence of any necrotic myocytes (Padua and Kardami 1993).

To better understand the function of hi versus lo FGF-2 in cardiomyocytes, Pasumarthi et al. (1994, 1996) used transient gene transfer of embryonic chicken and neonatal rat ventricular myocytes. In both cases overexpression of both isoforms caused an increase in proliferation, DNA synthesis and protein synthesis as well as a decrease in myosin levels, consistent with the dedifferentiation associated with induction of proliferation (Pasumarthi et al., 1994; Pasumarthi et al., 1996). Localization studies showed an accumulation of hi predominantly in the nucleus and lo FGF-2 in the cytoplasm and nucleus (Pasumarthi et al., 1994). Overexpression of hi but not lo FGF-2 also produced a very particular nuclear phenotype consisting of presence of several compacted chromatin 'clumps' encased by an apparently intact nuclear membrane (Pasumarthi et al., 1994; Pasumarthi et al., 1996). Hi FGF-2 expression was also associated with increased incidence of binucleation in rat cardiomyocytes (Pasumarthi et al., 1996). The effects of overexpressed hi or lo FGF-2 on proliferation were inhibited in the presence of added neutralizing anti-FGF-2 antibodies, indicating an autocrine or paracrine mechanism of action, likely mediated by plasma membrane FGF-2 receptors (Pasumarthi et al., 1996). In contrast, neutralizing antibodies

did not prevent chromatin clumping or binucleation, indicating an intracrine effect of hi FGF-2 (Pasumarthi et al., 1996).

Chromatin condensation and compaction caused by hi FGF-2 might be related to chromosome condensation during prophase of the cell cycle and/or to an apoptosis-like process. These questions were addressed by Sun et al. (2001) using neonatal rat ventricular myocytes transfected with human hi FGF-2. Mitotic chromatin condensation, detected by immunofluoresence with antibodies recognizing the phosphorylated histone H1 and H3, was not seen in myocytes presenting the hi FGF-2-induced compacted phenotype. The effects of hi FGF-2 on chromatin were therefore, not using a mitotic mechanism of condensation. In addition, the effects of hi FGF-2 on chromatin did not seem to be associated with a 'classic' apoptotic mechanism, for the following reasons: (1) they were not prevented by caspase inhibition or by Bcl-2 overexpression; (2) myocytes presenting the compacted phenotype (at least in the earlier stages) did not stain positive for TUNEL or the truncated P17 species of caspase-3; (3) they exhibited an intact nuclear lamina, assessed by continuous anti-laminB staining; (4) the presence of an apoptotic ladder was not detected (Sun et al., 2001). Since they found that purified hi FGF-2 could cause chromatin condensation in vitro, they concluded that the observed effects were likely caused by direct interaction of hi FGF-2 with chromatin (Sun et al., 2001).

The above studies have shown that endogenous hi FGF-2 is subject to developmental, thyroid hormone, and injury-dependent regulation in the heart, and that hi FGF-2 can exert autocrine-paracrine effects (stimulation of proliferation) as well as intracrine, nuclear effects in cultured neonatal cardiomyocytes. Further characterization of the nuclear effects of hi FGF-2 is the subject of this project.

1.9 Rationale for Project

Previously, we used transient transfections by calcium phosphate (10-20% efficiency) and showed that overexpression of hi FGF-2, but not lo FGF-2, caused chromatin compaction and separation within the nucleus in about 20% of overexpressing cells as well as multinucleation (Pasumarthi et al., 1994; Pasumarthi et al., 1996; Sun et al., 2001). Evidence was also provided supporting the notion that this nuclear phenotype does not share several features usually associated with apoptosis: specifically (1) cells presenting the nuclear phenotype were TUNEL-negative and had intact nuclear lamina; (2) Bcl2 (an anti-apoptotic protein), added lo FGF-2 or caspase 3 inhibitors did not prevent the hi FGF-2 effects; (3) no DNA ladder could be detected (Sun et al., 2001). Expression of both hi and lo FGF-2 stimulated DNA synthesis that was blocked by neutralizing antibodies, indicating a receptor-dependent paracrine effect of both hi and lo FGF-2 (Pasumarthi et al., 1996). Neutralizing anti-FGF antibodies did not prevent the appearance of the

compacted chromatin in hi FGF-2 expressing cells (Pasumarthi et al., 1996). Our previous studies: (a) were largely qualitative due to the relatively low transfection efficiencies (10-20%) achieved by the calcium phosphate method (To overcome this problem, we have now used adenoviral vectors expressing hi or lo human FGF-2 to achieve transfection efficiencies of >80%), (b) were conducted within 48 hours from transfection. We have now sought to determine the fate of overexpressing cells over a longer time period (72 hours post-transfection), (c) suggested that the appearance of compacted chromatin was dependent on the levels of hi FGF-2 expression (We have now tested this by using two m.o.i. (50 and 200) to achieve different levels of hi FGF-2 expression), and (d) employed neutralizing antibodies to block the effect of extracellular FGF-2. We have now used adenovirally-driven expression of a dominant-negative FGFR to specifically block membrane receptor-mediated effects. In addition, we examined the role of erk 1/2, a downstream target of FGFR1, in hi FGF-2-mediated nuclear compaction.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

A previously characterized site-specific polyclonal antibody highly specific for the phosphorylated form of the amino-terminus of histone H3 (ser10) was a gift from Dr. Jim Davie at the University of Manitoba in Winnipeg, Manitoba (Chadee et al., 1999; Hendzel et al., 1997); polyclonal rabbit phospho-p42/44 antibody was from Cell Signaling Technologies.

A guinea pig polyclonal antibody, specific for the human hi FGF-2 was a gift from Dr. Robert Florkiewicz at the Ciblex Corporation in San Diego, California (Florkiewicz and Sommer 1989). A mouse monoclonal anti-FGFR1 antibody was purchased from QED Bioscience Inc. A previously characterized, rabbit polyclonal antiserum S2 antibody was raised against a synthetic peptide containing residues 1-24 of the truncated, 146 amino acid, bovine brain bFGF (Kardami et al., 1991). A mouse monoclonal anti-human hi FGF-2 antibody was purchased from Upstate Biotechnology.

Secondary antibodies for immunofluorescence (anti-mouse Texas red, anti-guinea pig Texas red, anti-rabbit biotin, streptavidin fluorescein) were all purchased from Amersham Corp. Secondary antibodies for Western blotting (rabbit horseradish peroxidase and mouse horseradish peroxidase) were purchased from Biorad. The guinea pig secondary

antibody linked to horseradish peroxidase was purchased from Jackson Laboratories.

2.1.2 Adenoviral Vectors

The Ad.hi FGF-2 and Ad.lo FGF-2 viruses were gifts from Dr. Meenhard Herlyn at the Wistar Institute in Philadelphia, Pennsylvania and obtained as described by (Nesbit et al., 1999). The Ad.FGFR (dn) was a gift from Dr. Peter Cattini and Dr. Farah Sheikh at the University of Manitoba in Winnipeg, Manitoba. The Ad.MKK1(dn) and Ad. PKCε were gifts from Dr. Peipei Ping, produced by her laboratory at the University of Louisville in Louisville, Kentucky and tested previously in our lab (Doble et al., 2001).

2.2 Methods

2.2.1 Cell Culture

Primary cultures of neonatal rat ventricular myocytes were obtained using one-day-old Sprague-Dawley rat pups (36 pups/preparation) according to established procedures (Doble et al., 1996; Doble et al., 2000). The hearts were briefly minced in cold phosphate-buffered saline (PBS) containing 3.5g/L glucose and then dissociated into single cells in a collagenase (740 U/digest) / trypsin (370 U/digest) / DNase (2880 U/digest) (Worthington Biochemical Corp.) solution using a water-jacketed spinner flask at 37°C. Six consecutive 10-minute digestions were performed. Following each digestion, liberated cells were decanted off

from the undigested tissue. After the final incubation, the remaining undigested tissue was pipetted several times to assist in dissociation into single cells. The cell suspension was layered onto a discontinuous Percoll gradient (Amersham) to remove contaminating fibroblast cells. Myocytes were counted with a hemocytometer and plated at $0.7x10^6$/35mm dish on collagen-coated plates or coverslips in the presence of growth medium (10% FBS (Hy Clone); 10% horse serum (Gibco); 1000 units/mL penicillin/streptomycin (Gibco)) in F-10 medium (Gibco).

2.2.2 Gene Transfer

One day after plating, the myocytes were rinsed 3x with warmed Dulbecco's modified Eagle's medium (DMEM; Gibco) to remove dead cells and debris. They were then transfected with adenoviral vectors expressing hi FGF-2 or lo FGF-2 viruses at an m.o.i. of 50 or 200. Controls were obtained by transfecting with a non protein-expressing empty adenoviral vector. Cells were maintained in a humidified incubator (37°C, 5% CO_2) and analyzed at 24, 48, and 72 hours after transfection.

Adenoviral vectors expressing dominant negative variants of signal transduction intermediates, namely Ad.FGFR1(dn), Ad.MKK1(dn), Ad.PKCε(dn) were added to myocyte cultures 12 hours before transfection with Ad.hi FGF-2. They were used at m.o.i. of 50 (Ad.FGFR1(dn), Ad. MKK1(dn)) or 25 (Ad.PKCε(dn)). Parallel control plates were pre-

transfected with 25-50 m.o.i. of vector. Myocytes were then transfected with 175-150 m.o.i. of Ad.hiFGF-2 and analyzed after 72 hours.

2.2.3 DNA Ladder Analysis

According to previously published procedures (Gurevich et al., 2001; Kumar et al., 1999), myocytes were washed with cold PBS and scraped into eppendorf tubes. Cells were lysed in 500 μL of 0.1 M NaCl, 0.3 M Tris-HCl (ph 8.0), 0.2 M sucrose, and 20% SDS. The lysates were incubated for 30 minutes at 65°C. 8M potassium acetate (KOAc) was added and incubated at 4°C overnight. The cells were centrifuged at 14,000 RPM for 30 minutes at 4°C. The supernatant was collected and 10 μg/mL RNase A (Sigma) was added and incubated at 37°C for 1 hour. Genomic DNA was extracted using phenol/choloroform/isoamyl alcohol and loaded onto a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide. To obtain positive controls for DNA ladder formation, myocytes were treated with staurosporine (Sigma), a PKC inhibitor that has been shown to cause apoptosis (Yue et al., 1998).

2.2.4 Immunofluorescence

Myocytes were fixed in cold 4% paraformaldehyde in PBS for 15 minutes at 4°C, rinsed with PBS and permeabilized with cold 0.1% Triton X-100 (Roche) in PBS for 15 minutes at 4°C. The cells were washed extensively with PBS and incubated overnight at 4°C with a primary antibody diluted in

1% BSA in PBS. The primary antibodies were diluted as follows: 1) rabbit polyclonal anti-phospho H3 – 1:3000; 2) guinea pig polyclonal anti-human hi FGF-2 – 1:175; 3) mouse monoclonal anti-FGFR1 – 1:200; 4) anti-FGF-2 rabbit serum S2 – 1:2000. After washing 3x with PBS, the cells were incubated with a secondary antibody: purified anti-guinea pig or anti-mouse immunoglobulin (IgG) conjugated to texas red or purified anti-rabbit IgG conjugated to biotin diluted in 1% BSA/PBS (1:50, 1:20 and 1:20, respectively), for 1 hour at room temperature. The coverslips that were treated with the guinea pig or mouse antibodies were rinsed and mounted while the rabbit IgG-treated cells were rinsed and further incubated with streptavidin-fluorescein diluted 1:20 in 1% BSA/PBS for 1 hour at room temperature. All coverslips were counter-stained with 2.5 mM Hoechst 33342 (Calbiochem) for 1 minute and rinsed thoroughly. IgG ProLong antifade medium (Molecular Probes) was used to mount the coverslips onto slides. A Nikon Diaphot as well as a Zeiss Axiovert 200 epifluorescent microscope was used to view the coverslips.

2.2.5 Protein Extraction and Western Blotting

According to standard procedures (Doble et al., 1996), myocytes were lysed in 1% SDS buffer containing 50 mM Tris-HCl (ph 7.4), 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM PMSF and 2 μg/mL each of leupeptin, pepstatin, aprotinin and E-64 following transfection with the virus(es). Lysates were boiled for 5

minutes, sonicated and centrifuged for 10 minutes in a microcentrifuge. Nuclei were obtained using the Nuclei-EZ kit (Sigma) as per manufacturer's instructions. The BCA assay (Pierce) was used to determine protein concentration of the supernatants. 20 μg of protein were loaded onto a 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Roche) (Laemmli 1970). The membrane was blocked overnight at 4°C using 10% skim milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST) (Sigma) and then incubated with a primary antibody in 1% skim milk powder/TBST for 1 hour at room temperature with constant agitation. The various antibodies were diluted as follows: 1) monoclonal mouse anti-FGF-2 (UBI) – 1:750; 2) polyclonal guinea pig human anti-FGF-2 – 1:500; 3) polyclonal rabbit anti-FGF-2 (S2) – 1:1000; 4) anti-erk1/2 (both total and active) – 1:1000. The membrane was rinsed with TBST and depending on the species, either rabbit/mouse/guinea pig secondary antibody linked to horseradish peroxidase was added. The membrane was then incubated with SuperSignal Chemiluminescent Substrate (Pierce) for 5 minutes and exposed to X-ray film. The bands were quantitated using a Bio-Rad Imaging densitometer.

2.2.6 Tritiated Thymidine Assay

To determine the amount of DNA synthesis, a protocol developed by the laboratory of Dr. Peter Zahradka was used (Zahradka et al., 1993). After

transfection with the virus(es), 1 μCi/mL of tritiated thymidine was added to the wells and left for 24 hours. The cells were lysed overnight with 2 mL of cell lysis buffer containing 0.1 M NaCl, 10 mM Tris (ph 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% SDS and transferred into test tubes. In order to precipitate the DNA, an equivalent volume of 20% TCA was added to each tube and left on ice for 15 minutes. The precipitated DNA was filtered on a manifold apparatus and the filters were rinsed several times with 5% TCA. The filters were transferred into scintillation tubes and dried overnight. 3 mL of Cytoscint scintillation cocktail (ICN Biomedicals) was added to each tube and the amount of tritiated thymidine was measured in a Beckman scintillation counter.

2.2.7 Determination of Mitotic Index

After staining with anti-phospho-H3 antibody, coverslips were divided into 8 sections. One random field was observed per section and the total number of nuclei and phospho-H3 positive nuclei were counted. The percentage of phospho-H3–positive nuclei over the total nuclei in each field was determined and averaged to give us the mitotic index (MI). A total of 32 fields (4 coverslips per treatment) were examined. The MI from Ad.hi FGF-2 infected cultures at specific time points and doses were divided by the corresponding MI values from vector-infected cultures to obtain a normalized index which compensated for outside effects of the viral infection. These normalized values represent the changes in MI due

to expression of hi FGF-2 irrespective of the effects of the vector. To determine cell numbers, the average number of nuclei per field was calculated at each dose and time point and compared.

2.2.8 Incidence of Chromatin Compaction

Myocytes were infected at a low titre (m.o.i. 50) and a high titre (m.o.i. 200) and left for 24, 48 and 72 hours. They were stained with Hoecsht 33342 and each coverslip was divided into 16 sections. One random field was observed per section and the percentage of nuclei exhibiting the 'clumped' phenotype over the total number of nuclei was determined and averaged. Each timepoint and titre was done on 2 coverslips thus a total of 32 fields were counted.

Compacted chromatin exhibit multiple, small chromatin clumps (over 10 per nucleus) of similar size surrounded by an intact nuclear envelpe as described by Pasumarthi et al., (1994, 1996) and Sun et al., (2001); these do not stain for TUNEL. In contrast, cells characterized as apoptotic contained few chromatin clumps (approximately 2-5 per nucleus) of unequal size and stain positive for TUNEL.

2.2.9 Anti-FGF-2 Neutralizing Antibodies and the MEK-1 Inhibitor PD98059

Myocytes were plated at 0.7×10^6 cells / coverslip. The next day the cells were rinsed 3x with DMEM. Either 25 μg/mL of anti-FGF-2, type I

neutralizing antibodies (UBI) or 25 μM of the MEK-1 inhibitor, PD98059 (Cell Signaling Technology) was added to each well for 1 hour. Each well was then transfected with 200 moi of Ad.hi FGF-2 and left for 72 hours. The cells were then processed for immunofluorescent staining.

2.2.10 Statistical analysis

Differences between vector-infected or Ad.hi FGF-2-infected cultures or between Ad.hi FGF-2-infected cultures at different timepoints and dosages were examined for statistical significance by analysis of variance and the Tukey's post hoc test, using the GraphPad InStat 3.0 program, where $p<0.05$ was considered significant.

CHAPTER 3

RESULTS

3.1 Characterization Studies

3.1.1 Expression of human hi or lo FGF-2 on cardiomyocytes after adenoviral transfection

In order to verify that adenoviral vectors were functional in our system, isolated neonatal rat ventricular myocytes were transfected with Ad.hi FGF-2 or Ad.lo FGF-2 and were analyzed by Western blotting as shown in Figure 3. Lysates from myocytes transfected with Ad.lo FGF-2 presented one immunoreactive band at 18 kDa corresponding to the expected size for the AUG-initiated FGF-2. Myocytes transfected with Ad.hi FGF-2 presented immunoreactive bands at 22-24 kDa, corresponding to CUG-initiated hi FGF-2, as well as an 18 kDa band, corresponding to AUG or lo FGF-2. This is in agreement with the original report on the production and characterization of Ad.hi FGF-2 (Nesbit et al., 1999).

3.1.2 Accumulation of human hi FGF-2 in the nucleus

Hi FGF-2 is found predominantly in the nucleus (Bugler et al., 1991; Florkiewicz et al., 1991; Pasumarthi et al., 1994; Quarto et al., 1991; Renko et al., 1990). To test this in our system, we obtained nuclear extracts from myocyte cultures transfected with Ad.hi FGF-2 or vector, and analyzed them for presence of hi FGF-2 by Western blotting. Results are shown in Figure 4. Nuclear extracts from Ad.hi FGF-2 transfected

Figure 3. ***Expression of human hi or lo FGF-2 on cardiomyocytes after adenoviral transfection***

Western blot analysis of cardiomyocytes transfected with Ad.hi and lo FGF-2 (m.o.i. 25). Immunoreactive FGF-2 present in total myocyte culture lysates (20 μg/lane) was detected using the FGF-2 monoclonal antibody.

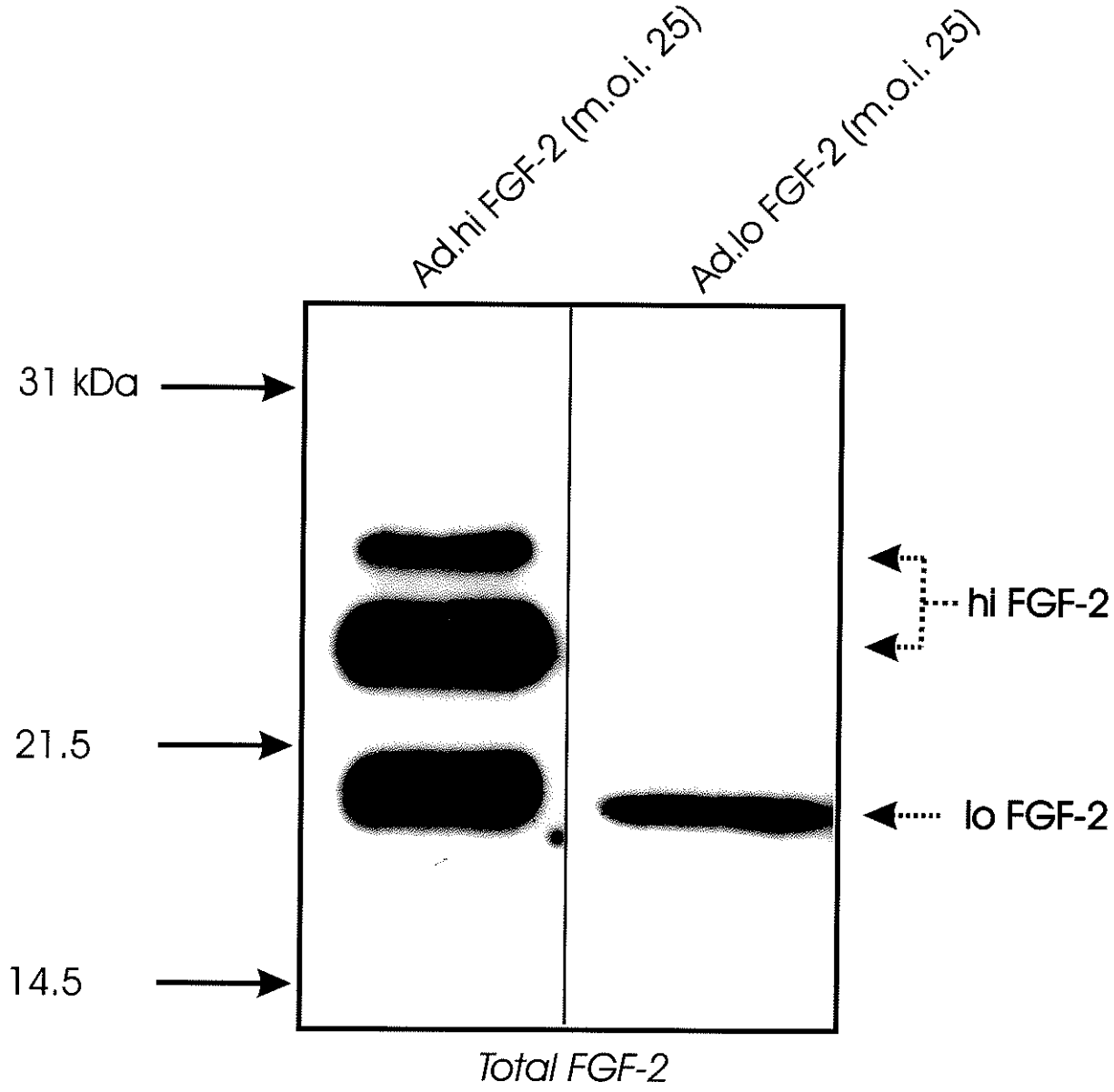
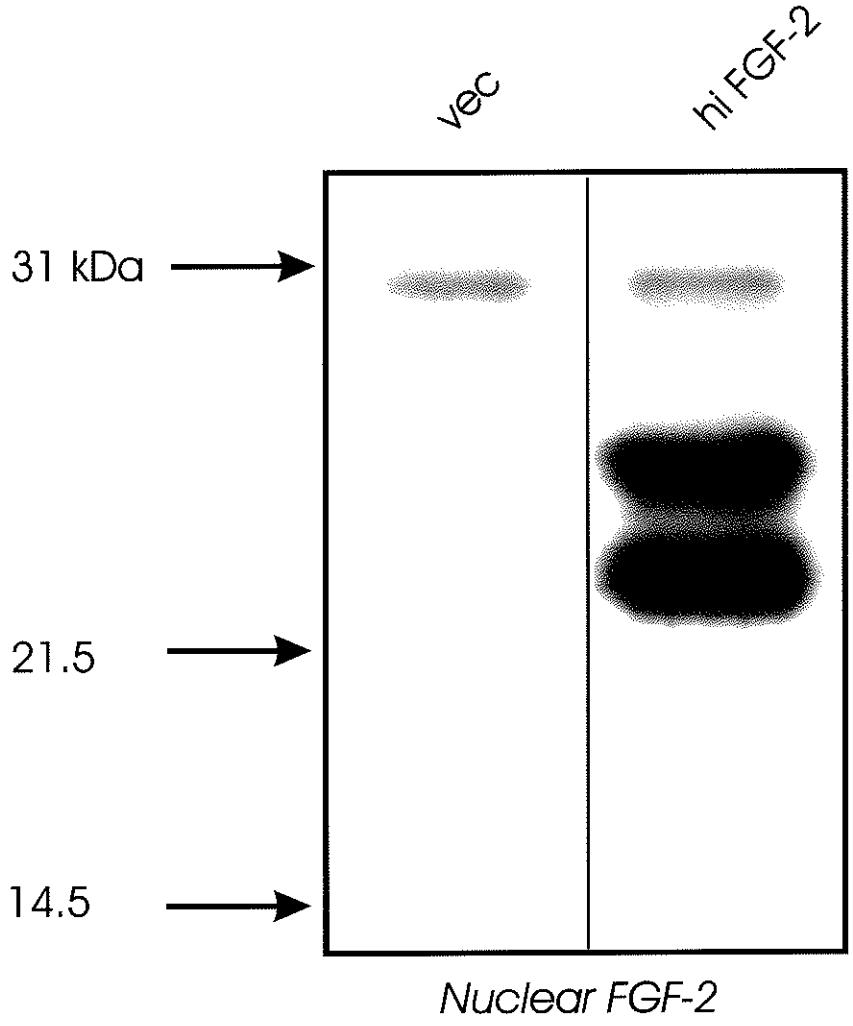

Ad.hi FGF-2 (m.o.i. 25)
Ad.lo FGF-2 (m.o.i. 25)
31 kDa
21.5
14.5
hi FGF-2
lo FGF-2
Total FGF-2

Figure 4. ***Accumulation of human hi FGF-2 to the nucleus***

Western blot analysis of FGF-2 present in nuclear protein (5 μg/lane) extracted from myocytes transfected with vector or Ad.hi FGF-2 using the Nuclei-EZ kit. Immunoreactive FGF-2 present was detected using a guinea pig polyclonal antibody. The ~30kDa band present is likely an endogenous FGF-2-like protein or it may represent non-specific reactivity. In the human, CUG-initiation produces bands of 22, 23, 34 kDa. It is possible that another CUG site may exist in the rat, thus producing the (minor) 30 kDa band.



Nuclear FGF-2

cultures contain only the 22-24 kDa bands of human hi FGF-2, while cells transfected with the vector only, do not.

3.1.3 Effect of dosage and time on hi FGF-2 expression

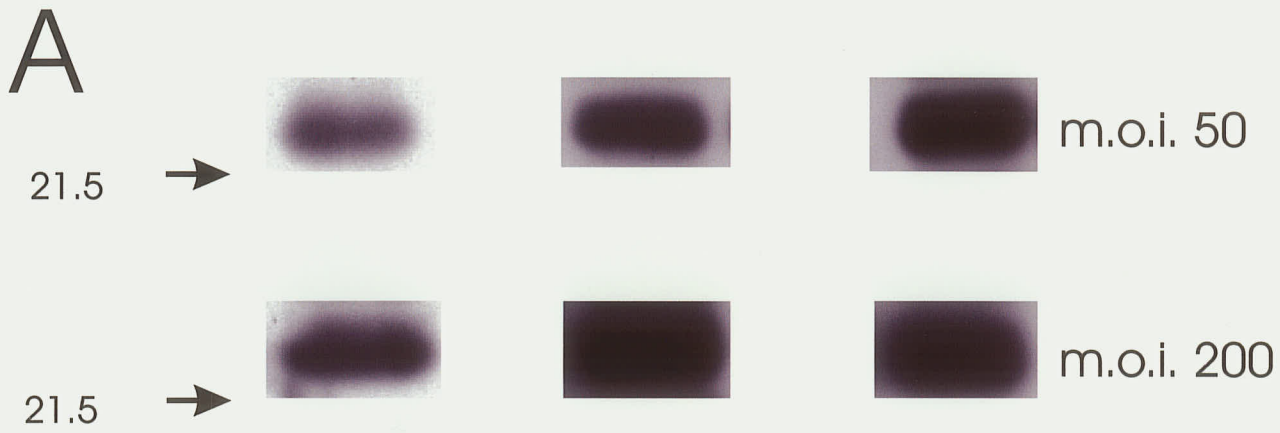
To assess the effects of dosage and time in culture on hi FGF-2 accumulation, myocytes were transfected with two different doses (m.o.i. 50 and m.o.i. 200) of Ad.hi FGF-2 and examined at 24, 48, 72 hours post-transfection. To control for non-specific effects of the virus, control cells were infected with 50 and 200 m.o.i. of vector alone. FGF-2 levels were examined by Western blotting using an anti-FGF-2 antiserum (S2) previously characterized in our lab (Kardami et al., 1991). Densitometry was used to obtain measurements for the hi FGF-2 at the different time points. As seen in Figure 5, relative levels of FGF-2 were maximal 48 hours post-transfection at 50 m.o.i. and then decreased slightly at 72 hours. This decrease was not statistically significant. This pattern was more pronounced at the higher, 200 m.o.i. dosage. In this case we observed a steep increase from 24 to 48 hours and a sharp decline from 48 to 72 hours. As expected, relative levels of hi FGF-2 at 200 m.o.i. were higher than those at 50 m.o.i., at all time points. These differences were statistically significant ($p<0.05$).

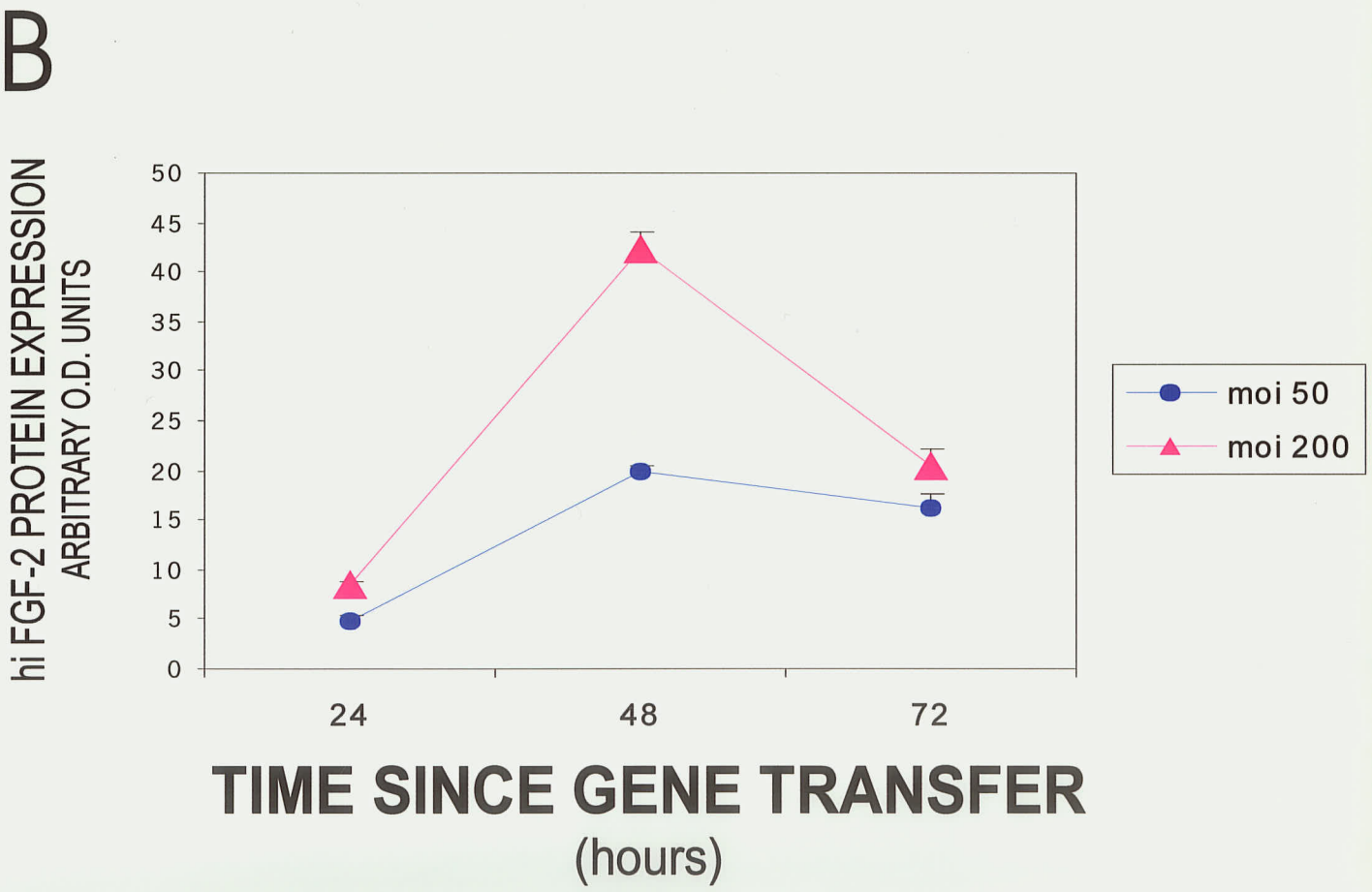
Figure 5. ***Effect of dosage and time on hi FGF-2 expression***

A. Representative Western blot analysis of hi FGF-2 expression of myocytes transfected with Ad.hi FGF-2 at an m.o.i. of 50 or 200 for 24, 48 or 72 hours in culture.

B. Relative levels of hi FGF-2 (in arbitrary O.D. units) expression of myocytes infected with Ad.hi FGF-2 depicted as a function of dosage (m.o.i.) and time in culture. The densitometric data are depicted as the mean $\pm$ S.E.M. of n=4.

- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 48 hr = $p<0.001$
- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 72 hr = $p<0.001$
- m.o.i. 50 at 48 hr compared to m.o.i. 50 at 72 hr = N.S.
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 48 hr = $p<0.001$
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 200 at 48 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 50 at 24 hr compared to m.o.i. 200 at 24 hr = N.S.
- m.o.i. 50 at 48 hr compared to m.o.i. 200 at 48 hr = $p<0.001$
- m.o.i. 50 at 72 hr compared to m.o.i. 200 at 72 hr = N.S.


A
21.5
m.o.i. 50
21.5
m.o.i. 200


B
hi FGF-2 PROTEIN EXPRESSION
ARBITRARY O.D. UNITS
50
45
40
35
30
25
20
15
10
5
0
24
48
72
TIME SINCE GENE TRANSFER
(hours)
moi 50
moi 200

3.1.4 Effect of dosage and time on mitotic index

To obtain an estimate of the fraction of myocytes in mitosis (ie. mitotic index, MI), an anti-phosphorylated histone 3 antibody (anti-P-H3) was used. Anti-P-H3 antibody is a marker associated with mitotic chromatin condensation; anti-P-H3 staining, therefore, was used to determine the mitotic index of cells transfected with the hi FGF-2 adenovirus (Chadee et al., 1999; Hendzel et al., 1997). Nuclei stained with anti-P-H3 and the total number of nuclei present in any given field were scored. A total of 32 fields were scored. The ratio of mitotic versus total nuclei was calculated. To compensate for any effects caused by viral infection, we obtained a 'normalized' index, by dividing the MI from Ad.hi FGF-2 infected cultures at specific time points and doses with the corresponding MI values from vector-infected cultures. Normalized index values, therefore, represent the changes in MI due to expression of hi FGF-2 irrespectively of effects of the vector and is shown in Figure 6. When graphed as a normalized labeling index, mitosis increased maximally from 24 to 48 hours and decreased from 48 to 72 at m.o.i. 50. This increase in mitosis coincides with increased levels of hi FGF-2 at 48 hours (Fig.3). At m.o.i. 200, mitotic fraction decreased from 24 to 48 hours and decreased even further from 48 to 72 hours.

Figure 6. ***Effect of dosage and time on mitotic index***

Normalized index (MI in Ad.hi FGF-2 transfected cultures/MI in vector-only transfected cultures) as a function of dosage (m.o.i.) and time in culture, as indicated. MI is the fraction of nuclei expressing phospho-H3. The data are depicted as the mean $\pm$ S.E.M. of n=4.

- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 48 hr = $p<0.001$
- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 72 hr = $p<0.05$
- m.o.i. 50 at 48 hr compared to m.o.i. 50 at 72 hr = N.S.
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 48 hr = N.S.
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 200 at 48 hr compared to m.o.i. 200 at 72 hr = $p<0.05$
- m.o.i. 50 at 24 hr compared to m.o.i. 200 at 24 hr = N.S.
- m.o.i. 50 at 48 hr compared to m.o.i. 200 at 48 hr = $p<0.01$
- m.o.i. 50 at 72 hr compared to m.o.i. 200 at 72 hr = $p<0.001$


NORMALIZED INDEX
hi FGF-2 Mitotic Index
vector Mitotic Index
180
160
140
120
100
80
60
40
20
0
24
48
72
moi 50
moi 200
TIME SINCE GENE TRANSFER
(hours)

3.1.5 Effect of dosage and time on nuclear number

To obtain an estimate of cell numbers at the various time points, we compared average numbers of total nuclei counted per field (n=32). Figure 7 shows that at m.o.i. 50, nuclear numbers remained unchanged at 24-48 hours, decreasing slightly at 72 hours. At 200 m.o.i., however, nuclear numbers dropped slightly from 24-48 hours and decreased considerably from 48-72 hours compared to the earlier time points or to any values of the 50 m.o.i. groups.

3.1.6 Effect of hi FGF-2 overexpression on chromatin compaction

We have previously shown that overexpression of hi FGF-2 results in a compacted nuclear phenotype characterized by multiple, condensed 'clumps' of chromatin in the nucleus (Pasumarthi et al., 1994; Pasumarthi et al., 1996; Sun et al., 2001). Figure 8 shows representative images of fluorescent labeling for nuclear DNA using Hoechst 33342. Myocytes infected with hi FGF-2 at an m.o.i. of 200 for 72 hours exhibited several 'clumped' nuclei (see arrows) compared to myocytes treated with vector, which did not. Figure 9 shows the percent of nuclei displaying chromatin compaction at 24, 48 and 72 hours post-infection, using two different doses. At 24 hours, no clumping could be observed at either dosage. Nuclear disruption was evident at 48 h at 200 m.o.i. (but not the 50 m.o.i.) dose, and reached maximal levels (nearly 50% of the total nuclei) at 72 h and 200 m.o.i. At this time point, even the 50 m.o.i. dose elicited some

Figure 7. ***Effect of dosage and time on nuclei number***

Average number of nuclei counted per random visual field (mean ± S.E.M., n=32) as a function of dosage (m.o.i.) and time in culture, as indicated (n=32).

- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 48 hr = N.S.
- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 72 hr = N.S.
- m.o.i. 50 at 48 hr compared to m.o.i. 50 at 72 hr = $p<0.05$
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 48 hr = N.S.
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 200 at 48 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 50 at 24 hr compared to m.o.i. 200 at 24 hr = N.S.
- m.o.i. 50 at 48 hr compared to m.o.i. 200 at 48 hr = $p<0.001$
- m.o.i. 50 at 72 hr compared to m.o.i. 200 at 72 hr = $p<0.001$


NUCLEI PER FIELD
70
60
50
40
30
20
10
0
24
48
72
TIME SINCE GENE TRANSFER
(hours)
moi 50
moi 200

Figure 8. ***Effect of hi FGF-2 overexpression on chromatin compaction***

Nuclear staining of myocytes transfected with 200 m.o.i. of:

(a) vector

(b) Ad.hi FGF-2

for 72 hours. Arrows indicate the compacted chromatin.

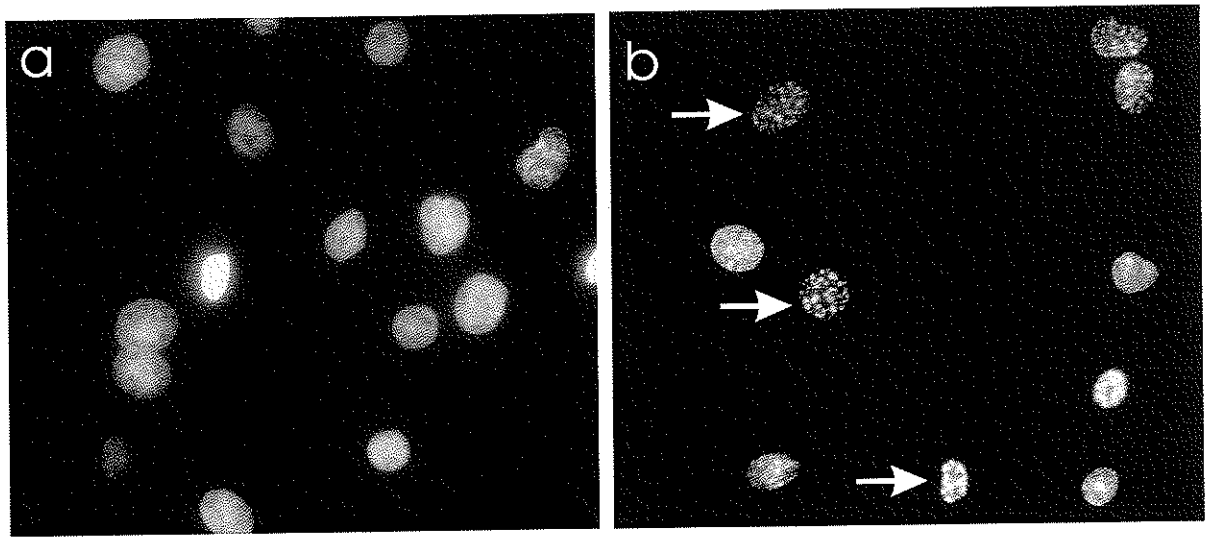
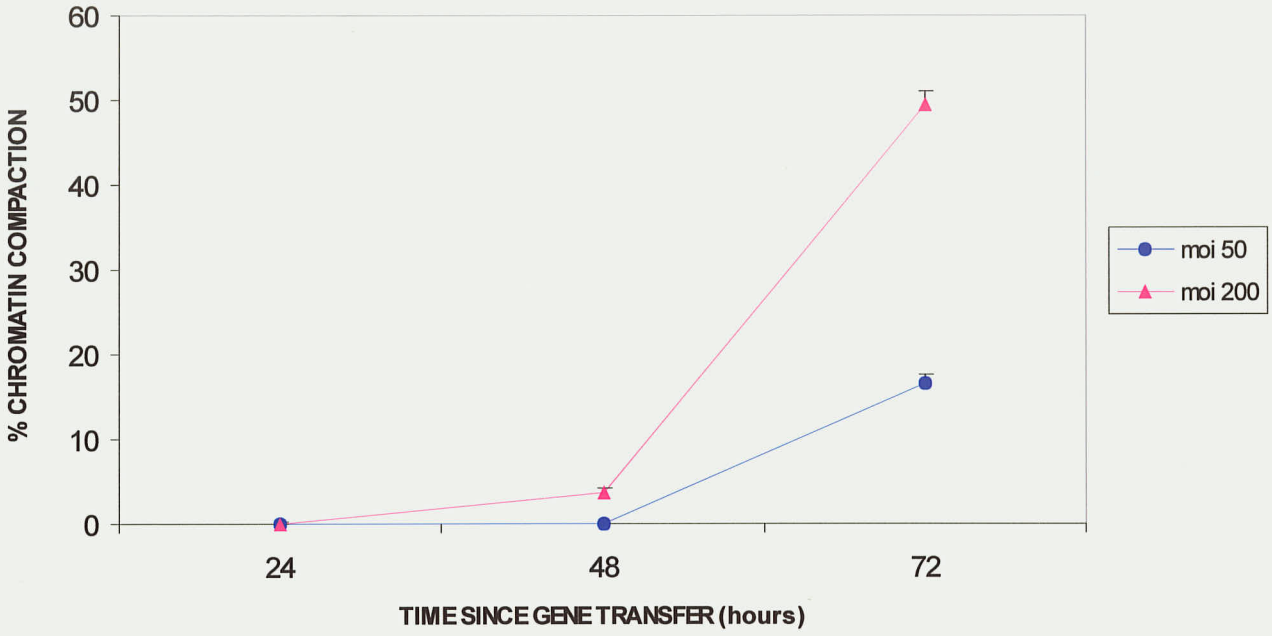

a
b

Figure 9. ***Effect of hi FGF-2 overexpression on chromatin compaction***

Percentage of nuclei displaying chromatin compaction as a function of dosage (m.o.i.) and time in culture, as indicated. Data are shown as the mean ± S.E.M. of n=4.

- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 48 hr = N.S.
- m.o.i. 50 at 24 hr compared to m.o.i. 50 at 72 hr = $p<0.001$
- m.o.i. 50 at 48 hr compared to m.o.i. 50 at 72 hr = $p<0.001$
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 48 hr = N.S.
- m.o.i. 200 at 24 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 200 at 48 hr compared to m.o.i. 200 at 72 hr = $p<0.001$
- m.o.i. 50 at 24 hr compared to m.o.i. 200 at 24 hr = N.S.
- m.o.i. 50 at 48 hr compared to m.o.i. 200 at 48 hr = $p<0.05$
- m.o.i. 50 at 72 hr compared to m.o.i. 200 at 72 hr = $p<0.001$


60
50
40
30
20
10
0
% CHROMATIN COMPACTION
24
48
72
TIME SINCE GENE TRANSFER (hours)
moi 50
moi 200

nuclear compaction (17%). Cells infected with vector-only (50 and 200 m.o.i.) did not exhibit chromatin clumping nor the decline in cell numbers/mitotic index induced by the high dose of hi FGF-2 at 72 h (data not shown).

3.1.7 Nuclear distribution of hi FGF-2 in relation to chromatin

We examined the distribution patterns of clumped chromatin and hi FGF-2 in the nucleus under high magnification. Figure 10a shows that hi FGF-2 is localized in a homogeneous pattern, while 10b shows that chromatin has formed distinct 'clumps'. At this level of analysis, there was no evidence of specific co-staining for FGF-2 and chromatin 'clumps'.

3.1.8 Effect of hi FGF-2 overexpression on DNA ladder formation

Genomic DNA was analyzed to determine presence of a DNA ladder upon transfection with Ad.hi FGF-2. The presence of small fragments of DNA producing a ladder-like pattern is an indicator of apoptosis. Genomic DNA isolated from myocytes infected with hi FGF-2 was examined at different time points and at different doses using agarose gel electrophoresis. Staurosporine was used to induce apoptosis in cells in order to produce a control DNA ladder. Figure 11 shows a classic DNA ladder that was observed at 72 hours (see arrows) in cells infected with the hi FGF-2 adenovirus at both m.o.i. 50 and m.o.i. 200. No ladder was detected in any of the vector-treated cells at any dose or time point or in cells infected

Figure 10. ***Nuclear distribution of hi FGF-2 in relation to chromatin***

Simultaneous fluorescence labeling for:

(a) FGF-2

(b) DNA

Anti-FGF-2 antibodies were used at a dilution detecting overexpressed hi FGF-2, but not endogenous FGF-2. Bar in (a) corresponds to 5 μM.

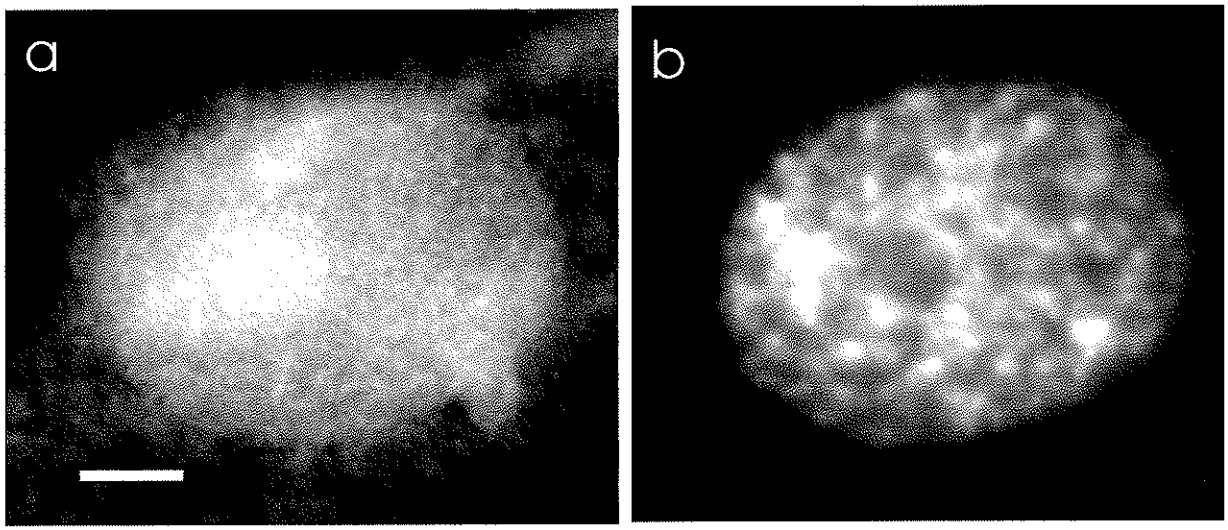
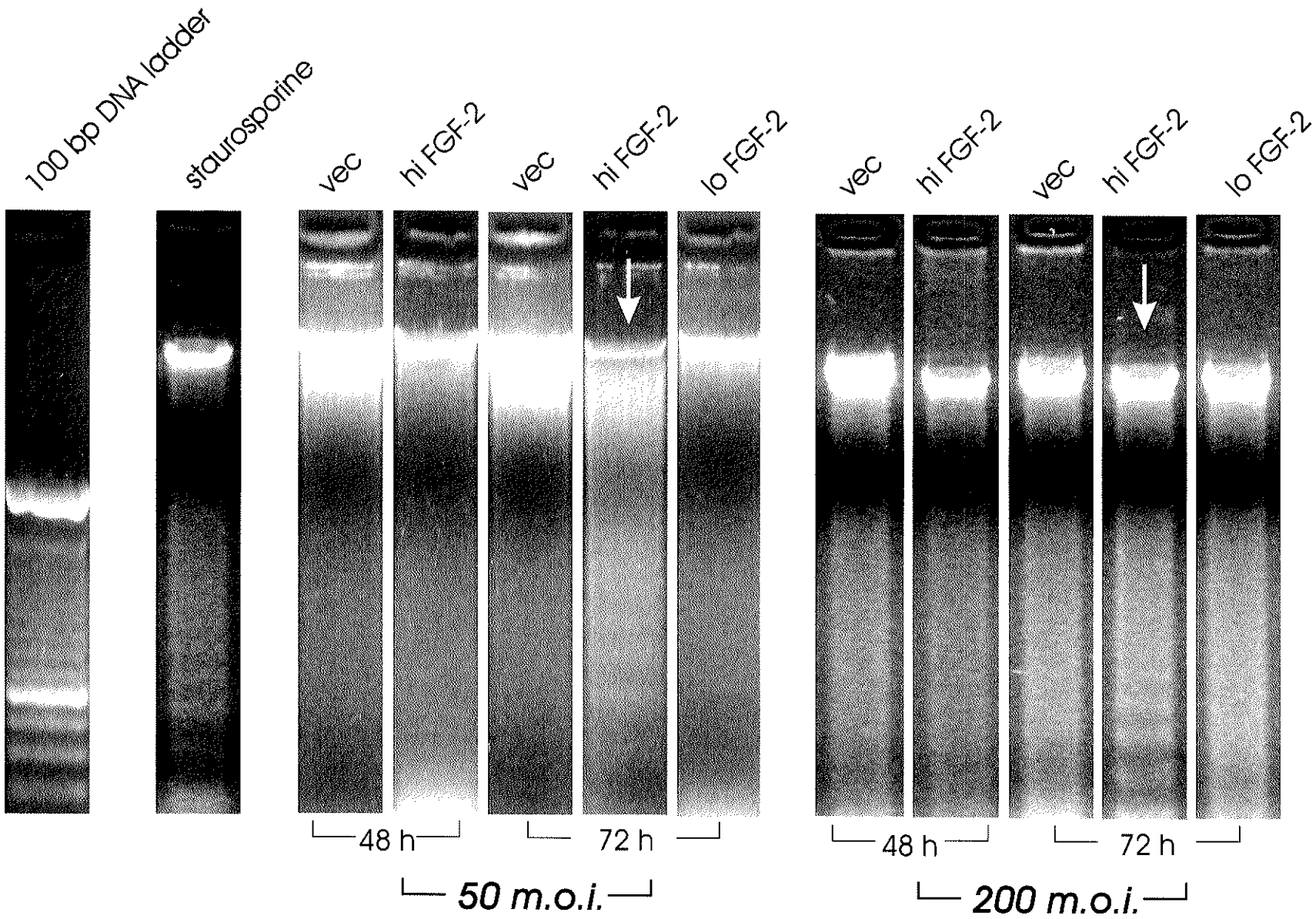

a
b

Figure 11. ***Effect of hi or lo FGF-2 overexpression on DNA ladder formation***

Electrophoretic analysis of genomic DNA from control, vector-transfected cells and hi FGF-2 transfected cells at 48 and 72 hours, and 50 – 200 m.o.i., as indicated. Cells transfected with lo FGF-2 are shown at 72 hours. Arrows show DNA ladder. DNA from cells treated with staurosporine (apoptosis-inducer) is used as a positive control for DNA ladder formation.


100 bp DNA ladder
staurosporine
vec
hi FGF-2
vec
hi FGF-2
lo FGF-2
vec
hi FGF-2
vec
hi FGF-2
lo FGF-2
48 h
72 h
50 m.o.i.
48 h
72 h
200 m.o.i.

with the Ad.lo FGF-2 adenovirus at 24 or 48 hours. This experiment was repeated three times using different myocyte preparations. Similar results were obtained in all three experiments.

3.2 Signal Transduction Pathway(s) Leading to CUG-FGF-2-induced Chromatin Clumping

The paracrine and autocrine effects of all FGF-2 species are mediated by binding to cell surface tyrosine kinase receptors, namely FGFR1-4 (Bernard et al., 1991; Bikfalvi et al., 1997; Engelmann et al., 1993; Jin et al., 1994; Liu et al., 1995; Partanen et al., 1992; Patstone et al., 1993; Speir et al., 1992; Yazaki et al., 1993). FGFR1 is the main FGF-2 receptor in cardiac myocytes (Liu et al., 1995; Speir et al., 1992; Yazaki et al., 1993). However, it is not as yet known if the intracrine effects of hi FGF-2 also require binding to FGFR1. This receptor is not only located to the plasma membrane but also the nuclear membrane and the nucleus (Maher 1996). To examine the role of FGFR1 we used an adenoviral vector to express a dominant negative FGFR1 species [FGFR1(dn)]. In addition, we used well-characterized anti-FGF-2 neutralizing antibodies, in order to distinguish between effects of extracellular versus intracellular hi FGF-2.

3.2.1 Effect of preventing FGFR1 activation

FGFR1(dn) is kinase deficient and while it can bind the FGF-2 ligand and dimerize with itself as well as FGFR1, it, unlike FGFR1, cannot activate downstream signal transduction pathways (Yayon et al., 1997). To verify that this is indeed so in our system, we tested the effect of FGFR1(dn) on an FGF-2-dependent end-point such as DNA synthesis. Cardiac myocyte cultures were pre-transfected with Ad.FGFR1(dn) or vector, and 12 hours later transfected with Ad.hi FGF-2 or vector. Four groups were therefore examined: Group 1: Ad.vector + Ad.vector; Group 2: Ad.FGFR1(dn) + Ad.vector; Group 3: Ad.vector + Ad.hi FGF-2; Group 4: Ad.FGFR1(dn) + Ad.hi FGF-2. DNA synthesis was assessed 24 h later by determining ^{3}H-thymidine incorporation, and the results are shown in Fig.12. As expected, hi FGF-2 expression resulted in net stimulation of DNA synthesis ($P<0.01$); this was completely prevented in cultures expressing FGFR1(dn) ($P<0.05$). We therefore conclude that FGFR1(dn) does indeed block FGFR1-mediated effects. We then examined whether blocking FGFR1 activation (by expressing FGFR1(dn)) would affect the hi FGF-2-induced chromatin disruption. Myocyte cultures were pre-transfected with Ad.FGFR1(dn) or Ad.Vector (at 50 m.o.i. each), and, after overnight incubation, transfected with Ad.hi FGF-2 or Ad.vector at 150 m.o.i. each. Each culture dish received therefore a total of 200 m.o.i. of virus. The four groups examined were similar to the groups listed in the preceding paragraph. Chromatin disruption was evaluated 72 hours after Ad.hi FGF-

2 transfection. Results are shown in Fig.13. Prevention of FGFR1 activation fully prevented chromatin disruption by hi FGF-2 ($p<0.0001$).

Figure 12. ***Effect of Ad.FGFR(dn) on DNA synthesis***

Myocytes were transfected with either 50 m.o.i. of vector or Ad.FGFR(dn) for 12 hours. The cells were subsequently transfected with either 50 m.o.i. of vector or Ad.hi FGF-2 for another 24 hours. Cells were then incubated for 8 hours with 1 μCi/mL of ^{3}H-thymidine and then lysed and processed for ^{3}H-thymidine incorporation determination. The data shown are the mean ± S.E.M. of n=4. *$p<0.05$ compared to Ad.vector + Ad.hi FGF-2 treated cells; **$p<0.01$ compared to Ad.vector + Ad.vector treated cells.

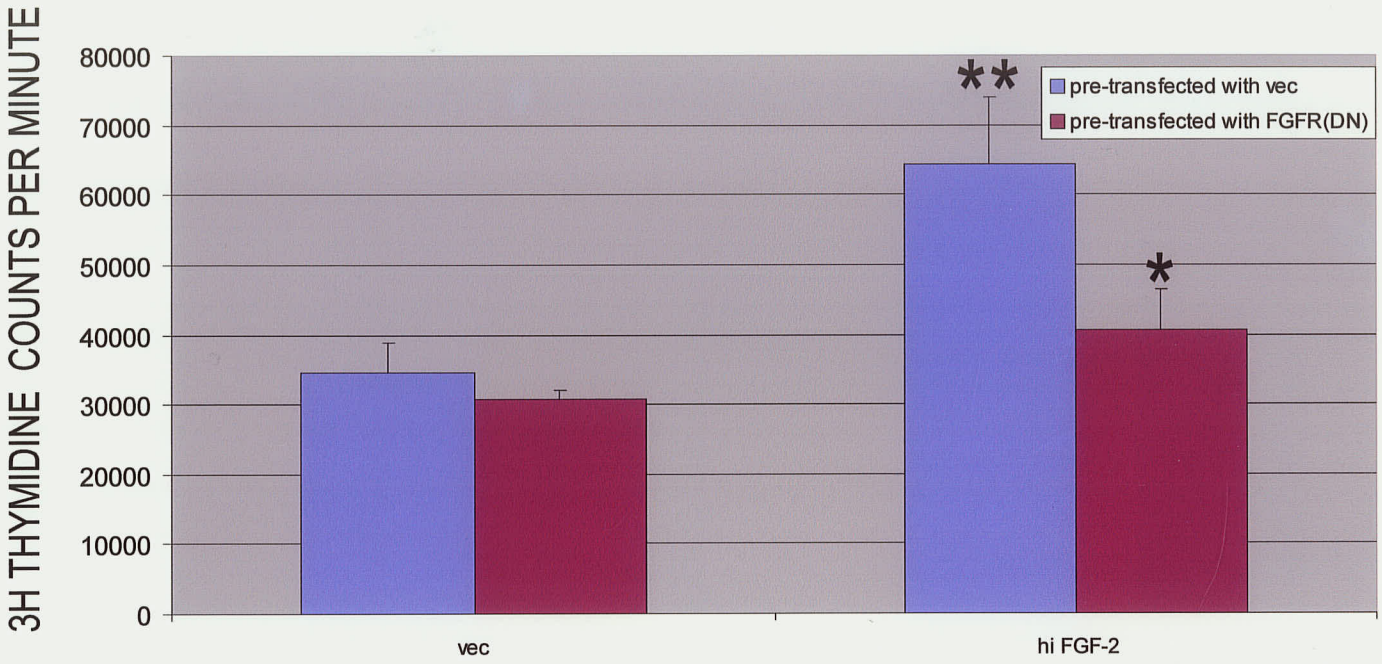
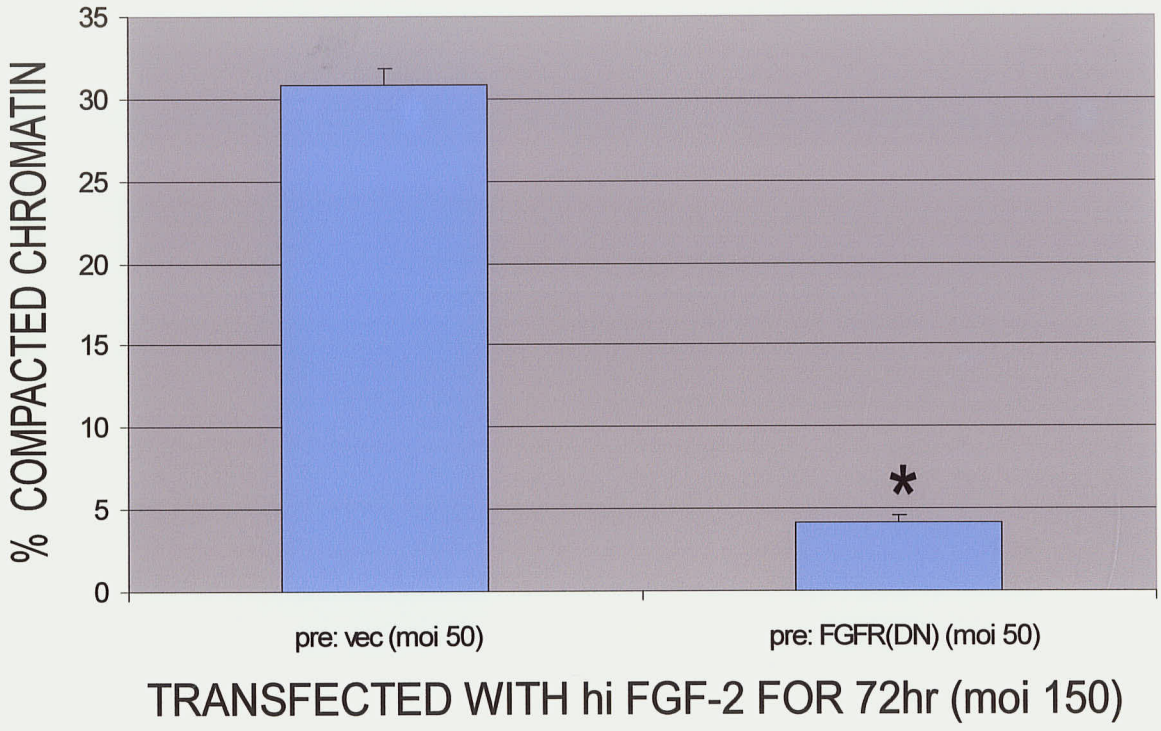

3H THYMIDINE COUNTS PER MINUTE
80000
70000
60000
50000
40000
30000
20000
10000
0
**
*
pre-transfected with vec
pre-transfected with FGFR(DN)
vec
hi FGF-2

Figure 13. ***Effect of Ad.FGFR(dn) on chromatin compaction induced by hi FGF-2***

Myocytes were pre-transfected with 50 m.o.i. or vector or Ad.FGFR(dn) for 12 hours prior to subsequent transfection with 150 m.o.i. of Ad.hi FGF-2 for an additional 72 hours. Data are shown as the mean $\pm$ S.E.M. of n=32. *$p<0.0001$ compared to vector-treated cells.


% COMPACTED CHROMATIN
35
30
25
20
15
10
5
0
*
pre: vec (moi 50)
pre: FGFR(DN) (moi 50)
TRANSFECTED WITH hi FGF-2 FOR 72hr (moi 150)

3.2.2 Effect of preventing erk 1/2 activation

Activation of FGFR1 by FGF-2 is linked to the activation of the ras-raf-(erk 1/2) MAPK pathway (Bogoyevitch et al., 1994; Doble et al., 2000; Padua et al., 1998). We therefore examined whether erk1/2 activation was involved in the hi FGF-2-induced chromatin disruption. We used two approaches to prevent erk1/2 activation: inhibition of the activating kinase MKK1 (mitogen kinase kinase 1) by expressing a dominant negative kinase (Ad.MKK1(dn)), and 2) pharmacological inhibition of MKK1, using the compound PD98059.

First, we verified that expression of Ad.MKK1 (dn) at 50 m.o.i. affected the activation of erk1/2. This was accomplished by Western blotting of cardiac myocyte lysates and antibodies specific for the active form of erk1/2, followed by densitometry. As shown in Fig.14A, a significant decrease ($P<0.0001$) in active (p42/p44) MAPK was observed in cultures expressing MKK1(dn) following stimulation with lo FGF-2. We then examined the effects of Ad.MKK1 (dn) on hi FGF-2 induced chromatin disruption. Myocyte cultures were pre-transfected with Ad.Vector or Ad.MKK1(dn); after overnight incubation they were transfected with Ad.hi FGF-2 or Ad.vector. Four groups were examined: Group 1: Ad.vector + Ad.vector; Group 2: Ad.MKK1(dn) + Ad.vector; Group 3: Ad.vector + Ad.hi FGF-2; Group 4: Ad.MKK1(dn) + Ad.hi FGF-2. As seen in Fig.14B, expression of MKK1(dn) significantly decreased the hi FGF-2-induced chromatin disruption.

It has been established previously that use of the pharmacological MKK1 inhibitor PD98059 fully blocks the activation of erk1/2 in cardiomyocytes (Doble et al., 2000). Myocyte cultures were pre-treated with 25 μM PD98059 and then transfected with Ad.hi FGF-2 or vector (200 m.o.i.). Chromatin disruption was assessed 72 hours later. As shown in Fig.14C, hi FGF-2-induced chromatin disruption was prevented by PD98059. This reduction in erk 1/2 activation by Ad.MKK1(dn) was not as prominent as the reduction in chromatin clumping (compare Fig. 14A with Fig. 14B). It is possible that even a small decrease in erk 1/2 activity is sufficient for preventing the effects on chromatin. It is also possible that the initial decrease in erk 1/2 activation was more pronounced at earlier time points when expression of Ad.MKK1(dn) might have been more robust. The fact remains, however, that Ad.MKK1(dn) did affect erk 1/2 activation.

To verify the link between FGFR1and erk1/2, we examined the effect of Ad.FGFR1(dn) on erk1/2 activation after stimulation with lo FGF-2. Results are shown in Fig.15. Activity was significantly reduced in cultures expressing Ad.FGFR1(dn) ($p<0.0001$).

Figure 14. ***Effect of preventing erk 1/2 activation on chromatin compaction***

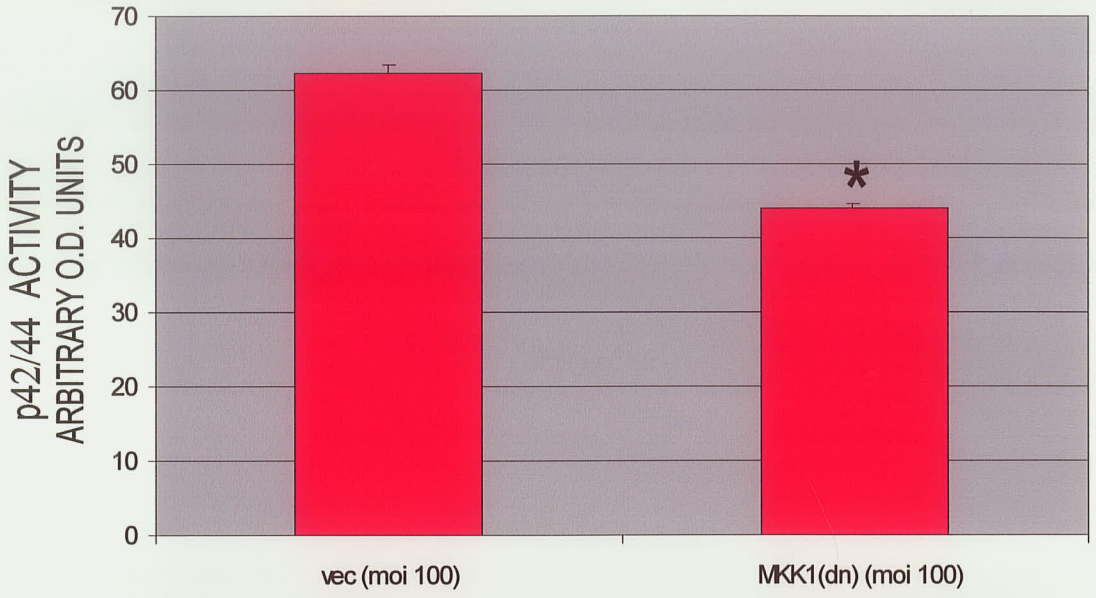
A. To determine the effect of Ad.MKK1(dn) on erk1/2 activity, myocytes were transfected with Ad.MKK1(dn) or vec for 24 hours prior to stimulation with 10 ng/mL of lo FGF-2 for 15 minutes. Active p42/44 (phospho erk1/2) protein levels were obtained by Western blotting using antibodies recognizing the dually phophorylated (active) erk1/2. Intensity of bands corresponding to active erk1/2 was assessed by densitometry. Data are shown as the mean $\pm$ S.E.M. of n=3. *$p<0.0001$ compared to vector-treated cells.

B. To determine the effect of Ad.MKK1(dn) on chromatin compaction induced by hi FGF-2, myocytes were pre-transfected with 50 m.o.i. of vector or Ad.MKK1(dn) for 12 hours prior to infection with 200 m.o.i. of Ad.hi FGF-2 for an additional 72 hours. Data are shown as the mean $\pm$ S.E.M. of n=32. *$p<0.0001$ compared to vector-treated cells.

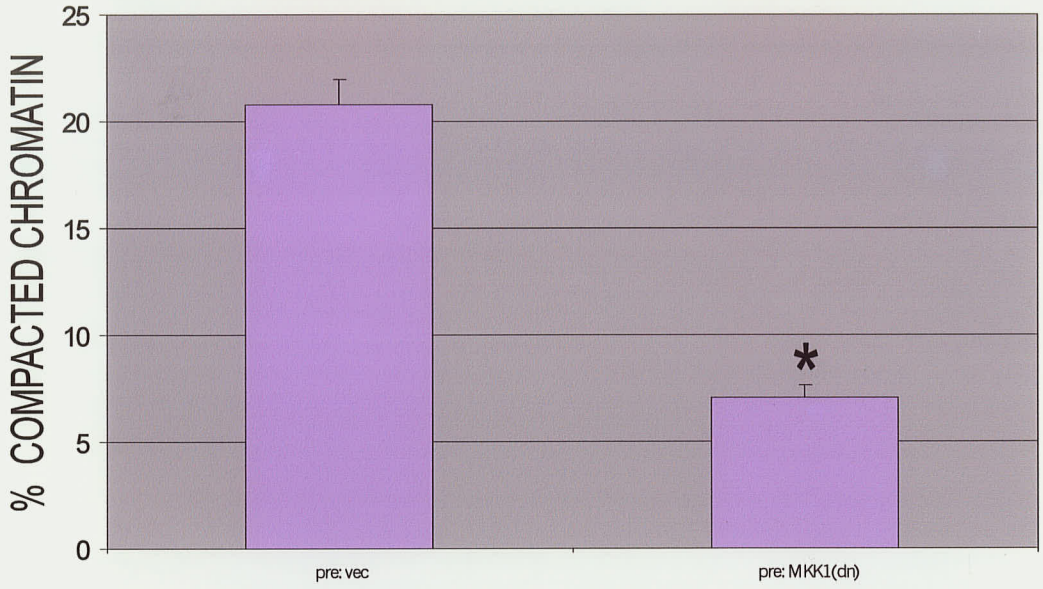
C. To determine the effect of the MEK-1 inhibitor PD 98059 on chromatin compaction induced by hi FGF-2, 25 μM of PD 98059 (MEK-1 inhibitor) was added 1 hour before myocytes were transfected with 200 m.o.i. of Ad.hi FGF-2. myocytes were incubated for an additional 48 hours after transfection. Data are shown as the mean $\pm$ S.E.M. of n=32.

$p<0.0001$ compared to control cells.

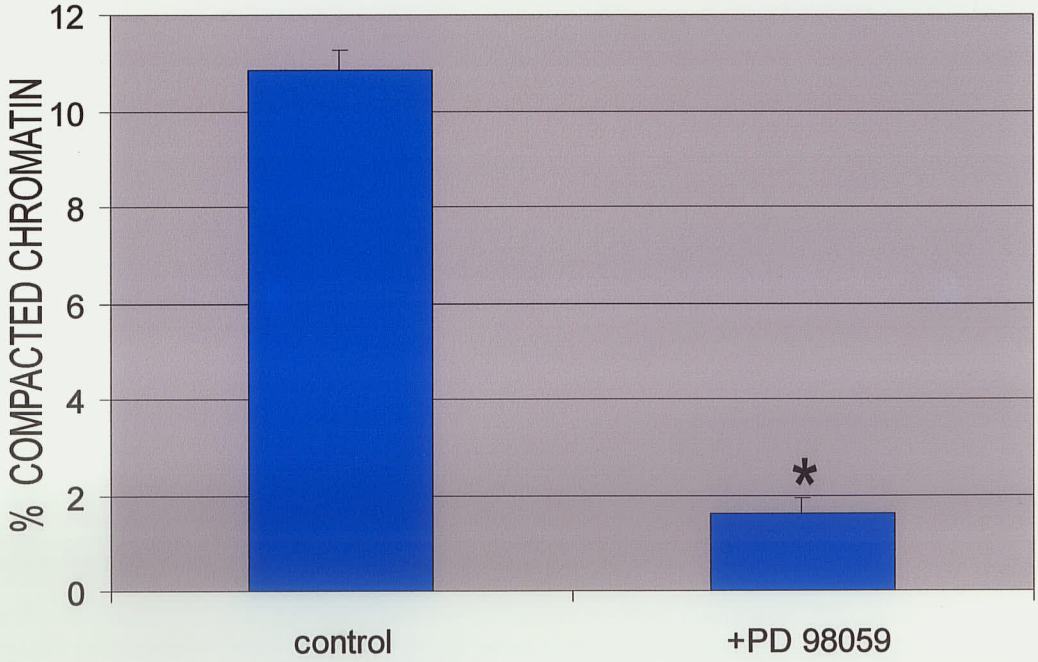
A



B



C

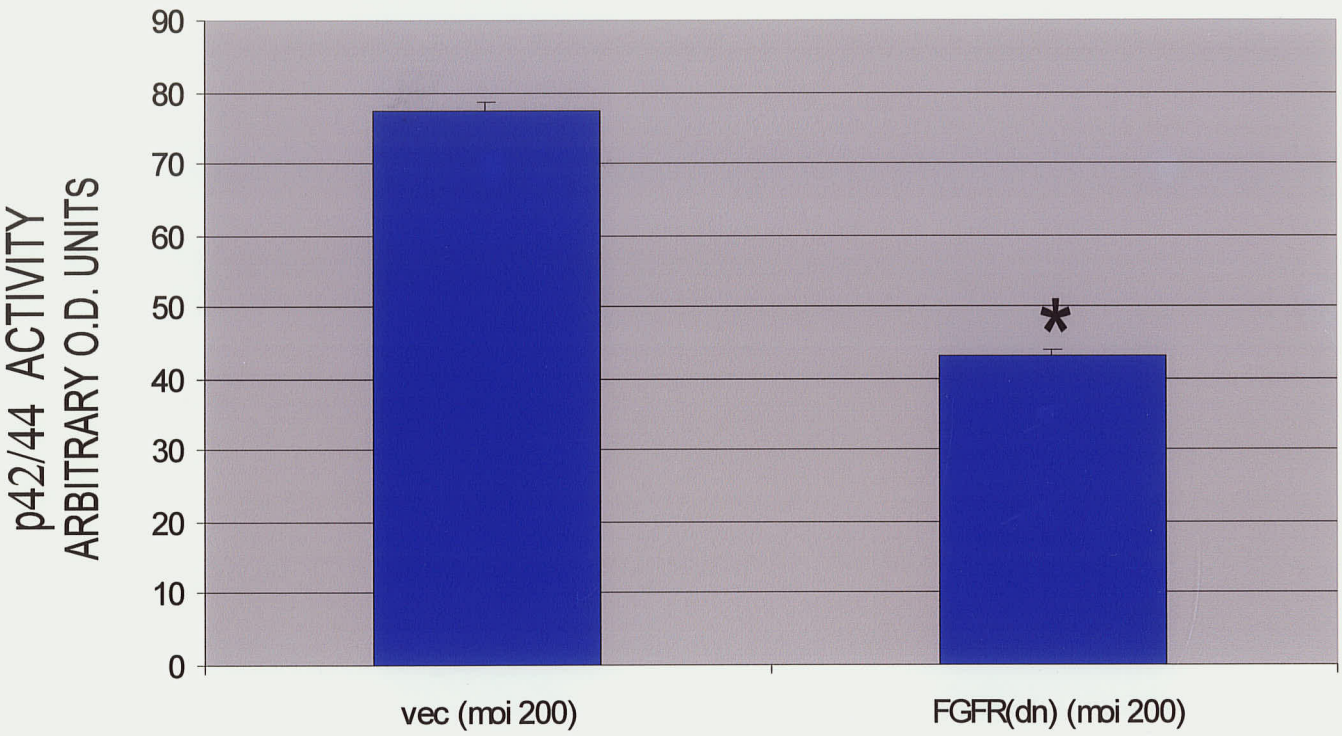


TRANSFECTED WITH hi FGF-2 FOR 72hr (moi 200)

Figure 15. ***Effect of Ad.FGFR(dn) on erk1/2 activity***

Myocytes were transfected with Ad.FGFR(dn) or vec for 24 hours prior to stimulation with 10 ng/mL of lo FGF-2 for 15 minutes. Active p42/44 (phospho erk1/2) protein levels were obtained by Western blotting using antibodies recognizing the dually phosphorylated (active) erk1/2. Data are shown as the mean $\pm$ S.E.M. of n=3.

*$p<0.0001$ compared to vector-treated cells.


p42/44 ACTIVITY
ARBITRARY O.D. UNITS
90
80
70
60
50
40
30
20
10
0
*
vec (moi 200)
FGFR(dn) (moi 200)

3.2.3 Effect of preventing the activation of PKCε

FGF-2 signal transduction includes activation of serine/threonine kinases of the protein kinase C family, such as PKCε (Powers et al., 2000). We used expression of a dominant negative species of PKC [Ad.PKCε(dn)] to prevent its activation, as described previously by Doble et al. (2000, 2002) from our laboratory, and examined its effects on chromatin disruption. Myocytes were pretreated with Ad.vector or Ad.PKCε (dn) (at 25 m.o.i each) and after overnight incubation, these cells were transfected with Ad.vector or Ad.hi FGF-2 (at 150 m.o.i. each). Each culture received therefore a total of 175 m.o.i. of virus. Degree of nuclear disruption was evaluated 72 h later. Because it was immediately evident that cultures treated with both Ad.PKCε(dn) and Ad.hi FGF-2 presented higher incidence of apoptotic-looking nuclei, we scored apoptotic nuclei separately from 'clumped' nuclei. The sum of 'clumped' and 'apoptotic' nuclei represented the fraction of nuclei presenting chromatin disruption. These results are shown in Fig.16. Inhibition of PKCε did not prevent hi FGF-2 induced chromatin disruption. In fact, cultures treated with Ad.PKCε (dn) and Ad.hi FGF-2 showed a trend towards increased overall nuclear disruption, although this did not reach statistical significance. The fraction of apoptotic-looking nuclei did however increase significantly, nearly 4-fold ($p<0.05$). Fig.17 shows typical nuclear images of cultures treated with (a) Ad.PKCε (dn): no nuclear disruption; (b) Ad.hi FGF-2:

nuclear disruption consisting mostly of 'clumping' and (c) Ad.hi FGF-2/Ad.PKCε(dn): chromatin disruption with increased apoptosis.

Figure 16. ***Effect of preventing the activation of PKCε on nuclei disruption***

Myocytes were pre-transfected with 25 m.o.i. of Ad.PKCε (dn) or vec and left overnight. They were subsequently transfected with 150 m.o.i. of Ad.hi FGF-2 or vec for an additional 72 hours. Data shown are the mean ± S.E.M. of n=4. *$p<0.05$ compared to vector-treated cells.

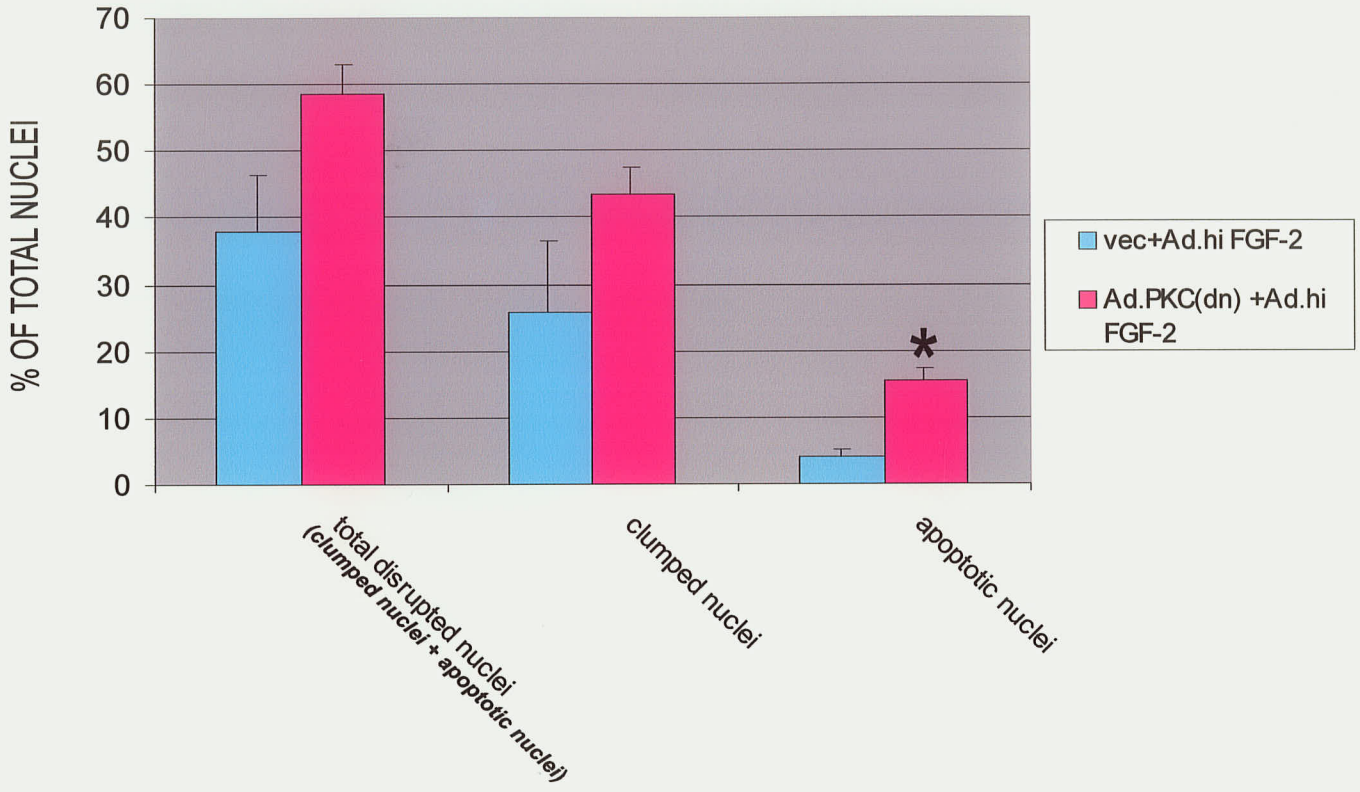

70
60
50
40
30
20
10
0
% OF TOTAL NUCLEI
total disrupted nuclei (clumped nuclei + apoptotic nuclei)
clumped nuclei
apoptotic nuclei
*
vec+Ad.hi FGF-2
Ad.PKC(dn) +Ad.hi FGF-2

Figure 17. ***Effect of preventing the activation of PKCε***

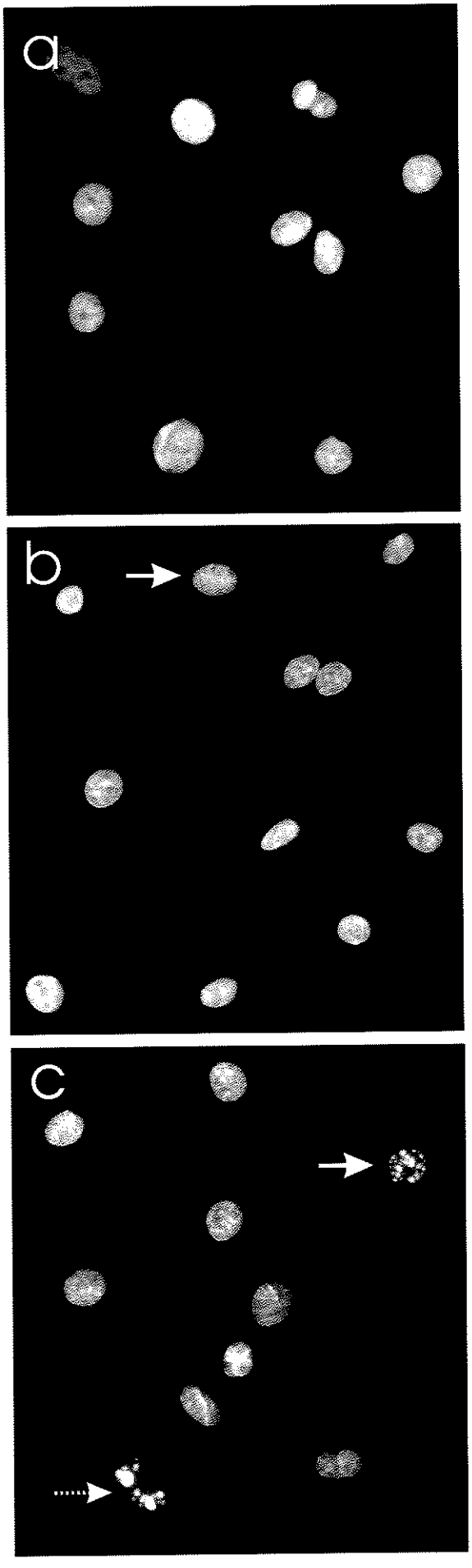
Nuclear staining of myocytes transfected with 200 m.o.i. of:

(a) Ad.PKCε(dn)

(b) Ad.hi FGF-2

(c) Ad.hi FGF-2 + Ad.PKCε(dn)

For 72 hours. Solid arrows indicate the compacted chromatin and the broken arrow indicates apoptosis.

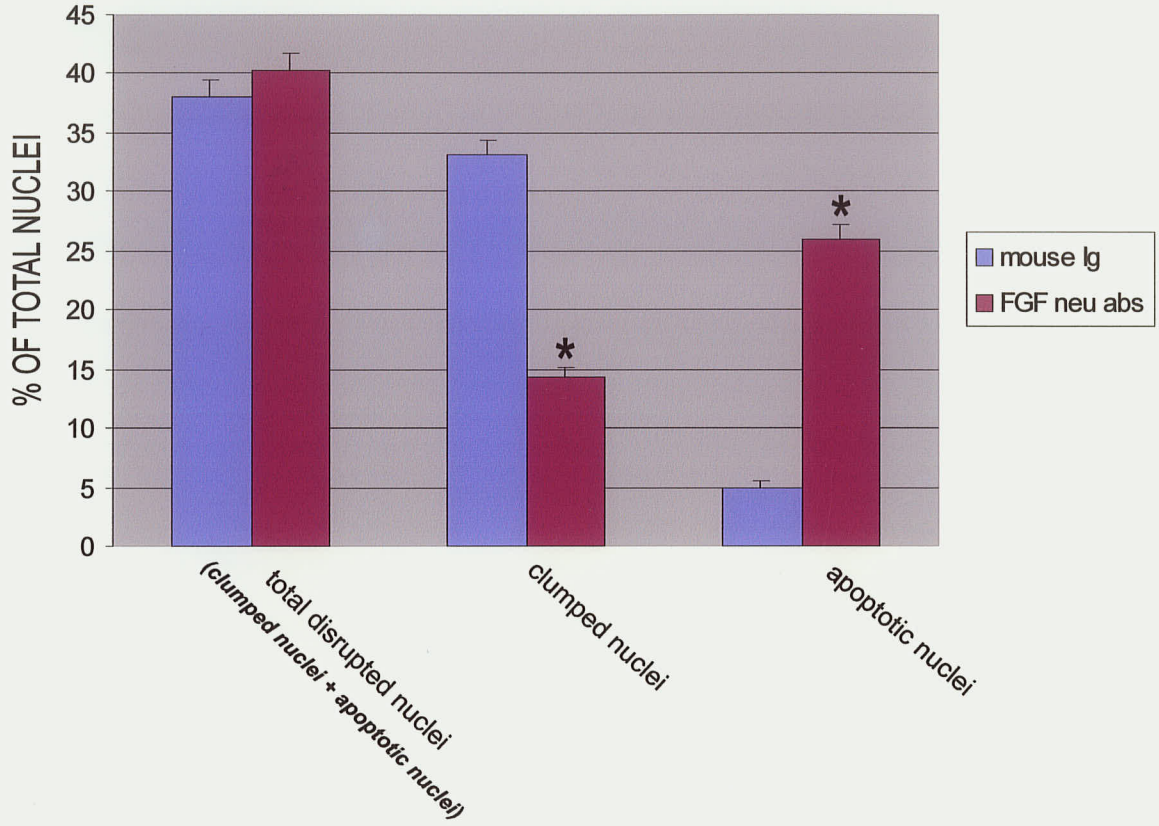

a
b
c

3.2.4 Effect of anti-FGF-2 neutralizing antibodies

Anti-FGF-2 neutralizing antibodies were used at a concentration of 25 μg/mL to block the effects of extracellular FGF-2; these antibodies recognize both hi and lo FGF-2 (Pasumarthi et al., 1996). Non-immune mouse IgG was used at similar concentrations, as a control for non-specific effects of the antibodies. Antibodies were added to the myocytes prior to transfection with Ad.hi FGF-2. We counted two categories of myocytes with disrupted nuclear morphology: those that presented the 'clumped' phenotype, and those that presented an apoptotic phenotype (i.e. a small number of large condensed chromatin clumps). Results are shown in Fig.18. In the presence of non-immune IgG, transfection with Ad.hi FGF-2 was associated with 33.2% 'clumped' and 5.0% 'apoptotic' nuclei. In the presence of neutralizing anti-FGF-2 IgG, 14.3% clumping was observed (significantly less than clumping observed with non-immune IgG, $p<0.01$); at the same time, 'apoptotic' nuclei reached 25.9% of the total, significantly higher than in the presence of non-immune IgG ($p<0.01$). The percentage of nuclei with disrupted phenotype (the sum of 'clumped' and 'apoptotic' nuclei) was not significantly different ($p>0.05$), reaching 38.1±1.4% and 40.2±1.5% in the presence of non-immune IgG or neutralizing anti-FGF-2 antibodies, respectively. Neutralizing anti-FGF-2 antibodies, used in the absence of hi FGF-2 expression, did not elicit nuclear disruption or apoptosis (data not shown).

Figure 18. ***Effect of FGF-2 neutralizing antibodies on nuclei disruption***

25 μg/mL of anti-FGF-2 type 1 neutralizing antibody was added for 1 hour before myocytes were transfected with 200 m.o.i. of Ad.hi FGF-2 for 72 hours. Mouse Ig was added to control plates. Data shown are the mean ± S.E.M. of n=32. *$p<0.01$ compared to mouse Ig control plates.



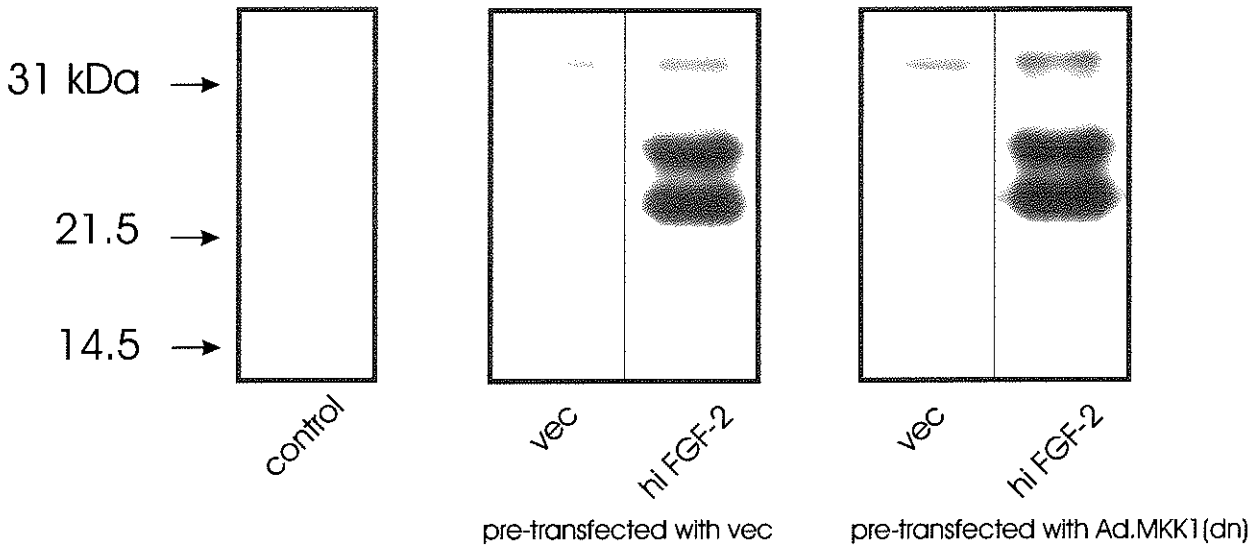
TRANSFECTED WITH hi FGF-2 FOR 72 hr (moi 200)

3.2.5 Effect on nuclear accumulation of hi FGF-2

Next, we wanted to determine the mechanism through which MAPK prevented nuclear disruption. MAPK has been implicated in the nuclear import of proteins (Agutter and Prochnow 1994) so inhibiting MAPK might prevent the nuclear accumulation of hi FGF-2. Myocytes were pre-transfected with 50 m.o.i. of Ad.vector or Ad.MKK1(dn) for 12 hours prior to subsequent infection with 200 m.o.i. of Ad.hi FGF-2 for 72 hours. Nuclear protein was extracted using the Nuclei-EZ kit and analyzed via Western blot. We found that inhibition of MAPK with Ad.MKK1(dn) did not prevent accumulation of hi FGF-2 to the nucleus. Results are shown in Fig.19.

Figure 19. ***Effect of Ad.MKK1(dn) on the accumulation of hi FGF-2 to the nucleus***

Western blot analysis of FGF-2 present in nuclear protein (5μg/lane) extracted from myocytes using the Nuclei-EZ kit. Myocytes were pre-transfected with 50 m.o.i. of vector or Ad.MKK1(dn) for 12 hours prior to subsequent infection with 200 m.o.i. of Ad.hi FGF-2 for an additional 72 hours. The ~30kDa band present is likely an endogenous FGF-2-like protein. Immunoreactive FGF-2 present was detected using a guinea pig polyclonal antibody.

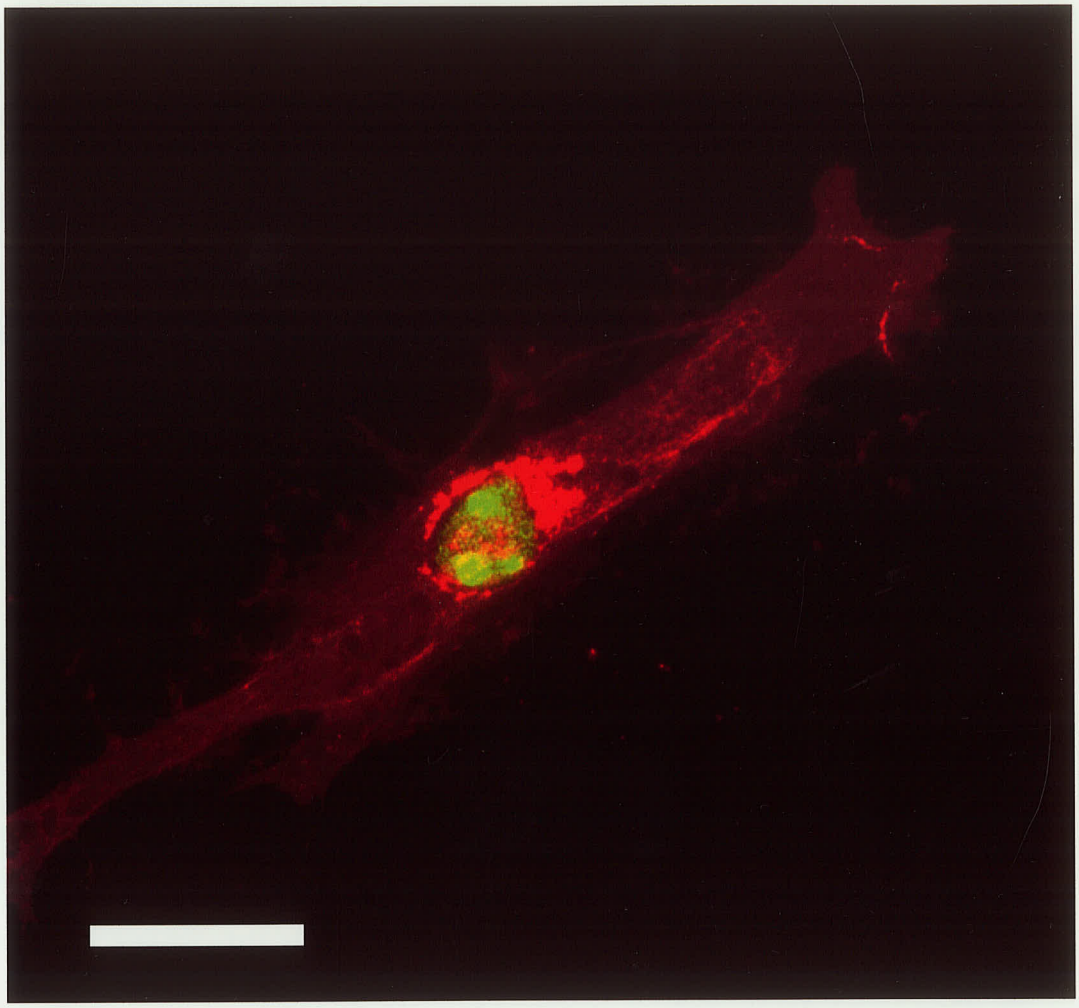

31 kDa
21.5
14.5
control
vec
hi FGF-2
pre-transfected with vec
vec
hi FGF-2
pre-transfected with Ad.MKK1(dn)

3.2.6 Localization of FGFR1(dn)

To determine FGFR1 localization in the cell, we infected myocytes with the Ad.FGFR(dn) and did immunofluorescent staining for FGFR1(dn) (red) and hi FGF-2 (green). Figure 20 shows that FGFR1(dn) localized to the plasma membrane as well as in and around the nucleus. Hi FGF-2 (green) was largely confined to the nucleus. Areas of yellow indicate co-localization of FGFR1 and hi FGF-2. Endogenous FGFR1 showed a similar localization pattern. All cells examined (in several fields, from at least three different coverslips of two different experiments) displayed the same pattern of localization. The perinuclear staining likely represents sites of synthesis of FGFR1 at the endoplasmic reticulum.

Figure 20. ***Localization of FGFR1***

Representative immunofluorescent staining of hi FGF-2 (green) and FGFR1(dn) (red) in rat cardiomyocytes transfected with Ad.FGFR1(dn). Hi FGF-2 and FGFR1 co-localize at yellow areas within the nucleus. Bar corresponds to 25 μM.



CHAPTER 4

DISCUSSION

4.1 Characterization of hi FGF-2

We have undertaken a characterization of the effects of hi FGF-2 overexpression in cardiac myocytes, made possible by the availability of adenoviral vectors expressing hi and lo FGF-2. These vectors allowed very high transfection efficiency, (as we have shown previously (Doble et al., 2000) and unpublished observations) as well as some regulation of dosage and thus, the level of expression. First, we established that this method of overexpression produced qualitatively similar changes in cardiomyocyte nuclear phenotype, as did our previous studies that used calcium phosphate transfections. Second, we were able to demonstrate that the appearance of hi FGF-2-induced chromatin disruption is both dose and time dependent. Elevated levels of hi FGF-2 expression (at 200 m.o.i) were more effective in promoting nuclear compaction compared to lower levels (at 50 m.o.i.) at the same time point. Thus at 48h, high (but not low) expression of hi FGF-2 was accompanied by clear evidence of chromatin compaction. Time in culture was also an important factor: the percentage of myocytes with compacted chromatin was maximal at 3 days post-infection in high expressors. However, even low expressors showed evidence of nuclear disruption at this time point. Furthermore, increased incidence of chromatin compaction at the later time point was

accompanied by the appearance of DNA laddering (a marker of apoptosis), as well as decreased cell number.

Changing the viral vector m.o.i. was successful in promoting different levels of hi FGF-2 expression. Thus, the relative levels of hi FGF-2 increased as a function of both dosage and time in culture, at least until the 48 h time point. Until then, we expect that the introduced gene continued to be expressed, resulting in increased protein accumulation. The decline in accumulated hi FGF-2 at 72 h, especially at the 200 m.o.i., could be a consequence of decreased expression at this time point and/or increased degradation, coinciding with decreases in cell number and DNA ladder formation.

Earlier work by Pasumarthi et al. (1994, 1996) demonstrated that hi FGF-2 can affect myocytes both in an autocrine/paracrine manner, exiting the cell and stimulating DNA synthesis, but also in an intracrine manner (not exiting the cell), causing the compacted nuclear phenotype. Our data are in agreement with these findings, since we found that hi FGF-2 (at a low level of expression) stimulated the mitotic index of cardiomyocytes and that at the 72 h time-point nuclear compaction was also clearly detected in both high and low expressors. Overall, higher levels of expression, may 'tip the balance' towards disruption of chromatin and cell death. Indeed loss of ability for cell division, and decreased cell numbers were observed at later time points. It is noteworthy that we did not detect chromatin compaction before the 48 h time point, despite high levels of hi

FGF-2 expression even at 24 h post infection. We did not obtain a simple relationship between hi FGF-2 levels and chromatin compaction: Similar levels of hi FGF-2 were observed at 50 m.o.i. and 48 h (no compaction), at 50 m.o.i. and 72 h (17% compaction) and at 200 m.o.i. and 72 h (56% compaction). These findings suggest that the development of the disrupted nuclear phenotype is not solely a consequence of increased expression and/or direct effects of hi FGF-2 on chromatin condensation. Myocytes needed to be exposed to this factor for a certain time (about 48 h in our experimental system) before they develop nuclear disruption. We conclude that the effects of hi FGF-2 are not instantaneous but rather activate a signal transduction pathway(s) that leads to chromatin disruption and eventually to cell death.

Previously, we showed that pure hi FGF-2 is capable of increasing histone-reconstituted chromatin compaction in the test tube (Sun et al., 2001) and thus we suggested that nuclear hi FGF-2 may cause 'clumping' by direct interaction with chromatin. This is not supported by our present findings. In addition, the distinct patterns of distribution of compacted chromatin and hi FGF-2 within the nucleus also argue against a direct interaction and point to an indirect mode of action. Nevertheless it may be that hi-FGF-2-chromatin interaction does occur at earlier time points before the effects on chromatin compaction are detectable; this is currently under investigation.

Because hi FGF-2 is detected by anti-hi FGf-2 antibodies that were raised against the N-extension of the molecue, and because nuclear FGF-2 has an apparent molecular weight of 22-25 kDa (Fig. 4), we presume that it remains "intact" with the nucleus.

4.1.1 Hi FGF-2 and apoptosis

Previous work from this lab concluded that the hi FGF-2-induced nuclear phenotype did not represent apoptotic cell death (Sun et al., 2001). These studies examined cells overexpressing hi FGF-2 for 48 hours, and reported that the chromatin phenotype caused by hi FGF-2 (appearance of multiple smaller clumps in the nucleus) were detected in the absence of disruption of nuclear lamina, staining for TUNEL, or for the active form of caspase 3 (P17). Furthermore, neither Bcl-2 overexpression, nor use of caspase inhibitors prevented the hi FGF-2 effects on chromatin (Sun et al., 2001). Finally, no DNA ladder was detectable in the DNA from cultures overexpressing hi FGF-2 for 48 h (Sun et al., 2001). Our present studies, while in broad agreement with the previous ones for the 48 h time point, have now provided evidence that the hi FGF-2 effects on the cell lead to apoptotic-like cell death at later time points. Our evidence for an apoptotic type of cell death after 72 h of hi FGF-2 overexpression can be summarized as follows: (a) presence of apoptotic DNA ladder (b) nuclear appearance, as seen by Hoechst staining, consisting of few large 'clumps' of chromatin resembling typical apoptotic nuclei, and (c) decreased cell number. Additional features of

apoptotic cell death have subsequently been detected at this time point such as positive TUNEL and activated caspase 3 staining (Kardami lab unpublished data). We suggest that high transfection efficiency achieved by adenoviral vectors made detection of the DNA ladder possible. This effect was not an artifact of adenoviral transfection as it was absent in both vector-transfected myocytes and those overexpressing lo FGF-2 at the same titre and the same time point. In the latter case, however, it may be that the known ability of lo FGF-2 to prevent cardiomyocyte apoptosis (Kazama and Yonehara 2000) may mask any adverse vector effects.

The effects of hi FGF-2 on chromatin are mediated by an intracellular intracrine mechanism. Studies by Pasumarthi et al. (1996) have shown that neutralizing anti-FGF-2 antibodies, used at 10 μg/ml, while capable of preventing the mitogenic effect of FGF-2, were unable to prevent its effects on chromatin. We were able to reproduce these data in our system as well, at both 10 and 25 μg/ml neutralizing antibody concentrations. Neutralizing anti-FGF-2 antibodies bind to both hi and lo FGF-2 at the extracellular space, and presumably prevent interaction with cell surface receptors. Neutralizing anti-FGF-2 antibodies did not prevent overall chromatin disruption. In fact, neutralization of extracellular FGF-2 seemed to potentiate or accelerate nuclear disruption, by increasing the percentage of 'frankly' apoptotic nuclei while decreasing the percentage of 'clumped' nuclei.

In conclusion, we have provided evidence that prolonged cardiomyocyte exposure to the intracrine action of hi FGF-2 leads to mitotic arrest, chromatin disruption and finally cell loss, presumbaly through some form of apoptotic cell death. This contrasts to the autocrine/paracrine effects of FGF-2, supporting mitotic stimulation and protection from injury (Detillieux et al., 2003). Preferential accumulation of hi FGF-2, occurring during stress (Vagner et al., 1996) might be expected to contribute to the development of cell destruction.

4.2 Intracrine Signal Transduction Mechanism

It is becoming increasingly evident that at least some of the effects of FGF-2 (hi or lo) are mediated by binding to intracellular FGFR. As mentioned in the introduction, FGFR1 (the main FGFR present in myocytes) is found in the nuclear membrane, as well as nuclear matrix sites (Stachowiak et al., 2003). It is thus possible that it may interact with, and mediate, the intracrine effects of hi FGF-2. To address this, we used overexpression of a kinase-deficient FGFR1 species (by adenoviral transfection), to block all receptor-mediated actions in a dominant-negative (dn) manner. The FGFR1(dn) distributed in a manner identical to the endogenous FGFR1, (i.e. at the plasma membrane) but also in association with the nucleus. We observed that overexpression of the FGFR1(dn) decreased DNA synthesis (similar to the effect of neutralizing antibodies), and at the same time (unlike the effect of neutralizing

antibodies), completely prevented chromatin disruption by hi FGF-2. We assume that inhibition of DNA synthesis occurred by blocking the action of plasma membrane receptors, as similar effects were obtained by extracellular anti-FGF-2 neutralizing antibodies. We suggest that inhibition of nuclear compaction occurred by preventing an intracrine pathway triggered by the activation of intracellular, or even nuclear, FGFR1 by hi FGF-2. There is evidence that such a pathway exists in cells: Peng et al. were able to block the intracrine activation of the tyrosine hydroxylase gene by hi FGF-2 by using an intracellular dominant negative FGFR1 (Peng et al., 2002). Extrapolating from these results, we suggest that an intact, fully-operational and nucleus-associated FGFR1 is mediating the intracrine effects of hi FGF-2. It follows that intracellular/nuclear FGFR1 likely mediates hi FGF-2-induced cell death. Therefore, while plasma membrane FGFR1 mediates mitotic stimulation as well as protection from cell death by FGF-2 (hi or lo), (Jiang et al., 2002; Padua et al., 1998) intracellular FGFR1, through its interaction with hi FGF-2, has diametrically opposite effects.

Several signal transduction pathways are activated downstream of plasma membrane-associated FGFR1: These include the ras-raf-MEK1-erk1/2 pathway and the PLC-PKC pathway (Klint and Claesson-Welsh 1999; Powers et al., 2000). Of these, erk1/2 (or MAPK) is implicated in nuclear import of proteins (Agutter and Prochnow 1994; Czubryt et al., 2000). We thus tested the hypothesis that blocking erk1/2 would affect

nuclear accumulation of hi FGF-2 and/or chromatin disruption. We used the inactivation of the upstream kinase MEK-1 (MKK1(dn)), pharmacological inhibition with PD98059 and overexpression of a dominant negative MEK1 kinase to block erk1/2 activity and verified that this was indeed so. We were thus able to show that erk1/2 activation was mediating the effects of hi FGF-2 on chromatin. Inhibition of FGFR1 by the kinase deficient FGFR1(dn) resulted in decreased erk1/2 activation, providing additional evidence for the link between FGFR1 and erk1/2 in our system.

Blocking the erk1/2 pathway prevented nuclear compaction, but did not affect nuclear accumulation of hi FGF-2. Nuclear accumulation of hi FGF-2 is not, therefore, sufficient, in and of itself, to cause chromatin compaction, but requires the presence of active erk1/2. It remains to be seen how erk1/2 may signal chromatin compaction. Erk1/2 activation leads to the activation of MSK1 (mitogen and stress activated protein kinase 1) that phosphorylates serine 10 on histone 3. Phosphorylated histone 3 is associated with mitotic chromatin compaction and remodeling (Thomson et al., 1999); however, Sun et al. (2001) have shown that phosphorylation of histone H3 does not occur in hi FGF-2 overexpressing cells and thus is not likely to be involved in the observed nuclear phenotype. Blocking MSK1 activation furthermore (Kardami Lab unpublished data) had no effect on hi FGF-2 induced nuclear compaction. It may be that erk1/2 activation is involved in the phosphorylation of other

histones (for example H2B) that are reported to be associated with apoptotic chromatin condensation (Cheung et al., 2003).

The PKC pathway is another pathway affected by the plasma membrane FGFR1. FGF-2 stimulates activation of several PKC isoforms including PKCε (Doble et al., 2000; Padua et al., 1998). Several of the effects of FGF-2 on cardiomyocytes are mediated by PKCε activation including DNA synthesis (Skaletz-Rorowski et al., 1999), cardioprotection (Doble et al., 2000; Jiang et al., 2002; Padua et al., 1998) and connexin-43 phosphorylation (Doble et al., 1996; Doble et al., 2000). We therefore used overexpression of a dominant negative version of PKCε (Ping et al., 1999) to investigate whether activation of this enzyne was required for the intracrine effects of hi FGF-2. Unlike erk1/2, inactivation of PKCε did not prevent the nuclear effects of hi FGF-2. In fact, PKCε inactivation seemed to exacerbate or accelerate the appearance of an apoptotic nuclear phenotype by hi FGF-2, in a manner similar to that of neutralizing anti-FGF-2 antibodies. We therefore conclude that the effects of hi FGF-2 on chromatin are not mediated by PKCε activation. More likely, endogenous PKCε activity prevents or delays the nuclear effects of hi FGF-2, in agreement with its known cytoprotective function (Ping et al., 2002; Vondriska et al., 2001; Zhang et al., 2003).

We conclude that the effects of hi FGF-2 on chromatin require activation of intracellular, possibly nuclear, FGFR1, and the erk1/2, but not the PKCε, pathway. Nuclear accumulation of hi FGF-2 is not sufficient for

promoting chromatin compaction. While plasma membrane FGFR1 mediates cardioprotection and stimulation of DNA synthesis, mediated by PKCε activation, the interaction of intracellular/nuclear hi FGF-2 and FGFR1 can lead to chromatin disruption and cell death.

CHAPTER 5

FUTURE DIRECTIONS

It is important to clearly differentiated between the actions of plasma membrane and nuclear FGFR1. Use of a modified as well as dominant negative FGFR1 that is forced to localize exclusively to the nucleus will be useful in such studies. It will also be important to delineate the complete signal transduction pathways triggered by hi FGF-2 leading to chromatin compaction and cell death. Closer examination of the effect of overexpression of hi FGF-2 on the histones and chromatin (i.e. determine what histone modifications occur, what is the 'histone' code) would be helpful. Employing the chromatin immunoprecipitation (ChIP) technique (Spencer et al., 2003) to identify regions of DNA that interact with hi FGF-2 may provide useful clues as to the mechanism involved.

Finally, it would be important to examine whether hi FGF-2-induced cell death is an event that occurs in vivo under conditions of stress. If so, it would be important to study the mechanisms regulating the balance between the pro-life effects of lo FGF-2 and the pro-death effects of hi FGF-2, such as their translational regulation and post-translational processing.

CHAPTER 6

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