EXPRESSION AND REGULATION OF BASIC FIBROBLAST GROWTH FACTOR AND ITS RECEPTORS IN CARDIAC MYOCYTES

BY

LEI LIU

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EXPRESSION AND REGULATION OF BASIC FIBROBLAST GROWTH FACTOR AND ITS RECEPTORS IN CARDIAC MYOCYTES

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LEI LIU

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

DOCTOR OF PHILOSOPHY

Dedicate to my motherland, China.

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ABSTRACT

Cardiac myocytes lose their ability for hyperplastic and hence regenerative response soon after birth. As a consequence, damaged myocardial tissue as might occur after ischemia becomes replaced by scar. The heart hypertrophies in order to meet the functional demand; beyond a certain potential for adaptation, congestive heart failure ensues. Elucidating the mechanisms controlling cardiac myocyte proliferation may provide means to stimulate regeneration, reduce scar size and prolong cardiac lifespan after injury.

Control of cardiac myocyte hyperplastic and hypertrophic growth may be exercised, in part, at the level of expression and/or activity of endogenous growth factors, such as bFGF. As a first step in understanding the role of this factor in cardiac growth we examined: (a), bFGF subcellular distribution in cardiomyocytes during the cell cycle, (b), regulation of bFGF expression and activity by thyroid hormone, (c), expression of bFGF in the mdx mouse model of muscle degeneration, and, (d), expression of bFGF receptors by cardiomyocytes in culture and in vivo.

(a). Using triple indirect immunofluorescence staining for bFGF, myosin and nuclear DNA on cultured embryonic chicken cardiomyocytes, we showed increased accumulation of bFGF in a fraction of myocyte nuclei. These nuclei were more abundant in more immature myocytes, were generally larger in diameter and their intensity of anti-bFGF staining was similar to that of prophase nuclei, findings

consistent with traversing the S or G2 stage of the cell cycle. Distribution of bFGF changed dramatically during mitosis, from being closely associated to chromosomes in prophase and early metaphase, to complete dissociation from chromosomes and localization to the area of cleavage furrow formation and the midbody in anaphase and telophase. It appears therefore that bFGF or closely related proteins play a dynamic role during the cell cycle and participate in cytokinesis. The pattern of cell-cycle dependent bFGF localization is likely species specific, since it was not observed in rat cardiomyocytes. In the latter, bFGF was localized to the nucleus, the perinuclear region and gap junctions, and did not localize to the midbody[1,2].

(b). We have found that bFGF stimulated cardiomyocyte labelling index (LI; proportion of myocytes capable of DNA synthesis), thus prolonging the ability of these cells for cell division in culture. Thyroid hormone, on the other hand, decreased the stimulating effect of bFGF on LI. This inhibition of bFGF action may be of physiological significance since levels of thyroid hormone increase after birth at the same time that myocytes transition from hyperplastic to hypertrophic growth. Using biochemical fractionation and immunoblotting we have shown that thyroid status regulates composition and abundance of bFGF in cardiac ventricles and other organs, in an organ-specific manner. Hypothyroidism stimulated a 5-fold increase in the 21.5 - 22 kD bFGF, an isoform predominating in neonatal ventricles. Thyroid hormone therefore exerts a negative regulatory effect on the accumulation of 21.5 -22 kD bFGF in the heart *in vivo*. It is proposed that the effects of thyroid hormone are directly exercised on the myocytes: thyroid hormone-treated cultured cardiomyocytes

displayed a statistically significant reduction in the proportion of 21.5 - 22 kD bFGF compared to cultures maintained in thyroid-hormone depleted media. Our data suggest for the first time that one of the ways by which thyroid hormone affects cardiac muscle growth and maturation may be by modulating the expression and activity of bFGF[3].

(c). To investigate a potential role of endogenous bFGF in the injury-repair response we investigated its localization in cardiac muscle of mdx and control mice. Actively degenerating myocytes displayed intense cytoplasmic anti-bFGF staining prior to cellular infiltration. In addition, strong nuclear anti-bFGF staining was detected in cardiac myocytes at the borders of the necrotic regions. In agreement with immunolocalization data, hearts from dystrophic mice contained consistently higher bFGF levels compared to control. Our data therefore pointed to a participation of bFGF in the injury-repair response of the heart[4]. Hearts from hyperthyroid mdx mice displayed reduced anti-bFGF immunostaining and more extensive scarring compared to controls, suggesting that thyroid hormone aggravated the dystrophic phenotype. We hypothesize that this may be due, at least in part, to a reduction in bFGF levels[5].

(d).Finally we investigated expression of bFGF receptors as a function of developmental transitions *in vitro* and *in vivo*. Unexpectedly, we found that adult cardiomyocytes expressed not only low- but also high affinity bFGF receptors. This conclusion was supported by several approaches: (i), Affinity cross-linking of ^{125}I-

bFGF to purified cardiac sarcolemmal membranes revealed presence of a 134 kD bFGF receptor. (ii), Equilibrium binding studies demonstrated the presence of high and low affinity binding sites on purified sarcolemma. (iii), The cytotoxin bFGF-saporin, which is lethal to cells possessing high affinity bFGF receptors, induced a significant loss in the viability of isolated adult cardiomyocytes, consistent with the presence of functional bFGF receptors in these cells. (iv), Isolated adult cardiomyocytes expressed the gene for FGFR1, as well as for N-syndecan, a proteoglycan considered as a low affinity bFGF receptor. Expression of bFGF receptors indicates a role for bFGF in adult cardiomyocyte physiology[6]. Since bFGF receptors comprise a large family, it is possible that different receptors are linked to a different cellular response to bFGF. We have shown that proliferative myocytes, responding to bFGF by increased DNA synthesis, as well as post-mitotic cultured cardiomyocytes (which do not respond to bFGF by increased DNA synthesis within the timeframe of the experiment) express comparable levels of a 134 kD bFGF receptor and are capable of heparin-resistant (high-affinity receptor mediated) and heparin-sensitive (low-affinity receptor mediated) bFGF internalization. A 105 kD bFGF receptor however was expressed predominantly in proliferative myocytes. These data are consistent with the notion that one of the ways by which bFGF can exert mitogenic as well as non-mitogenic activities on myocytes could be by using different bFGF receptors[7].

In conclusion, we have shown that bFGF participates in several aspects of cardiac myocyte biology. In addition to acting as a classic mitogen, stimulating cell

division by binding to cell surface receptors, bFGF may also participate directly in the mechanics of the cell cycle and cytokinesis. In addition to direct action, thyroid hormone could exert some of its effects on muscle differentiation/growth and repair by regulating the activity as well as expression/composition of bFGF. Finally, expression of bFGF receptors by adult myocytes is consistent with a non-mitogenic role in the heart. These findings have confirmed the complexity of bFGF in the heart and, I hope, have challenged or enlarged the scope of previously held beliefs.

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ACE	Angiotensin converting enzyme
aFGF	acidic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
ANF	atrial natriuretic factor
Ang	Angiotensin
ATP	adenosine triphosphate
B nuclei	brightly staining nuclei
bFGF	basic fibroblast growth factor
Bis	N-N-methylene-bis-acrylamide
BSA	bovine serum albumin
CA	catecholamine
cdc	cell-division cycle
CDK	cyclin dependent kinase
cDNA	complementary deoxynucleic acid
CMF	calcium and magnesium-free
Co.	company
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGTA	ethylen glycol-bis (ß-aminoethyl ether) N,N,N'N'-tetraacetic acid

F nuclei	faintly staining nuclei
F-12	Ham's F-12 medium
FGF	fibroblast growth factor
FGFR	FGF receptor (high affinity)
Fig.	Figure
GAPDH	glyceraldehyde phosphate dehydrogenase
G protein	GTP-binding protein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMW	high molecular weight
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
IGF	insulin-like growth factor
Inc	incorporated
IP3	1,4,5-triphosphate
IS2	rabbit anti-bFGF serum characterized in reference Kardami et al, 1990
Kd	dissociation constant
LI	labelling index
	= labelled PAS^+ cells/total number of PAS^+ cells
MAP	mitogen-activated protein
MAPK	MAP kinase
Mek	MAPK kinase
MEM	Minimum Essential Medium
MHC	myosin heavy chain

Mr.	molecular weight
mRNA	messenger ribonucleic acid
N-	amino-
NTS	nuclear translocation sequence
PAGE	polyacrylamide gel electrophoresis
PAS	Periodic Acid Schiff staining
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pI	isoelectric point
PIP2	phosphotidylinositol 4,5-bisphosphate
PKC	protein kinase C
PL	phospholipase
PMSF	phenylmethyl-sulfonyl fluoride
PtdIns	phosphatidylinositide
PTU	6-n-propylthiouracil
RAS	renin-angiotensin system
Rhi	Ratio of high molecular weight bFGF =(21.5-22 kDa)/total bFGF X 100
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SEM	standard error of mean
SDS	sodium dodecyl sulphate

SkA	α-skeletal actin
SL	sarcolemma
SmA	α-smooth muscle actin
SR	sarcoplasmic reticulum
T3	triiodothyronine
T3R	T3 receptor
T3RE	T3 responsive element
T4	thyroxine
TEMED	N,N,N',N'-tetra-methyl-ethylenediamine
TGF	transforming growth factor
TRAPs	T3 receptor auxiliary proteins

UNITS OF MEASUREMENT

k-	kilo-
m-	mill-
μ-	micro-
n-	nano-
p-	pico
f-	fempto
g	gram
l	liters
M	molar
bp	base pair

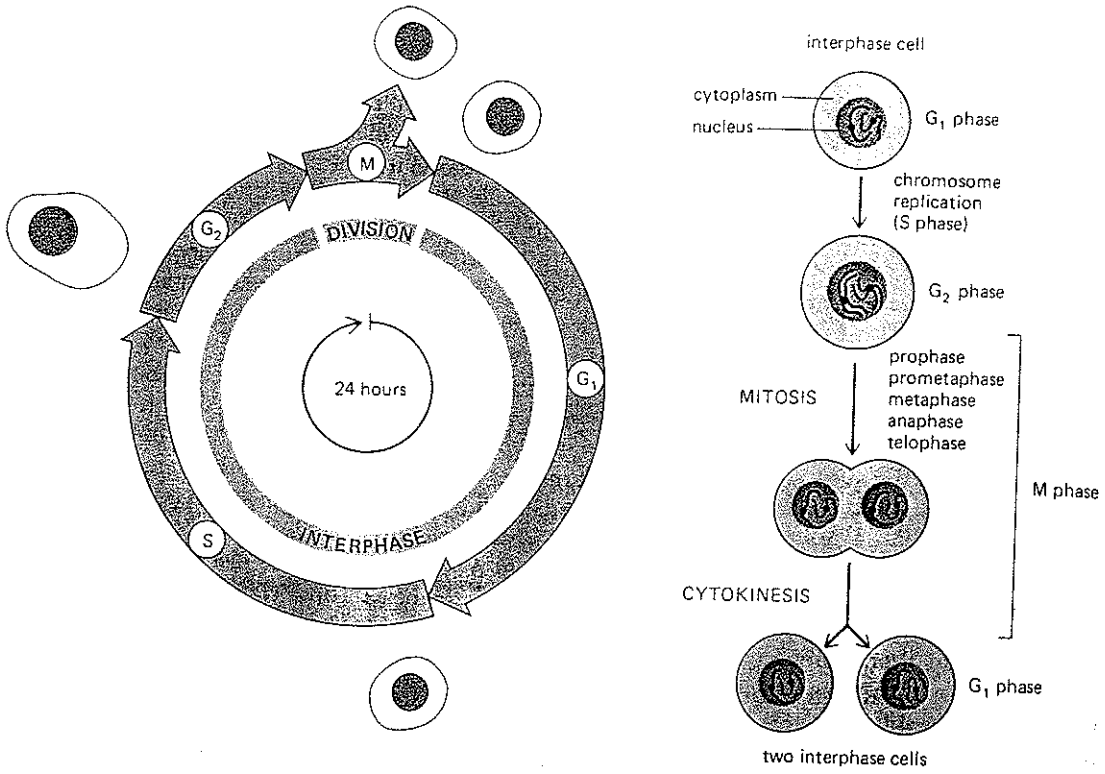
Ci	curie
Da	dalton
A	amperes
sec	seconds
min	minutes
hr	hours
wk	weeks
°C	degrees celsius
DPM	disintergrations per minute
cpm	count per minute
rpm	revolution per minute

CHAPTER 1. INTRODUCTION

Division and proliferation of the differentiated ventricular heart muscle cell in the adult mammal is not activated after injury such as that caused by a myocardial infarction (Rumyantsev, 1991). Following irreversible injury and death of cardiomyocytes, fibrotic scar replaces the damaged muscle cells (Fletcher, 1993). The remaining myocardium hypertrophies to meet the need for extra work (White, 1992; Sharpe, 1992) but beyond a certain potential for adaptation, cardiac failure ensues (Dhalla *et al.*, 1991). It is not known how mitosis is blocked in these myocytes during development. Knowledge of the mechanisms that control the cardiac myocyte cell cycle would allow us to design reagents or procedures to induce regeneration of the adult myocardium following injury. An optimal aim would be to revert cardiac muscle cells in intact heart muscle to the biochemical states they were in during early fetal growth when they were actively dividing and proliferating. Here, we will briefly review the mechanism(s) for cell cycle control, growth of cardiac myocytes and possible factors controlling postnatal growth and regeneration of the myocytes. A more detailed discussion will be concentrated on two of those factors, basic fibroblast growth factor (bFGF) and thyroid hormone.

1.1 CELL CYCLE CONTROL

A typical eukaryotic cell cycle consists of four successive phases (Fig 1.1a), known as G1 (Gap 1), S (DNA synthesis), G2 (Gaps) and M (Mitosis). The M phase



a b

Fig.1.1 a. The four successive phases of a typical eukaryotic cell cycle. A typical 24-hour cycle is illustrated here, although cell-cycle times in eucaryotic cells vary widely, with most of the variability being in the length of the G1 phase.

b. The M phase of the cell cycle starts at the end of the G2 phase and ends at the start of the next G1 phase. It includes the five stages of nuclear division (mitosis) and cytoplasmic division (cytokinesis). From Albert et al, 1989

(Fig.1.1b), which includes five stages of nuclear division (mitosis), namely prophase, prometaphase metaphase anaphase and telophase, and cytoplasmic division (cytokinesis), occupy a brief period in the cell's reproductive cycle. The much longer time that elapses between one M phase and the next is known as interphase, which includes G1, S and G2 phase. Interphase starts with the G1 phase, in which the biosynthetic activities of the cells, which proceed very slowly during mitosis, resume at a high rate. The S phase begins when the DNA synthesis starts, and ends when the DNA content of the nucleus has doubled and the chromosomes have replicated (each chromosome now consists of two identical "sister chromatids"). The cell then enters the G2 phase, which continues until mitosis starts (Albert *et al.*, 1989).

Cells in different tissues, species, and at different stages of embryonic development have division cycles that vary enormously in duration. Although all phases of the cell cycle vary to some extent, by far the greatest variation occurs in the duration of G1. In some cells the G1 is so long that the cell appears to have altogether ceased progressing through the division cycle and to have withdrawn into a quiescent states. Cells in such a quiescent G1 state are often said to be in the G0 state and many types of cells enter G0 as a result of terminal differentiation (Albert *et al.*, 1989).

Cell-cycle regulation is exerted at two points, the G1-S (including G0-S) or START, which is where a cell commits itself to DNA replication, and G2-M transition. The importance of each regulatory step varies among organisms and among

developmental stages within the same organism. For most proliferating cells, both *in vivo* and *in vitro*, cell-cycle regulation is exerted at the START (Murray and Kirschner, 1989). In eukaryotic cells, the ordered progression of events in the division cycle is controlled by a family of protein kinases encoded by cell-division-cycle (cdc) genes. These kinases function in association with phase specific sets of proteins known as cyclins, which are thought to regulate their catalytic activity, and thus they are called "cyclin-dependent kinases" or CDKs (Pines, 1993a).

A 34 kDa serine/threonine kinase (p34) encoded by the cdc2 gene ($p34^{cdc2}$) is highly conserved through evolution. In yeast, $p34^{cdc2}$ was responsible for regulating the G1-S and the G2-M of the cell cycle. It seems that $p34^{cdc2}$ is bound and activated by different members of the cyclin family of proteins in different parts of the cell cycle (Pines, 1993b). G1 cyclins regulate $p34^{cdc2}$ activity at START, whereas mitotic (B-type) cyclins regulated $p34^{cdc2}$ at mitosis (Lew and Reed, 1992). In animal cells there are at lease 4 types of cyclins, each binding to different CDKs and regulating different parts of the cell cycle (see Fig. 1.2; Sherr, 1993).

In addition to regulation by cyclin accumulation, activation of the $p34^{cdc2}$ protein kinase in many cases appears to be controlled by regulatory phosphorylation and dephosphorylation state of both $p34^{cdc2}$ and cyclin (Draetta, 1990; Rowley *et al.*, 1992). For example, phosphorylation of $p34^{cdc2}$ on threonine-161 may stabilize its binding to cyclin B and is required for the subsequent activation of the enzyme. Other

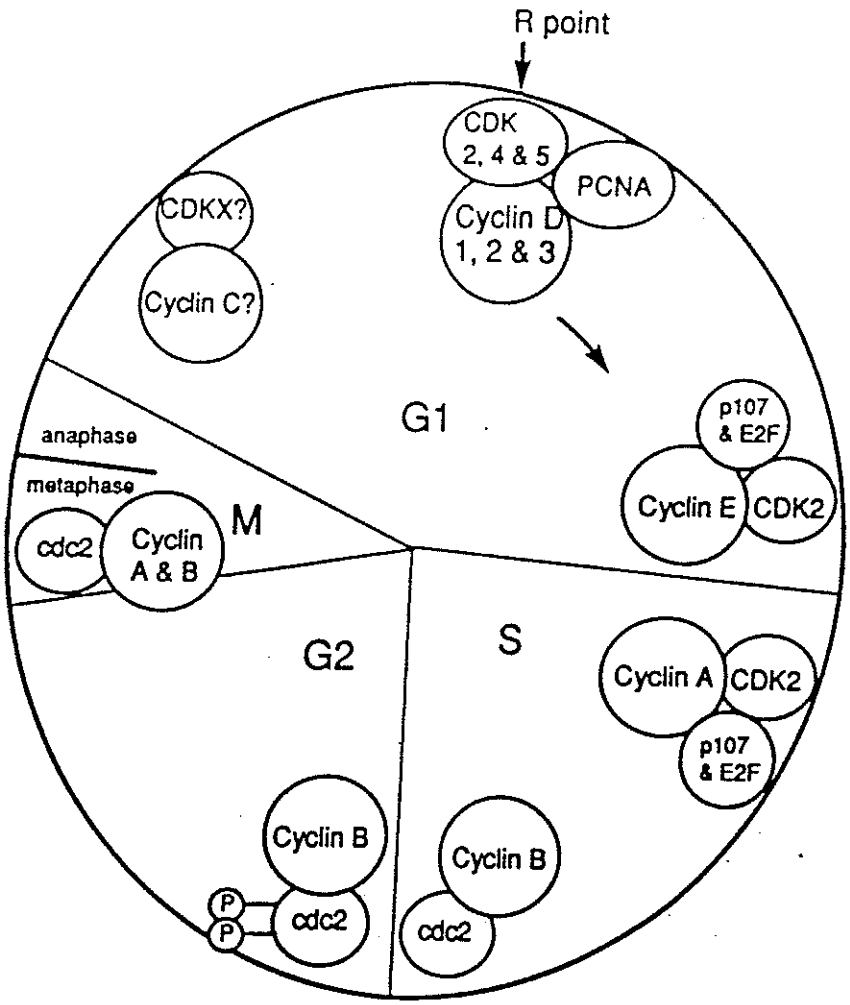


Fig.1.2 Cyclins and cyclin-dependent kinases: Which protein binds to what and when in the cell cycle. CDKX binds cyclin C and has kinase activity, but the CDK-type has not yet been determined. The R point is the restriction point, at which the cell cycle can proceed in a serum-independent manner. The cyclin D complex formed around this point is detected through the G1-S boundary and into S phase. From Pines, 1993.

phosphorylation at threonine-14 and tyrosine-15 within the $p34^{cdc2}$ ATP-binding site maintain the kinase in an inactive form throughout S and G2. Removal of the inhibitory phosphates from cyclin B-associated $p34^{cdc2}$ at the G2-M transition activates the $p34^{cdc2}$ kinase and triggers entry into mitosis (Sherr, 1993).

Many substrates for $p34^{cdc2}$ have now been identified (Table 1.1) (Nigg, 1993). In G1-S transition, one substrate is the retinoblastoma tumor suppressor gene product pRb, which acts as a suppressor of cell proliferation during the G1 phase of the cell cycle (Weinberg, 1991) by sequestering multiple cellular proteins, particularly transcription factors involved in controlling the expression of "S-phase genes" (Nevins, 1992). These proteins are thought to be released from pRb in response to phosphorylation of pRb by CDKs (Nigg, 1993). After traversing of G1, CDK function is again required during S phase. Two other substrates, the SV40 large T antigen and RPA, which are required for the initiation of DNA replication are shown to be phosphorylated by cdk (Dutta and Stillman, 1992; Fotedar and Roberts, 1992; McVey *et al.*, 1989).

In G2-M transition and during M phase, $p34^{cdc2}$-cyclin-B is thought to be controlling entry into mitosis. In mammalian cells, entry into mitosis involves in numerous events, including chromosome condensation, mitotic spindle formation, reorganization of the microfilament and nuclear envelope breakdown. All those events appear to be controlled by the direct or indirect phosphorylation effect of activated

Table 1.1 EXAMPLES OF CANDIDATE CDK SUBSTRATES

Substrate	Proposed role of phosphorylation
G1-S and S phases	
pRb	Release of transcription factors
PRA	Initiation of DNA replication
SV40 T ag	Initiation of DNA replication
p53	Regulation of nuclear localization
G2-M and M phases	
Tyrosine kinases ($p60^{src}$,$p150^{abl}$)	Cytoskeletal reorganization?
Serine/threonine kinase (casein kinase II)	?
Histone H1	Chromosome condensation
HMG	Chromosome condensation
Nucleolin	Disassembly of nucleoli
Caldesmon	Microfilament reorganization
Myosin II light chain	Delay of cytokinesis
Lamin	Lamina depolymerization
Vimentin	Vimentin redistribution
MAP4	Microtubule disassembly
cdc25	Activation of phosphatase
SWI5	Regulation of nuclear localization
Unrelated to cell cycle?	
Neurofilament M/H	Neurofilament stabilization

From Nigg, 1993

$p34^{cdc2}$ (Nigg, 1993). The $p34^{cdc2}$-cyclin-B complex phosphorylates histones H1 and certain types of major chromatin-associated non-histone proteins of the high-mobility group family, whose hyperphosphorylation is known to accompany chromosome condensation (Th'ng *et al.*, 1990; Bradbury, 1992). Mitotic reorganization of the microtubule network depends on changes in the stability of microtubules, as well as the nucleation characteristics of centrosomes (Albert et al, 1989), in which processes $p34^{cdc2}$ has been shown to play a key role (Verde *et al.*, 1990).

During cytokinesis, assembly of an actomyosin system is required for formation of a contractile ring (Cao and Wang, 1990). Two actomyosin-associated proteins have been identified as likely substrates of $p34^{cdc2}$: first, phosphorylation of the actin-binding protein caldesmon caused it to become dissociated from microfilaments, thus potentially contributing to cell rounding (Yamashiro and Matsumura, 1992); second, phosphorylation of the regulatory light chain of myosin II inhibits actin-dependent myosin ATPase, and this could delay cytokinesis until $p34^{cdc2}$-cyclin-B is inactivated at the onset of anaphase (Satterwhite *et al.*, 1992).

In conclusion, the CDK-cyclin complexes are responsible for driving the eukaryotic cell cycle, controlling key steps at G2-M and to some extent G1-S transition (McKinney and Heintz, 1991). Upstream regulatory signals (growth factors, nutrients, intracellular processes such as DNA replication) converge on specific cdks to effect progression through a given cell cycle phase (Forsburg and Nurse, 1991).

One of the mechanisms by which cells respond to extracellular signals and proceed to the G0-S transition, involves activation at the membrane of receptor tyrosine kinases (RTKs). Many growth factors activate RTKs (Fig 1.3). Molecules involved in RTK signaling have been highly conserved during evolution. It appears that all RTKs activate the mitogen-activated protein (MAP) kinase pathway (Perrimon, 1993; Blenis, 1993; Pouysségur and Seuwen, 1992).

MAP kinases (MAPKs) are serine/threonine protein kinases that are activated in response to a wide variety of stimuli. Activation of MAPKs has been observed during growth factor stimulation of DNA synthesis and during differentiation (Crews *et al.*, 1992). MAPK activation is an important event whenever cells re-enter the cell cycle from a quiescent state (Pelech and Sanghera, 1992), regardless of whether arrest occurred in G0 or G2 (Nigg, 1993).

Activation of MAPKs by growth factors can proceed via a common set of molecules that includes Grb2, p21ras, Ras-associated regulatory proteins, Raf, MAPK kinases or Mek (Blenis, 1993; Perrimon, 1993), as summarized in Figure 1.4. Activated Ras then activates c-Raf (by an as yet unknown mechanism) and the serine/threonine/tyrosine phosphorylation cascade within which reside MEK, MAPK, and RSK (Blenis, 1993). The activation of MAP kinases has at least two consequences: the direct phosphorylation of substrates, such as the transcription factors c-myc, c-jun, and $p62^{TCF}$ and the activation of other kinases such as $p90^{rsk}$ (Davis,

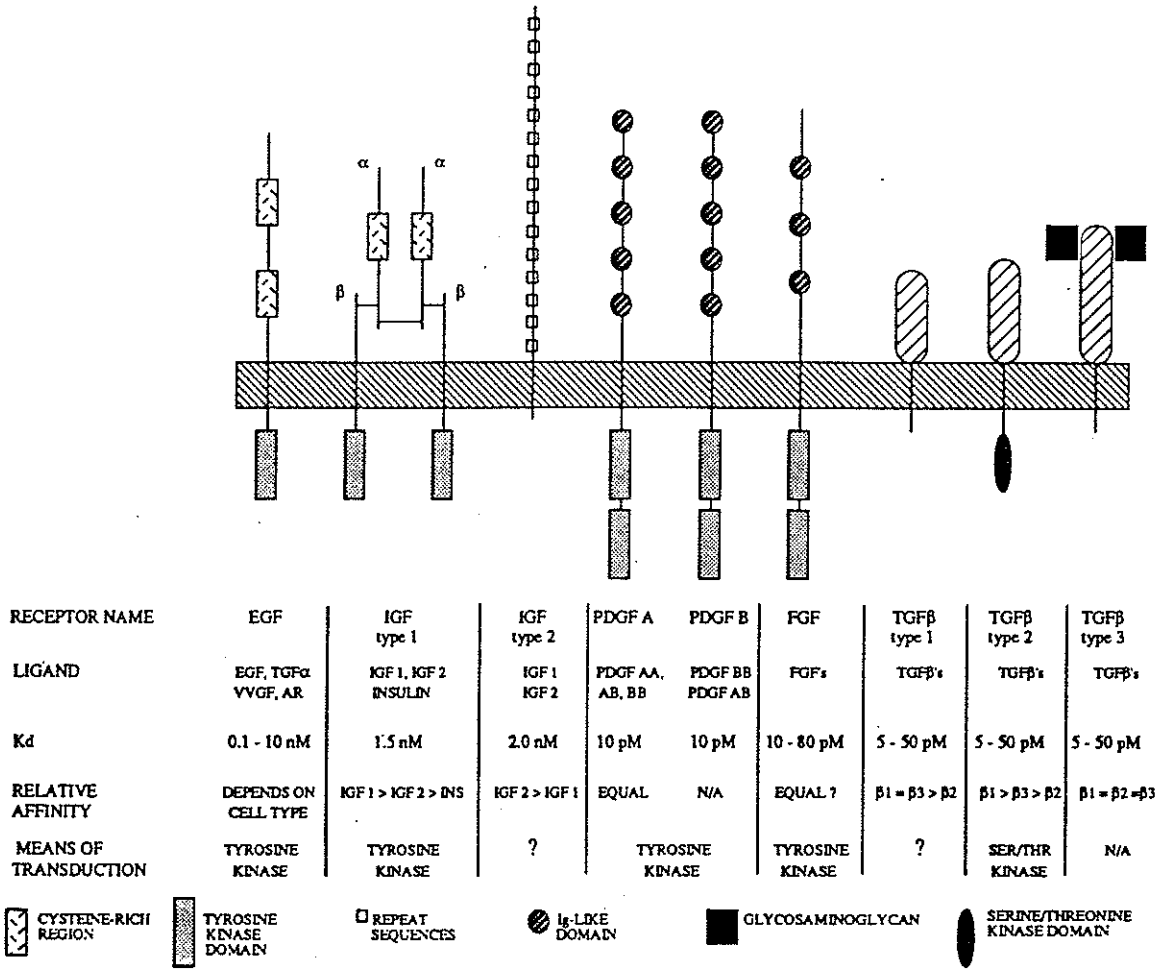


Fig.1.3. Receptors of peptide growth factors. The biologic effects of peptide growth factors are mediated by receptor proteins present in the plasma membrane of target cells. abbreviations: EGF = epidermal growth factor; TGF = transforming growth factor; IGF = insulin-like growth factor; PDGF = platelet-derived growth factor; AR = amphiregulin. From Bennett and Schultz, 1993

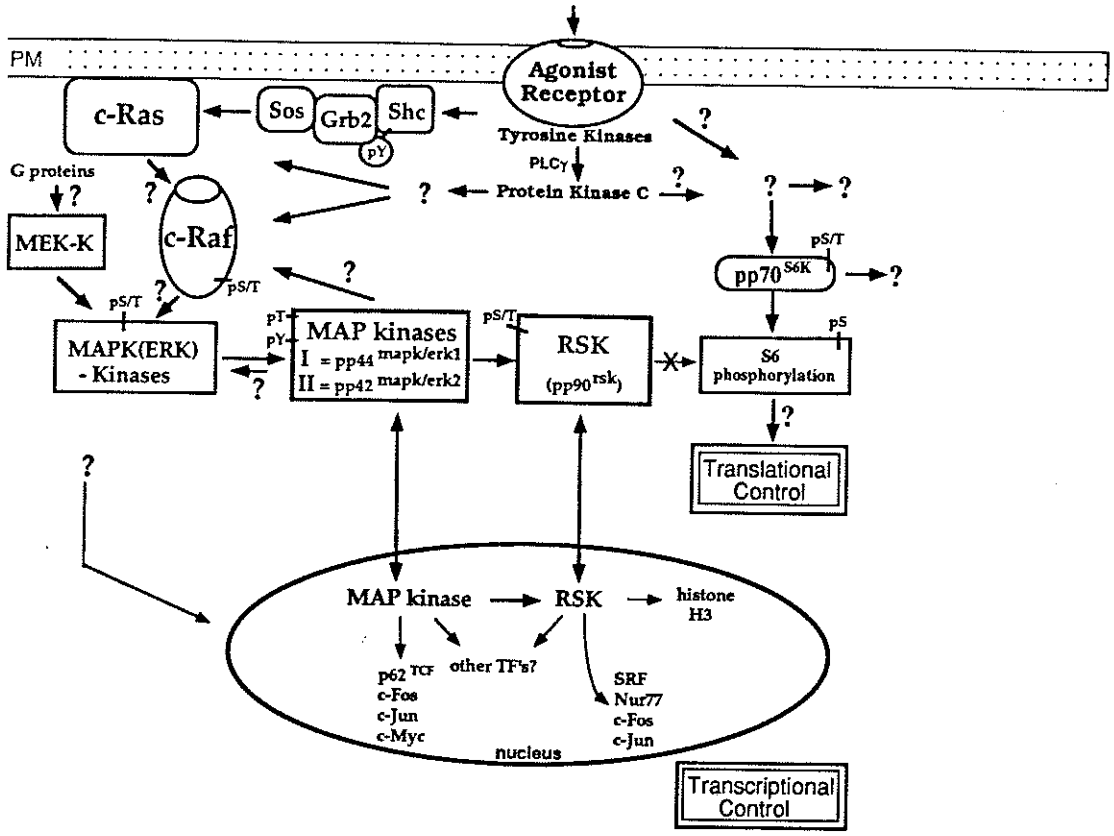


Fig.1.4 Model for signal transduction pathways regulating MAP kinases and RSK. Abbreviations not appeared in the text: TF = transcription factor; SRF = serum respond factor; PM = plasma membrane. From Blenis, 1993

1993; Blenis, 1993).

This cascade may also be regulated by heterotrimeric G proteins, activated by thrombin and angiotensin II receptors, via activated MEK kinase (Lange-Carter *et al.*, 1993; Gardner *et al.*, 1993). However, cyclic AMP, which serves as a second messenger for transmitting the signals of a diverse group of hormones including ß-adrenergic receptors, blocks transmission of signals from Ras to Raf-1 and thereby prevents activation of the MAPK cascade in some cells (Wu *et al.*, 1993; Cook and McCormick, 1993). How protein kinase C participates in the activation of this cytoplasmic phosphorylation cascade is not clear. It is not yet clear whether Ras, MAX, or MEK can signal directly to the nucleus independently of MAP kinase and RSK.

1.2 GROWTH OF CARDIAC MYOCYTE

1.2.1 Cardiac myocyte growth in development

Cardiac growth involves proliferation of cardiac myocytes (hyperplasia) during fetal and early post-natal development, followed by a transition whereby proliferation ceases and further growth occurs through an increase in myocyte size (hypertrophy) (Anversa *et al.*, 1980 & 1992; Bugaisky and Zak 1989; Claycomb, 1992). Clubb and Bishop (1984) described three phases of postnatal myocardial cell growth in the rat model (Fig.1.5). In the first phase (birth to 4 days of age) sustained hyperplastic

growth of myocytes is observed, i.e. cardiomyocytes synthesize DNA and divide. In the second phase (6-14 days of age) the nuclei of myocardial cells continue to synthesize DNA but there are few cell divisions. As a result, a rapid transition from mono- to binucleated myocardial cells occurs, interpreted as failure of cytokinesis following karyokineses. The third phase (14 -21 days of age) represents hypertrophic myocardial cell growth, i.e. little or no DNA synthesis, no cell division, high protein synthesis and continuous increase in heart weight.

The differentiated ventricular cardiac myocyte of the adult mammal has traditionally been considered to be permanently postmitotic, in that it has apparently lost the capacity to replicate DNA, undergo mitosis, and divide *in vivo* (Claycomb, 1983 & 1991, Rumyantsev, 1991). As a result, any subsequent increase in functional demand is met by hypertrophy (Zak, 1984; Morgan and Baker, 1991). Atrial myocytes display a much higher replicative activity. Some atrial cardiac muscle cells of the adult mammal have been shown to replicate their DNA and re-enter the mitotic cycle in response to ventricular infarction or functional overload (Oberpriller 1983; Borisov and Rumyantsev 1991; Rumyantsev 1991). Transgenic mice, carrying the gene for the viral SV40 antigen driven by the atrial natriuretic factor (ANF)
promoter developed tumors in the atrium due to uncontrolled cardiac myocyte division (Field, 1988); these myocytes appeared normal in all respects. There is increasing evidence showing that mammalian ventricular myocytes do not lose their ability for DNA replication and cell division irreversibly. Adult cardiac myocytes in long term

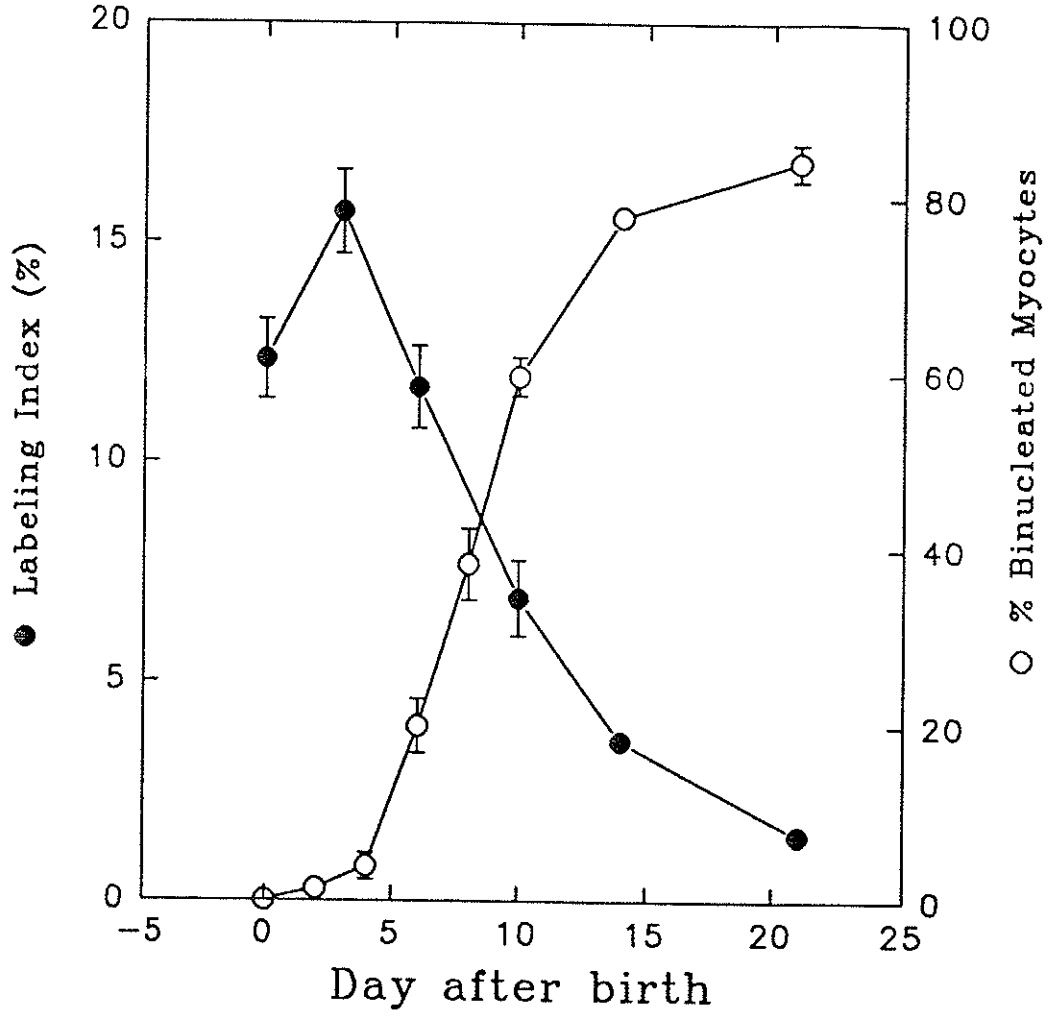


Fig.1.5 Postnatal development of cardiomyocytes in rat ventricle: DNA synthesis (circle) and binucleation (square). Plotted according to data from Clubb and Bishop, 1984.

culture regain the ability for DNA synthesis (Nag and Cheng, 1986; Claycomb and Bradshaw, 1983). *In vivo*, it has been shown that the numbers of myocyte nuclei increase in response to pressure overload on the left (Anversa *et al.*, 1990a) or right (Olivetti *et al.*, 1987) ventricle of the adult rat heart. It has also been demonstrated that myocyte cellular hyperplasia occurs in the aging heart (Anversa *et al.*, 1990b). Myocyte nuclei proliferation has been documented in humans under a variety of pathological conditions that impose a large and prolonged stress on the myocardium (Astorri *et al.*, 1971 & 1977). All these indicate that the inability of cardiac myocytes to undergo cell division is a property that may be repressed and not irreversibly lost during maturation (Marino *et al.*, 1991).

1.2.2 Cardiac hypertrophy

In response to hormonal, genetic, and mechanical stimuli, the myocardium adapts to increased workload through hypertrophy, characterized by an increase in the total mass of myocytes and in the total amount of cellular and extracellular components of connective tissue. The increase in myocyte mass is mainly mediated by hypertrophic growth of individual cells (Morgan *et al.*, 1987), though there are some reports that hyperplastic growth may also contribute to cardiac hypertrophy (Grajek *et al.*, 1993; Astorri *et al.*, 1971 & 1977; Anversa *et al.*, 1990a). Cardiac hypertrophy is not only characterized by an additional increase in cell size, but also a selective up- and down-regulation of the expression of distinct genes. At the molecular level, switches take place with respect to contractile protein isoforms and other muscle specific protein

expression (Parker and Schneider, 1991). As shown in Table 1.2, hemodynamic overload-induced hypertrophy is accompanied by a tendency to return to a fetal-resembling gene program, ie. an up-regulation of ß-myosin heavy chain (MHC), α-skeletal actin (SkA) and α-smooth actin (SmA) and down-regulation of α-MHC and cardiac sarcoplasmic reticulum Ca^{2+} ATPase. In addition, ventricular myocytes re-express atrial natriuretic factor, a feature that is lost during normal postnatal development. Work overload also produces the rapid expression of a set of proto-oncogenes involved in normal cell growth such as c-fos and c-myc. So in a general biological context, cardiac hypertrophy could be considered as equivalent to the growth response exhibited by most cell types in response to mitogens (Nadal-Ginard and Mahdavi, 1993).

It has been recently reported (Sadoshima and Izumo, 1993a) that mechanical stretch activated many second messenger pathways, including tyrosine kinase, $p21^{ras}$, MAPK, $pp90^{RSK}$, phospholipase (PL) C and PKC, which is highly reminiscent of the complex cellular response to growth factors (Williams, 1989; Ullrich and Schlessinger, 1990; Rozengurt 1991 and Fig. 1.4). Stretch is believed to be the trigger of mechanical overload of myocytes (Moalic *et al.*, 1993), which results in hypertrophic phenotype

of culture cardiac myocytes (Sadoshima *et al.*, 1992a,b; Komuro *et al.*, 1991).

In vitro studies have shown that overload hypertrophy can be mimicked by the

Table 1.2 Regulation of cardiac gene expression

	Embryonic	Adult	TH	bFGF	Overload	
MHC						
α	-	+++		↑	↓	↓
ß	+++	-		↓	↑	↑
α-Actin						
Cardiac	++	+++		↑↔	↔	↔
Skeletal	++	-/+		↑↔	↑	↑
Smooth	++	-		?	↑	↑
ANF	+++	-		↑	↑	↑
SR Ca^{2+}ATPase	+	+++		↑	↓	↓

Abbreviations: MHC = myosin heavy chain; SR = sarcoplasmic reticulum; TH = thyroid hormone. (+,++,+++)relative or (-) absent expression in normal myocardium; ↓, down-regulation; ↑, up-regulation; ↔ little or no change

From Schneider and Parker, 1990 and Parker and Schneider, 1991 with modification

administration of a variety of stimuli, among which are catecholamines (Ikeda *et al.*, 1991; Zierhut and Zimmer, 1989; Simpson *et al.*, 1991), endothelin-1 (Shubeita *et al.*, 1990), and angiotensin II (Baker and Aceto, 1990), while part of the changes in gene expression in overload is similar to cardiac response to growth factors, such as aFGF, bFGF and TGF-ß (Table 1.2 and Schneider *et al.*, 1992). In contrast, administration of thyroid hormone leads to a form of hypertrophy that is different from those caused by overload and will be reviewed in paragraph 1.3.2.

Cardiac hypertrophy can be a physiological adaption of the heart to chronic mechanical overload. Cardiac failure, possibly caused by contractile disfunction as a result of increased microtubule formation (Tsutsui *et al.*, 1993), indicates the limits of the process.

1.3. FACTORS AFFECTING CARDIOMYOCYTE HYPERPLASTIC AND HYPERTROPHIC GROWTH

Although little information exists on the molecular triggers that control myocyte proliferation and the extent of the regenerative response, or about the transition from hyperplastic to hypertrophic growth *in vivo*, local polypeptide growth factors and circulating hormones have been suggested to be involved in the normal signalling process (Zak 1984, Schneider & Parker 1990).

Polypeptide growth factors are known to play a critical role in the cycle of

normal cell division, differentiation, and growth of cells from a variety of organs and tissues. The heart expresses a variety of growth factors thought to act locally, in an autocrine or paracrine manner (Parker and Schneider, 1991) and cardiac myocytes have been shown to be the target of selected growth factors, at least in culture (Kardami, 1990; Parker *et al.*, 1990b). Among them, two classes of growth factors, namely the transforming and fibroblast growth factors, have been implicated in signalling processes involving the cardiac myocytes (Schneider and Parker, 1991; Cummins, 1993).

Hormones like thyroid hormone, catecholamines, growth hormone, and steroid hormones have been also shown to affect cardiac growth or hypertrophy directly or indirectly. Circulating and local angiotensin II, which is considered now as a cardiac growth factor, promotes cardiac hypertrophy as well.

1.3.1 BASIC FGF

Acidic and basic FGF are the best characterized members of the FGF family which contains at least eight structurally related peptides, FGF-1 (aFGF), FGF-2 (bFGF), FGF-3 (hst/K-fgf), FGF-4 (int-2), FGF-5, FGF-6 and FGF-7 (KGF) and FGF-8 (Androgen inducible growth factor)(Baird, 1993). The term fibroblast growth factor was first used in 1974 by Gospodarowicz to describe a cationic polypeptide with a pI of 9.6 found in pituitary and in brain that stimulated cell division in 3T3 cells (Gospodarowicz, 1974; 1975; Gospodarowicz *et al.*, 1978). Subsequently, brain was

found also to contain an anionic FGF with a pI of 5.6 that was a potent mitogen for endothelial cells (Maciag *et al.*, 1979; Thomas *et al.*, 1980 & 1984; Lemmon *et al.*, 1982). These two growth factors were eventually named bFGF and aFGF. They bind heparin with high affinity, and this property has been used for their isolation and characterization (Burgess and Maciag, 1989).

In the heart aFGF is less well studied compared to bFGF, however, a similar distribution of aFGF and bFGF during embryonic and neonatal development in the rat heart from 10-20 days gestation and up to five weeks after birth has been reported (Speir *et al.*, 1990). The effects of aFGF and bFGF on their target cells are very similar (Klagsbrun, 1989) and all the *in vivo* and *in vitro* role bFGF may play have also be suggested for aFGF. Since the biology and function of bFGF is reviewed in detail in the following paragraphs, here we will only mention the aspect of functional difference between aFGF and bFGF. An interesting difference has been reported by Parker *et al.* (1990a, 1990b & 1992), who have found that (i) aFGF down-regulates the gene encoding cardiac α-actin, whose transcription is unaffected by bFGF, (ii) aFGF down-regulates skeletal muscle actin, whose transcription, antithetically, is increased by bFGF.

1.3.1.1 Distribution and release

Basic FGF is probably the most widely distributed mitogen yet characterized. It has been found without exception in all tissues, organs, tumors and cells so far

examined, even though the amount accumulated may vary greatly (Basilico and Moscatelli, 1992). The majority of the factor was found to remain cell-associated or in the extracellular matrix (Schweigerer *et al.*, 1987; Kardami and Fandrich, 1989; DiMario *et al.*, 1989).

Basic FGF is synthesized by normal cells, as well as a variety of tumor cells (Burgess and Maciage, 1989; Gospodarowicz, 1989; Lobb, 1988; Eggo *et al.*, 1991; Floege *et al.*, 1992). It is believed to act through autocrine (Sato and Rifkin, 1988; Mignatti *et al.*, 1991; Sumitani *et al.*, 1993) and paracrine (Eguchi *et al.*, 1992) pathways. Recently an intracrine interaction of bFGF has been proposed (Sherman *et al.*, 1993; Nakanishi *et al.*, 1992), meaning that bFGF remains within the cell of origin and acts directly as intracellular messenger to regulate cellular function. Acting in this way, bFGF needs not be secreted, nor does it require cell surface receptors to mediate its activity (Logan, 1990). Basic FGF is not secreted conventionally like other secreted proteins, i.e. via the Golgi apparatus (D'Amore, 1990), since it lacks an N-terminal hydrophobic signal peptide sequence (Klagsbrun, 1989). The mechanism for bFGF release is not clear. It has been shown that bFGF can be released upon cell lysis during tissue injury and cell death (Gajdusek and Carbon, 1989), or by gentle mechanical wounding (Kardami *et al.*, 1991a; McNeil *et al.*, 1989) and antibody-complement-mediated injury (Floege *et al.*, 1992). It may also be released from cell- and matrix-associated sites by heparin, heparan sulfate and heparinases (D'Amore, 1990; Thompson *et al.*, 1990), or by plasminogen activator-mediated proteolysis

(Saksela and Rifkin, 1990). Recently, it is reported that bFGF can be released via a mechanism of exocytosis independent of the endoplasmic reticulum-Golgi pathway (Mignatti *et al.*, 1992). Considering the paucity of bFGF mRNA in most tissues (Shimasaki *et al.*, 1988) and the relative abundance of bFGF protein, the biological function of bFGF has been proposed to be controlled by its degree of bioavailability (Logan and Hill, 1992).

1.3.1.2 Biological function

At the present time, the physiological function(s) of bFGF has not been clearly established, nevertheless, this molecule displays a wide spectrum of effects both *in vitro* and *in vivo*.

Mitogenesis and differentiation: Basic FGF is a multi-potential factor that regulates cell proliferation, migration and differentiation. It is a mitogen for a variety of cells of mesodermal and neuroectodermal origin, including fibroblasts, vascular endothelial cells, vascular smooth muscle cells, myoblasts, epithelial cells (Klagsbrun, 1989) and cardiac myocytes (Kardami, 1990).

Besides being mitogenic, bFGF is a chemotactic factor for a number of cell types including endothelial cells (Presta *et al.*, 1986; Connolly et al, 1987), fibroblasts (Senior *et al.*, 1986) and corneal cells (Grant *et al.*, 1992), which support the concept

that bFGF may play a key role in wound healing.

Basic FGF promotes the differentiation of a number of cell types, such as endothelial cells and neurons (Klagsbrun, 1989). On the other had, bFGF can also inhibit differentiation. Specifically, bFGF repressed the terminal differentiation of myoblasts into myotubes and repressed the expression of muscle-specific gene and the commitment to the post-mitotic states (Clegg, *et al.*, 1987).

Angiogenesis is one of several proliferative processes which can occur in the fully differentiated heart, during atherosclerosis (Ross 1986), hypertrophy (Tomanek, 1990), and infarction (Casscells *et al.*, 1990a). The establishment of an effective alternative blood supply, or collateral circulation, plays a critical role in the prognosis of ischemic diseases. Basic FGF has been shown to stimulate most of the individual components of new capillary growth (Folkman and Klagsbrun, 1987; Schott and Morrow, 1993), including: (1) endothelial cell proliferation (Lindner *et al.*, 1990) and regeneration (Brindle, 1993), (2) release of proteases which digest the basement membrane (Saksela *et al.*, 1988; Mignatti *et al.*, 1989), (3) endothelial cell migration (Mignatti *et al.*, 1991), and (4) endothelial cell invasion (Pepper *et al.*, 1993) and organization into tubules (Montesano *et al.*, 1986). It has been shown that in a canine experimental myocardial infarction model, intracoronary injection of bFGF increased the numbers of arterioles and capillaries in the infarct (Yanagisawa Miwa *et al.*, 1992)

Embryogenesis: Perhaps, the most intriguing role proposed for bFGF *in vivo* is that of an inducer during embryonic development. Mescher and Gospodarowicz (1979) proposed that FGF enhanced limb regeneration in amphibians. Slack *et al.* (1987) observed that purified bFGF mimicked the mesenchymal inducer in *Xenopus* embryos. Larson *et al.* (1992) reported that TGF-ß and bFGF synergistically promoted development of bovine embryos *in vitro*. The reports that bFGF mRNA and/or protein are present in the embryos of chick (Parlow *et al.*, 1991; Joseph Silverstein, 1989) and rat (Weise *et al.*, 1993; Burton *et al.*, 1993) are consistent with the hypothesis that endogenous bFGF may act as an embryonic inducer.

Wound healing: The biological properties of bFGF, including the ability to stimulate angiogenesis, chemotaxis, cell proliferation and differentiation, suggest a possible role in wound healing.

Increased bFGF has been detected in various models of injury. Gomez-Pinilla and Cotman (1992) reported a transient lesion-induced increase of bFGF and its receptor in the rat cerebellum. Increased bFGF immunoreactivity was observed at the borders of lesions following injury to spinal cord (Koshinaga *et al.*, 1993), brain (Finklestein *et al.*, 1988; Kiyota 1991 *et al.*,), myocardium (Padua and Kardami, 1993) and skeletal muscle (Anderson *et al.*, 1991). Induction of bFGF mRNA has been shown in skin epithelia cells after acute cutaneous injury (Antoniades *et al.*, 1993), in the brain after cortical brain injury (Frautschy *et al.*, 1991), during skeletal muscle

regeneration caused by ischemia and glycogen depletion (Guthridge *et al.*, 1992), and in isoproterenol-induced cardiac injury (Padua and Kardami, 1993) .

Basic FGF has been shown to promote wound healing in several experimental wound models. Purified bFGF stimulated the formation of granulation tissue in subcutaneously implanted sponges, and increased the protein and DNA content of the sponges (Broadley *et al.*, 1989a). Application of bFGF induced significant dermal wound healing in healing-impaired diabetic mice (Tsuboi *et al.*, 1992; Greenhalgh *et al.*, 1990). Implantation of specific bFGF antibodies during the subcutaneous wound process caused significant reductions in DNA, protein, and collagen content (Broadley *et al.*, 1989b). Human recombinant bFGF stimulated corneal endothelial wound healing in rabbits (Rieck *et al.*, 1992a,b) and cat (Rich *et al.*, 1992). Intra-articular administration of partially purified brain FGFs into the joints of rabbits with standardized cartilage lesions resulted in significant healing (Cuevas *et al.*, 1988). Direct injection of FGF into the site of rat femoral shaft fractures can significantly stimulate repair, primarily by enhancing cartilage formation (Joyce *et al.*, 1991).

1.3.1.3 High molecular weight forms of bFGF

Basic FGF was originally purified as a 16- to 18- kDa peptide, composed of 146 and 154 amino acids, respectively (Klagsbrun, 1989). Subsequently, a 157 amino acid form of bFGF extended at the N-terminus was isolated from placenta (Sommer *et al.*,

1987) and a 163 amino acid form of bFGF extended at the N-terminus was isolated from human hepatoma cells (Prats *et al.*, 1989). In addition, 20-25 kDa forms of bFGF have been isolated from guinea pig brain, hepatoma cells, h-ras-transformed Rat-1 cells (Klagsbrun, 1989), rat, bovine, mouse and human brain tissues (Doble *et al.*, 1990; Brigstock *et al.*, 1990), thyroid tissue (Emoto, 1991), human pituitary adenomas (Li *et al.*, 1992) and bovine aortic endothelial cells (Maier *et al.*, 1990). The high molecular weight (HMW), ie. 20-25 kDa, forms of bFGF result from the use of alternative CUG (Leu) translation initiation codons 5' to the usual AUG (Met) codons (Prats *et al.*, 1989; Florkiewicz and Sommer, 1989). The relative usage of CUG versus AUG initiation codons is modulated by cis-acting element corresponding to secondery or tertiary RNA stucture of the bFGF mRNA (Prates et al, 1992). Transcription and translation of a 7 kb hepatoma bFGF cDNA or a 832 bp rat bFGF cDNA *in vitro* generated three polypeptides with molecular weights of 18, 21, and 22.5 kDa. The 21 kDa and 22.5 kDa forms of bFGF are N-terminal extensions of the 18 kDa bFGF (Prats *et al.*, 1989; Powell and Klagsbrun, 1991). The high molecular weight forms of bFGF (20- to 25- kDa) can be N-terminal truncated into the 18 kDa form by tryptic proteolysis (Doble *et al.*, 1990; Gualandris *et al.*, 1993; Liu *et al.*, 1993).

Limited information exists as to the functional significance of the presence of different bFGF forms. All forms of bFGF purified so far have the same specific activity in mitogenic assays, binding, presumably, to the same cell surface receptors (Florkiewicz and Sommer, 1989; Iberg *et al.*, 1989; Prats *et al.*, 1989; Amalric *et al.*,

1991). However, bFGF is primarily an intracellular molecule which may exert some of its effects in an "intracrine" fashion (Logan, 1990; Sherman *et al.*, 1993) without exiting the cell, through intracellular associations. In this respect different physiological roles may be envisaged for the lower and higher molecular weight forms of bFGF.

The N-extension of HMW-bFGF confers a nuclear localization signal (Quarto *et al.*, 1991a). Though both the 18 kDa and HMW forms of bFGF can be found in the nucleus (Gualandris *et al.*,1993), and the N-terminal sequence is not obligatory for transport of bFGF into nucleus (Powell and Klagsbrun, 1991), several investigators have shown that the 21- to 24-kDa human bFGFs are preferentially localized in the nucleus and the ribosomal fraction of cells, the 18-kDa bFGF is primarily cytosolic (Renko *et al.*, 1990; Quarto *et al.*, 1991a; Florkiewicz *et al.*, 1991). Similarly it has been shown that the 18-kDa as well as the 21.5- to 22-kDa forms of rat bFGF (derived from translation initiation from CUG start sites and introduced by gene transfer) localized in the nucleus of cultured cardiac myocytes while the 18-kDa bFGF was also localized in the cytosol (Pasumarthi *et al.*, 1992).

The significance of the subcellular localization is not clear at present, however, increased nuclear bFGF localization has been reported for proliferative compared to growth arrested cells (Cattini *et al.*, 1991). The alternative initiation of bFGF translation might serve as a post-transcriptional mechanism for regulating expression,

intracellular localization and biological activity of this growth factor. For instance, expression of 18 kDa or the HMW forms of bFGF may differently affect the phenotype and the tumorigenic potential of transfected NIH 3T3 cells (Quarto *et al.*, 1991b). Also, the expression of HMW-bFGFs, but not of 18 kDa-bFGF, allows the immortalization of bovine aortic endothelial cells (Couderc *et al.*, 1991). A rat pancreatic acinar cancer cell line (AR4-2J) transfected by HMW-bFGF cDNA and expressing 22.5 kDa bFGF was able to grow in serum-free medium (Estival *et al.*, 1993). Levels of a 25 kDa-bFGF appear to be higher in embryonic versus newborn or adult rat liver, and increase 4-fold in regenerating rat liver after partial hepatectomy (Presta *et al.*, 1989). Similarly, a transient increase in 21.5-22 kDa bFGF was detected in the heart after isoproterenol-induced cardiac injury (Padua and Kardami, 1993). In addition, we reported that neonatal rat heart contains predominately 21.5-22 kDa bFGF while adult ventricle contains predominately the 18 kDa form (Fig.1.6). A relationship therefore, may exist between HMW-bFGF and a proliferative cell phenotype on one hand, and between 18 kDa-bFGF and a less proliferative or mature phenotype on the other.

1.3.1.4. Basic FGF in the heart

Expression and localization: Using heparin-chromatography and immunoblotting, bFGF was detected in cardiac extracts of many vertebrate species, such as bovine, chicken, sheep and cow (Kardami & Fandrich, 1989), porcine and canine (Quinkler *et al.*, 1989), mice (Anderson *et al.*, 1992), rat (Liu *et al.*, 1993), and human (Casscells

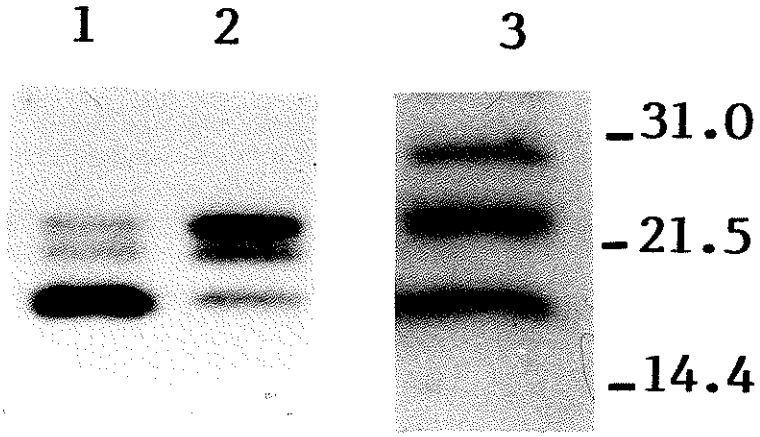


Fig.1.6 Basic FGF composition in neonatal and adult hearts. Cardiac ventricular extract heparin-binding fractions from 5 week old (lane 1) and 1 day old (lane 2) rat. Bovine pituitary bFGF (lane 3) is used as a standard. Each lane is loaded with the heparin-binding fraction from 30 mg extract (3 μg protein).

From Liu, Doble, Kardami, 1993.

et al., 1990b). It has also been detected in dissociated adult cardiac myocytes (Speir *et al.*, 1992) as well as cultured embryonic or neonatal myocytes (Kardami *et al.*, 1990). The paucity of bFGF mRNA in myocardium, as in most tissues (Shimasaki *et al.*, 1988), contrasts with the relative abundance of this peptide in cardiac myocytes and extracellular matrix (Kardami and Fandrich, 1988, Padua and Kardami, 1993). Basic FGF is more highly expressed in the atrium than the ventricles (Kardami and Fandrich, 1989). Using immunofluorescence and immunohistochemistry, bFGF was localized to the nucleus, cytoplasm, cell membrane, gap junctions and basement membrane of the cardiomyocyte (Fig.1.7; also Kardami and Fandrich, 1989; Anderson *et al.*, 1992; Liu *et al.*, 1993).

Expression of bFGF is regulated during chicken heart development: During embryogenesis, bFGF at first was restricted to developing myocardial cells and appears subsequently in the extracellular matrix (Stages 9 and 15, respectively, Parlow *et al.*, 1991). The intensity of bFGF-like stain decreases in the later compared to the earlier stages of embryogenesis (Joseph-Silverstein, 1989). A switch from the high (21.5-22 kDa) to low (16-18 kDa) molecular weight bFGF occurs during the transition from neonate to adult stage of development (Liu *et al.*, 1993). Increased bFGF accumulation was observed in isoproterenol-induced cardiomyocyte injury (Padua and Kardami, 1993).

Biological effects of bFGF in the heart are not clear. The relatively abundant

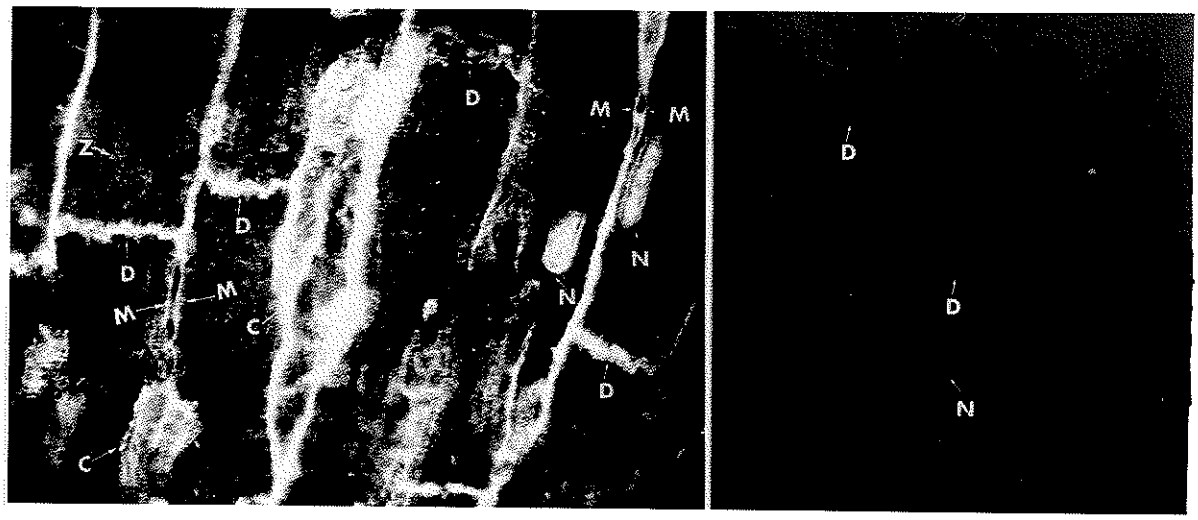


Fig.1.7 Localization of bFGF in section from normal adult rat ventricle. Immunofluorescence staining with bFGF anti-serum IS1 (1:3000) (a) and preimmune rabbit serum at an equivalent dilution (b), respectively. C = capillaries, D = intercalated discs, M = basement membrane, N = muscle nucleus, and Z = muscle z-line (Bar = 20 μm). From Kardami et al, 1990

amount of bFGF in chick (Parlow *et al.*, 1991; Josheph-Silverstein, 1989) and rat (Spirito, 1991) embryonic heart during embryogenesis sugests the possibility that bFGF may be an important stimulator of cardiac morphogenesis and cytodifferentiation. Basic FGF does stimulate DNA synthesis and proliferation of chick and rat embryonic cardiac myocytes in culture (Kardami, 1990). It also promotes expression of embryonic isoforms of muscle specific genes and inhabits expression of those of adult isoforms (Parker *et al.*, 1990b; Schneider *et al.*, 1992). That bFGF plays an important role in early cardiogenesis is supported by the report that an antisense oligodeoxynucleotide complementary to bFGF mRNA caused inhibition of precardiac mesoderm cell proliferation (Sugi *et al.*, 1993). Basic FGF also stimulates the DNA synthesis of dissociated myocytes from adult rat maintained in culture (Claycomb and Moses, 1988; Speir *et al.*, 1992). The effect of bFGF in repressing terminal differentiation of skeletal muscle myoblasts into myotubes has been well documented (Kelvin *et al.*, 1989). Basic bFGF, therefore, has the potential to act as a regeneration factor after cardiac injury. An increase in cytoplasmic and nuclear bFGF in areas bordering the injury sites was observed in the mdx mouse model of injury and regeneration (Anderson *et al.*, 1992), in the coronary occlusion model of infarction and in an isoproterenol model of cardiac injury (Padua and Kardami, 1993).

Basic FGF may also play a role in cell-cell recognition, adhesion and communication. Evidence for these possible functions comes from Kardami *et al.* (1991), who reported that bFGF is localized along the intercalated disc region in

cardiomyocytes (Kardami and Fandrich, 1989). Further research using immuno-electromicroscopy revealed that bFGF was associated with the cytoplasmic face of cardiac as well as astrocyte gap junctions (Kardami *et al.*, 1991; Yamamoto *et al.*, 1991). Thus, bFGF may play a role in modulating gap junctional intercellular communication. Finally, recent evidence from our laboratory points to a cardioprotective role for bFGF (Padua *et al.*, 1993; Kardami *et al.*, 1993).

1.3.1.5 FGF receptors

Two classes of FGF binding sites have been characterized on cell surfaces (Moscatelli, 1987). The so-called high affinity, low capacity receptors (FGFR) bind FGFs with a Kd of 20-600 pM. These receptors are transmembrane glycoproteins containing an intrinsic tyrosine kinase activity (Coughlin *et al.*, 1988). The low affinity (Kd 2-20 nm), high capacity binding sites are cell surface heparan sulfate proteoglycans (HSPG, Burgess and Maciag, 1989). Other, as yet less well characterized receptors have also been reported. Recently, a 150 kDa proteoglycan of rat parathyroid cells was described which binds aFGF with very high affinity (Sakaguchi *et al.*, 1991). Whether this proteoglycan is related to the high affinity tyrosine kinase receptors is not known yet. A high affinity receptor containing no tyrosine kinase domain has also been isolated from chicken (Olwin *et al.*, 1991).

FGFR gene family

FGF binding sites were first detected on the surface of FGF-responsive cells in

experiments using radiolabeled aFGF or bFGF and chemical cross-linking reagents. These studies detected high affinity receptors with reported molecular weights ranging from 110 kDa to 150 kDa (Neufeld and Gospodarowicz, 1985 & 1986). Further work indicated that FGFRs are associated with protein tyrosine kinase activity (Friesel *et al.*, 1989; Coughlin *et al.*, 1988; Huang and Huang, 1986). Four distinct gene products, namely FGFR1-4, have been reported so far which encode highly homologous FGFRs sharing 56-92% amino acid sequence identity and similar overall structure (Jaye *et al.*, 1992). The characteristics of these receptors and their genes are shown in Table 1.3.

The cloning and sequencing of the FGFR cDNAs has revealed considerable information concerning the overall structure of the FGFR proteins (Figure 1.8). The longest receptor forms predicted by the cDNAs contain 3 immunoglobulin (Ig) like domains in the extracellular ligand binding region of the receptor. The Ig-like domains are inferred from the presence and location of cysteines and other specific conserved amino acids (Dionne *et al.*, 1991). Between the first and second Ig domains is a region containing a high concentration of acidic residues, referred to as the "acid box". The receptors contain 8-9 N-linked glycosylation sites and at least some of these are utilized since tunicamycin treatment of cells results in lower apparent molecular weight forms of the receptors (Dionne *et al.*, 1991). A single transmembrane segment joins the extracellular region to the cytoplasmic region which is notable in that it contains a relatively long juxtamembrane sequence and an insertion in the conserved tyrosine kinase domain. Alternative exon usage, differential splicing and poly-adenylation

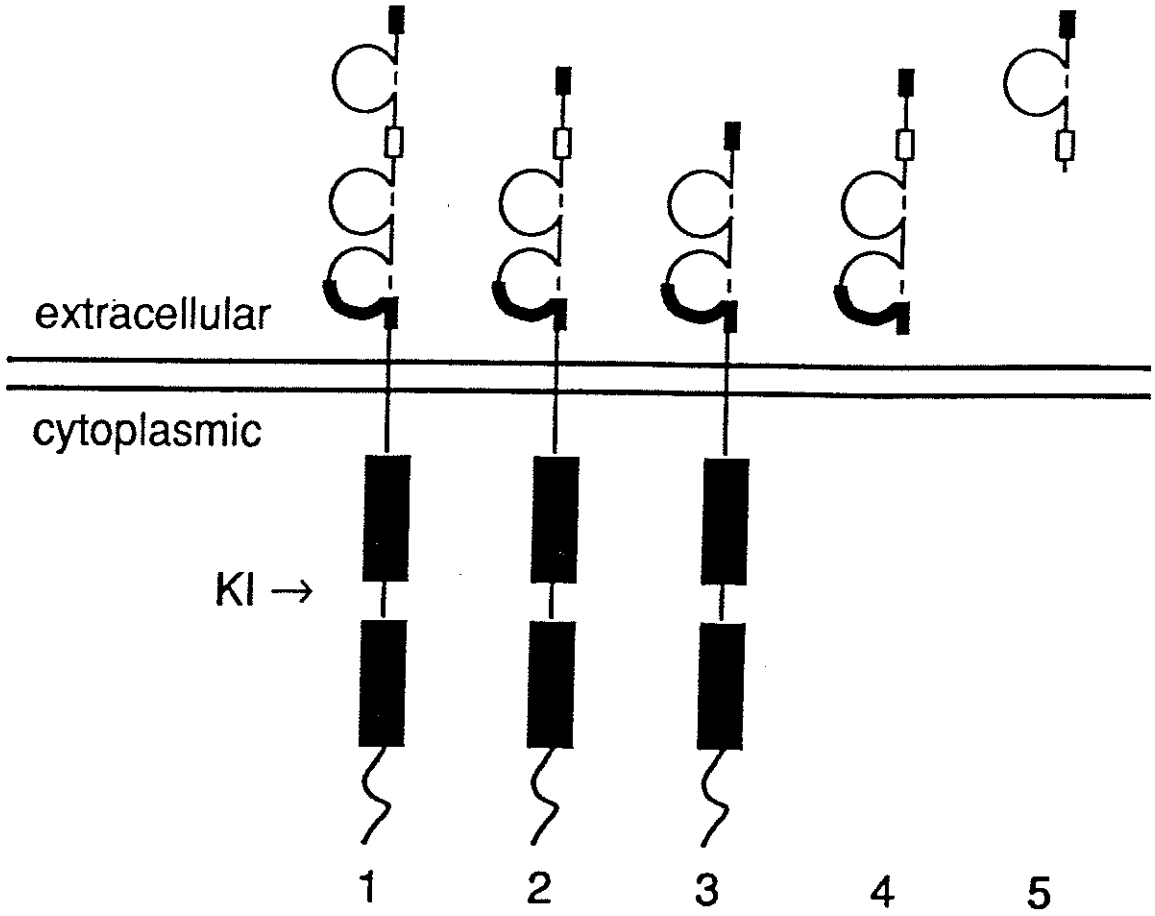


Fig.1.8 Schematic structure or polypeptides encoded by the various forms of FGFR RNAs. The extracelluar regions consist of one to three Ig-like domains and may contain a hydrophillic "acid box" (open box) in the region between the first and second Ig-like domain. The cytoplasmic region, when present, consists of a kinase domain (large shaded box) with a kinase insert (KI). There is alternative usage of exons corresponding to the second half of the third Ig-like domains (thick line). From Dionne et al, 1991)

Table 1.3 The FGFR family

	Other name	mRNA length	Apparent MW	Ligand binding
FGFR1	flg cek1	4.2, 4.3 kb	150 kDa	aFGF, bFGF, K-FGF
FGFR2	bek cek3 KGFR	4.4 kb	135 kDa	aFGF, bFGF, KFGF
FGFR3	cek2	4.5, 7.5 kb	135, 125,97 kDa	aFGF, bFGF
FGFR4		3.0 kb	110,95 kDa	aFGF, not bFGF

MW = molecular weight

From Partanen et al, 1993

Table 1.4 Binding of FGFs to FGFR1 and FGFR2 forms containing different sequences in the third Ig domain (III)

	Relative affinity for different FGFs
FGFR1	
IIIa	bFGF > aFGF
IIIb	aFGF > bFGF
IIIc	aFGF = bFGF
FGFR2	
IIIb	aFGF >KGF > bFGF
IIIc	aFGF = bFGF (KGF does not bind)

From Johnson and Williams, 1993

result in numerous forms of FGFR1 and FGFR2 variants, which differ in their extracellular and cytoplasmic domains (Partanen *et al.*, 1992). A secreted form of human FGFR1, lacking transmembrane segment and cytoplasmic domain has been reported to generate by alternative splicing of the RNA (Duan *et al.*, 1992). This FGFR1 bound bFGF with high affinity and oligomerized upon ligand binding (Duan *et al.*, 1992). Little is known about the *in vivo* functions of the secreted FGFR. It may bind to FGFs and function as a specific antagonist of the full length FGF receptor, as reported for the secreted form of platelet derived growth factor receptor ß (Duan *et al.*, 1991).

Ligand binding characteristics of FGF receptor

The various FGF receptors bind different FGFs in a partially overlapping and complex manner. Initial studies demonstrated that FGFR1 binds aFGF and bFGF with similar high affinity and FGF-3 with about 15-fold lower affinity (Dionne *et al.*, 1990; Mansukhani *et al.*, 1990). FGFR2 appears to bind aFGF, bFGF, and FGF-3 with similar high affinity (Johnson and Williams, 1993). The human FGFR3 expressed in Xenopus oocytes is activated with both aFGF and bFGF. Human FGFR4 expressed in COS cells can only bind aFGF but not bFGF (Partanen *et al.*, 1991). However, Ron *et al.* (1993) isolated another human FGFR4, whose amino acid sequence is identical to that of the one reported by Partanen *et al.*, (1991) and which binds both aFGF and bFGF with high affinity. Recently, it was found that alternative splicing in the third Ig domains is important for determining ligand binding specificities (Werner

et al., 1992). As show in Table 1.4, binding specificity for FGFs can be determined on the basis of which exon (IIIa, IIIb or IIIc) is used to code for the second half of the third Ig domain (Johnson and Williams, 1993).

FGFs also bind to cells at low affinity sites corresponding to surface associated HSPG. Heparin and heparan sulfate present in the side chains of cell surface proteoglycans have been shown to protect bFGF from proteolytic degradation (Saksela *et al.*, 1988) and have been suggested to act as a storage reservoir for FGFs (Moscatelli *et al.*, 1991). These low affinity FGF binding sites have been shown to play a direct role in FGF signal transduction (Klagsbrun and Baird, 1991; Ruoslahti and Yamaguchi, 1991). Digestion of cell surface heparan sulfate or prevention of its sulfation abolished bFGF binding to its high affinity receptors and thus inhibited the biological activity of bFGF (Rapraeger *et al.*, 1991). Furthermore, heparan sulfate-deficient CHO mutant cells transfected with the FGFR1, are not capable of bFGF binding but high affinity binding can be reconstituted by addition of exogenous heparin or heparan sulfate (Yayon *et al.*, 1991).

The binding of FGF to low affinity HSPG is abolished and reversed by high salt (2M NaCl, pH 7.5) or soluble heparin, while the binding to high affinity FGFR is reversed by high salt in acidic conditions (2M NaCl, pH 4.0; Moscatelli, 1987).

FGFR in striated muscle

FGFR1 mRNA and high affinity FGF binding proteins have been detected in skeletal muscle myoblasts (Moore *et al.*, 1991; Templeton and Hauschka, 1992; Olwin and Hauschka, 1988), and tissues (Wanaka *et al.*, 1991; Olwin and Hauschka, 1990; Patstone *et al.*, 1993). Differentiation of skeletal muscle myoblasts, which require bFGF for growth and repression of differentiation, results in down-regulation of FGFR1 mRNA and a permanent loss of cell surface FGF receptor, following a time course parallel to that of differentiation (Olwin and Hauschka, 1988; Moore *et al.*, 1991; Templeton and Hauschka, 1992).

Using *in situ* hybridization or polymerase chain reaction (PCR) techniques, FGFR1 mRNA, but not other forms of FGFRs, has been detected in the myocardium of embryonic chick (Patstone *et al.*, 1993) and rat (Wanaka *et al.*, 1991; Engelmann *et al.*, 1991 & 1993; Yazaki *et al.*, 1993). Engelmann *et al.* (1992) has reported that the level of FGFR1 transcripts dropped to virtually undetectable levels shortly before and/or after birth; though there was an increase in FGFR1 mRNA during the period 2-7 weeks postnatally, limited or undetectable expression was noted in mature myocardium of rats. In chick embryos, ^{125}I-aFGF binding was detectable in day-17 but not day-19 embryonic hearts (Olwin and Hauschka, 1990). Low affinity HSPG bFGF receptors have been detected and purified from mature bovine heart sarcolemma (Ross and Hale, 1990; Ross *et al.*, 1993). Very low levels of ^{125}I-bFGF binding were reported for isolated adult cardiomyocytes (Speir *et al.*, 1992). There are as yet no detailed studies on the presence, characteristics or regulation of FGFR in cardiac

sarcolemma.

FGFR-mediated signal transduction

The binding of aFGF or bFGF to its FGFR induces receptor dimerization (Bellot *et al.*, 1991; Ueno *et al.*; 1992), similar to what has been observed for several other growth factor receptors (Williams, 1989; Ullrich and Schlessinger, 1990). Interestingly, both homodimeric and heterodimeric receptor species can be formed between the FGFR1, the FGFR2 and the FGFR3 proteins (Bellot *et al.*, 1991; Ueno *et al.*, 1992). Binding also leads to activation of FGF receptor tyrosine kinase activity and receptor autophosphorylation (Huang and Huang, 1986; Coughlin *et al.*, 1988; Mansukhani *et al.*, 1990). Phosphorylation of dimerized receptors appears to occur via an intermolecular transphosphorylation mechanism (Bellot *et al.*, 1991). Activation of the receptor tyrosine kinase also leads to increased tyrosine phosphorylation of a number of cellular proteins (Huang and Huang, 1986; Bottaro *et al.*, 1990; Burgess *et al.*, 1990; Miki *et al.*, 1991; Peters *et al.*, 1992). Phosphorylation of a 90-kDa protein (and possibly others) may be unique to FGFR signaling pathways (Coughlin *et al.*, 1988).

Currently, only one protein, phospholipase C (PLC)-τ, has been identified as a candidate substrate for FGFR. PLC-τ is phosphorylated on tyrosine residues following FGF stimulation (Burgess *et al.*, 1990) and direct association with the receptor has been demonstrated (Mohammadi *et al.*, 1991; Peters *et al.*, 1992). However, the

precise role of PLC-τ in FGFR-mediated signaling remains unclear. In CCL39 cells, for instance, FGF does not stimulate hydrolysis of poly-phosphoinositides (PtdIns) (Magnaldo *et al.*, 1986), indicating that the FGFR on these cells do not couple to PLC-τ signaling pathways. Furthermore, FGFR point mutants, in which tyrosine-phosphorylation of PLC-τ or hydrolysis of PtdIns are prevented, can be autophosphorylated and phosphorylate several cellular proteins as well as stimulate DNA synthesis (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). These results indicate that PLC-τ activation and PtdIns hydrolysis is not required for FGF-induced mitogenesis. FGF-induced PtdIns hydrolysis could be involved in other non-mitogenic responses mediated by FGF, such as cellular differentiation during embryogenesis (Amaya *et al.*, 1991; Maslanski *et al.*, 1992) and controlling cell shape and morphology (Goldschmidt-Clermont *et al.*, 1991).

As reviewed in Paragraph 1.1 and shown in Figure 1.4, RTK triggers a cascade of a signaling pathway of activating MAP kinase through Ras and Raf (Pazin and Williams, 1992). Experiments using dominant-negative c-Ras alleles have indicated that Ras plays and important role in FGF-induced mitogenesis (Cai *et al.*, 1990). Dominant-negative c-Ras alleles also block FGF-induced neurite outgrowth (Wilden *et al.*, 1990) and c-fos expression (Szeberenyi *et al.*, 1990).

1.3.2 TGF-ß

The TGF-ß family is comprised of TGF-ß 1-5, but only TGF-ß 1-3 are found in

mammalian cells and they have similar biological activities (Bennett and Schultz, 1993). The range of cell types that respond to TGF-ß and the variety of cellular responses elicited is wide. It can either stimulate or inhibit cell proliferation and growth (Sporn *et al.*, 1987) and it regulates skeletal myogenesis in *in vitro* models (Sporn and Robert, 1990). The antiproliferative effects of TGF-ß are well described for epithelial cells; yet mesenchymal cells such as skeletal muscle cells, fibroblasts, hepatocytes and endothelial cells are also sensitive to TGF-ß inhibition (Sporn and Roberts, 1990). Elevated levels of TGF-ß1 mRNA are found in regenerating liver at a time when DNA synthesis begins to diminish, possibly acting as a locally generated regulatory signal involved in the inhibition of further hepatocyte replication (Mead and Fausto, 1989; Strain *et al.*, 1987). Studies in the heart have focused mainly on TGF-ß1 and to a much lesser extent on TGF-ß2. Levels of TGF-ß1 mRNA were much more abundant in cardiac ventricles than the liver of one week old rat (Engelmann *et al.*, 1992). This may reflect the proliferative status of the two organs since neonatal hepatocytes actively proliferate for several weeks, while cardiomyocytes are virtually non-proliferative by this age (Zak, 1984). The level of expression of TGF-ß1 is negligible to non-detectable in the fetal myocardium (Akhurst *et al.*, 1990; Schmid *et al.*, 1991; Choy *et al.*, 1990 & 1991) and increases with myocardial maturation (Engelmann *et al.*, 1992). TGF-ß1 gene transcripts are present in relatively high abundance in the one-week-old rat heart (Engelmann *et al.*, 1992) compared to fetal heart. In adult heart, there is little mitotic activity but relatively high levels of TGF-ß RNA and peptide immunoreactivity (Sporn *et al.*, 1986; Cummins *et al.*, 1993; Roberts

and Sporn, 1993).

While TGF-ß has no direct effect on DNA synthesis by itself, it inhibits the stimulation of DNA synthesis induced by bFGF and the IGFs on embryonic or neonatal cardiac myocytes (Kardami, 1990; Engelmann *et al.*, 1992 & 1987).

Little is known so far about the physiological role of TGF-ß in the heart. The developmental regulation in the myocardium together with the *in vitro* effects of this factor on cardiac myocytes are consistent with the hypothesis that TGF-ß inhibits myocyte proliferation. However, TGF-ß promotes re-expression of the genes of the fetal isoforms of myosin heavy chain (MHC) and actin contractile proteins at a time when these cells are normally expressing the adult isoforms (Parker and Schneider, 1990). These transitions are similar to those following hemodynamic overload. This finding suggests the cardiac protein isoforms which have been shown to undergo well defined transitions during both development and hemodynamic overload may be linked in some way to growth factor expression, perhaps by sharing regulatory gene elements involved in cell proliferation and growth (Schneider and Parker, 1990).

Cardiac infarction induced a quick loss of immunohistochemical staining for TGF-ß in the ischemic areas accompanied by a slow increase in cells around the margins of the infarction, leading to the suggestion that TGF-ß1 may play some role in the response of the adult heart to injury after acute myocardial infarction or ischemia

(Roberts and Sporn, 1993; Casscelles *et al.*, 1990a; Lefer *et al.*, 1990; Thompson *et al.*, 1988). TGF-ß was shown to mediate cardiac protection in ischemic-reperfusion injury (Lefer *et al.*, 1990; Lefer, 1991). TGF-ß has also been implicated in numerous other actions having little or no relationship to cardiac growth (Sporn *et al.*, 1987), which will not be discussed here.

Members of the TGF-ß family bind to many different types of receptors (Massagué, 1992). The known receptors which have roles in signal transduction have intrinsic serine/threonine kinase activities (Fig. 1.3; Lin and Lodish, 1993). Thus, the downstream signalling most likely involves the phosphorylation of specific substrates on serine/threonine residues, but knowledge about the identity of such substrates is still lacking.

1.3.3 The IGFs

IGF-I and II are small peptides with similar amino acid structures and overlapping biological activities (Sara and Hall, 1990). Both IGFs are found in the circulation but they are bound to high molecular weight binding proteins which modulate their activities. Many organs and tissues produce IGFs; their local paracrine and autocrine actions may be more significant than their endocrine activities. Heart expresses IGF-I and IGF-II mRNAs (Claycomb *et al.*, 1989; Han *et al.*, 1988; Englemann *et al.*, 1989; D'Ercole *et al.*, 1984; Turner *et al.*, 1988). The presence of IGF receptors on rat myocardium and cultured rat cardiomyocytes have been

demonstrated (Engelmann *et al.*, 1989; Ito *et al.*, 1993). Lowe and co-workers (1987) described that in the rat heart, expression of IGF-I increases substantially during the first post-natal days indicating its possible importance during development. IGF-1 mRNA increases rapidly after coarctation of rat, in parallel with the increased protein synthesis of the hypertrophying heart (Czerwinski *et al.*, 1993) and in the right ventricular hypertrophy induced by chronic hypoxia (Russell *et al.*, 1993). IGF-I has a direct hypertrophic effect on cultured rat cardiomyocytes (Ito *et al.*, 1993). IGF I and II have also been shown to stimulate ^{3}H-thymidine incorporation and hyperplastic growth of cultured embryonic (Kardami, 1990) and neonatal cardiac myocytes (Engelmann *et al.*, 1992). The IGFs and insulin produce their effects by binding to specific receptors, which are also RTK-associated.

1.3.4 Other growth factors

The presence of other growth factors including PDGF, TGF α and heparin binding EGF are also reported in the myocardium (Whitman and Melton, 1989; Engelmann *et al.*, 1989; Abraham *et al.*, 1993). EGF significantly decreases the number of cells expressing sarcomeric actin in cultured human fetal ventricular myocytes, suggesting that EGF inhibits or reverses cardiac differentiation in those cells (Goldman and Wurzel, 1993).

1.3.5. THYROID HORMONE

Thyroid hormone exerts profound effects on the growth, development and

homeostasis of vertebrate organisms (Moore and Brent, 1991).

1.3.5.1 Receptors and signal transduction

The physiological effects of thyroid hormone are primarily mediated by triiodothyronine (T3) via the nuclear T3 receptor (T3R) proteins that act to increase or decrease the rates of transcription of target genes (Fig 1.9; Glass and Holloway, 1990). T3Rs are the gene products of c-erbA and closely related to the steroid hormone and retinoic acid receptors (Fig 1.10; Lazar and Chin, 1990). The nuclear T3R contains a DNA-binding domain, which is rich in cysteine and forms two zinc fingers and interacts with T3 responsive elements (T3RE) on responsive genes. The T3R also contains a ligand binding domain, and binding of ligand with this domain will activate or inhibit the transcription of specific genes and change the amount of specific mRNA (Beato, 1989). It has been established that in mammals at least two distinct but closely related genes encoded T3Rs. These have been termed c-erbA-α (T3R-α) and c-erbA-ß (T3R-ß) genes on the basis of sequence similarities and chromosomal localization (Spurr *et al.*, 1984; Thompson *et al.*, 1987; Dayton *et al.*, 1984). So far, four receptors have been described. Three of them, α1-, ß1-, and ß2-receptors, have a high binding affinity for T3 and can recognize the same DNA binding sites (Polikar *et al.*, 1993), whereas the α2-receptor does not display any known ligand binding specificity and when co-expressed, can prevent the action of α1-, β1- and β2-receptors in a dominant negative fashion (Privalsky, 1992). T3 α and ß has different tissue specific distribution (Glass and Holloway, 1990) and functional

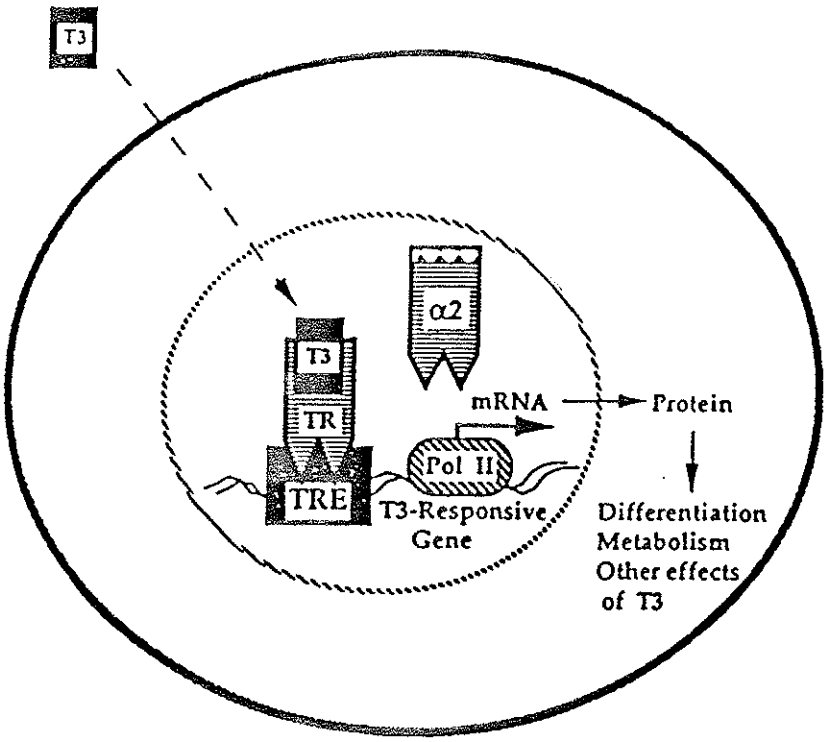


Fig.1.9 Model of the mediation of T3 action by nuclear thyroid hormone receptors (TR). T3 either enters the cell (as depicted) or is derived from intracellular deiodination of T4. Nuclear interaction between a T3-bound TR and a thyroid hormone-responsive element (TRE) results in increased or decreased activity of RNA polymerase II (pol II) on a T3-responsive gene. The TRE is indicated as containing two half-sites and the TR may bind as a dimer. Effects on mRNA levels are translated into increased or decreased cellular concentration of proteins so as to promote differentiation, metabolic processes, and other cell-specific effects of T3. In the absence of T3, the TRE-bound TR may repress basal transcription. c-erbA-α2 (α2), the non-T3-binding splice variant, can inhibit the effects of T3-bound TRs by a mechanism which has not been established. From Lazar and Chin, 1990

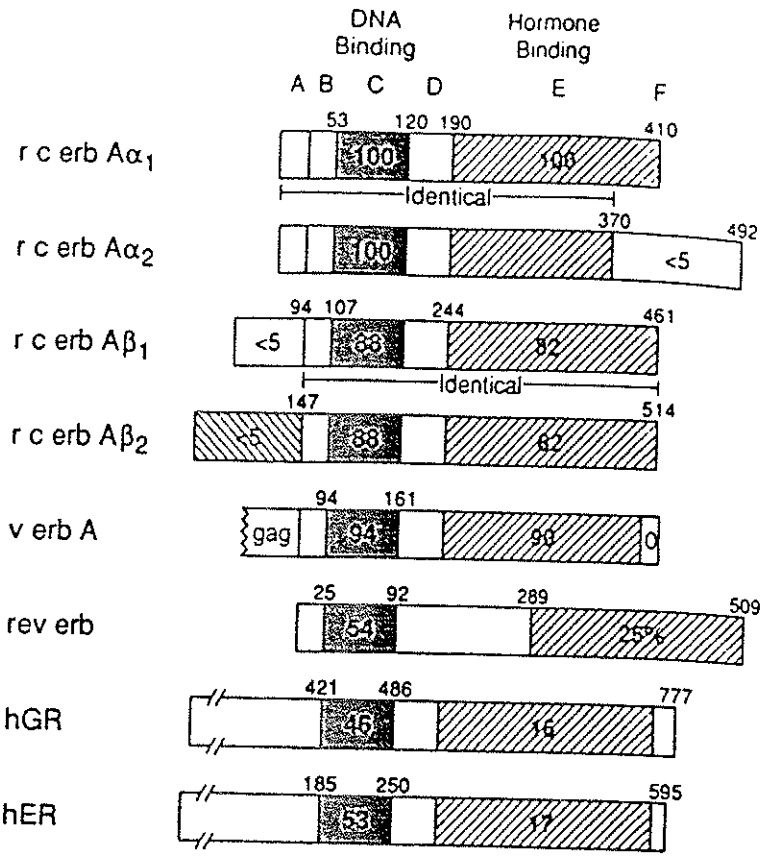


Fig.1.10 Schematic representation of the primary structures of *erb*A gene products and other selected members of the ligand-dependent family of transcription factors. Protein alignment is based on the highly conserved cysteine-rich DNA-binding (shaded box) and C terminal ligand-binding (stripped box) domains. Numbers within these domains represent percent amino acid identity when compared to the rat c-erbA-α1 thyroid hormone receptor. Numbers above receptors indicate amino acid boundaries of each DNA-binding and hormone binding domains, as well as the total amino acid length of the protein. Receptors diagrammed are the rat α1, α2, ß1, ß2 thyroid hormone receptors, the viral erbA protein, rev erb, human glucocorticoid (hGR) and the human estrogen receptor (hER). From Glass and Holloway, 1990.

heterogeneity has been suggested for the various T3R (Thompson *et al.*, 1987; Hodin *et al.*, 1989). Although T3Rs can bind to T3REs as monomers or homodimers (Williams *et al.*, 1991), they require T3 receptor auxiliary proteins (TRAPs) for efficient DNA binding (Zhang *et al.*, 1991b; Glass *et al.*, 1990). Retinoid X receptors constitute one group of these TRAPs (Zhang *et al.*, 1992). The R3R/TRAP heterodimer binds to T3REs with a higher affinity than does T3R alone (Burnside *et al.*, 1990; Lazar and Berrodin, 1990) and enhances the transcriptional activity of T3R (Zhang *et al.*, 1992).

T3R regulates gene transcription not only through the classical protein-DNA interaction mechanism, but also by inhibiting induction of AP-1 site-dependent gene activation (Zhang *et al.*, 1991c). AP-1 is a transcriptional factor composed of the two subunits, c-Fos and c-Jun, that binds to specific DNA sequences outside of the coding region and controls genetic expression (Ponta *et al.*, 1992). T3Rs inhibit DNA binding of AP-1 in the presence of thyroid hormone, thus repressing transcriptional activation by AP-1 (Schmidt *et al.*, 1993; Zhang *et al.*, 1991c; Lopez *et al.*, 1993). Since AP-1 activation is the signal transduction pathway used by many growth factors (Schneider and Parker, 1990), the interaction of thyroid hormone with AP-1 provides a pathway for the cross-talk between the two major factors regulating cell growth, development and differentiation. Such a mechanism for the regulation of gene expression has been shown for the activity of the interstitial collagenase promoter (Desbois *et al.*, 1991).

Some functions of thyroid hormone are mediated by extranuclear receptors, which have been found in the atria and ventricles of rat (Osty *et al.*, 1988) and other tissues (Lennon *et al.*, 1980). Binding of thyroid hormone with those extranuclear receptors directely influences the transport of amino acids, sugars and calcium across the membrane without affecting protein synthesis (Segal, 1990; Dillmann, 1990).

1.3.5.2 Effects of thyroid hormone on development

Thyroid hormones were found to exert dramatic effects on amphibian metamorphosis, as shown by Gudernatch, Allen and others in the period between 1910-1930 (Glass and Holloway, 1990). Administration of thyroid hormone extracts to the aquatic environment of tadpoles accelerated tail resorption and leg growth. Evidence that onset of spontaneous metamorphosis was dependent on the production of endogenous thyroid hormones was provided by experiments using inhibitors of thyroid hormone synthesis. These compounds blocked spontaneous metamorphosis and their effects could be overcome by administration of exogenous thyroid hormones.

Although less striking than in amphibians, thyroid hormones also exert profound effects on mammalian development. A large increase in the serum levels of thyroid hormones takes place in mammals shortly after birth (Dubois and Dussault, 1977) and has been related to a number of physiological and structural changes occurring in various tissues during the postnatal period (Greenberg *et al.*, 1974; Schwartz and Oppenheimer, 1978). The increase in thyroid hormone levels after birth parallels

terminal differentiation of skeletal and cardiac muscle. In skeletal muscle, it has been shown that the transition from the neonatal ATPase myofibrillar profile and myosin isoforms into the adult ones is dependent on thyroid hormone (Samuel *et al.*, 1989; Chizzonite and Zak, 1984). Hypothyroidism induces a complete inhibition of postnatal muscle differentiation, while hyperthyroidism significantly accelerates terminal differentiation (D'Albis *et al.*, 1987). In rat cardiac ventricles, hypothyroidism induces a shift toward the fetal isomyosin (V3, Schwartz *et al.*, 1982). Higher thyroid hormone levels stimulate the expression of rat adult isomyosin (Mahdavi *et al.*, 1987), i.e., myosin V1, as well as other muscle specific genes, such as cardiac Ca^{2+} ATPase (Dillmann, 1990; Nagai *et al.*, 1989, Lomprè *et al.*, 1989), as shown in Table 1.2. As mentioned earlier, mature cardiomyocytes have been considered as terminally differentiated and post-mitotic (Zak, 1984). The question arises as to whether thyroid hormone promotes the terminal differentiation and/or inhibition of proliferation of cardiomyocytes. Gerdes *et al.* (1983) reported that injection of thyroid hormone into neonatal rats resulted in a 19% decrease in ventricular myocyte number, suggesting that the transition of cardiac myocytes from hyperplastic to hypertrophic growth was accelerated by thyroid hormone. Kardami (1990) also showed that thyroid hormone cancelled the stimulatory effect of bFGF on the proliferation of cardiomyocytes in culture.

Experimentally induced hyperthyroidism is associated with cardiac hypertrophy (Oppenheimer, 1990; Bedotto *et al.*, 1989), though the mechanism(s) remains

controversial. Hyperthyroid heart performs increased work, a stress similar to that shown to produce cardiac hypertrophy (Morgan *et al.*, 1987). Evidence for indirect effects of thyroid hormone on cardiac hypertrophy came from Korecky *et al.*, (1987) and Klein and Hong (1986), who reported that T3 did not stimulate cardiac growth or attenuate the rate of atrophy in heterotopically isotransplanted "nonworking" rat heart in spite of its direct effect on stimulating α-MHC expression. They suggested that hyperthyroidism-induced cardiac hypertrophy is mediated indirectly via changes in cardiac work. However, Bedotto and co-worker (1989) reported that cardiac hypertrophy was produced by T4 treatment despite ß-blockade with propranolol, alteration of cardiac load with captorpril or hydralazine and the combination of ß-adrenoceptor blockade and captopril. Therefore, according to these authors, cardiac hypertrophy induced by thyroid hormone is independent of loading conditions and ß-adrenoceptor blockade. Additional information is required to solve these conflicting results, which, to a large degree reflect limitations of the different systems used. It should be noted that a hypertrophic effect of thyroid hormone is not in conflict with a role in promoting differentiation, since differentiated cardiomyocytes readily undergo hypertrophy, even though they apparently lose their ability for hyperplasia.

1.3.6 Angiotensin II

The renin-angiotensin system (RAS) traditionally has been considered as an endocrine system whose effects are exerted entirely through the blood-borne peptide hormone angiotensin II (Ang II). Ang II results from two proteolytic steps in the

processing of the precursor angiotensinogen. The action of renin produces the decapeptide Angiotensin I (Ang I). The subsequent cleavage of Ang I by angiotensin converting enzyme (ACE) produces the active octapeptide Ang II. Besides its potent vasoconstrictive effect, Ang II has been suggested to work as an autocrine/paracrine factor regulating growth of local tissues including heart (Baker *et al.*, 1992; Dzau and Pratt, 1993; Lindpaintner and Ganten, 1991; Dzau, 1993a). The transcripts and corresponding translation products of endogenous angiotensinogen and renin have been identified in myocardium, cultured cardiac myocytes and fibroblasts (Dostal *et al.*, 1992a; Dzau and Re, 1987). Ang I, II and ACE were detected in cultured cardiac myocytes and fibroblasts (Dostal *et al.*, 1992b). Ang I to Ang II conversion has been reported in isolated perfused hearts (Lindpaintner *et al.*, 1988). These data suggest the existence of a intracardiac RAS with Ang II acting as a local growth factor in the heart (Baker *et al.*, 1992).

It has been demonstrated that under pressure, there is an increased expression of ACE mRNA and subsequent increases in ACE and Ang II; myocardial Ang II in turn can play a role in adaptive hypertrophy through its growth promoting effect (Dzau, 1993b). *In vivo* studies have suggested a direct effect of angiotensin on cardiac hypertrophy. ACE inhibitors prevented or regressed hypertension-induced hypertrophy of the heart (Makino *et al.*, 1993), a phenomenon which was not mediated through antihypertensive effects (Linz *et al.*, 1989). In cultured cardiac myocytes, Ang II caused an increase in protein synthesis and a rapid induction of immediate-early genes,

i.e. c-fos, c-jun, jun B, egr-1 and c-myc as well as late markers of cardiac hypertrophy, SkA and ANF (Sadoshima and Izumo, 1993b). While Ang II is mitogenic for rat cardiac fibroblasts in culture, it does not stimulate DNA synthesis in cultured cardiac myocytes (Schorb *et al.*, 1993; Sadoshina and Izumo, 1993b).

The cardiac hypertrophic effect of Ang II is mediated primarily by the AT1 receptor subtype, and possible through phospholipid-derived second messengers. Ang II activates PL-C, PL-D and possible PL-A. Inhibition of PL-C or PKC significantly suppressed Ang II-induced c-fos expression. (Sadoshima and Izumo, 1993c). PKC-activation is associated with the activation of MAP kinase (Molloy *et al.*, 1993). Ang II can also activate the MAPK pathway through MEK kinase (Tsuda *et al.*, 1992).

1.3.7 Catecholamines

The role of catecholamines (CA) in the growth of the myocardium has been investigated extensively, especially in the hypertrophic model. Increased plasma levels of CA are mostly found under conditions leading to myocardial hypertrophy *in vivo* (Morgan and Baker, 1991; Bugaisky *et al.*, 1992). Since CA increase arterial pressure and body metabolism, most studies were performed on cultured or isolated cardiac myocytes to distinguish between the direct effects of CA on myocardium and indirect effects caused by increased work load. Noradrenergic agonists can stimulate neonatal cardiomyocyte growth in size. This growth in size is characterized by increases in cell volume, surface area and protein content (Simpson *et al.*, 1982) as well as in protein

synthesis (Meidell *et al.*. 1986). In fetal cardiac myocytes, norepinephrine stimulates cell proliferation without significant increase in cell size (Marino *et al.*, 1989). In ventricular cardiac myocytes from adult rat, α1-adrenergic agonists resulted in not only an increased protein and RNA content, but also selective up-regulation of fetal/neonatal mRNA encoding skeletal actin (SkA), ß-MHC (Decker *et al.*, 1993; Long *et al.*, 1992), and a fetal shift in the expression of creatine kinase isoforms (Pinson *et al.*, 1993), resembling the response of myocytes to other mitogens and to overload. It is conceivable that CA stimulate different growth responses in the cardiac myocytes of different ages. Fetal cardiomyocytes respond differently compared to either adult or neonatal cardiac myocytes and this likely involves the ability of fetal cells to grow by cell division, while adult cardiomyocytes grow only by increasing their volume.

Those growth responses are not dependent upon cardiomyocyte beating, i.e. stretch (Simpson, 1985) and are predominately an α1-adrenergic response (Simpson, 1983, Simpson *et al.*, 1986), specifically α1A-adrenergic receptor subtype (Knowlton *et al.*, 1993). Although ß-adrenergic stimulation led to increased total protein synthesis (Pinson *et al.*, 1993), and a moderate degree of hypertrophy, this response largely depended upon myocyte contractile activity and involved the presence of non-muscle cells (Long *et al.*, 1992). Alpha-adrenergic receptor stimulation leads, via a GTP-binding protein, to the activation of PtdIns-specific PL-C which breaks down phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from intracellular stores, Ca^{2+} and DAG

activate PKC (Lefkowitz and Caron, 1988). Recent studies have implicated the Ras proto-oncogene in the signalling pathway which activate genetic makers of the hypertrophic response (Thorburn *et al.*, 1993). In the adult mammalian heart, α1-adrenergic receptor density is low (Bristow *et al.*, 1988), however, it is up-regulated in pathological conditions such hypothyroidism (Fox *et al.*, 1985), in ischemic or hypoxic myocardium (Heathers *et al.*, 1987; Corr *et al.*, 1980; Maisel *et al.*, 1987) and in infarct-induced congestive heart failure (Dixon and Dhalla, 1991).

1.3.8 Other hormones

Growth hormone *in vivo* stimulates cardiac hypertrophy, and most of its growth promoting effects are mediated by IGF-1 (Lindahl *et al.*, 1991; Fisher, 1991), which has been discussed previously in paragraph 1.3.3. Steroid hormones can also induce hypertrophic growth of the heart, which appears to be secondary to pressure and/or volume overload (Mangrulkar and Nigrovic, 1993) and will not be discussed here. Insulin produces a very similar but weaker effect on cardiac growth as the IGFs, and they use the same receptors (Fig.1.3).

To summarize, the ability of cardiac myocytes to undergo hyperplastic or hypertrophic growth is under the control of environmental factors. Most hormonal or growth factors, including CA, Ang II, IGFs, stimulate myocardium and/or cardiomyocytes hypertrophy. TGF-ß possibly promotes the differentiation of cardiomyocytes, however it also promote the expression of some fetal contractile

proteins. Acidic and basic FGF are factors which can stimulate embryonic myocyte hyperplastic growth and induce adult myocytes to express a partial fetal phenotype. Thyroid hormone promotes the differentiation of most tissues and organs including the heart. It is possible that the ability for adult myocytes to undergo cell division and proliferation is controlled by regulating the availability and/or function of stimulating factors, such as bFGF. Understanding the regulation of these factors may lead to a better understanding of cardiac growth control and to the development of interventions in the growth and regeneration processes.

CHAPTER 2. EXPRESSION OF BASIC FGF IN THE HEART

2.1 INTRODUCTORY REMARKS

Both bFGF and thyroid hormone are important regulators of cardiac myocyte growth and maturation. Basic FGF favors the expression of fetal contractile proteins and phenotype (Schneider and Parker, 1990), while thyroid hormone promotes the expression of adult isoforms of contractile proteins (Lomprè *et al.*, 1991). It is also known that a switch from the 21.5-22 kDa to 16-18 kDa molecular weight bFGF occurs as the neonate developes into the adult (Liu *et al.*, 1993), and compared to the adult animal, the neonatal rat is physiologically hypothyroid (D'Albis *et al.*, 1987), which suggests a direct effect of thyroid hormone on bFGF expression.

To understand the role and regulation of bFGF in the context of cardiac physiology and growth, we investigated: (a) Localization of bFGF in cultured cardiac myocytes from rat and chick. (b) Effects of bFGF on DNA synthesis of cultured myocytes. (c) Accumulation and composition of bFGF in cardiac ventricles as a function of thyroid status, and (d) Distribution of bFGF in the heart of the mdx mouse model of Duchenne muscular dystrophy. Data from these studies are presented in this chapter and strongly suggest that bFGF actively participates in cardiac developmental, physiological and pathological processes, and that thyroid hormone may exert some of its effects on the myocytes by affecting bFGF expression.

2.2 MATERIAL AND METHODS

2.2.1 Cell cultures

All the culture media and sera were obtained from GIBCO Laboratories (Life Technologies Inc, Grand Island, NY) unless otherwise indicated. Calcium and magnesium-free phosphate buffered saline (CMF-PBS) and phosphate buffered saline with calcium and magnesium (PBS) were also obtained from GIBCO Laboratories. Trypsin Type III, Collagenase type I, Pancreatin, Insulin, T3 and DNase were obtained from Sigma Chemical Co. (St. Louis, MO). Culture dishes and plates were from Corning Glass Works (Corning, NY)

Seven- or 5-day old chicken embryos (Animal Science, University of Manitoba, Winnipeg, MB) and 19- to 21-day old fetuses of Sprague Dawley rat (Central Animal Care, University of Manitoba, Winnipeg, MB) were used. Ventricles of the embryos were cleaned as much as possible of connective tissues, and minced. Ventricular minces were washed with CMF-PBS several times. For chicken myocytes, minces were digested with an enzyme mixture containing 0.083% trypsin, 0.0083% collagenase I, 0.0018% pancreatin and 1.3% chicken serum in CMF-PBS in 37°C for 8 minutes under continuous gentle agitation. After stopping the digestion by adding an equal amount of culture medium containing 10% calf serum, tissue pieces were rinsed 3 times with Ham's F-12 medium and were subsequently dispensed into a single cell suspension in Ham's F-12 medium and 0.00002% DNase. For rat myocyte

cultures, the minced ventricles were first digested with 0.05% trypsin in CMF-PBS for 5 minutes at 37°C, then the digestion was stopped by adding a culture medium containing 10% calf serum and rinsed 3 times with CMF-PBS. The procedure were repeated 3 to 4 times. The supernatants from digestion and washes, containing liberated cells were pooled, with the exception of the first one. The suspension was then filtered through a Swiss Nitex Nylon Monofilament HD3-85 (Thompson Co LTD, Scarborougn, ONT). Isolated cells were pelleted by centrifugation at 700 rpm at a Centaur 2 Centrifuge (Johns Scientific Inc, Toronto, ONT) for 10 minutes. The cells were plated on plates coated with rat tail collagen type I (Upstate Biological Inc, Lake Placid NY) at various densities as required.

To remove fibroblasts and enrich in cardiac myocytes, we used a differential adhesion procedure. The cell suspension was initially plated on 100 X 20-mm culture dishes and incubated for 30 minutes. Non-attached cells (mostly myocytes) were collected, counted and plated. Nonmuscle cells were essentially eliminated from the final culture because they remained attached to the initial dishes (pre-plates).

Cultures were kept in a humidified atmosphere of 5% CO_2 in air. The medium for muscle cells was a mixture (1:1) of Ham's F-12 medium (F-12) and Minimum Essential Medium (MEM) with 0.4% to 10% fetal calf serum for chick cultures and 0.5% to 5% calf serum for rat cardiac myocytes. In some experiments, serum was partially depleted of thyroid hormone by incubating with charcoal (Norit A) at 56°C

for 30 min, followed by centrifugation to remove the charcoal and sterilization by filtration through 0.22 μm filter (Westphal *et al.*, 1975; Loo *et al.*, 1990). Serum T3 and T4 levels, tested by St. Boniface General Hospital Biochemistry Laboratory, showed that about 80% of the T4 and more than 90% of the T3 were removed from sera treated by the above method. In cultures using charcoal-absorbed, heat inactivated serum, insulin (0.5μg/ml) was supplied to the medium.

2.2.2 Labelling index (LI)

Cardiomyocytes from 21-day rat fetuses were maintained in 5% calf serum/F12 medium. T3 (1 nM), bFGF (12 ng/ml, Pepro Tech, Inc., Rocky Hill, NJ) and cardiac extracts (0.3 mg/ml) prepared as described by Kardami (1990) were added to cultures 48 hrs after plating. LI was determined after 5 days in culture. Myocytes were incubated with 5 μCi/ml of ^{3}H-thymidine (Amersham Co., Arlington Heights, IL) for 48 hr at 37°C before fixation in 90% ethanol, 10% formalin at 4 °C overnight. Cells were stained histochemically for glycogen by periodic acid schiff (PAS, Sigma) stain according to the manufacture's instruction (Polinger, 1973). They were subsequently processed for autoradiography, using Kodak NTB emulsion, as described (Nag *et al.*, 1983; Kardami, 1990). Slides were developed 5 days later, in D19 Kodak developer, fixed, dehydrated and mounted with Permount (Fisher Scientific, Ottawa, ONT). A total of 1000 cells of labelled or unlabelled PAS^{+} cells were scored per slide. LI= Labelled PAS^{+} cells/ Total number of PAS^{+} cells. Mean and SEM values of 4 slides in each group were calculated and compared.

2.2.3 Animal Models

Sprague-Dawley rats (Central Animal Care, University of Manitoba, Winnipeg, MB) were used. Age and body weight matched male rats (200-225 g body weight) were divided into three groups: euthyroid, hyperthyroid and hypothyroid. Hyperthyroidism was induced by daily subcutaneous injection of L-thyroxine (Sigma Chemical Co, St. Louis, MO), 1mg/Kg body weight for 10 days. Hypothyroidism was induced by addition of 0.1% of 6-n-propylthiouracil (PTU, Sigma Chemical Co, St. Louis, MO) in the drinking water for 3 weeks. Mdx mice (C57BL/10ScSn mdx) and their normal age-matched control mice (C57BL/10ScSn) were bred by brother-sister matings from original breeding pairs (Bulfield *et al.*, 1984) and housed according to the Canadian Council on animal Care in the University of Manitoba Central Animal Care Facility.

The animals were sacrificed and hearts, brains, livers, spleens, kidneys and skeletal muscles were dissected, frozen on dry ice and stored in -70°C as soon as possible. Sera were collected and serum T3/T4 levels were determined by St. Boniface General Hospital Biochemical Laboratory using DEFLIA (Wallac Oy, Turku, Finland) Time-resolved fluoroimmunoassay (Hemmilä *et al.*, 1984). Animal body weights and heart weights were also determined.

2.2.4 Extraction

All the electrophoresis chemicals and apparatuses were obtained from Bio-Rad

Laboratories, (Richard, CA). All the other chemicals were ordered from Sigma Chemical Co, (St Louis, MO), unless otherwise indicated.

To minimize degradation, all procedures were performed at 4°C unless otherwise specified and protease inhibitors, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin were extensively used in every step of extraction of bFGF from tissues and cells.

Tissues were weighed, minced with scissors and homogenized in 3 volumes (over weight) of 0.15 M ammonium sulfate $[(NH_4)_2SO_4]$ pH 4.5, with a Polytron homogenizer (Brinkmann, Rexdale, ONT) at medium setting. Insoluble residue, obtained by centrifugation in a Beckman L7 Ultracentrifuge (Beckman Instruments, Inc, Palo Alto, CA) at 100,000 xg, 45 min, 4°C, was re-extracted in the same manner. The supernatant from the two extractions were combined. The pH was adjusted to 7.0, using 5 M NaOH, protein concentration was determined colorimetrically by BioRad protein determination kit (Bio-Rad Laboratories, Richmond, CA). The salt concentrations were adjusted to 0.6 M by adding solid NaCl. The extract was then incubated with heparin-sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden), prepared according to the manufacture's instructions, in 0.6 M NaCl, 10 mM Tris-HCl, pH 7.0 at 4°C, overnight, under gentle agitation. Approximately 1 μl beads were used for each 1 mg protein in the extract. After extensive washing with 0.6 M NaCl, 10 mM Tris-HCl, pH 7.0, acidic FGF-like proteins were eluted with 1.1 M NaCl 10mM

Tris-HCl, pH 7.0. The beads were then equilibrated in 0.11 M NaCl, 10 mM Tris-HCl, pH 7.0 and heparin-bound peptides were eluted by boiling in electrophoresis sample buffer [2% sodium dodecyl sulphate (SDS), 0.1 M NaCl, 0.1 M Tris-HCl pH 7.0, 5% ß-mercaptoethanol, 10% glycerol) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The amount of heparin-sepharose used here (40 µl) was found to bind at least 400 ng of human recombinant bFGF (Upstate Biotechnology Inc,. Lake Placid, NY); this is well above the expected range of bFGF amounts in 40 mg of extracted protein (5-20 ng). Pilot experiments established that 40 µl of heparin-sepharose removes all of the heparin-binding bFGF present in 40 mg of extracted protein.

Cell culture extracts were prepared from confluent monolayers on 100-mm dishes after 3-4 days in culture. Cells were washed 3 times with ice-cold PBS, scraped and pelleted by centrifugation. Cells were resuspended in 0.6 M NaCl, 10 mM Tris-HCl, pH 7.4, and disrupted by three cycles of quick freezing and thawing followed by sonication on a Braun-Sonic 200 sonicator at high setting, 3x15 sec. Cell extracts were centrifuged (25,000xg, 20 min) and the supernatant was incubated with 30 µl (packed) heparin-Sepharose for 1 hr at room temperature. The heparin-Sepharose was then processed for SDS-PAGE, using the same methods as for tissue extracts.

Total tissue lysates were obtained by complete homogenization of 3 mg of tissue in a final volume of 300 µl of 2% SDS, 100 mM Tris-HCl, pH 7.0, 20% ß-mercaptoethanol, followed by boiling for 5 min. Ten (10) µl of total homogenate

(about 100 µg of protein) was loaded onto one gel lane.

2.2.5 SDS-PAGE, Western blotting and autoradiography

Basic FGF-containing samples were analyzed in 1.5-mm-thick polyacrylamide slab gels (Kardami & Fandrich, 1989) with the lower gel containing 0.4% N-N'-methylene-bis-acrylamide (Bis), 12.5% acrylamide, 3.5 mM SDS, 0.375 M Tris-HCl, pH 8.8, 0.067% ammonium persulfate and 0.067% N,N,N',N'-tetra-methyl ethylenediamine (TEMED); and the upper gel containing 0.12% Bis, 4.5% acrylamide, 0.125 M Tris-HCl, pH 6.8, 3.5 mM SDS, 0.067% ammonium persulfate and 0.067% TEMED. Protein molecular mass standards (10-100 kDa) as well as pure recombinant human bFGF (1-50 ng/lane) were included in each analysis. The gel was run in electrophoresis migration buffer (25 mM Tris-HCl, 192 mM glycine and 3.5 mM SDS) using a Bio-Rad electrophoresis apparatus. The temperature was maintained at 18°C. To compare bFGF levels and composition as a function of thyroid status, each gel lane was loaded with the heparin-bound fraction (approximately 2-3 µg of protein) from 30 or 40 mg of extract, respectively, and for cultured cell extracts, each lane was loaded with the heparin-bound fraction from extracts of two 100-mm culture dishes. Protein concentrations of tissue extracts were determined by the Bradford method according to manufactures' instruction (Bio-RAd). Protein concentrations in cell lysates were determined by the micro bicinchoninic acid protein assay (Redinbaugh and Turley, 1986). Silver staining of a duplicate gel was also performed as described (Morissey, 1981) to check for possible loading variation.

Gels were transferred onto Immobilon-P-membranes (Millipore Co., Bedford, MA) by electrophoretic transfer (Burnette, 1981). Briefly, after completion of SDS/PAGE, gels were carefully removed from the plates and the stacking gels cut off. The lower gel was placed in transfer buffer (0.325 M glycine, 0.1 M Tris-HCl, 0.2 M methanol) for 20 min. A sandwich setup (Bio-Rad) was made according to the manufacture's instruction. The electrophoretic transfer was performed in a transblot cell connected to a power supply [Model 200/7.0], and the transfer was run at 0.55 mA for 2 hrs. A Haake D8 waterbath (Haake Co. Berlin, Germany) was used to maintained the transfer buffer at 10°C.

After transfer, nonspecific protein-binding sites were blocked by incubation with 1% gelatin-PBS with gentle agitation for 30 min, at room temperature. The membrane was then incubated with rabbit anti-[1-24]bFGF serum IS2 (1:5000, Kardami *et al.*, 1991) in TBST (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.05% Tween 20) overnight at 4°C. Antigen-antibody complexes were visualized by incubating the membrane with 0.1 μCi/ml of ^{125}I-protein A (Amersham Co.) in TBST for 1 h. Autoradiography was performed with X-omat film (Eastern Kodak Co, Rochester NY) and intensifying screens (Lighting plus model, Dupont Cronex Co.) at -70°C for 1-7 days. Autoradiograms were scanned with a LKB 2202 Ultroscan laser densitometer and 2220 Recording Integrator (LKB-Produkter AB, Bromma, Sweden) to determine the relative intensity of the bands. Amounts of bFGF were estimated by comparing to the standard bFGF loaded on the gel at 1, 5, 10, 20 and 50 ng/lane.

2.2.6 Immunofluorescence microscopy

Cardiac tissue was frozen into Tissue Tek OCT glycerol mix (Miles Inc, Elkhart, IN) in a dry ice ethanol bath and used immediately for cryosectioning. Transverse sections of 7 μm thickness were routinely obtained, using a Leitz 1720 Kryostate (Leitz Instroment Co., Wetzlar, Germany). Sections were collected on gelatin coated glass slides, placed in humid chambers and incubated with primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS and 0.02% (w/v) sodium azide, overnight at 4°C. The primary antibodies used and their concentrations were: rabbit anti-[1-24]bFGF serum IS2, 1:3000; purified anti-[1-24] bFGF IgGs (Kardami and Fandrich, 1989), 1:10; mouse anti-vinculin IgG (Sigma Chemical Co. St. Louis, MO), 1:100; and mouse anti-myosin (a generous gift from Dr. Zak, University of Illinois at Chicago, IL), 1:3000. After washing the sections with cold PBS, they were incubated with fluorescein conjugated donkey anti-rabbit IgGs (Amersham Co.) and Texas Red conjugated sheep anti-mouse IgGs (Amersham) at 1:20 dilution in 1% BSA-PBS, for 1 h at room temperature. Following extensive washing with cold PBS, sections were fixed in cold 95% ethanol for 10 min, washed with PBS, and immersed for 30 seconds in 1μg/ml of Hoechst Dye 33342 (Behring Diagnostics, San Diego, CA). Well-washed sections were mounted with glycerol-PBS or Vectashield mounting medium (Vector Laboratories, Inc., Burlingam, CA), sealed with colorless nail varnish and stored at 4°C until observation. A Nikon Labophot microscope equipped with epifluorescence optics and appropriate filters was used for observation and microphotography of the sections. The UFX-IIA Nikon system and Kodak Tri-X-Pan 400 were used to view and

photograph selected fields. The anti-bFGF immunofluorescence intensity of the slides was also monitored with a SPEX Fluorolog Spectrofluometer (SPEX Industries Inc., Edison, NJ) by setting the excitation wavelength at 480 nm and slit width at 4mm. Six fields on each slide were measured and recorded. Background fluorescence of each tissue section was obtained from slides incubated with non-immune rabbit serum.

Myocytes cultured on coverslips were rinsed with PBS and fixed in 1% paraformaldehyde in PBS for 10 min, followed by 0.1% Triton X-100 in PBS for 10 min, or 50% methanol in PBS for 10 min, and 50% methanol, 50% acetone for 10 min. All fixation procedures were performed at 4°C and produced similar staining patterns. Fixed cultured cells were processed for immunofluorescence following the same procedure as for tissue sections.

2.2.7 Calculation and Statistical Analysis

All experiments were repeated at least three times, unless otherwise indicated. Quantitative data were expressed as mean ± SEM. Statistical evaluation of difference between results of various treatments was done by analysis of variance, and followed, when required, by Duncan's new multiple range post hoc test. To evaluate the differences in high molecular weight bFGF between T3 treated and untreated cultures, Paired Student's t-test was used. When comparing frequency data of two groups, a X^2 median test was used. In all cases, a probability of $P<0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 Localization of bFGF in the nuclei of cultured chick cardiomyocytes in interphase and M phase

Distribution of bFGF in the nuclei of chicken myocytes was examined by immunofluorescence. Myocytes obtained from 5- or 7- day old chick embryo ventricles were maintained in culture for 48 hours in 2% charcoal-absorbed, heat-inactivated fetal calf serum. After fixation, cultures were analyzed by simultaneous fluorescent staining for bFGF (rabbit anti-bFGF serum IS2), for myosin (anti-striated myosin mouse monoclonal #52) and for nuclei (Hoechst 33342). Typical results from 5-day old cultures are presented in Figure 2.1. Strong anti-bFGF staining was observed in a percentage of interphase cardiomyocyte nuclei. This percentage was found to be 26% in cultures from 5-day old hearts compared to 10% in those from 7-day old hearts. The majority of myocyte nuclei appeared to stain weakly or non-detectably for bFGF under our experimental conditions (Fig. 2.1).

Morphological observation indicated that nuclei staining brightly for bFGF (B nuclei) tended to appear larger than those which either did not stain at all or stained very faintly (F nuclei). To examine the relationship between anti-bFGF staining intensity and size, we measured the diameters (average of the largest and smallest diameter of each nucleus) of B and F nuclei. In one representative experiment, out of 504 nuclei, 88 and 416 were counted as B or F, respectively. Diameters of F nuclei

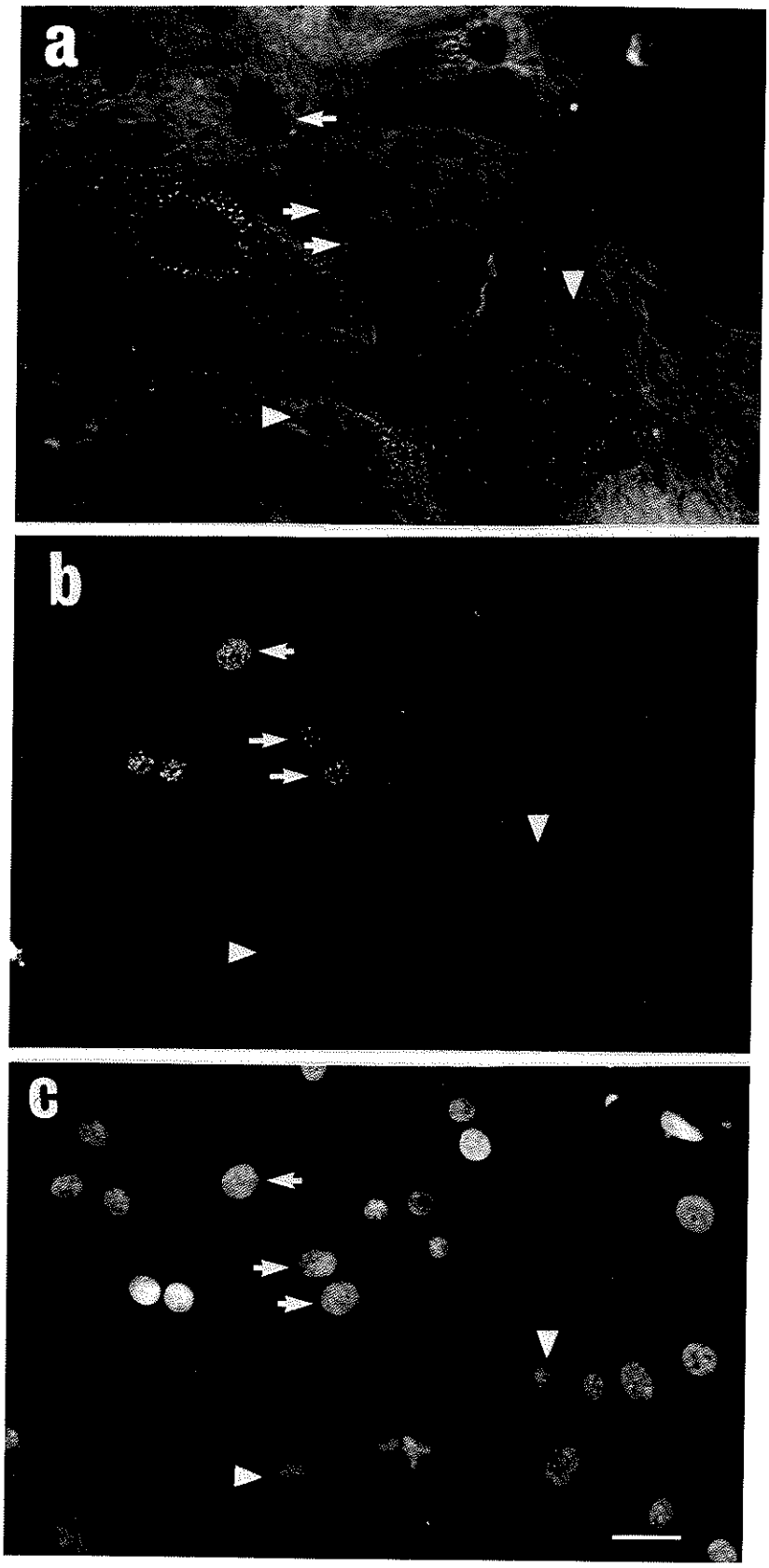


Fig. 2.1
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Figure 2.1. Near-confluent cultures of fetal cardiomyocytes from 5-day chick embryos. Triple fluorescence labelling for (a) striated-muscle myosin (antibody #52, 1:5000), (b) bFGF (IS2 1:4000) and (c) nuclear DNA. Short arrows indicate interphase nuclei that stain intensely for bFGF. Arrowheads indicate some of the nuclei that do not stain for bFGF. (Bar = 20 μm).

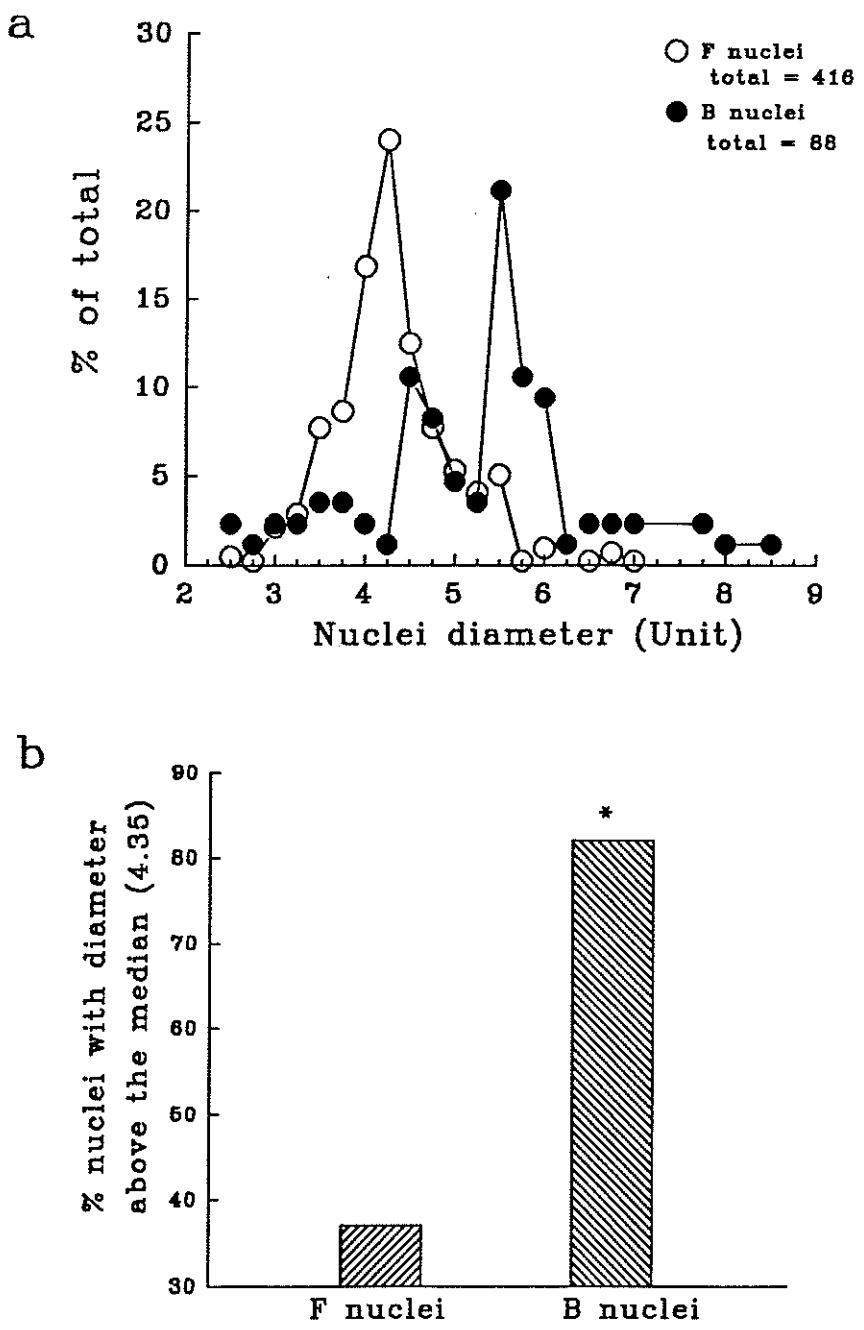

Figure 2.2. Relationship between average myocyte nuclear diameter and intensity of anti-bFGF staining. (a), incidence of myocyte nuclear diameter between 2.50 to 8.50, for either F (hollow circles) or B (solid circles) nuclei. (b), percentage of nuclei with a diameter above 4.35 (the median value) in B vs F nuclei. * $P<0.001$

varied from 2.5 to 7 mm (measured on 3.5 X 5" prints, corresponding to X400 magnification). When the frequency of each diameter was examined, a maximum was discerned at 4.25 mm. Frequency of F nuclei diameters showed an approximately normal distribution (Figure 2.2a, hollow circles). Diameters of B nuclei varied from 2.5 to 8.5, with a maximal frequency at 5.5 (Figure 2.2a, solid circles). The percentage of nuclei with diameters larger than the median (4.35 mm) are 82% and 37% in B and F nuclei, respectively (Figure 2.2b). This difference is statistically significant ($P<0.001$) tested by X^2 median test.

In prophase, the chromatin, which is diffuse in interphase, slowly condenses into well-defined chromosomes (Fig. 2.3c). Each chromosome has duplicated during the preceding S phase and consists of two sister chromatids which are held together at the centromere. We compared the pattern of myocyte nuclear bFGF distribution in prophase cells (Fig.2.3 c,d,e,f) with that of myocytes at interphase (Fig. 2.3 a,b). In all prophase myocytes, identified by the appearance of chromosome condensation, a strong punctate anti-bFGF stain was discerned, similar in intensity and overall shape to the pattern of B interphase nuclei (Figure 2.3, compared d, prophase, to b, interphase). This anti-bFGF staining appeared to be associated with chromosomes throughout prometaphase. However, as the chromosomes condensed further, the punctate foci of staining and the uneven distribution of bFGF became more visible compared to prophase (Fig.2.3f). In both prophase and prometaphase, the staining pattern of DNA and bFGF were found to be largely superimposed, although not

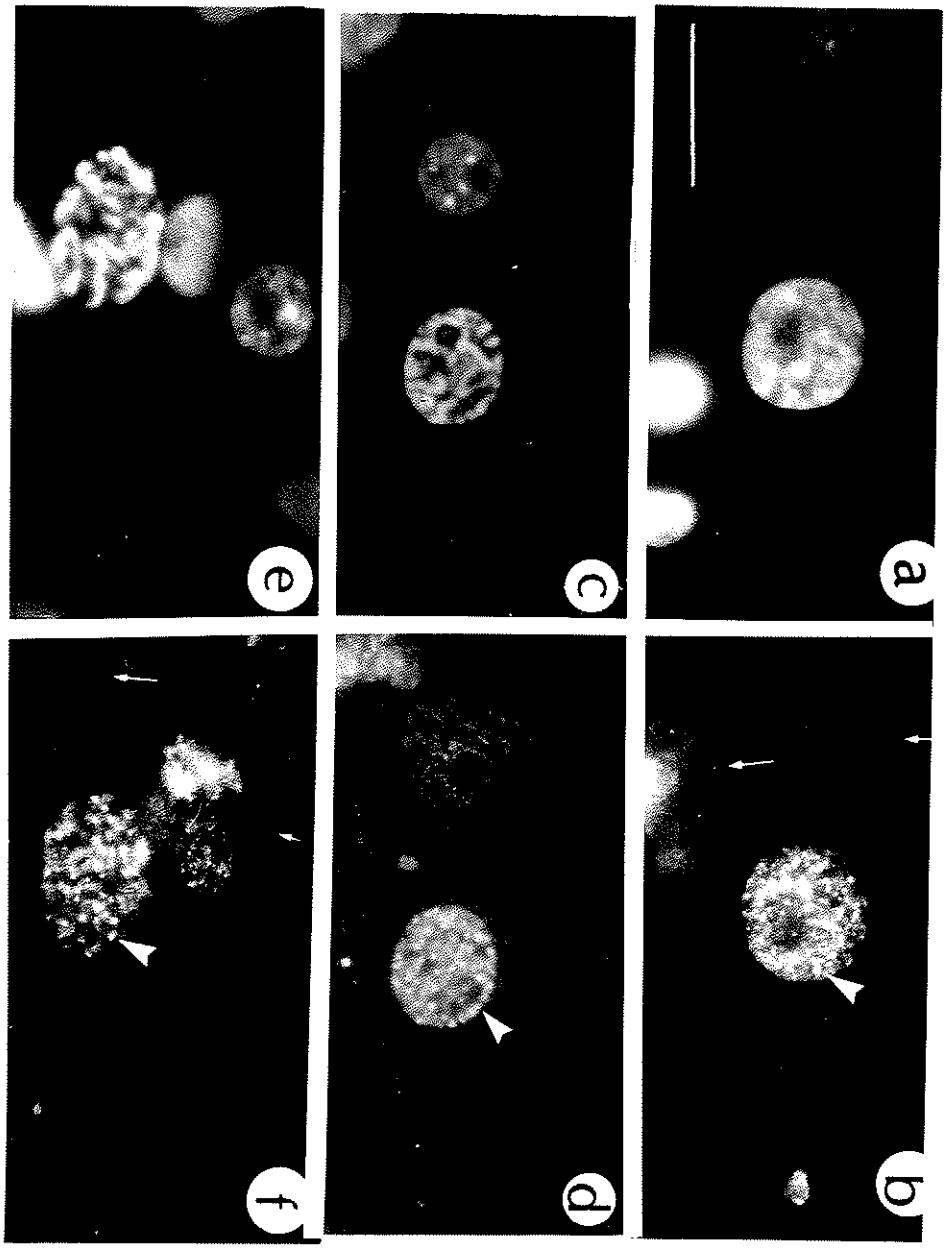

Fig. 2.3
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Figure 2.3. Localization of bFGF in myocytes in interphase and undergoing mitosis. Double-fluorescence staining of embryonic chick cardiac myocytes for (a,c,e), DNA, and (b,d,f), bFGF. a,b, interphase nuclei; c,d, prophase nuclei; e,f, prometaphase nuclei. Arrows indicate myofibrillal structures in the cytoplasm. Arrowheads point to the punctate nuclear staining. Bar = 50 μm

absolutely identical (compare the anti-bFGF labelling in Figure 2.3d,f with the chromosomal DNA-stain in Figure 2.3c,e). Certain regions of the chromosome apparently stained more intensely with anti-bFGF antibodies than others. In metaphase, the chromosomes are highly condensed and align at the metaphase plate which is perpendicular to the spindle and located half way between the spindle poles (Fig. 2.4a). The close association of anti-bFGF and chromosomal staining persists in metaphase (Figure 2.4a,b). Basic FGF appears confined to the metaphase plate. Some punctate foci of staining are still observed. However, most of them are intermingled with streaks that appear to traverse the plate parallel to the spindle axis (Fig.2.4b). Anaphase starts when the two sister chromatids are pulled toward each pole by the shortening of kinetochore microtubules (Fig.2.4c). During anaphase the anti-bFGF staining completely differentiated from that of chromosomes and appeared associated with coarse fibers forming a band between the two daughter cells (Figure 2.4d). The cleavage of cytoplasm also usually starts sometime during anaphase. In telophase, while the separated daughter chromatids arrive at the poles and a new nuclear envelope re-forms around each group of daughter chromosomes, the cleavage furrow forms. The membrane around the middle of the cell, perpendicular to the spindle axis and between the daughter nuclei, is drawn inward to form the cleavage furrow. Anti-bFGF staining during that period is intensely and sharply localized to the region of the only remaining connection between the two daughter cells, the midbody (Figure 2.4f), and persist until cytokinesis is almost finished (Figure 2.4h).

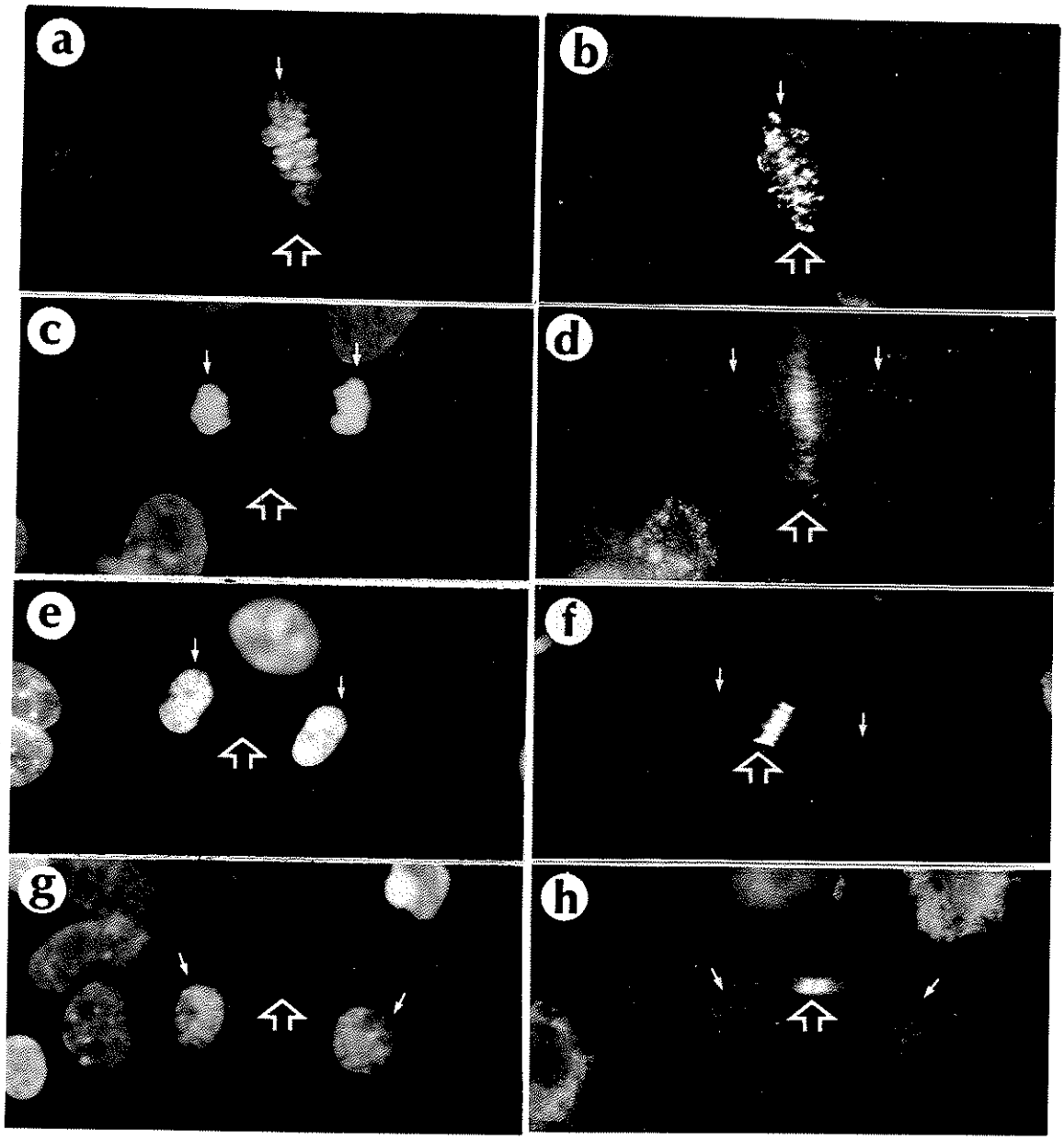

Fig. 2.4
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Figure 2.4. Localization of bFGF in mitotic chick myocytes. Double-fluorescence staining of embryonic chick cardiomyocytes at various stages of mitosis, for, (a,c,e,g), DNA, and (b,d,f,h), bFGF. Small arrows indicate location of chromosomes and/or nuclei, while double arrows point to the plane of cellular division and the midbody. (Bar = 50 μm)

2.3.2 Localization of bFGF in cultured rat cardiomyocytes

Rat cardiomyocytes were obtained from 18-day fetuses and maintained in media supplemented either with 5 % calf serum for 48 hours (Figure 2.5a,c,e) or 0.5% calf serum for 1 week (Figure 2.5b,d,f). These conditions favor a more proliferative or more differentiated phenotype, respectively (Ueno *et al.*, 1988). This is confirmed by nuclear staining of myocytes as shown in Figure 2.5e,f. Cultures maintained in high serum for a short time consisted of mononucleated cells and displayed a fraction of cells (approximately 10%) actively undergoing mitosis (Figure 2.5e). Myocytes in metaphase and anaphase can be identified in Figure 2.5e. Myocytes cultured in low serum for 1 week displayed no cells in active mitosis, and a high incidence of binucleation (about 90% of total myocytes, Figure 2.5f). Binucleation is a characteristic of a more advanced developmental stage (Clubb and Bishop, 1984; Ueno *et al.*, 1988). Staining for myosin showed well-organized myofibrils in the mature myocytes (Figure 2.5d). In both proliferative and differentiated myocytes IS2 stained the nuclei. Basic bFGF was localized in association with all myocyte nuclei in interphase (Fig 2.5b, double arrows) as well as during mitosis (Figure 2.5a, arrowhead, big arrows). However, bFGF appeared to have primarily a perinuclear localization, although a punctate staining can also be seen inside the nuclei (Fig.2.5a, arrowhead, Fig.2.5b double arrows). Anti-bFGF staining could be seen in association with myofibrils (likely at the Z-lines) especially in more differentiated myocytes (Figure 2.5b, small arrows). Finally, bFGF was localized to what appeared to be myocyte gap junctions (Fig.2.5b, curved arrows), as shown previously (Kardami *et al.*, 1991a).

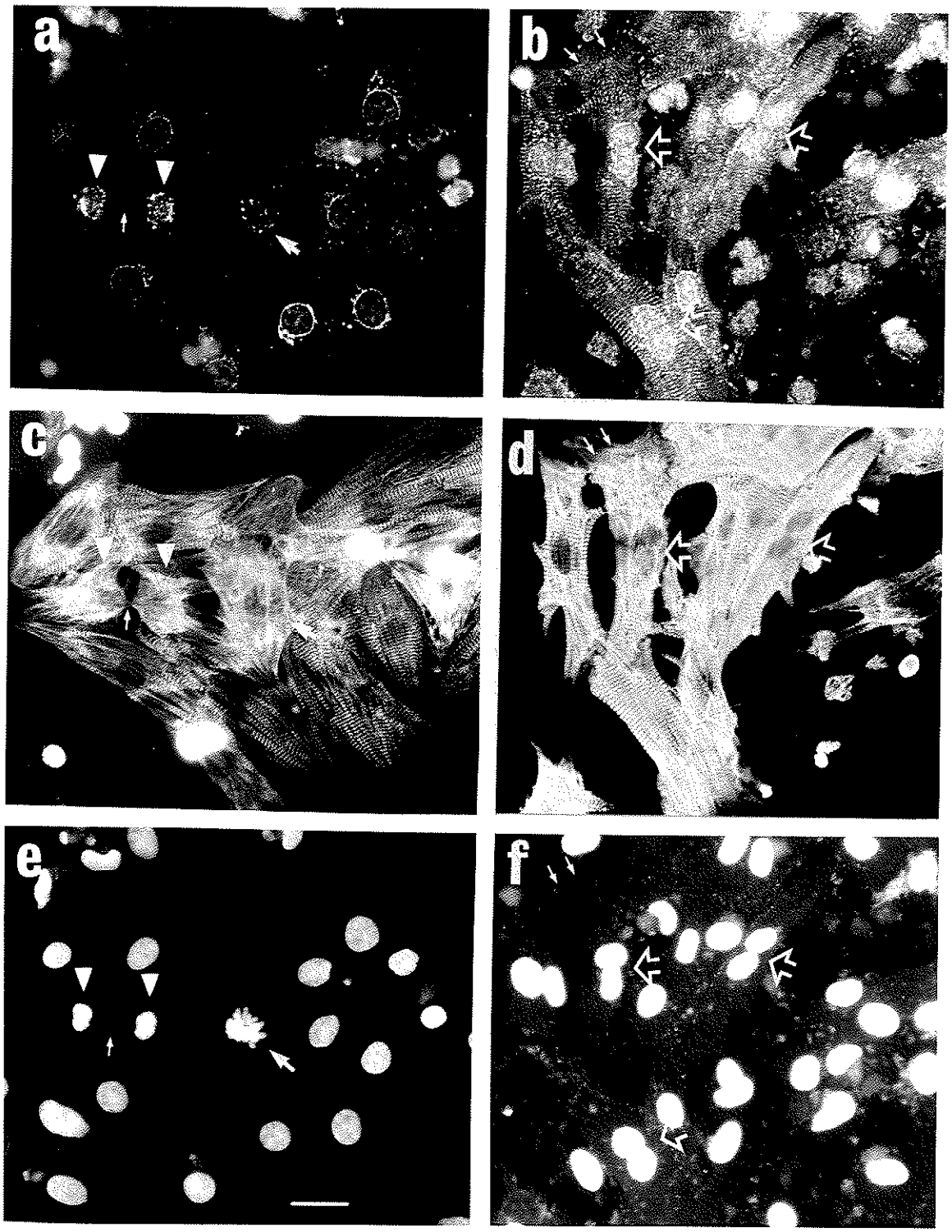

Fig. 2.5
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Figure 2.5 Localization of bFGF in rat myocytes. Triple fluorescence staining of embryonic rat cardiac myocytes for (a,b), bFGF, (c,d), myosin and (e,f), nuclear DNA. Myocytes cultured for,(a,b,c), 48 hours in 5 % calf serum and, (b,d,f), 1 week in 0.5% calf serum. Arrowheads point at dividing myocytes in telophase, arrows at mitotic myocyte in metaphase, double arrows at binucleated myocytes, and curved arrows at myocyte gap junctions. (Bar = 50 μm)

2.3.3 Effect of bFGF and T3 on myocyte DNA synthesis

To investigate the effect of bFGF on the ability for DNA synthesis and the influence of this effect by thyroid hormone, we determined the proportion of muscle cells that were capable of synthesizing DNA (Labeling Index, LI) in culture, in the presence or absence of bFGF (12 ng/ml) and/or T3 (1 and 10 nM). Myocytes were identified by muscle- detecting histochemical staining, Periodic Acid Schiff stain (PAS, Fig.2.6, arrows). Cells synthesizing DNA during labelling incorporated ^{3}H-thymidine (Figure 2.6, big arrows for myocyte nuclei and arrowheads for non-muscle nuclei). Absence of ^{3}H-thymidine labelling leads to a "ghost" image of the nuclei (small arrows). Results, shown in Figures 2.6 and 2.7, are summarized as follows: (a) After 5 day in culture, control myocytes have a LI of 0.13, i.e. 13% of the total myocytes were still capable of synthesizing DNA. (b) Basic FGF (12 ng/ml) induced a 2.6-fold increase in LI compare to control, and the increase was statistically significant ($P<0.01$). (c) T3 (1 and 10 nM) itself had no significant effect on myocyte LI, however 1 nM T3 decreased the stimulating effect of bFGF from LI of 0.35 ($\pm$0.01) bFGF alone, to 0.24 ($\pm$0.019) bFGF plus T3. Similar results were also obtained by counting cell number as well as total ^{3}H-thymidine incorporation (Kardami, 1990). (d) All cardiac extracts induced increases in LI for ventricular myocytes. This increase was the highest in the presence of extract from hypothyroid rats (about 3.1-fold compared to control), while extracts from hyperthyroid rat induced a 1.9-fold increase over control.

Figure 2.6. Effect of bFGF and T3 on cardiac myocytes DNA synthesis. Rat cardiac myocytes from 20-day fetuses cultured in (a), control medium (5% calf serum), (b), with 12 ng/ml bFGF, (c), 1 nM T3, and (d), bFGF (12 ng/ml) plus T3 (1nM). Black nuclei are those capable of DNA synthesis, labelled with tritiated thymidine. Muscle cell cytoplasm is visualized using PAS+ staining (see material and methods.) Arrows point at muscle nuclei with (big arrows) and without (small arrows) tritiated thymidine labelled DNA, while arrowheads point at non-muscle nuclei. (Bar = 20 μm)

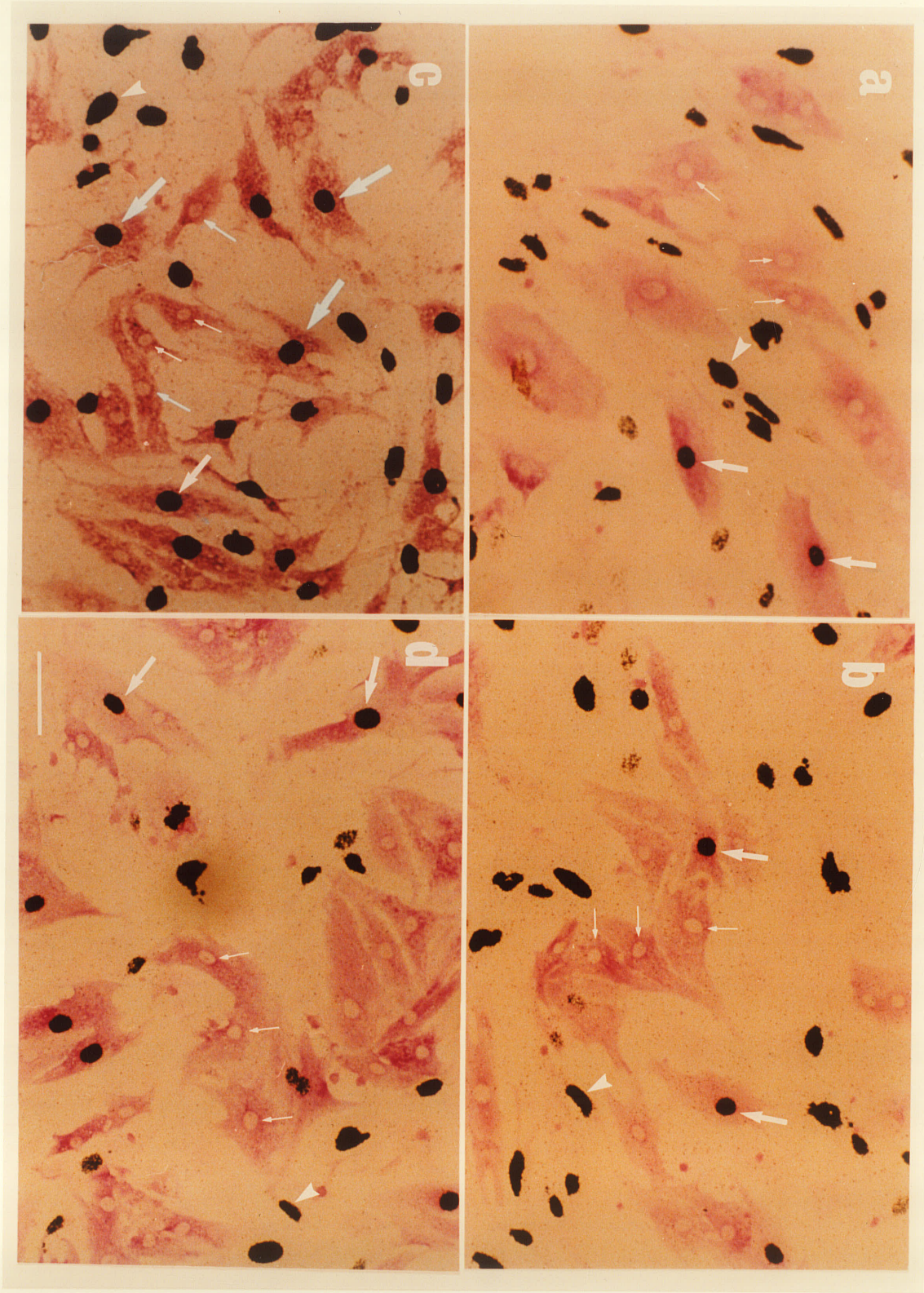

Fig. 2.6
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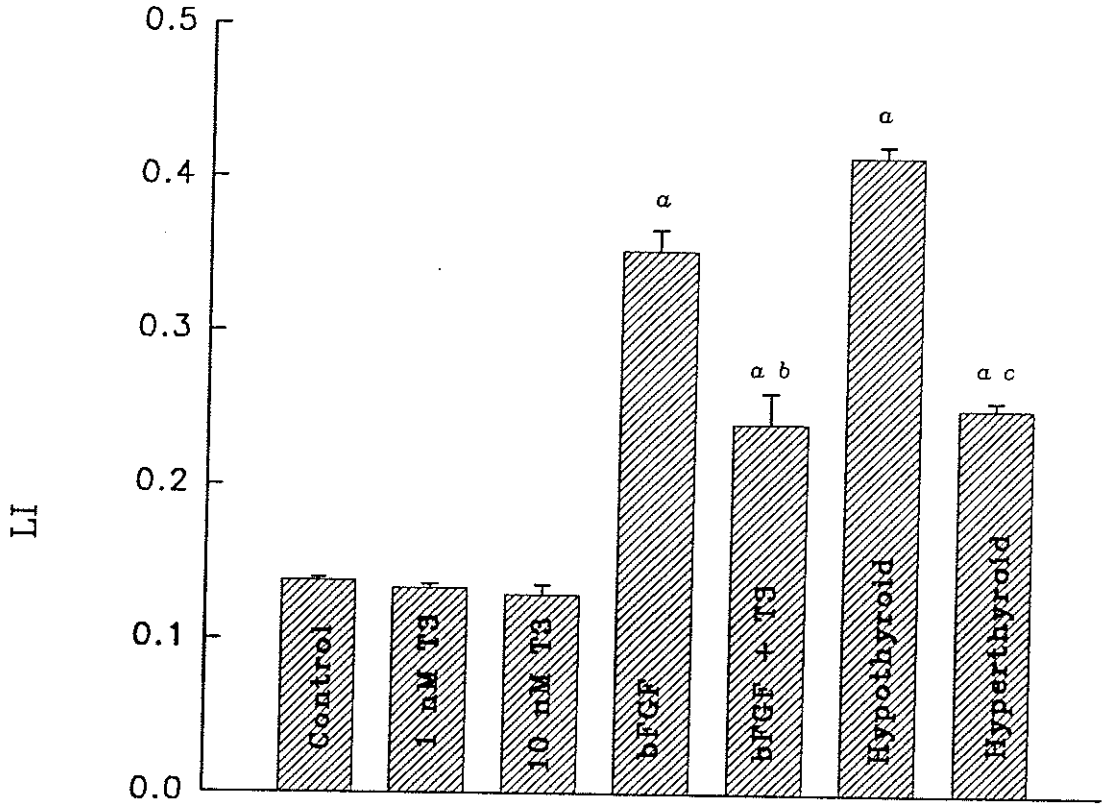

Figure 2.7. Effect of bFGF, T3 on cardiac myocyte labelling index. Rat cardiomyocytes from 20-day fetuses were cultured in 5% calf serum/F12 (control), in the presence of 12 ng/ml bFGF (bFGF), T3 (1 nM and 10 nM), bFGF (12ng/ml) plus T3 (1nM), and cardiac extract (final concentration 0.3 mg/ml) from hypothyroid and hyperthyroid rat.

Labelling Index = [labelled muscle nuclei/total muscle nuclei]

[a] $P < 0.01$ compare to control

[b] $P < 0.01$ compare to bFGF-treated

[c] $P < 0.01$ compare to hypothyroid-extract-treated

2.3.4 Effect of thyroid status on bFGF accumulation in the heart.

Table 2.1 summarizes the general characteristics of thyroxin and PTU treated rats. The plasma of euthyroid rats contained 110.6 ± 2.56 nM and 3.84 ± 0.28 nM T4 and T3, respectively. Administration of exogenous T4 to rats led to a more than 3-fold elevation of T4 and almost 2-fold increase in T3. The ventricle to body weight ratio [ventricle weight (mg)/body weight (g)] increased to 3.96 ± 0.25 (n = 20) compared to the euthyroid value of 2.68 ± 0.13 (n = 30). PTU treatment of rats for 4 weeks resulted in significant decrease in thyroid hormone contents in the blood, to 18.33 ± 1.69 nM for T4 and 1.73 ± 0.42 nM for T3. PTU treatment caused a decrease in ventricular weight to 0.756 ± 0.02 g (n = 60) compared to 1.381 ± 0.067 (n = 30) in age-matched euthyroid controls, while the ventricle to body ratio was at 2.74 ± 0.09).

Equivalent amounts of pooled extracts from hypo-, eu- and hyper-thyroid rat heart ventricles were fractionated by heparin-sepharose and analyzed by immunoblotting for their bFGF content and composition. Results from one representative set of experiments are shown in Figure 2.8a. Euthyroid cardiac extracts contained mostly the 18 kDa bFGF (85% of the total) (Figure 2.8a, lane 2). Hypothyroid ventricular extracts displayed about a 5-fold increase in 21.5-22 kDa bFGF compared to controls (Figure 2.8a, lane 3); overall bFGF levels were increased approximately 2-fold. A minor (1.2-fold) increase in the 18 kDa bFGF was also detected in hypothyroid extracts (Figure 2.8a, lane 3). As a consequence of these

Table 2.1. Body weight, ventricular weight and serum thyroid hormone of experimental animals

	CONTROL n=60	PTU TREATED n=30	T4 TREATED n=20
Body Weight (BW g)	515.0 ± 8.7	281.1 ± 7.6*	456.8 ± 5.0
Ventricle Weight (VW mg)	1381.1 ± 67.2	756.7 ± 20.6*	1813.1± 127.6*
VW/BW (mg/g)	2.68 ± 0.13	2.74 ± 0.09	3.96 ± 0.25*
Serum T4 (nM)	110.6 ± 2.56	18.33 ± 1.69*	412.4± 106.07*
Serum T3 (nM)	3.84 ± 0.28	1.73 ± 0.42*	6.13 ± 0.60*

* $P < 0.01$ treated group vs control group

changes, the 21.5-22 kDa bFGF comprised nearly 50% of total heart bFGF in hypothyroid rats. Some increase in overall bFGF (1.5-fold) was also seen in extracts from hyperthyroid hearts (Figure 2.8a, lane 1). This increase was mostly due to the 18 kDa species, although a small increase in the 21.5-22 kDa bFGF was sometimes seen (see composite data in Figure 2.11a). Omission of protease inhibitors during extraction resulted in the complete absence of the 21.5-22 kDa bFGF from these extracts (Figure 2.9, lane 2). Treatment of the heparin-binding fraction from hypothyroid heart extracts with trypsin (0.0025%, w/v, 10 minutes at 37 °C), in the presence of heparin-sepharose, resulted in the complete disappearance of the 21.5-22 kDa bands and augmentation of the 18 kDa bFGF (Figure 2.9, compare lanes 4 and 5), indicating a precursor-product relationship between the 21.5-22 and 18 kDa immunoreactive peptides.

Brain extracts from hypo-, and hyperthyroid rats were also analyzed for their bFGF composition in comparison to euthyroid extracts, and results are shown in Figure 2.8b. Euthyroid brain controls displayed primarily the 21.5-22 kDa forms of bFGF (Figure 2.8b, lane 2). Hypothyroid (Fig.2.8b, lane 3) as well as hyperthyroid (Fig.2.8, lane 1) brain bFGF composition was comparable to that of the controls. Similar data were obtained in three different sets of experiments. Combined results are illustrated in Fig.2.11a, where the ratio of densitometric values of the 21.5-22 kDa bFGF over total bFGF X100 (= R_{hi}) is shown as a function of thyroid status. Compared to the large increase in R_{hi} in the cardiac ventricles of hypothyroid animals, changes in the

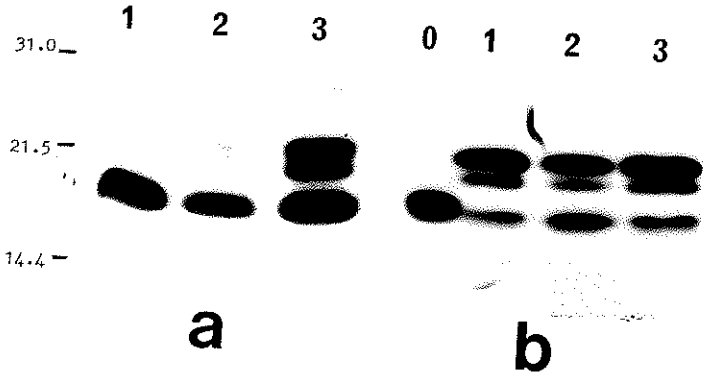

Figure 2.8. Effect of thyroid status on bFGF expression in heart and brain. Analysis of bFGF composition in heparin-binding tissue fractions from, (a), cardiac ventricles and, (b),brain tissues by immunoblotting. Lane *0* (b), 5 ng of human recombinant bFGF, Lane *1* (a,b), tissue from hyperthyroid animals, lane *3* (a,b), tissue from hypothyroid animals. Lane *2* (a,b), tissues from age matched control euthyroid animals. Each lane is loaded with the heparin-binding fraction (approximately 3 μg of protein) from 40 mg of extract. Migration of molecular weight markers is indicated in kDa.

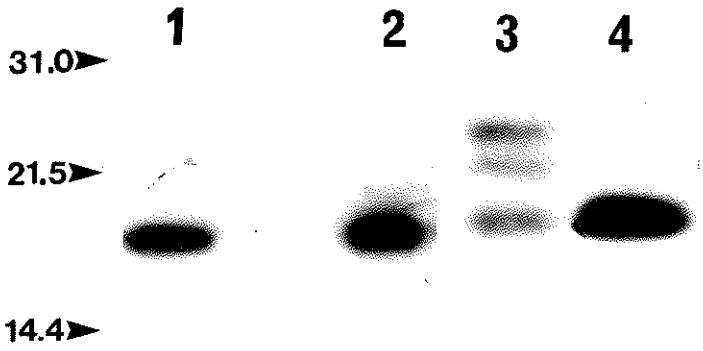

Fig. 2.9 Influence of proteolysis on bFGF composition. Analysis of bFGF in tissue heparin binding fraction from hypothyroid cardiac ventricles extracted in the absence (lane *2*) or presence (lanes *3,4*) of proteases inhibitors. Lane *1*, 5 ng of human recombinant bFGF, land *3* and *4*, heparin binding fraction before and after treatment with 0.0025% trypsin, respectively. Each lane is loaded with the heparin-binding fraction from 20 mg of extract. Migration of molecular weight markers is indicated in kDa.

brain were minor; R_{hi} was consistently high. On the other hand, in cardiac atria R_{hi} was consistently low in euthyroid as well as hypo- or hyperthyroid animals, although a significant ($P<0.01$) increase was observed in hypothyroidism. Other tissues were also examined for their bFGF composition, and similar results from two experiments were averaged and plotted in Figure 2.11b. As for brain, spleen extracts had comparatively high R_{hi} values irrespectively of thyroid status. In skeletal muscles (either predominantly red, slow-twitch, or predominantly white, fast twitch), R_{hi} was elevated in hypothyroid animals (Fig.2.11b). In the kidney and liver, hypothyroidism as well as hyperthyroidism was associated with elevated R_{hi} compared to controls. Finally, in aorta, hyperthyroidism resulted in significantly increased R_{hi} compared to controls. Figure 2.10 shows the autoradiographs of the first set of experiments.

Sections were obtained from rat ventricles and examined for bFGF localization, using affinity purified anti-bFGF IgG derived from antiserum IS2. (Figures 2.12 and 2.13). To identify cardiomyocytes, sections were simultaneously stained for vinculin, localizing to the intercalated discs and the lateral plasma membrane of myocytes (Steenbergen *et al.* 1987; Padua *et al.*, 1993). To identify nuclei, sections were counterstained with a nuclear fluorescent dye (Hoechst 33342; Kardami and Fandrich, 1989, Kardami *et al.*, 1990). Bright anti-bFGF staining was observed in association with myocardial nuclei irrespectively of thyroid status. This is illustrated in Fig.2.12 d,e and f, which shows bFGF localization in nuclei (arrows) of cardiac sections from hypothyroid, euthyroid and hyperthyroid rats, respectively. Nuclear anti-bFGF staining

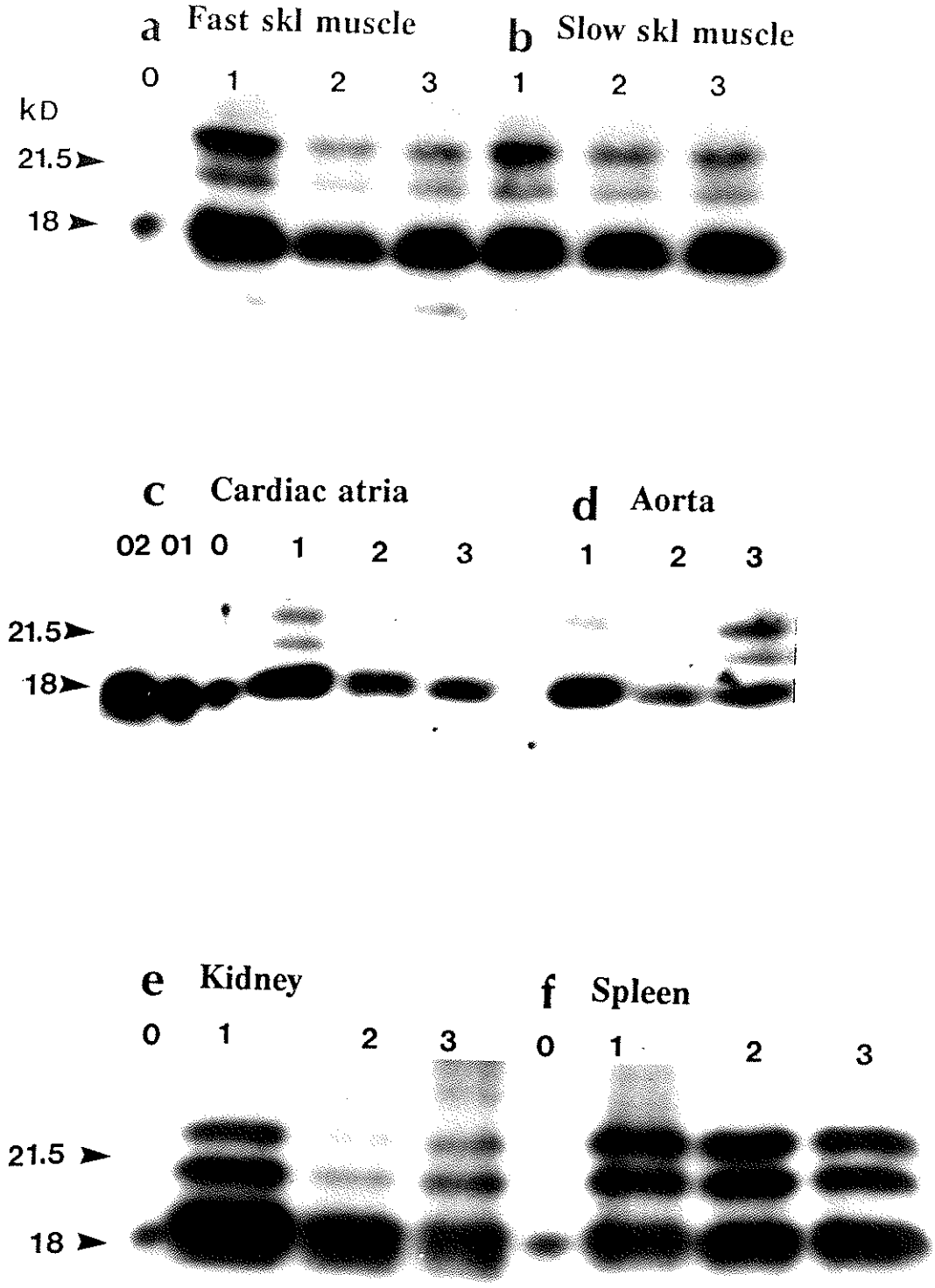

Fig. 2.10
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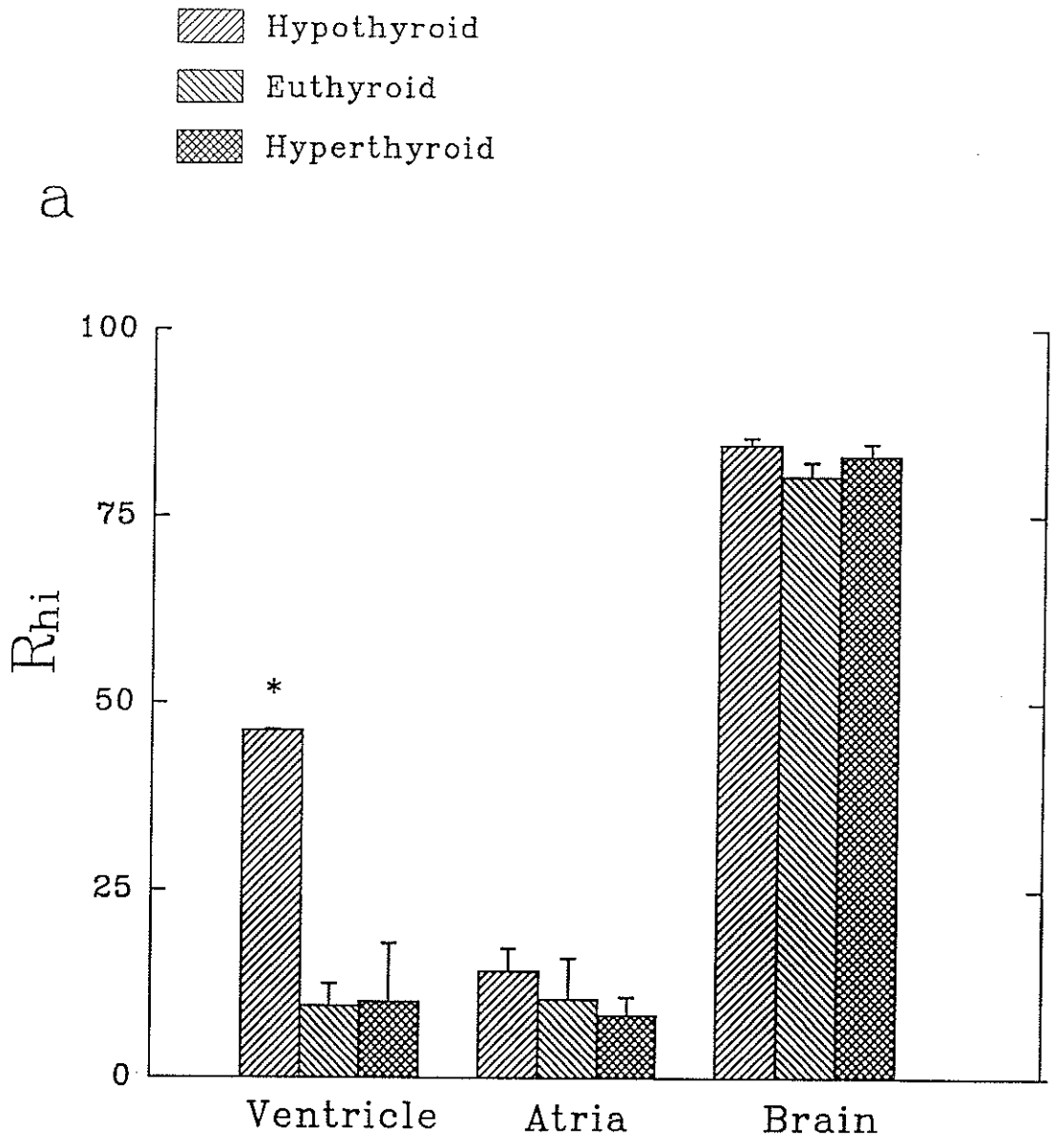
Figure 2.10. Effect of thyroid status on bFGF expression. Analysis of bFGF composition in heparin-binding tissue fractions from, (a), fast skeletal muscle; (b), slow skeletal muscle; (c), cardiac atria; (d), aorta; (e), kidney ; and (f), spleen by immunoblotting. Lane *0* (a,c,e,f) 2 ng of human recombinant bFGF, lane *01* and *02* (c), 10 and 5 ng of human recombinant bFGF, respectively. Lane *1*, tissues from hyperthyroid animals, Lane *3*, tissues from hypothyroid animals. Lane *2*, tissues from age-matched control euthyroid animals. Each lane is loaded with the heparin-binding fraction (approximately 3 μg of protein) from 40 mg of extract. Migration of molecular weight markers is indicated in kDa.



Rhi = (21.5–22 kDa/total)bFGF x 100%

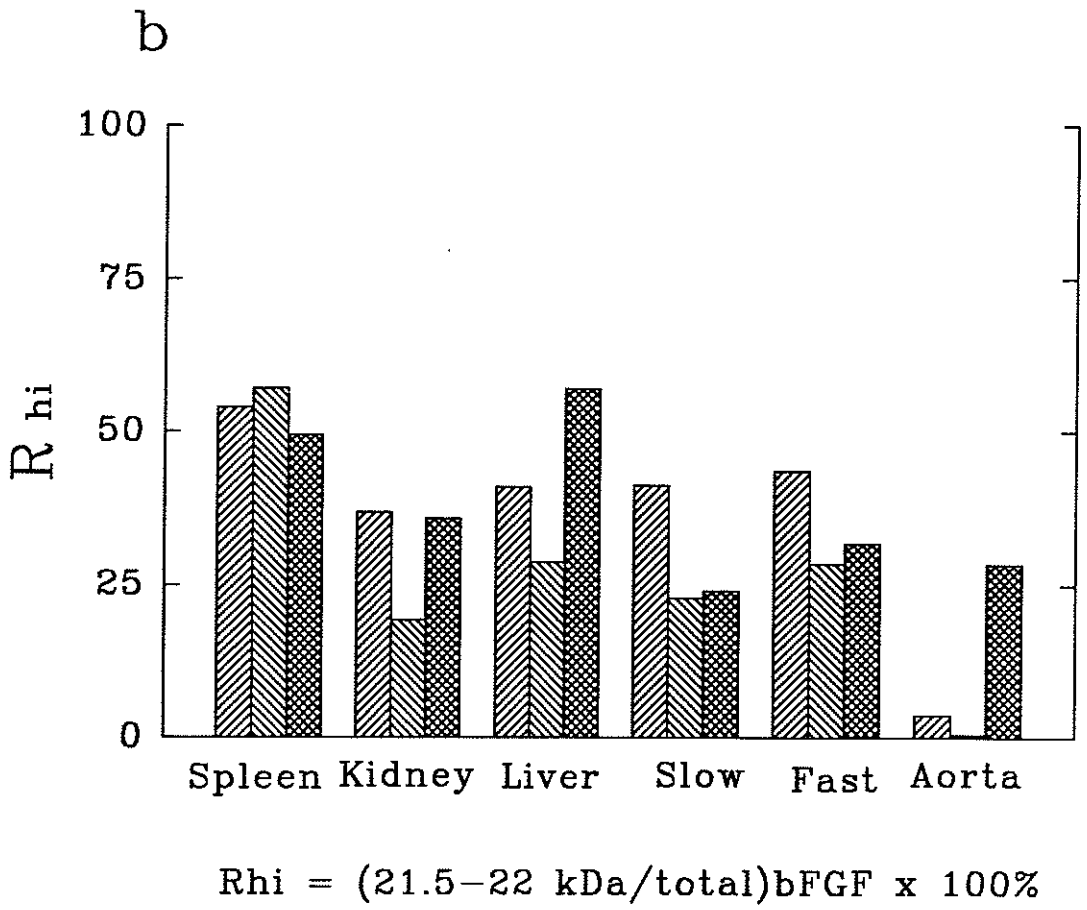


Figure 2.11. Rhi plotted as a function of thyroid status. (a): Cardiac ventricles and atria, brain. Results showinn represent mean ± SEM from 4 experiments (ventricle and brain) and 3 experiments (atria). * $P<0.01$ compared to euthyroid. Statistic evaluation was done by a multiple analysis of variance test followed, when appropriate, by Duncan's new multiple range post hoc test. (b): Spleen, kidney, aorta and skeletal muscle. Results shown represent the mean value from 2 experiments. Rhi = ([21.5-22 kDa bFGF]/[Total bFGF]) x 100.

is also indicated in Figure 2.13. It should be noted that, in all cases, an undetermined proportion of myocardial nuclei did not stain brightly or detectably for bFGF.

Hypothyroid ventricles (Fig.2.12d and Fig.2.13a,d) displayed more intense overall anti-bFGF staining compared to euthyroid (Fig.2.12e and Fig.2.13b) or hyperthyroid (Fig.2.12f and Fig.2.13c,e) hearts. Staining was localized in the intercalated disc areas, as expected (Kardami *et al.* 1991a,b). Anti-bFGF staining was also seen in connections apparently between the lateral surfaces of adjacent myocytes; this was more evident in hypothyroid ventricles (Fig.2.13d, arrowheads). Local anti-bFGF staining of the intercalated disc regions appeared more intense in sections from hypothyroid compared to eu- or hyper-thyroid rats. The anti-bFGF immunofluorescence intensity of the slides measured by spectrofluometer was shown in Figure 2.14. Slides obtained from hyperthyroid rat heart showed a 45% reduction in the anti-bFGF fluorescence intensity compared to those of the euthyroid, while those from hypothyroid heart showed a 28% increase.

2.3.5 Effect of T3 on bFGF composition of cultured cardiomyocytes

To investigate whether the change in bFGF composition with thyroid status reflects a direct effect of thyroid hormone on the myocytes, we used cultured cardiac myocytes obtained from 7-day-old chick embryos. Cultures were maintained in 4% thyroid hormone-depleted fetal calf serum (see Material and Methods), for 4 days. T3 (10 nM) was added 24 hours before the cells were harvested. Presence of T3 did not

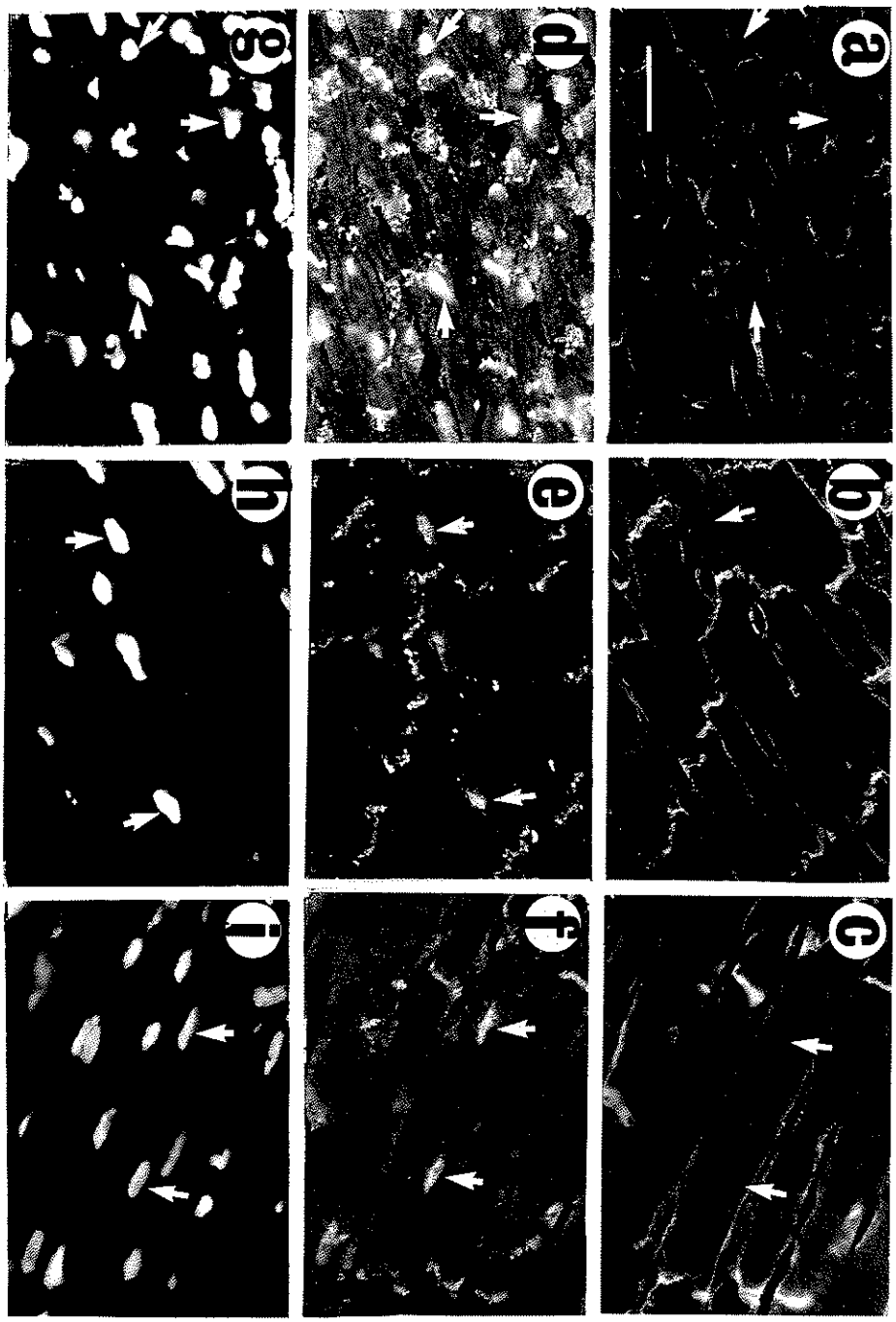


Fig. 2.12
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Figure 2.12. Simultaneous fluorescence staining of cardiac ventricular tissue sections for (a,b,c), vinculin, (d,e,f), bFGF. (g,h,i), nuclear DNA (see methods). Sections from hypothyroid (a,d,g), euthyroid (b,e,h) and hyperthyroid (c,f,i) rats. Arrows point at nuclei which stain for bFGF. Bar=50 μm.

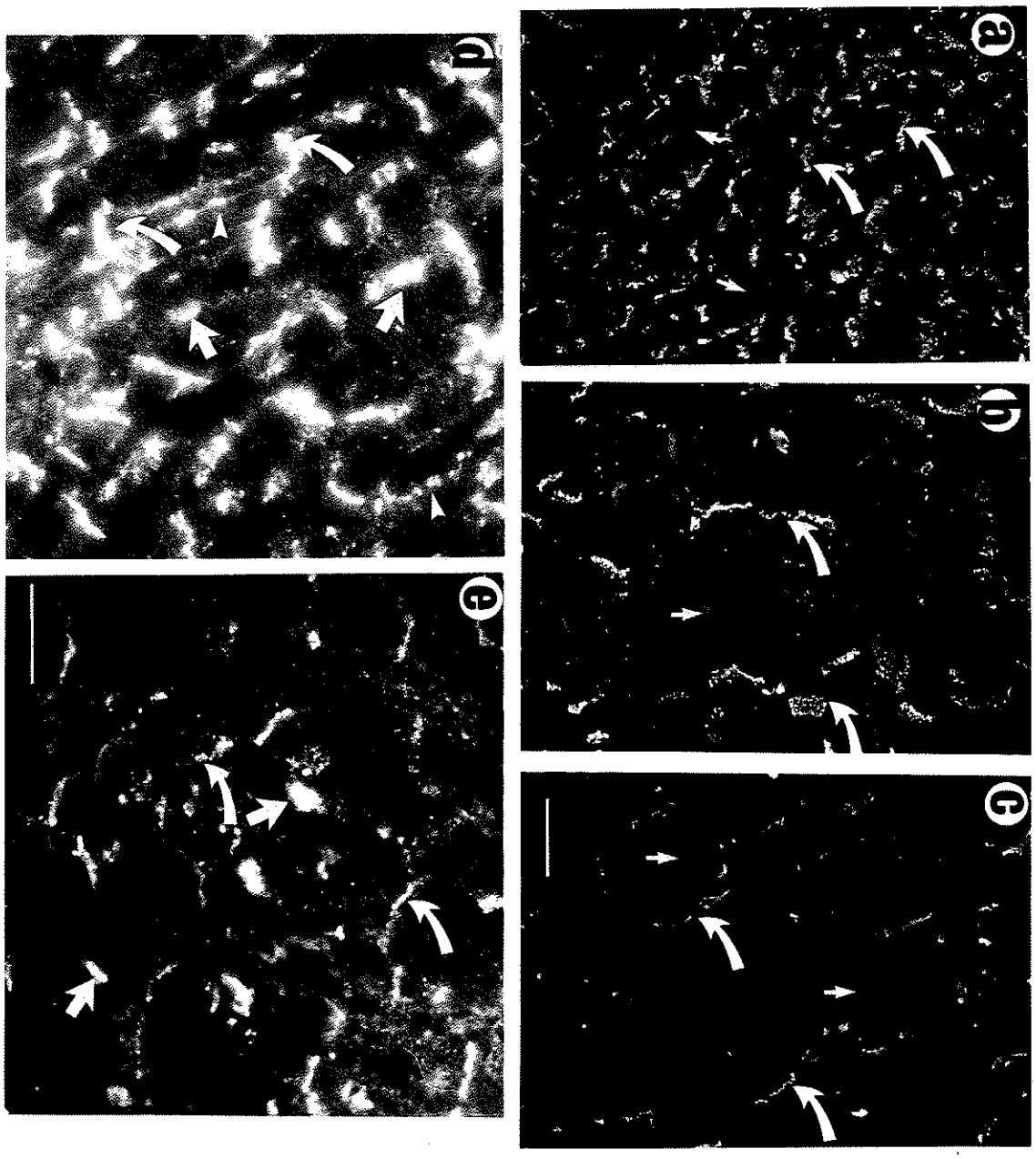

Fig. 2.13
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Figure 2.13 Effect of thyroid status on cardiac bFGF expression. Indirect immunofluorescence for bFGF of tissue sections from hypothyroid (a,d), euthyroid (b) and hyperthyroid (c,e) rat ventricles. Results from two different sets of experiments (first shown in a,b,c, second shown in d,e). Curved arrows point at intercalated discs, straight arrows at nuclei and arrowheads indicated apparently lateral connections between myocytes. Bar=50 μm.

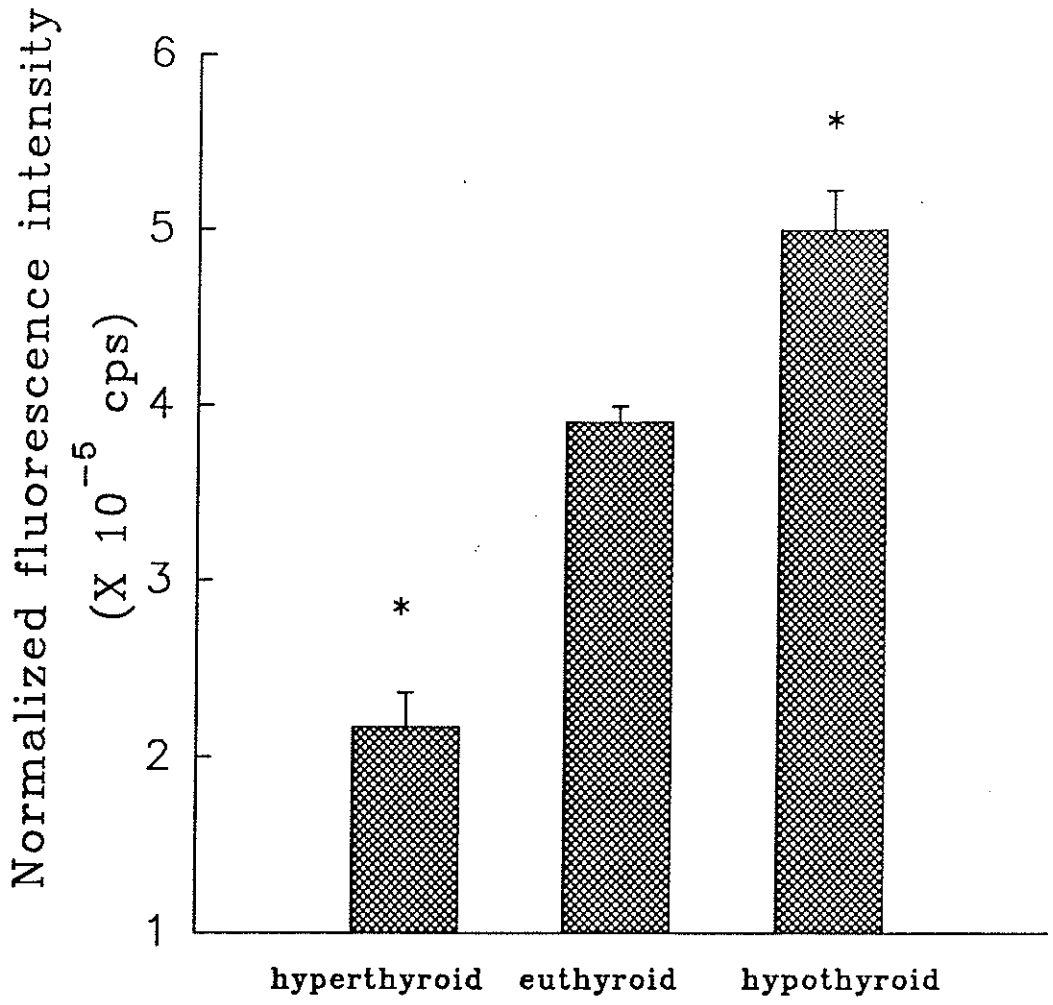

Figure 2.14 Relative immunofluorescence intensity of bFGF in cardiac sections from hypothyroid, euthyroid and hyperthyroid rats. Background fluorescence (obtained from slides reacted with non-immune serum) was subtracted and data were presented as mean $\pm$ SEM of 5 sections.

* $P < 0.01$ compare to euthyroid

changed cardiomyocytes morphology or contractility. Cell extracts from two 100-mm confluent culture dishes were analyzed for their bFGF content by immunoblotting of heparin-bound fractions. Results are shown in Figure 2.15. Lane 1 contains 5 ng recombinant bFGF used as positive control. The bFGF from control cell extract (lane 2) was composed of equal amount of 21.5 kDa bFGF and 18 kDa bFGF, while trace amount of bFGF lower than 18 kDa were also present. Cells treated with 10 nM T3 for 24 hours (lane 3) contained primarily the 18 kDa bFGF and increased amount of lower molecular weight bFGF. These experiments were repeated three times. In each case, treatment of the cells with T3 reduced the percentage of 21.5-23 kDa bFGF over total bFGF by 20-30% (25.3 ± 4.1) compared to controls. This difference was statistically significant ($p<0.025$, paired t-test).

2.3.6 bFGF in cardiac muscle of control and mdx mice (This work was done in collaboration with Dr. J. Anderson, Department of Anatomy, Unversity of Manitoba)

The distribution of bFGF in normal mouse cardiac muscle was identical to that of rat. Briefly, bFGF was localized pericellularly and in association with myocyte intercalated discs, as well as nuclei of muscle and non-muscle origin (Anderson, Liu and Kardami, 1991).

Groups of myocytes with intense cytoplasmic anti-bFGF staining were observed in sections of mdx cardiac muscle (Fig.2.16, arrows). These myocytes observed under

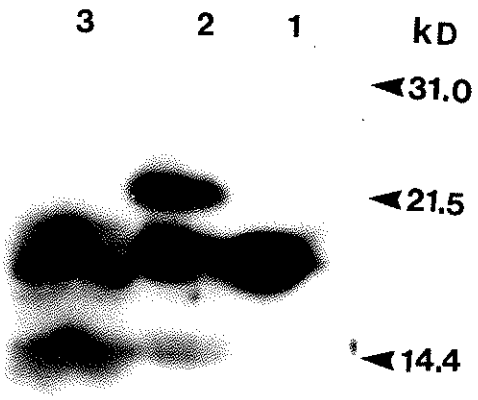

Figure 2.15. Effect of thyroid hormone on bFGF accumulation of cultured chick myocytes. Western blotting for bFGF. Cardiac myocytes from 7-day chick embryos were maintained for 4 days in 4% thyroid-hormone-depleted fetal calf serum. Thyroid hormone (10 nM) was then added to one half of the cultures. Basic FGF was analyzed in control and treated cultures 48 hour after T3 addition. Lane *1*: Recombinant human bFGF (5 ng) used as a positive control. Lane *2*: Heparin-binding peptides from control cell lysate. Lane *3*: Heparin-binding peptides from T3-treated cell lysate.

phase contrast optics and low magnification exhibited a clearly different texture (Figure 2.16b, arrows) compared to adjacent fibers (Fig.2.16b, arrowheads). Intense cellular infiltration associated with these areas was indicated by nuclear counterstaining (Fig.2.16c, arrows), and confirmed that they represented regions of active degeneration (Bridges, 1986). Another staining pattern frequently encountered in sections from mdx but not normal ventricles was associated with areas of complete myocyte loss. Myocytes in these areas were fully replaced by small, single cells (Fig.2.16d, crossed arrow), assumed to be inflammatory cells or fibroblasts in scar tissue. Intact myocytes adjacent to these scars exhibited increased nuclear anti-bFGF staining (Fig.2.16d, curved arrows) compared to myocytes located further away (Fig.2.16d, straight arrows).

Basic FGF levels in extracts from mdx and control cardiac and fast-twitch skeletal muscles (2.5 ml, 8 mg/ml each) were determined by SDS-PAGE and immunoblotting. Mdx cardiac muscles (Fig.2.17, lane1) had greater levels of immunoreactive bFGF (about 2-fold) compared to control heart extract (Fig.2.17, land 2). Cardiac extracts from either mdx or control tissue had higher bFGF levels than their fast-twitch skeletal muscle counterparts (Fig.2.17, compare lanes 1 and 2 with lanes 3 and 4, respectively). Table 2.2 provides a comparison of the relative density measurements of the autoradiogram bands corresponding to all immunoreactive bFGF peptides in extracts from normal and mdx striated muscles. Cardiac muscles from both mdx and control appeared to contain the 21 kDa as well as the 18 kDa bFGF.

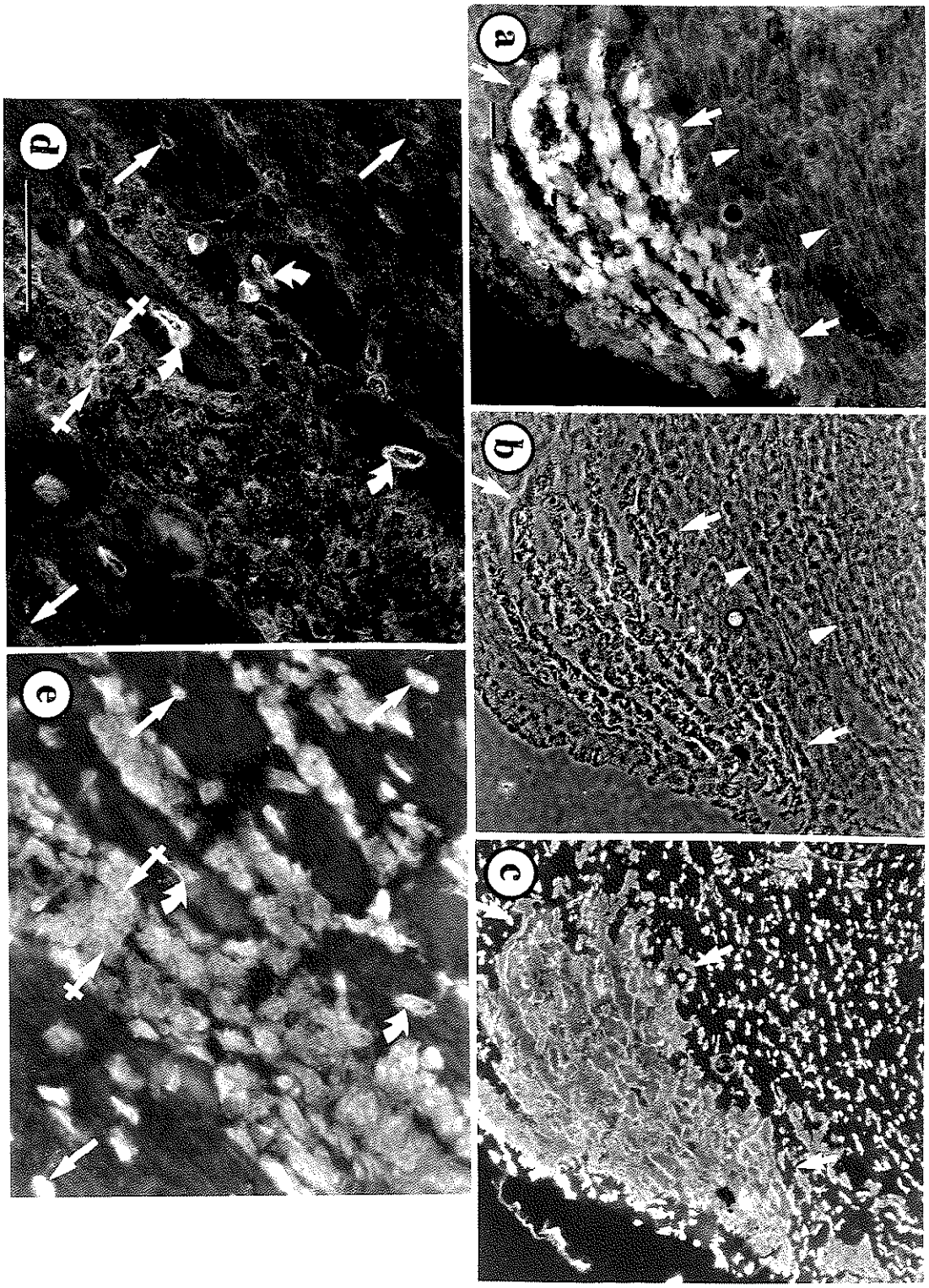

Fig. 2.16
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Figure 2.16. Location of bFGF in cardiac sections from mdx mouse. (a) Indirect immunofluorescence for bFGF. (b) Phase-contrast view of the field shown in a. (c) DNA staining for nuclei. (d,e) Double fluorescence for (d), bFGF, and (e), nuclei. Arrows in a,b, and c point to degenerating myocytes. Arrowheads in a and b point to normal myocytes. Large curved arrows in c point to normal nuclei. Crossed arrows in d and e indicate an area of scar. Small curved arrows in d and e point to the nuclei of myocytes adjacent to the scar which label intensely of bFGF. Straight arrows point to the nuclei of myocytes located some distance from the scar. (bar = 50 μm)

Distribution of bFGF in cardiac sections from T3-treated mdx mice was similar as that of untreated ones (Fig.2.18c), though the general intensity of staining was reduced (Fig.2.18b). T3 treatment of mdx mice resulted in more frequent and larger areas of intense intracellular staining from young (5-week-old, Fig. 2.18d) and older (10-week-old, Fig. 2.18e) mdx mice, indicating an increase in the recent fiber damage in T3-treated mdx heart tissue. Histological morphometry has shown that cardiac tissue in T3-treated mdx mice exhibited a dramatic increase in the number and size of lesions in young animal, and large areas of inflammation and scarring in older animals, compared to the untreated mdx mice (Anderson *et al.*, in press).

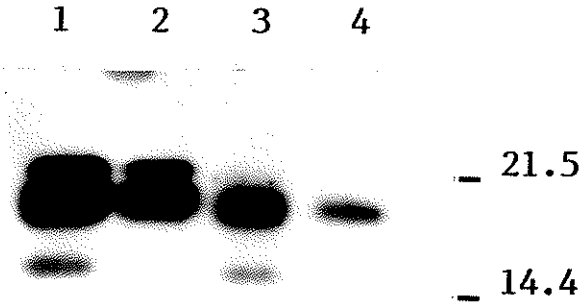

Figure 2.17. Basic FGF content in mdx and normal cardiac and skeletal muscle. Lanes *1-4* contain heparin-binding peptides present in 2.5 ml of extract from mdx cardiac tissue (lane *1*), control cardiac tissue (lane *2*), mdx fast-twitch skeletal muscle (lane *3*), and control fact-twitch muscle (lane *4*). Relative migration of molecular weight markers is indicated in kD.

Table 2.2. Normalized densitometric values corresponding to bFGF bands present in equivalent amounts of muscle extract.

	control mice	mdx mice
Fast-twitch skeletal muscle	1.0	2.5
Slow-twitch skeletal muscle	5.3	6.0
Cardiac muscle	4.3	8.0

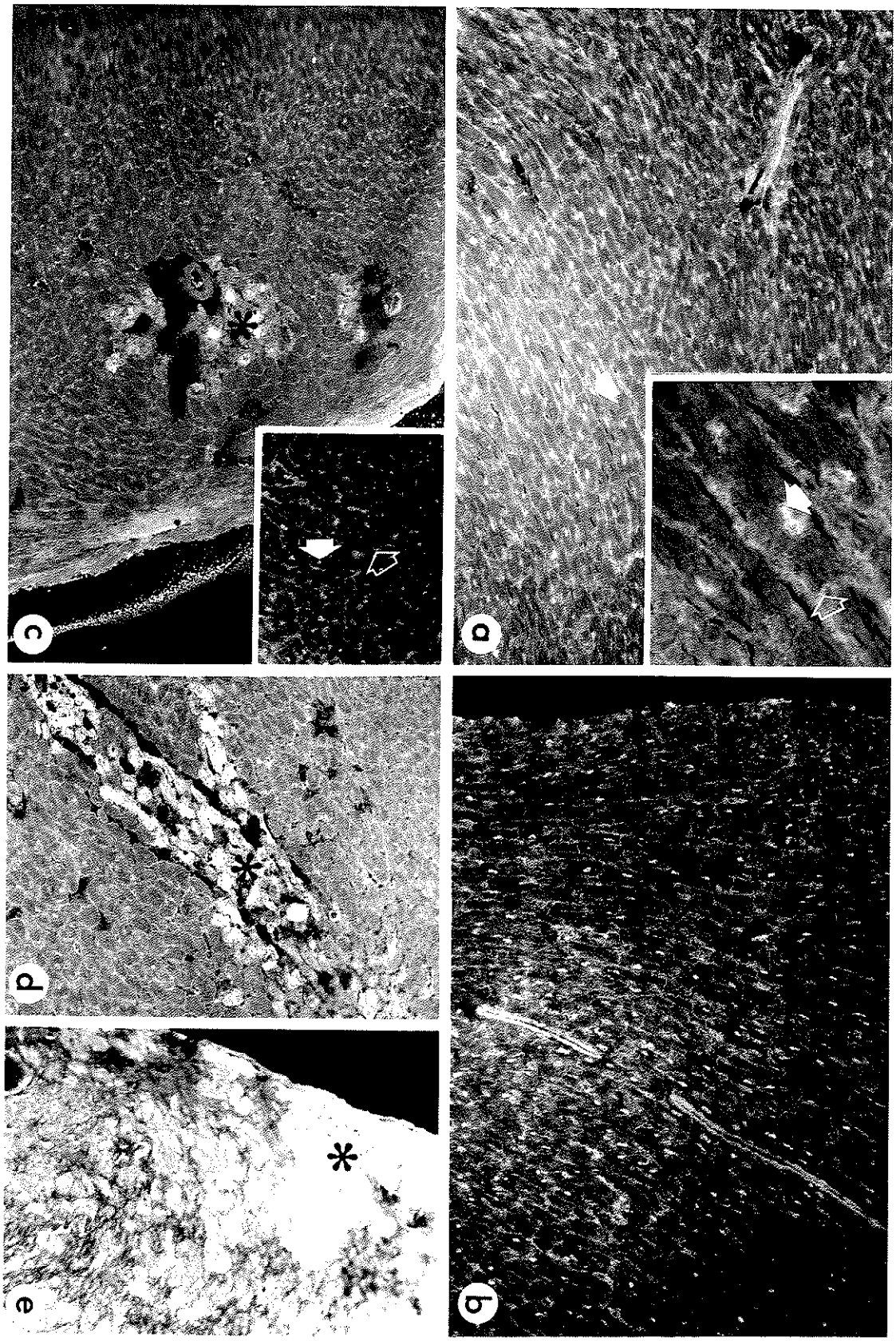

Fig. 2.18
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Figure 2.18 Immunofluorescence for bFGF in myocardium from control (a,b) and mdx (c-e) mice. b,d,e, T3-treated 5-week-old (b,d) and 10-week-old (e) mice. a and c, untreated 5-week-old mouse. Double arrows point to intercalated discs, solid arrows, central nuclei and Asterisk, foci of damaged myofibers.

2.4 DISCUSSION

2.4.1 Technical considerations

2.4.1.1 Identification of bFGF

The 18-22.5 kDa peptides in rat tissue fractions are identified as cardiac bFGFs based on the following: First, they are recognized by several different preparations of bFGF-specific antibodies, raised against N-terminal (residues 1-24), internal (residues 106-120) and C-terminal (residues 139-146) domains of bFGF (Doble *et al.* 1990, and unpublished observations of our laboratory). Antibodies raised by several groups against residues 1-24 of the truncated, 146 amino acid bovine bFGF are highly specific for bFGF (Gonzalez *et al.* 1990, Kardami *et al.* 1990, Kardami *et al.* 1991, Riss and Sirbasku 1989), as expected, since this region has negligible sequence homology with the other members of the FGF family of growth factors (FGF-1,-3,-4,-5,-6,-7) (Coulier *et al.* 1991). Secondly, they bind to heparin-sepharose with affinity similar to that of bFGF, i.e. they remain bound to heparin at 1.1 M NaCl and start eluting at 1.5 M NaCl. Thirdly, they are in the anticipated range of molecular mass for bFGF (Basilico and Moscatelli, 1992). Finally, heparin-purified peptides from either neonatal (predominately 22 kDa bFGF) or adult (predominantly 18 kDa bFGF) are mitogenic (Kardami *et al.*, 1993).

2.4.1.2 Localization of bFGF by immunofluorescence

Basic FGF is believed to act locally, in a paracrine or autocrine manner.

Localization therefore of bFGF in tissue sections or cultured cells by immunofluorescence is an approach increasingly favored by many investigators, providing evidence for potential cellular sites of synthesis, storage as well as action for this factor. Unfractionated anti-bFGF IS2 as well as affinity-purified anti-bFGF IgG used here for immunolocalization of bFGF have been shown to be specific for bFGF in several previous studies (Kardami and Fandrich, 1989; Kardami *et al.*, 1990; Kardami, 1990b; Cattini *et al.*, 1991). IS2 was raised against the N-terminal region of bFGF [residues 1-24] of the 146 amino acid form (Esch *et al.*, 1985). This region of the molecule is available for antibody interaction even when bFGF is complexed with molecules of the extracellular area of cardiomyocytes such as heparin-like components (Kardami *et al.*, 1990). These studies indicated that significant epitope masking does not normally occur in that region of bFGF and were confirmed further by 3-D reconstruction of bFGF sequence showing that the N-terminal region "sticks out" of the bFGF molecular main core which has the heparin-binding, cell attachment and receptor sites (Zhang *et al.*, 1991a). It is unlikely therefore that the increased anti-bFGF staining seen in hypothyroid ventricles or the rat, and necrotic regions of mdx mouse is caused primarily by unmasking of bFGF epitopes. Furthermore, subjecting control heart sections in a variety of proteolytic conditions, an approach used to unmask hidden epitopes, did not result in enhanced cytoplasmic ant-bFGF staining (Padua and Kardami, 1993). It is concluded therefore that differences in immunofluorescence intensity parallel differences in bFGF abundance in tissues and myocytes in culture.

2.4.1.3 Quantitation/Composition of bFGF

To obtain an estimate of bFGF accumulation and composition we have used, (a), two cycles of vigorous tissue extractions followed by, (b), heparin-sepharose affinity fractionation, and, (c), western blotting with specific anti-bFGF serum. The approach used here has been found to produce maximal yield of soluble bFGF from cardiac tissues (Anderson *et al.* 1991, Padua and Kardami, 1993). Relative bFGF yields were comparable to those mentioned here when extraction buffers of higher ionic strength (up to 2.0 M NaCl) were used (Anderson *et al.* 1991). Heparin-sepharose is widely used for the fractionation of FGF-like growth factors from extracts, serving also to protect bFGF from degradation (Rifkin and Moscatelli, 1989; Doble *et al.*, 1990). This protection is partially lost upon elution of bFGF with high salt (Doble *et al.*, 1990). It has been demonstrated that proteases which are attacking the N-terminal of bFGF co-elute with this factor at high salt (Doble *et al.*, 1990). According to these data, while the overall yield in bFGF may be comparable, the composition in bFGF species may differ in different preparations; bFGF appears in 18 kDa or 18-25 kDa forms, depending on whether protease inhibitors are absent or present, respectively, in the elution step. We have therefore made extensive use of protease inhibitors at all steps of the preparation, ensuring a more realistic estimate of bFGF yield as well as composition. Storage of extracts or fractions was avoided. To maximize yield, heparin-binding proteins were eluted directly by boiling the heparin-sepharose beads in standard electrophoresis buffer and processed immediately for immunoblotting. As mentioned, antibodies used in our blots were specific and highly sensitive for bFGF.

This made possible speedy analysis of the bFGF content of relatively small amounts of heart extracts. Our method of determination does not detect insoluble or non-heparin binding (presumably inactive or denatured) bFGF. We have however found that, in the mouse, determination of comparative bFGF levels in total tissue homogenate gave the same results as those obtained by the extraction procedure described here (Anderson *et al.* 1991). Extraction of hearts from hypo-, hyper- and eu-thyroid animals produced comparable protein yields (42 ± 2 mg/g wet tissue), suggesting that the differences in bFGF yield detected are not an obvious consequence of different overall extractability.

2.4.2 Localization of bFGF in cultured cardiomyocytes

Localization of bFGF in cardiac myocytes was examined using immunofluorescence. As discussed previously, IS2 used here is highly specific for bFGF *in vitro* (Kardami and Fandrich, 1989; Kardami *et al.*, 1990). A total loss of bFGF staining in chick myocytes was observed when IS2 was absorbed with immobilized recombinant bFGF (Kardami *et al.*, 1991). It is impossible to completely eliminate the possibility that IS2 may recognize bFGF-like epitopes present in an otherwise unrelated protein in those experiments. However, the fact that IS2 recognizes proteins in the same molecular weight range and having similar biological (heparin affinity, mitotic activity) properties as bFGF in extracts of those cultures argues against this suggestion.

In near confluent, proliferative chick cardiomyocytes strong anti-bFGF staining was observed in a fraction of interphase cardiomyocyte nuclei. Nuclei staining brightly for bFGF, i.e. B nuclei, tended to be larger than those which either did not stain at all or stained very weakly. It was assumed that larger nuclear size within a homogenous cell population likely reflects increased DNA content. Increased DNA content can be expected in nuclei actively synthesizing DNA (i.e., in the S-phase) or in the G2-phase (preceding mitosis). It can also be expected in cases of multiploidy, a phenomenon encountered usually in older cells. Since myocytes used in our studies are still at an early developmental stage, it is likely that increased DNA content would reflect cells in S- or G2-phase. It has been shown that bFGF was translocated into the nucleus, in late G1-phase, in bovine aortic endothelial cells (Baldin *et al.*, 1990) and ovine fetal chondrocytes (Hill and Logan, 1992). Basic FGF accumulated only in the nucleus of exponentially growing cells (Baldin *et al.*, 1990), and increased nuclear bFGF has been reported for proliferative compared to growth arrested cells (Cattini *et al.*, 1991). We therefore propose that IS2 detected increases in nuclear bFGF in myocytes that are actively synthesizing DNA and are about to undergo mitotic division. If this is true, myocytes cultured from younger hearts should contain more B nuclei, because cardiomyocytes at early development stages are considered more proliferative than those at later development stages (Zak, 1984). In agreement, we found that the percentage of B nuclei was 26% in cultures from 5-day old hearts compared to 10% in those from 7-day old hearts.

We also compared the pattern of nuclear bFGF distribution between interphase and prophase myocytes. In all myocytes in prophase a strong punctate anti-bFGF staining was discerned, similar in intensity and overall shape to the pattern of B interphase nuclei. This in agreement with our suggestion that the B nuclei belong to cells traversing the S- or G2-phase of the cell cycle and are about to enter prophase.

Although nuclear localization of bFGF is well established, its exact role in the nucleus is far from clear. Basic FGF was shown to associate with chromatin (Brigstock *et al.*, 1991), possibly binding to specific DNA sequences. It has been reported that bFGF can bind to nucleotides like ATP and GTP (Baird 1993). Added to nuclei isolated from G1-arrested cells, bFGF stimulated r-DNA transcription (Bouche *et al.*, 1987). It has also been shown to directly affect gene transcription in a cell-free system (Nakanishi *et al.*, 1992).

Most previous studies have examined bFGF localization in interphase nuclei. Nothing is known about what happens to bFGF during mitosis. Studies therefore presented here have examined the fate of bFGF during the various stages of the mitotic cycle. We found that the localization of bFGF changed during mitosis of chicken cells from being closely associated with chromosomes in prophase, prometaphase and metaphase, to a complete dissociation from the chromosomes and localization to the midbody of the cytoplasm during anaphase and telophase. Several nuclear proteins have been shown to transfer to the spindle during anaphase, in a manner similar to that

of anti-bFGF staining reported here. These include the INCENPs (Cooke *et al.*, 1987), an Mr. 38 kDa protein identified by human antibodies (Kingwell *et al.*, 1987), an Mr 140 kDa and 153 kDa doublet identified with monoclonal antibodies (Pankov *et al.*, 1990), CENP-E (Yen *et al.*, 1991), a 250 kDa centromere protein (Compton *et al.*, 1991) and MSA-36 (Rattner *et al.*, 1992). Two members of those proteins, the INCENPs and MSA-36 showed identical localization as bFGF detected by IS2 here. The INCENP antibodies were raised against chicken bulk proteins of the mitotic chromosome scaffold fraction, and detected two inner centromere proteins INCENP A (155 kDa) and INCENP B (135 kDa, Earnshaw and Cooke, 1991). MSA-36 is a Mr. 36 kDa spindle-associated protein, recognized by human autoantibodies (Rattner *et al.*, 1992). They were detected in the nucleus in the G2-phase, along chromosomes during prophase, metaphase, separated from chromosome during late stage of mitosis, persisted during the furrowing process being confined to the midbodies. It is interesting that these autoantibodies recognized peptides of a similar size to bFGF, i.e. 18-20 kDa in addition to the 36 kDa protein, in immunoblots of isolated nuclei, metaphase chromosomes, spindle and midbodies (Rattner *et al.*, 1992). We cannot therefore exclude the possibility that MSA-36 recognized bFGF-like proteins, and that the 36 kDa antigen is actually a bFGF dimer. Our unpublished data did show that IS2 can recognize the 36 kDa dimmer of human recombinant bFGF in Western blot analysis. Similar immunostaining patterns for bFGF and the INCENPs or MSA-36 would imply that these proteins have similar localization and, by implication, function during mitosis. An alternative explanation of the staining patterns could be that the

IS2 serum somehow cross-reacts with epitopes on the INCENPs. This is unlikely, since the sequence of the bFGF peptide against which our antibodies were made shares no similarity with that of the INCENPs (Drs. Mackie and Earnshaw, personal communication). Several possible *in vivo* roles for the above mentioned cell-cycle dependent proteins have been suggested: regulation of sister chromatic pairing (Earnshaw and Cooke, 1991), chromosomal movement, structural contribution to the anaphase spindle and cleavage furrow (Earnshaw and Bernat, 1991). Basic FGF, or immunologically-related peptide(s) therefore may play similar roles in the nucleus. The dissociation of anti-bFGF staining from chromosomes during anaphase, and its corresponding deposition at the midbody implies a possible function during cytokinesis. Localization of bFGF at metaphase may be important for the following stage. It is known that the furrowing of the plasma membrane invariably occurs in the plane of the metaphase plate, at right angles to the mitotic spindle (Albert *et al.*, 1989). According to our data, bFGF moves with the chromosomes to the metaphase plate and is released from chromosomes at the metaphase to anaphase transition. Thus released bFGF could serve to mark the position of the metaphase plate once chromosomes move away from it during anaphase.

The M phase of a cell cycle is composed of karyokinesis, in which duplicate chromosomes are pulled apart into two equal sets, and cytokinesis, in which the cells itself splits into two daughter cells, each receiving one of the two sets of chromosomes. Cell division is not completed until the finishing of cytokinesis.

During the developmental transition from hyperplastic (increase in cell number) to hypertrophic growth (increase in cell size), cardiomyocytes lose their ability for cytokinesis (Zak, 1973) before they lose the ability for karyokineses. This indicates that cytokinesis plays a crucial role in the proliferation of cardiomyocytes during development. Nothing is known about what regulates cytokinesis of cardiac myocytes. Identification of bFGF or bFGF-like molecules in the areas of cleavage furrow and the midbody suggests a role in this process. It would be important to examine the possibility that bFGF regulates cytokinesis and hence cell division, since this may provide a clue for inducing myocyte regeneration.

The cell cycle dependent anti-bFGF staining seen in cardiomyocytes from chicken was not observed in cardiomyocyte cultures from rat. In the rat, IS2 serum stained nuclei relatively uniformly, in either proliferative or differentiated stages. In addition, bFGF appeared to have a distinct perinuclear localization, resembling that of the Golgi apparatus and the endoplasmic reticulum (McMorrow *et al.*, 1990; Albert *et al.*, 1989); this perinuclear localization was not seen in the chick. Cytoplasmic staining in apparent association with myofibrilla Z-lines was detected in myocytes from both species. No changes in anti-bFGF staining were observed in the rat during mitosis, neither any staining of the cleavage furrow or the midbody regions. These differences in anti-bFGF staining between chick and rat myocytes may represent genuine, species-related differences in bFGF localization, and by inference, function. It is unlikely that they can be accounted for by differences between rat and chick bFGF, since their

respective cDNAs are 90.5% homologous (Kurokawa *et al.*, 1988; Shimasaki *et al.*, 1988; Zùñiga *et al.*, 1993). The [1-24]bFGF peptide against which IS2 was raised is also highly conserved amongst species, displaying 92% homology between chicken and rat. Finally, IS2 cross-reacts strongly with chick as well as rat bFGF (Kardami and Fandrich, 1989; Kardami *et al.*, 1990). It is also unlikely that the movement of protein(s) from the chromosomes to the midbody is a phenomenon uniquely encountered in chick, since it has been reported for antigen MSA-36 in human cells (Rattner *et al.*, 1992), indicating that similar mitotic mechanisms exist between the two species. An alternative explanation for these differences in bFGF localization between chick and rat is that different sets of bFGF epitopes are exposed or masked in the two species, resulting in different localization patterns. Finally the possibility that IS2 recognizes related albeit distinct protein(s) in the two species, sharing bFGF epitopes, cannot be excluded at this point. Detailed biochemical analysis of the structures involved in the various cellular sites would be a formidable task, but the only way to obtain conclusive evidence about these issues.

2.4.3 Thyroid hormone and bFGF

Ventricular myocytes of adult mammals are considered to be terminally differentiated cells, incapable of proliferation (Zak, 1984). However, increasing evidence indicates that this depends on external factors rather than a rigid intrinsic program. Basic FGF and thyroid hormone are two compelling candidates for the regulation of proliferation and differentiation of cardiac myocytes. Thyroid hormone

regulates cardiac growth and muscle gene expression. In rat cardiac ventricles, hypothyroidism induces a shift toward the fetal isomyosin (V3, Schwartz *et al.*, 1982; Chizzonite and Zak, 1984). Higher thyroid hormone levels stimulate the expression of adult isomyosin, i.e., myosin V1, (Mahdavi *et al.*, 1987; Lompré *et al.*, 1984; Gustafson *et al.*, 1986; Morkin *et al.*, 1983), as well as other muscle specific genes, such as cardiac sarcoplasmic reticular Ca^{2+} ATPase (Schwartz *et al.*, 1992; Dillmann, 1990; Nagai *et al.*, 1989; Rohrer and Dillmann, 1988). In contrast to thyroid hormone, bFGF promotes the fetal program of muscle gene expression, by favoring expression of myosin isoform V3, α-skeletal actin (SkA), α-smooth muscle actin (SmA) while inhibiting expression of myosin isoform V1, α-cardiac actin and Ca^{2+} ATPase (Parker *et al.*, 1990). Thyroid hormone levels in the blood are developmentally regulated, increasing dramatically soon after birth (Dubois and Dussault, 1977; D'Albis *et al.*, 1987), in a manner which parallels the transition of cardiomyocytes from proliferative to hypertrophic growth (Club and Bishop, 1984). The abundance, composition and localization of bFGF are also developmentally regulated (see Chapter 1). To understand the role of bFGF and thyroid hormone in cardiac myocyte growth, we studied their effect on DNA synthesis of cultured rat cardiomyocytes. In addition we examined regulation of bFGF expression by thyroid hormone.

2.4.3.1 Effect of bFGF and T3 on myocyte DNA synthesis

The ability of cardiac myocytes for DNA synthesis and cell division decreases rapidly after a week in culture, in a manner resembling developmental changes *in vivo*

(Bogenman and Eppenberger, 1980). In agreement, only 13% of rat myocytes could synthesize DNA after 5 days in cultures, in our system. However, cardiomyocytes cultured with exogenous bFGF retained to a considerable degree (35.3%) their ability for DNA synthesis after 5 days in culture. These data suggest that bFGF prevented the differentiation of cardiomyocytes into a post-mitotic phenotype. T3 significantly inhibited the bFGF-induced prolongation of DNA synthesis by myocytes. It is possible that the increased thyroid hormone levels in development affect the ability for DNA synthesis ability of cardiac myocytes *in vivo*. Considering the effect of thyroid hormone on cardiac muscle gene expression and on DNA synthesis, it is reasonable to say that thyroid hormone plays a impotant role in promoting terminal differentiation of cardiomyocytes, and that autocrine and paracrine bFGF may be one of the targets of thyroid hormone regulation.

2.4.3.2 Basic FGF as a function of thyroid status, in the heart

While all cardiac extracts induced increases in LI for ventricular myocytes, this increase was highest in the case of extracts from hypothyroid rats. Since the extracts were dialyzed before being added to the cell culture, excess thyroid hormone should not be responsible for the reduced stimulating effect of extracts from hyperthyroid rats. It has been shown that bFGF is responsible for cardiac extract induced stimulation on cardiac myocytes (Kardami, 1990; Kardami and Fandrich, 1989), so one possible explanation for the stronger stimulating effect of extract from hypothyroid ventricles is that the bFGF content or composition is changed in the ventricles of those animals.

We therefore examined bFGF abundance in euthyroid, hyperthyroid and hypothyroid rats. Furthermore, since neonatal rat myocardium , which is physiologically hypothyroid (Dubois and Dussault 1977), has relatively high levels of 21.5-22 kDa bFGF (Liu *et al.*, 1993; Kardami *et al.*, 1993), we also investigated the possibility that thyroid hormone affects bFGF composition, i.e. that low thyroid hormone levels favor expression of the 21.5-22 kDa bFGF in the heart. Adult rats were successfully rendered hypothyroid and hyperthyroid according to established procedures. Age-matched euthyroid animals were used as controls. In agreement with our hypothesis, hypothyroid rat heart ventricles consistently displayed a clear increase in relative as well as absolute levels of 21.5-22 kDa bFGF, which constituted approximately half of all bFGF, compared to about 10% in euthyroid controls.

It has been shown that 21.5-22 kDa rat bFGFs are the result of translation from CUG start sites found upstream of the conventional AUG start site (Florkiewicz *et al.*, 1989); the latter produces the 18 kDa species (Powell and Klagsbrun 1991, Pasumarthi *et al.* 1992). It is concluded that the 21.5-22 kDa forms detected here represent these N-extended, CUG-initiated, rat bFGFs with which they share identical electrophoretic mobility and immunoreactivity (Pasumarthi *et al.* 1992). The 18 kDa form of rat bFGF may result either from direct translation from the AUG initiation codon, or from limited proteolysis of the CUG-initiated forms. Conversion of cardiac 21.5-22 kDa rat peptides to an 18 kDa form by proteolysis, as shown here, is consistent with such a precursor-product relationship. It is not likely that the 21.5-22 kDa peptides represent

post-translational modifications and/or aggregation of the 18 kDa bFGF. Phosphorylation does not alter bFGF mobility (Feige *et al.*, 1991) while bFGF aggregates migrate with a mobility of over 30 kDa (Kardami *et al.*, 1991a). It is theoretically possible that the 21.5-22 kDa peptides in rat tissues are very closely related to, but not CUG-initiated forms of, rat bFGF. Amino acid sequencing is required to provide conclusive proof for their identity.

An increase in overall bFGF in cardiac ventricles of hypothyroid animals was indicated not only by immunoblot analysis of extracts but also by immunolocalization of bFGF and spectrofluorometric measurement of the fluorescence intensity in tissue sections. The increase may reflect increases in bFGF synthesis or accumulation (decreased degradation). Increased epitope accessibility may also contribute to increased immunostaining.

Several investigators have shown that while the 21-24 kDa human bFGFs are localized in the nucleus and the ribosomal fraction of cells, the 18 kDa bFGF is primarily cytosolic but can also be found in the nucleus (Renko *et al.*, 1990, Quarto *et al.* 1991a, Florkiewicz *et al.* 1991). Based on these studies it is reasonable to hypothesize that the levels of 18 kDa or 21.5-22 kDa bFGF represent contributions mainly from the cytosolic or nuclear fractions of cardiac cells, respectively. Cardiac myocytes of the adult, euthyroid (or hyperthyroid) rat have a high cytoplasm-to-nucleus ratio and comprise at least 70% of the heart mass (Nag 1980). A cytosolic localization

for the 18 kDa bFGF (Pasumarthi *et al.* 1992) therefore would lead to the prediction of increased relative levels for this species in extracts from adult, euthyroid (and hyperthyroid) rat hearts, and decreased relative levels in hearts with smaller myocytes, such as those from newborn or hypothyroid rats, in agreement with our findings.

The purified anti-bFGF antibodies used here localize this factor in cardiac intercalated discs (Kardami *et al.* 1991a,b). Because of smaller myocyte size, density of intercalated discs would be increased per unit area of hypothyroid (compared to eu- and hyperthyroid) ventricles, resulting in overall increased anti-bFGF staining of sections, as indeed was observed. In addition, the intercalated disc areas of hypothyroid animals appeared on the whole to stain stronger than equivalent areas in euthyroid or hypothyroid hearts, suggestive of increased local presence for bFGF, and raising the possibility of involvement of this factor with any changes in intercellular communication which might occur in the ventricles of hypothyroid animals.

2.4.3.3 Regulation of bFGF accumulation in other tissues

Different organs were found to have different ratios of the 18 and 21.5-22 kDa bFGF. The effect of thyroid status on the relative accumulation of the various bFGF forms (R_{hi}) was also found to be organ-dependent. Based on data from three sets of experiments for atria and brain, two sets of experiments for liver, kidney, skeletal muscle and aorta, three kinds of responses were observed: (i). Brain or spleen displayed consistently high R_{hi}, independently of thyroid status. (ii) Hypothyroidism

induced an increase in R_{hi} in all striated muscles including cardiac atria and skeletal muscles. Hypothyroidism also increased R_{hi} in aorta, liver and kidney. (iii). Hyperthyroidism induced an increase in R_{hi}, for aorta but also for liver and kidney.

Thyroid hormone is involved in a complex array of developmental and physiological responses in many tissues of high vertebrates (Oppenheimer, 1990). As for the multiple, yet tissue-specific thyroid hormone responses, the mechanism underlying the complicated regulation pattern of R_{hi} by thyroid hormone is not clear. Structurally and functionally distinct thyroid hormone receptors (Thompson *et al.*, 1987; Hodin *et al.*, 1989; Glass and Holloway, 1990) may be one of the explanation.

2.4.3.4 Thyroid hormone regulates bFGF composition of cardiomyocyte cultures

Thyroid hormone is a multifunctional hormone, affecting the heart through direct and indirect pathways. It affects growth, differentiation and function of the heart by directly regulating gene expression and membrane transportation (Dillmann, 1990; Segal, 1990) and regulating adrenergic activity (Beekman *et al.*, 1989; Limas and Limas, 1987; Hohl *et al.*, 1989), muscle metabolism and hence hemodynamics (Klein, 1990). To examine whether the regulation of bFGF composition observed in myocardium is through a direct effect on the myocytes, we investigated the effect of T3 on bFGF accumulation of cultured chick myocytes. Myocytes were cultured in media containing thyroid-hormone-depleted serum to simulate a hypothyroid condition. Thyroid hormone-treated cultures displayed a significant reduction in the percentage

and absolute amount of 21.5-22 kDa bFGF compared to cultures maintained at low thyroid hormone levels. These data showed a direct effect of thyroid hormone on the relative accumulation of 21.5-22 kDa and 18 kDa bFGF by chick cardiac myocytes in culture and suggest that a similar effect is operating *in vivo*.

We have been unable to extract sufficient bFGF for comparative quantitative analysis from rat cardiomyocyte cultures. The experiments were repeated three times using different extraction and cell lysis techniques such as freezing and thawing, high salt concentration and sonication. We also increased the overall amount of cell extract used for bFGF fractionation by heparin-sepharose chromatography. It is possible that in these cultures bFGF becomes tightly bound to membranous cellular structures (Golgi apparatus or endoplasmic reticulum, as indicated by immunolocalization) and is not sufficiently solubilized. It is not likely that cultured rat myocytes do not express bFGF, since it has been detected by immunofluorescene of these cells using several antibodies against different residues of bFGF (Fig 2.5 a,b; Pasumarthi, *et al.*, 1992; Speir *et al.*, 1992). No report has yet been made on quantitation of bFGF in cultured rat myocytes by SDS-PAGE.

The functional significance of the changes in bFGF composition with thyroid status in the heart is not clear at present since the actual role of bFGF in the adult organs is not established. Basic FGF is primarily an intracellular molecule which may exert some of its effects in an "intracrine" fashion (Logan 1990; Sherman *et al.*, 1993)

without exiting the cell, through intracellular associations. In this respect different physiological roles may be envisaged for the 18 or 21.5-22 kDa bFGFs. The N-extension bFGF confers a nuclear localization signal (Quarto *et al.*, 1991a) and HMW bFGFs have been shown preferably located in the nuclei compare to the primarily cytosolic localization of 18 kDa bFGF (Renko *et al.*, 1990; Bristock *et al.*, 1991; Florkiewicz *et al.*, 1991). As we discussed in paragraph 1.3.1.3, increased nuclear bFGF localization is related to increased cell proliferation and replication/transcription function.

2.4.3.5 Increased bFGF in mdx cardiac muscle

Numerous studies have shown bFGF to be a potent chemoattractant for endothelial cells and fibroblasts, a mitogen for many cell types, including cardiac myocytes and non-muscle cells (Kardami, 1990a; Rifkin and Moscatelli, 1989), to affect differentiation and to protect cells from degeneration (Westerman *et al.*, 1990; LaVail *et al.*, 1991). All these injury-repair response related properties indicate that bFGF might participate in this process, and as reviewed in Chapter 1, bFGF does play a crucial role in regeneration and wound healing of many tissues. To investigate the role of bFGF in cardiac injury-repair-associated responses, the distribution and relative levels of bFGF were examined in the myocardium of dystrophic mice. An intense cytoplasmic anti-bFGF immunolabeling was noted in degenerating mdx cardiac tissues compared to non-degenerating fibers. Nuclear bFGF accumulation was clearly enhanced in surviving cardiomyocytes adjacent to the degenerating or necrotic foci in

the mdx ventricles. As discussed previously, increases in nuclear bFGF have been detected in normal cells in the G1 phase shortly before entering S phase of the cell cycle (Baldin *et al.*, 1990), in cultured embryonic chicken heart cells or human tumor cells which are about to undergo mitosis (Kardami *et al.*, 1991, Cattini *et al.*, 1991) as well as in rat cardiomyocytes adjacent to experimentally-induced cardiac injury (Padua and Kardami, 1993). It is likely that cardiac myocytes attempt to regenerate after injury, and that bFGF plays a favorable role in this context. T3 treated mdx hearts exhibited a general lower level of anti-bFGF immunolabeling, in agreement with the data obtained from rat (Anderson, Liu and Kardami, 1993). A dramatic increase in the number and size of lesions and scars in T3 treated mdx hearts might be the result of interference of T3 with the function and availability of bFGF in the mice. Thus, lower thyroid hormone level might be helpful in repairing cardiac damages by favoring the function and accumulation of bFGF. Interestingly, a brief hypothyroidism did appear in a patient following a heart attack (Ohyama *et al.*, 1987; Wiersinga *et al.*, 1981).

2.4.3.6 Possible mechanisms for the regulation of bFGF activity and accumulation by thyroid hormone

To understand how thyroid hormone can interfere with bFGF function, we should first know the signal transduction pathway for bFGF. Unfortunately, the signal transduction mechanism for bFGF remain as yet largely unknown. We have tried to piece the available evidences together: It seems that intracellular and extracellular bFGF are using overall different mechanisms in signal transduction. Extracellular

bFGF can mediate cellular responses by binding and activation of specific cell surface receptors (FGFR, Korhonen *et al.*, 1992). A low-affinity receptor, i.e., heparan sulfate proteoglycan, is a prerequisite for bFGF binding to the high-affinity tyrosine kinase receptor (Yayon *et al.*, 1991; Rapraeger *et al.*, 1991). FGFRs belong to the receptor tyrosine kinase (RTK) subclass IV (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990) and binding of bFGF to the high affinity receptors leads to activation of FGFR tyrosine kinase activity and receptor autophosphorylation (Huang and Huang, 1986; Coughlin *et al.*, 1988; Mansukhani *et al.*, 1990). Activation of the receptor tyrosine kinase also leads to increased tyrosine phosphorylation of a number of cellular proteins, including phospholipase C-γ1 (PLC-γ1, Jaye *et al.*, 1992; Bottaro *et al.*, 1990; Burgess *et al.*, 1990)). PLC-γ1 catalyzes the breakdown of the membrane phospholipid, phosphatidylinostitol biphosphate to inositol 1,4,5-triphophate (IP3) and diacylglycerol (DAG). These second messengers release calcium from intracellular stores, and activate protein kinase C (PKC), respectively (Berridge, 1987; Logan and Logan, 1991; Logan *et al.*, 1990). Transient expression of early response protooncogenes (c-fos and/or c-jun) is triggered by bFGF (Okazaki *et al.*, 1992; Isozaki *et al.*, 1992; Feldman *et al.*, 1992; Nielsen *et al.*, 1991; Hall *et al.*, 1991). This effect might be mediated by PKC since the expression of protooncogenes was stimulated by both bFGF and other PKC activators (Bardoscia *et al.*, 1992; Janet *et al.*, 1992), and this stimulation was inhibited by PKC inhibitors (Okazaki *et al.*, 1992; Bhat *et al.*, 1992). On the other hand, exogenous bFGF, internalized via high affinity FGFR or HSPG (Gannoun-Zaki *et al.*, 1991), either translocated to the nuclei, or remained in the

cytosol (Baldin *et al.*, 1990; Hill and Logan, 1993; Hawker and Granger, 1992). The translocation of exogenously added bFGF to the cell nucleus is possibly cell surface receptor dependent, since the nuclear localization of another member of the FGF family, FGF-1, through the exogenous pathway could be significantly reduced by suramin, an inhibitor of the interaction of FGF-1 with its receptor (Zhan *et al.*, 1992). Nuclear-binding of bFGF protects it from degradation (Hawker and Granger, 1992). In the nucleus, bFGF may affect DNA replication/transcription as we discussed previously. Endogenous, intracellular bFGF may be transported to the nucleus and/or remain bound in the cytosol and/or be exported to the cell surface, depending on the presence of N-terminal extension. N-terminal extended bFGF (21-24 kDa), which possesses a nuclear translocation sequence (NTS; Quarto *et al.*, 1991a), may be directly translocated to the nuclei where it presumably interacts with DNA and thus play an intracrine role (Rifkin *et al.*, 1991b). The 16-18 kDa bFGF, lacking NTS, may be released somehow into the extracellular space (Rifkin *et al.*, 1991a) and enter nuclei via the pathway for extracellular bFGF. Although there is uncertainty at present as to the link between the two pathways, both nuclear translocation and RTK pathways may be related to cell growth and proliferation.

Thyroid hormones mediate most of their physiological effects by binding to nuclear receptors (T3R; Dillmann, 1990). By binding to nuclear T3R, either positive or negative effects on gene transcription can be generated by the hormone/receptor/DNA complex, depending on the gene and target tissue (De Nayer,

1987). A novel pathway for T3R action through protein/protein interaction with transcription factor AP-1 (c-jun/c-fos, Ponta *et al.*, 1992) has been reported (Zhang *et al.*, 1991c). T3Rs inhibit DNA binding of AP-1 in the presence of thyroid hormone, thus repressing transcriptional activation by AP-1 (Schmidt *et al.*, 1993; Zhang *et al.*, 1991c; Lopez *et al.*, 1993). Since AP-1 activation is the signal transduction pathway used by many growth factors including bFGF (Schneider and Parker, 1990; Pertovaara *et al.*, 1993; Curran and Franza, 1988; Bravo, 1990), the interaction of thyroid hormone with AP-1 provides a pathway for the cross-talk between the two major factors regulating cell growth, development and differentiation.

The composition and accumulation of bFGF in the heart and cultured myocytes may be regulated by thyroid hormone through several possible ways. (1). Thyroid hormone may affect bFGF expression by regulating bFGF gene transcription. In all the genes regulated by T3, a T3 responsive element (T3RE) has been identified (Samuels *et al.*, 1989; Glass and Holloway, 1990). No such a T3RE has so far been identified in up to 1 kb of the 5'-flanking region of human bFGF (Shibata *et al.*, 1991). According to this data, it does not, therefore, appear that thyroid hormone regulates bFGF gene transcription directly; though the possibility that a T3RE located in the further upstream region can not be excluded. However, an indirect transcriptional regulation via AP-1 is possible: As mentioned, ligand-activated T3R represses the AP-1 mediated transcriptional activation of genes (Desbois *et al.*, 1991; Zhang *et al.*, 1991c), and there is a potential AP-1 binding site within the core promoter region of

human bFGF gene (Shibata *et al.*,1991). (2). The increase in 21.5-22 kDa as well as total bFGF content upon hypothyroidism might be induced by inhibition of proteolytic activity (slower bFGF turnover). In fact, trypsin and other serine proteases did convert the 21.5-22 kDa bFGF into the 18 kDa form as shown by our data and also by Doble *et al.* (1990). Hyperthyroidism has been associated with increased muscle proteolysis (Tauveron *et al.*, 1992; Angeras and Hasselgren, 1987). Keratinocytes cultured without T3 in the medium showed 70-80% decrease in plasminogen activator protease compare to controls (Isseroff *et al.*, 1989), indicating that thyroid hormone can regulate proteolytic activity. (3). The change in bFGF composition and abundance in hypothyroidism may also caused by increased bFGF mRNA stability or translation. One preliminary set of experiments did not indicate any changes in bFGF mRNA levels in the heart as a function of thyroid status (our unpublished observations); however, more experiments are needed to test this hypothesis.

In rat cardiac myocytes we showed a reduction of bFGF effects by thyroid hormone. Inhibition of bFGF-induced DNA synthesis by thyroid hormone could be mediated by: (1), the T3/R3R repression of transcriptional activation by AP-1 which would blocks the stimulating effects of bFGF (Schneider and Parker, 1990). (2), increased proteolysis. It has been proposed that bFGF is regulated mainly at the level of bioavailability rather than gene expression (Baird and Walicke, 1989). Increased proteolysis might result in the degradation of 18 kDa bFGF into smaller, non-active fragments. Finally, thyroid hormone may induce expression of other factors which

cancel the bFGF-derived stimulation. For example, T4 treatment increased TGF-ß1 mRNA in rat myocardium by about 53% (Yao and Eghbali, 1992), and it is known that TGF-ß inhibits the stimulating effect of bFGF on many cells, including cardiomyocytes (Kardami, 1990).

2.5 CONCLUDING REMARKS

In conclusion, we have shown increased bFGF localization to the nucleus of proliferating chick myocytes in the later portion of the cell cycle, prior to the onset of cell division. We also report for the first time that bFGF or bFGF-like antigens changed from being chromosome-associated during early stages of mitosis to a complete dissociation from chromosome and localized to the midbody during anaphase and telophase. This cell cycle dependent localization of bFGF suggests a possible role of bFGF in cytokinesis. The LI of cultured rat cardiac myocytes showed that bFGF stimulates DNA synthesis of cardiomyocytes and postponed the terminal differentiation of those cells; an effect blunted by thyroid hormone. It has been recently reported that immature rat cardiac muscle, which retains a proliferative phenotype, expresses predominantly that 21-23 kDa bFGF forms (Liu *et al.*, 1993). We provide evidence for the first time that thyroid status exerts a (negative) regulatory effect on the accumulation of 21.5-22 kDa bFGF in cardiac ventricles as well as other striated muscles, suggesting a relationship of the various bFGF forms with the myogenic program of differentiation. Similar changes of bFGF composition were also observed in cultured chick cardiomyocytes, showing that thyroid hormone can act directly on

these cells and down regulate the 21.5-22 kDa bFGF. We also show that the relative levels of 18 kDa and 21.5-22 kDa bFGF in eu-, hypo- and hyper-thyroid animals are characteristic for different organs. Finally, we investigated the distribution of bFGF in mdx mouse cardiomyopathy as a function of thyroid hormone treatment. T3-treated mdx hearts exhibited a general low level of anti-bFGF immunolabeling, and a dramatic increase in the number and size of lesions and scar. To summarize in a somewhat simplified scheme, in cardiac myocytes bFGF can be taken as factor promoting the genetic program that supports proliferation and regeneration. Thyroid hormone, which triggers or promotes muscle differentiation may do so partially by regulating the expression as well as activity of bFGF.

CHAPTER 3. FGF RECEPTORS IN THE HEART

3.1. INTRODUCTORY REMARKS

High affinity FGF receptors (FGFRs) have been identified in many cell types and tissues (Klagsbrun, 1989). The FGFRs are tyrosine kinases belonging to a family of five members (FGFR1-5), each of which exists in several variants (Jaye *et al.*, 1992). Low affinity receptors are cell surface heparan sulfate proteoglycans (HSPGs), considered essential for bFGF binding to high affinity receptors (Yayon *et al.*, 1991). Low affinity binding sites in bovine cardiac sarcolemma have been detected (Ross and Hale, 1990) and purified (Ross *et al.*, 1993). The mRNA for FGFR1 (but not other FGFRs) has been detected in young rat hearts (Engelmann *et al.*, 1993), while bFGF is continually present in cardiac development (Kardami *et al.*, 1993). FGFRs have not been reported for the adult myocardium. Since another type of striated muscle, skeletal muscle, loses its FGFR upon differentiation (Olwin and Hauschka, 1988), it has been generally accepted that the loss of cell division in cardiac muscle upon maturation is due at least in part to the loss or decrease of receptors for mitogens like FGFs (Schneider & Parker, 1990). This however has not been experimentally established. We therefore undertook to examine the expression and potential hormonal and/or developmental regulation of FGFR in cardiac myocytes. Our data, presented in this chapter, established that FGFR is expressed by adult as well as immature cardiomyocytes. We also provide evidence that the overall levels as well as receptor isoform may be regulated by developmental stage and/or thyroid hormone.

3.2. MATERIALS AND METHODS

Materials and apparatuses for cell culture, gel electrophoresis, autoradiography, protein determination are as described in Chapter 2. Other chemicals wrere ordered from Sigma Chemical Co. (St. Louis, Mo) unless otherwise indicated.

3.2.1 Cross-linking of ^{125}I-bFGF to cardiac membranes

Pure cardiac sarcolemma preparations were prepared as discribed by Lonsberry et al (1992), with instructions and assistance from Dr. G. Pierce (Department of Physiology, University of Manitoba), and by Pitts (1979). 100 µg of membrane protein were used per sample. The membranes were incubated with 3.5 ng ^{125}I-bFGF (DuPont Canada Inc., Mississauga, ONT) at 22°C, in 100 µl PBS containing 2 mM MgCl2, 0.2% gelatin, in the presence or absence of 5 µg/ml "cold" (non-radioactive labelled) bFGF (Pepro Tech, Inc., Rocky Hill, NJ) for 60 min. The mixture was diluted 10-fold with cold PBS and centrifuged at 16,000 xg for 10 min at 4 °C. The pellet was washed with PBS and then resuspended in 200 µl PBS. The cross-linker disuccinimidyl suberate (DSS, Pierce Chemical Co., Rockford, IL), freshly dissolved in dimethyl sulfoxide to a concentration of 50 mM, was added to the suspension at a final concentration of 0.30 mM. Samples were incubated for 20 min at 4 °C and cross-linking was terminated by the addition of 10 µl of 0.5 M Tris-HCl, pH7.4. Membranes were centrifuged for 10 min at 16,000 xg. The pellets were resuspended in 30 µl of homogenizing buffer (20 mM Hepes pH 7.4, 0.25 mM sucrose, 1 mM EDTA, 1µg/ml aprotinin and 1µg/ml of leupeptin) containing 1% Triton x-100 by

vortexing and triturating, and then incubated at 4°C for 15 min. Insoluble materials were removed by centrifugation at 16,000 xg for 10 min. The supernatant was prepared for SDS-PAGE.

3.2.2 Cross-linking of ^{125}I-FGF to cardiac myocytes

Cross-linking of 125I-bFGF to cardiac myocytes was carried out according to the methods described by Neufeld & Gospodarowicz (1986) with minor modifications. Proliferating or non-dividing, postmitotic rat cardiac myocytes were obtained as described in "Material and Methods" of Chapter 2. Myocytes, grown in 60 mm dishes, were washed twice with 5 ml cold Dulbecco's modified Eagle medium (DMEM). After washing, 0.75ml DMEM supplemented with 20 mM Hepes, pH7.4, 0.2% gelatin (Bio-Rad) was added to each dish. ^{125}I-bFGF was added to the dish to a final concentration of 2 ng/ml, in the presence or absence of 100 ng/ml cold bFGF. The dishes were then incubated at 4°C, under mild agitation for 90 min. At the end of the incubation, the dishes were washed twice with 5 ml of cold PBS, then 0.75 ml of PBS with protease inhibitors (1 mM PMSF, 5 mM EGTA, 10 μg/ml aprotinin and 10 μg/ml leupeptin) were added. The cross-linking reaction was initiated by the addition of 200 μl PBS containing 0.15 mM DSS (diluted from a 20 mM stock in dimethyl sulfoxide). Dishes were incubated at room temperature for 15 min,and the reaction was terminated by the addition of 20 μl 0.5 M Tris-HCl, pH7.4. The dishes were washed twice with cold PBS containing protease inhibitors, and subsequently 0.7 ml of the same solution were added to each dish. Cells were scraped off with a rubber

policeman and centrifuged for 10 min at 16,000 x g at 4°C. The pellet was resuspended and solubilized in 40 µl of 1% Triton X-100 in PBS with protease inhibitors for 10 min, centrifuged as before and protein concentration of the supernatant determined as previously described (Chapter 2). Aliquots from the supernatant were analyzed by SDS-PAGE.

3.2.3 Electrophoresis and autoradiography

The supernatant from the cross-linking experiments was diluted with an equal volume of H_2O and brought up to a final concentration of 12 mM Tris, 100mM glycine, 1% SDS, 10% glycerol, 0.004% bromphenol blue and 1% ß-mercaptoethanol using stock SDS-PAGE sample buffer. Samples were then boiled for 5 min and analyzed on a gradient (5-12.5%) polyacrylamide SDS slab gel, with a 3 % stacking gel. After electrophoresis, gels were fixed and stained with 0.1% Coomassie Blue in 50% tri-chloroacetic acid for 1 hr and destained overnight with 7% acetic acid. Gels were then dried on a Bio-Rad gel dryer (Model 583) using a gradient circle and subjected to autoradiography at -70°C using Kodak X-Omat R film and Dupont Lighting Plus intensifying screens. Broad range molecular weight protein markers (200-14.4 kDa, Bio-Rad) were used to determine the molecular weight of the labeled bands. A Bio-Rad Model GS-670 Imaging Densitometer and microanalysis programme was used to estimate the relative intensity of the labelled bands.

3.2.4. Binding of ^{125}I-bFGF to cardiac sarcolemma preparations.

20 μg of cardiac sarcolemmal membrane protein were used per binding assay. The final volume was 100 μl. Binding buffer contained 300 mM sucrose, 100 mM NaCl, 50 mM Hepes, pH 7.4, 1.2mM $CaCl_2$, 1.2 mM $MgCl_2$ and 0.5% (w/v) BSA. ^{125}I-bFGF was added to the desired concentration (1-50 ng/ml) and binding was done at 22°C. Binding was started by adding the sarcolemmal preparation to the system. After 90 min, 0.5 ml of cold binding buffer was added to stop the binding reaction. Two methods were used to separate bound from free ^{125}I-bFGF. In the tube method, the membrane suspension was centrifuged at 16,000 xg for 10 min, and the pellet was washed again with the binding buffer. The tips of the tubes were cut and counted in a τ-Counter (model 5500, Beckman Instruments, Inc., Mississauga, ON). In the filter method, the membrane suspension was filtered through Gellman GF/B filters, which had been pre-soaked in cold binding buffer for 30 min. Each filter was washed three times with 3 ml of cold binding buffer. The filters were then counted. The background of free ^{125}I-bFGF absorbed non-specifically to the tubes and filters were determined by controls that contained the same amount of ^{125}I-bFGF, but no sarcolemmal membranes. This background was not competed by the addition of cold bFGF. Binding values resulting from subtracting the background were analyzed by LIGAND computer program (Elsevier-BIOSOFT, Cambridge UK) to calculate the dissociation constant (Kd) and binding capacity (Bmax).

A different approach was also used to obtain an estimate of the amount of ^{125}I-bFGF bound to high and low affinity binding sites: after the binding reaction the filters

were washed 3 times with 1 ml of 2 M NaCl, 20 mM Hepes, pH 7.4, to elute the ^{125}I-bFGF bound to HSPGs (low-affinity sites) and counted. The filters were then washed again 3 times with 1 ml of 2 M NaCl, 20 mM sodium acetate, pH 4.0 to elute ^{125}I-bFGF bound to the high affinity receptors (Moscatelli, 1987). Controls were treated identically to estimate the background from each step. Results are presented as specific binding after subtracting the background.

3.2.5 Competition of ^{125}I-FGF binding to cardiac sarcolemma by "cold" bFGF

This was performed using the same binding conditions as for the tube method. ^{125}I-bFGF was added to a concentration of 85 pM. The background of free ^{125}I-bFGF absorbed to the tubes at each point was determined in controls that contained 85 pM ^{125}I-bFGF, various concentration of "cold" FGF, but no membranes. Non-specific binding of ^{125}I-bFGF to the membranes was determined in the presence of 1 μM (18 μg/ml) recombinant bFGF, and represented less than 20% of the total binding, in agreement with values reported previously (Olwin and Hauschka, 1988; Gospodarowicz and Neufeld, 1985, 1986). Values resulting from subtracting the background and nonspecific binding were considered as representing specific binding of ^{125}I-bFGF to the membranes.

3.2.6 Isolation of Cardiomyocytes

Cardiomyocytes were isolated from 300 - 350 g male Sprague-Dawley rats, as described by Liu and Pierce (1993). Briefly, the rat heart was removed and perfused

in a retrograde Langendorff model with a Ca^{2+}-free MEM-Joklik's medium supplemented as described (Langer *et al.*, 1987) for approximately 10 min. The perfusion solution was then changed to one which also contained 0.5 mg/ml hyaluronidase and 1.0 mg/ml collagenase (Cooper, class 1). After approximately 15-20 min, the heart was removed from the perfusion apparatus and the tissue gently teased apart in a petri dish to release the cardiomyocytes. The cells were harvested by gravity as described (Langer *et al.*, 1987) and were used either for RNA preparation or the bFGF-Saporin assay.

3.2.7 *In vitro* bFGF-saporin assay

Isolated ventricular myocytes were placed in 35 mm culture dishes previously coated with 0.2 mg/ml laminin. After 20 min, the dishes were carefully washed once to remove dead cells from the viable myocytes attached to the laminin coated dishes and 1 ml of MEM-Joklik medium was added to each dish. Dishes were divided into four experiment groups, namely (1), those treated with PBS only, (2) those treated with recombinant bFGF at 1, 5, and 10 nM concentrations, (3) those treated with saporin (a gift from Drs. Lappi and Baird, the Whittier Institute, La Jolla, CA) at 1, 5, and 10 nM, and, (4) those treated with the bFGF-saporin conjugate (a gift from Drs. Lappi and Baird) at 1, 5, and 10 nM. All the reagents were dissolved in PBS and the total volume added was 22.5 µl per dish. The viable, rod-like shaped cardiomyocytes with no spontaneous contractions were counted under the microscope before and 30 to 60 min after adding the reagents, to calculate the percentage of surviving myocytes.

3.2.8 Internalization of bFGF by cardiomyocytes

Cardiomyocytes from 19-day rat fetuses were plated on collagen-coated 24-well culture plates at a density of 500,000 cells/well. Internalization experiments were performed according to Roghani and Moscatelli (1992) with minor modification. Briefly, proliferative or non-dividing cardiomyocyte cultures were washed twice with PBS, and the medium was replaced with 0.5 ml DMEM containing 0.15% gelatin, and 0 or 10 μg/ml heparin. ^{125}I-bFGF was added to each dish at a final concentration of 5 ng/ml and the cells were incubated at 37°C for 5 hours. After incubation, the cells were washed twice with PBS, twice with 2 M NaCl in 20 mM HEPES, pH 7.5, to remove heparan sulfate-bound radioactivity, and twice with 2 M NaCl in 20 mM sodium acetate, pH 4.0 to remove receptor-bound radioactivity (Moscatelli, 1987). At the end of the washes, the cells were incubated for 3 min at room temperature with 0.5 ml 1% trypsin-EDTA, the released cells were collected and radioactivity was assessed. Nonspecifically associated radioactivity was assessed in parallel cultures incubated with ^{125}I-bFGF-containing medium at 4°C (a condition preventing internalization). Nonspecific radioactivity was subtracted from the experimental values.

3.2.9 Northern blot analysis.

Total RNA was prepared from adult rat ventricles or isolated ventricular myocytes using a single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Samples

of 100-200 µg of RNA were separated by formaldehyde-agarose gel electrophoresis as described (Lehrach *et al.*, 1977) and transferred to Nitrocellulose-plus membrane under standard conditions (Sambrook *et al.*, 1989). The cDNA probes were rat FGFR1 (a gift from Dr. J. Milbradt, Washington University School of Medicine, St. Louis, MO), homologous to the human *flg* gene (Wanaka, *et al.*, 1990) and rat N-syndecan (a gift from Dr. D.J. Carey, Sigfried & Janet Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania), which encoded a protein identified as one of the low affinity FGF binding sites. The cDNAs were labeled by random primer methods (Feinberg and Vogelstein, 1983 & 1984) with ^{32}P-deoxy ATP, for 5 hours, at room temperature. The specific activities cDNA were 1-3 x 10^9 cpm/µg DNA for labelled FGFR1 and 8-9 x 10^8 cpm/µg DNA for labelled N-syndecan. Hybridization was carried out for 16-18 hours at 42°C in hybridization buffer containing 10% (w/v) dextran sulfate, 5x SSC (1x SSC is 0.15 M NaCl, 0.015 M Na_3Citrate), 0.01 M $NaPO_4$, pH 6.5, 50% (v/v) formamide (diionized) and 10% (v/v) 50x Denhardt's solution. To compare FGFR and N-syndecan levels among samples, each lane was loaded with 100-200 µg total RNA. Membranes were also stripped by washing in boiling distilled water for 3 times, 5 min each, under gentle agitation and re-hybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (a gift from Dr. I.M.C. Dixon, Department of Physiology, University of Manitoba, Winnipeg, MB), a constitutively expressed protein, to check for possible loading variation. After hybridization, the membrane was washed at 65°C in 1x SSC, 0.1% SDS and air dried. Autoradiography and densitometry of the blots were performed as described in

paragraph 3.2.3.

3.3 RESULTS

3.3.1 Binding of ^{125}I-bFGF to cardiac myocytes.

^{125}I-bFGF was added to highly purified sarcolemmal vesicles from rat heart in the presence and absence of a 100-fold molar excess of non-radiolabelled bFGF; the complex was cross-linked with a zero length heterobifunctional cross-linker, DSS, and analyzed by SDS/PAGE and autoradiography. Characteristic results are shown in Fig.3.1: a 150 kDa labelled band was consistently observed in these plasma membrane preparations (Fig.3.1, lanes 1,2). Since this included bound bFGF, the apparent Mr. of the labeled macromolecule prior to cross-linking is estimated at 134 kDa. Specificity of the cross-linking was demonstrated by the complete disappearance of this band by incubation with non-radioactive labelled bFGF (Fig.3.1., Lane 3). ^{125}I-bFGF, which bound to the membrane, but did not cross-link, migrated at the front of the gel (the bottom band). A 36 kDa band was observed, which possibly represents a dimer of ^{125}I-bFGF. The minor band around 80 kDa was not observed consistently and may represent a proteolytic product (Fig.3.1, Lane 1).

Cross-linking of ^{125}I-bFGF to the myocyte cell surface was also done using monolayers of cultured embryonic or neonatal myocytes. Covalent cross-linking of ^{125}I-bFGF to proliferative cardiomyocytes yielded two major broad bands, with an apparent size of 150 kDa and 120 kDa, corresponding, after subtraction of the

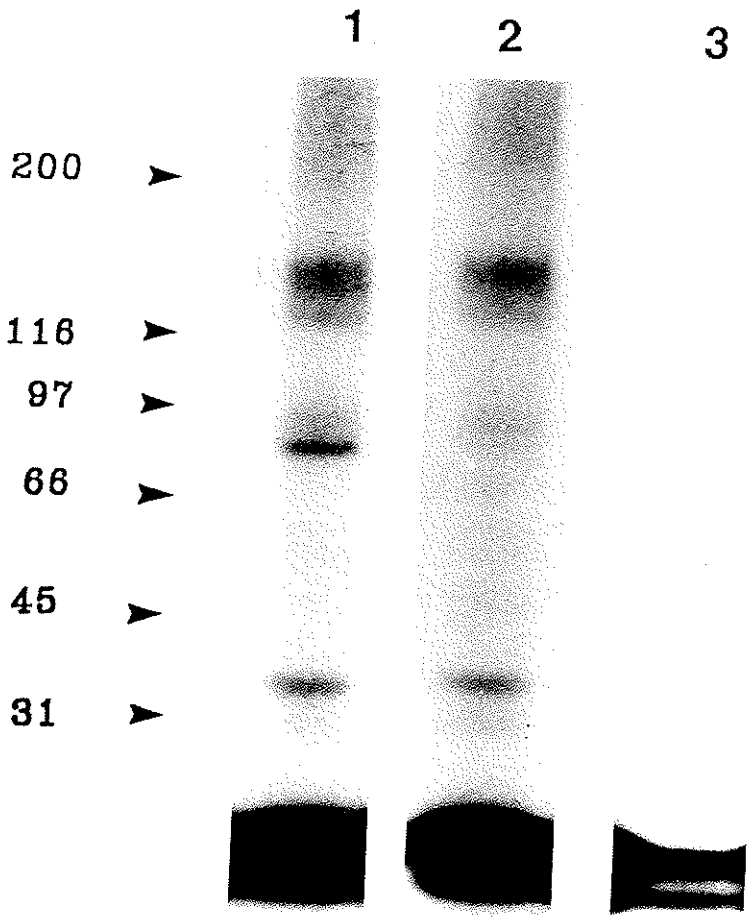


Fig.3.1 Affinity labelling of bFGF receptors in sarcolemmal (SL) preparations from adult rat heart.

Cardiac SL preparations (100 μg) were incubated with 3.5 ng ^{125}I-FGF in the absence (lanes 1,2) or presence (lane 3) of 5 μg/ml cold bFGF for 60 min at 22°C and then covalently cross-linked with DSS as described under "Materials and Methods". After completion of the cross-linking reaction, membranes were solubilized with 1% Triton X-100, separated by 5-12.5% gradient SDS-PAGE, and subjected to autoradiography for 7 days. Molecular weight standards are indicated at the left.

contribution by ^{125}I-bFGF, to proteins of 134 and 104 kDa respectively (Fig.3.2, lanes 1-4). These bFGF-binding proteins are, presumably, bFGF receptors. Both of these binding proteins (receptors) were also detected in non-dividing myocytes (Fig.3.2, lanes 5-10). In the latter case however, while levels of the 134 kDa receptor were comparable to those from proliferative myocytes, those of the 104 kDa receptor were reduced (Table 3.1). The relative densities of the two ^{125}I-bFGF-labelled proteins were examined by densitometry: the ratio of the 104/134 kDa ^{125}I-bFGF-receptors was about 0.63 ± 0.03 in the proliferating myocytes and 0.46 ± 0.02 in non-dividing myocytes (Fig.3.3 and Table 3.1). The difference between those two groups was statistically significant ($P<0.01$). An excess of "cold" bFGF fully competed with ^{125}I-bFGF binding, resulting in disappearance of the cross-linked bands (Fig.3.2, Lane 11). Treatment of the proliferative cardiomyocytes cultures with T3 resulted in a slight increase in the 104 kDa bFGF binding protein (104/134 kDa ratio is 0.71 ± 0.02 in T3 treated compared to 0.63 ± 0.03 of control), however this difference was not significant ($P>0.1$). T3-treatment of post-mitotic, non-dividing cardiomyocytes cultures resulted in a statistically significant decrease of the 104 kDa bFGF binding protein. As a result, the ratio of 104/134 kDa bFGF-receptors was 0.34 (± 0.06) in T3 treated non-dividing myocytes compared to 0.46 ± 0.02 in non-treated non-dividing myocytes ($P<0.01$).

The interaction of ^{125}I-bFGF with sarcolemmal vesicles was also examined by equilibrium binding studies, and results are shown in Figure 3.4. Scatchard plot

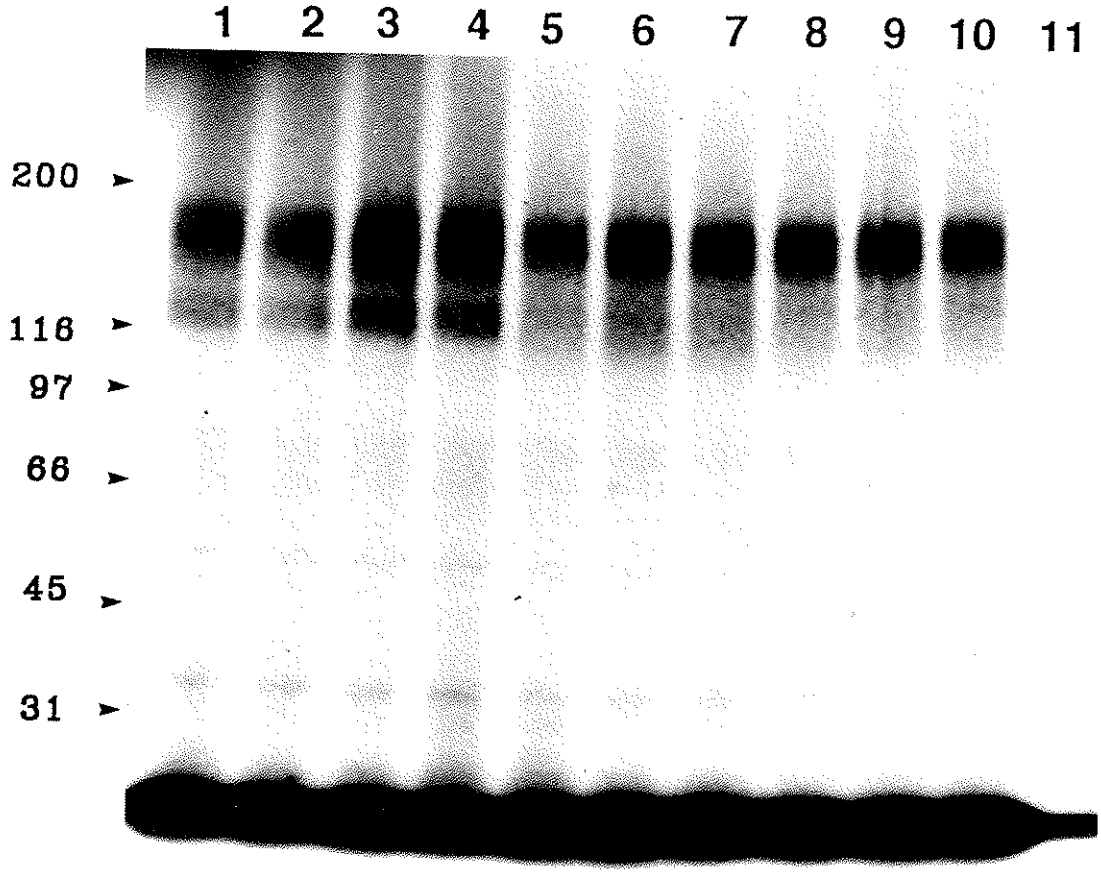


Fig.3.2 Affinity labelling of bFGF receptors on cultured cardiomyocytes

Cardiac proliferative (maintained in culture for 48 h in high serum) and non-dividing (maintained in culture for 1 wk in low serum) myocytes were incubated with 2 ng/ml ^{125}I-bFGF in the absence (lanes 1-10) or presence (lane 11) of 100ng/ml non-radioactive bFGF; cross-linking was performed as described under "Materials and Methods". Equal amounts of cultured cell proteins, solubilized with 1% Triton X-100, were then analyzed by SDS/PAGE and autoradiography. Lanes 1-4, proliferative myocytes, maintained in the absence (lanes 1,2) or presence (lanes 3,4) or T3, for 24 hours. Lanes 5-10, non-dividing myocytes, maintained in the absence (lanes 5-7) or presence (lane 8-10) of 10 nM T3, for 3 days. Molecular weight standards are indicated at left.

Table 3.1 Densitometry of cross-linked FGF receptors in cultured cardiomyocytes

Cardiac myocytes	Density of 134 kDa FGF receptor [a,b]	Ratio of 105/134 kDa FGF receptors [b]
proliferative (P)	29.4 ± 1.2	0.63 ± 0.03
non-dividing (ND)	29.4 ± 1.4	0.46 ± 0.02 *
T3-treated P	29.9 ± 4.2	0.71 ± 0.02
T3-treated ND	30.9 ± 1.0	0.34 ± 0.06 **

[a] Density of 134 kDa FGF receptor was normalized according to protein load, determined by densitometry measurement of the gel.

[b] Values shown represent mean ± SEM of four repeated sets of experiments.

* $P<0.01$ Non-dividing vs Proliferative

** $P<0.001$ T3-treated vs non T3-treated

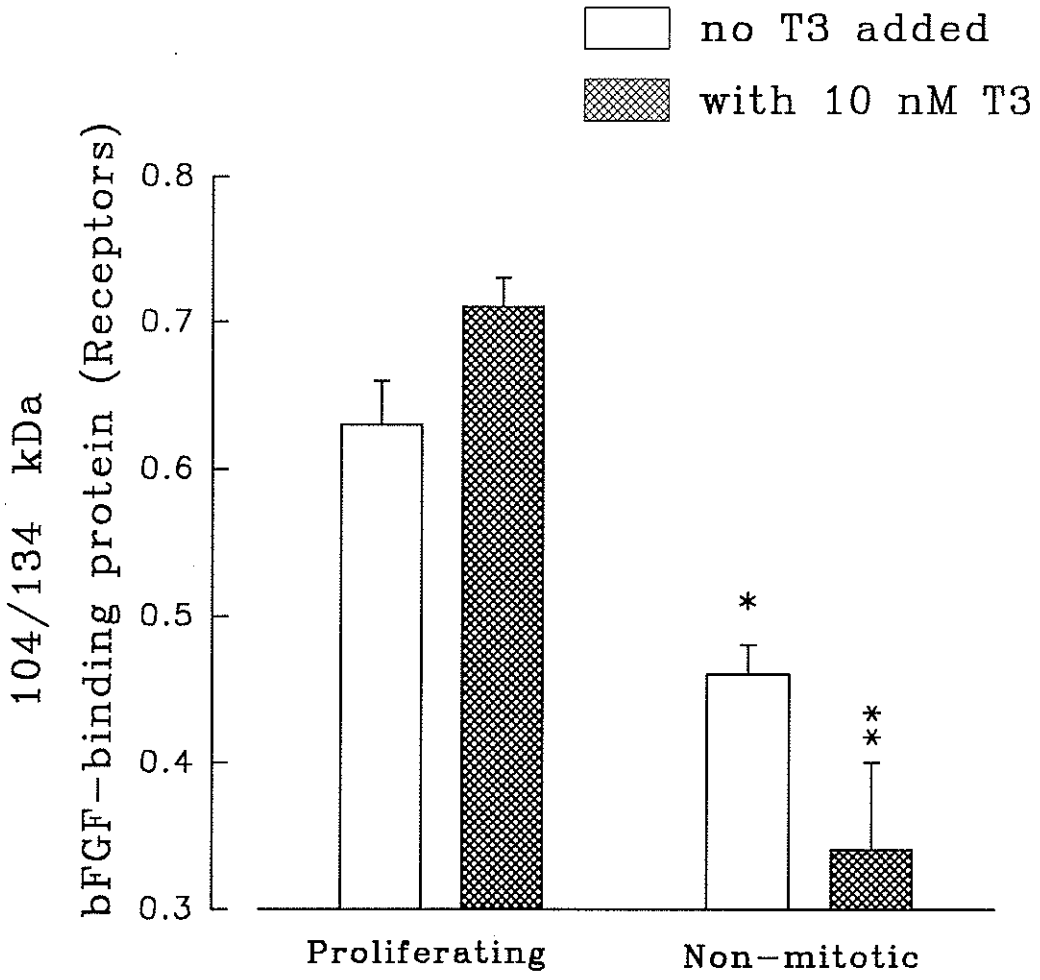


Fig.3.3 Effect of thyroid hormone and development in culture on the relative levels of the 104 kD and 134 kD bFGF-receptors in cardiac myocytes

The ratio of the 104 kDa to the 134 kDa bFGF receptors was determined by densitometry of autoradiograms from four distinct sets of experiments and plotted as a function of proliferation and/or T3 addition.

* $P < 0.01$ non-dividing vs proliferating myocytes

** $P < 0.01$ T3-added vs no T3 added

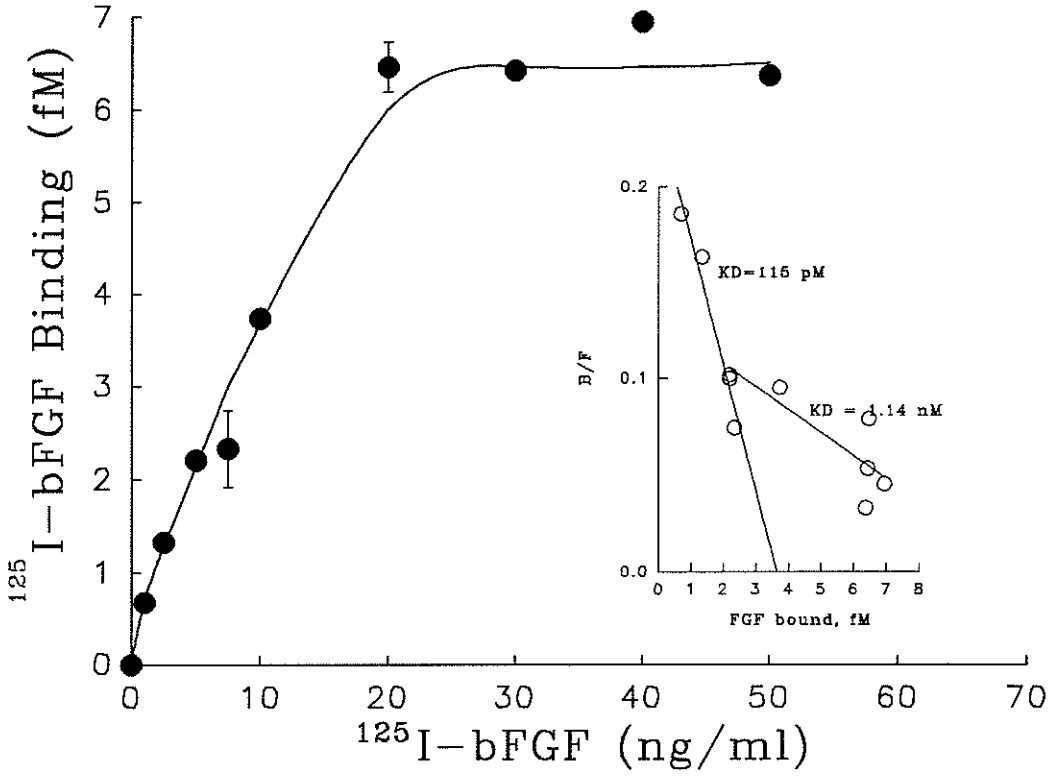
analysis indicates the presence of two classes of binding sites, with dissociation constants of 1.14 nM and 115 pM ("low" and "high" affinity), respectively. The high affinity binding sites had a Bmax of 170 pM/mg (or 2.8 ng/mg) protein, and the Bmax for low affinity binding sites was 670 fM/mg (or 11.4 ng/mg) protein.

To discriminate between binding to the low or high affinity sites, ^{125}I-bFGF-membrane complexes were incubated with 2M NaCl, pH 7.5, conditions which dissociate bFGF from the low affinity sites. Subsequent incubation with 2M NaCl, pH 4.0 liberated bFGF from the high affinity sites (Moscatelli, 1987). ^{125}I-bFGF released under these incubation conditions was plotted as a function of total added ^{125}I-bFGF (Fig. 3.4 b, and c). Dissociation constants derived from these data were 2.2 nM (Fig 3.4 b inset) and 254 pM (Fig 3.4 c inset). Binding of ^{125}I-bFGF to the sarcolemmal membranes was inhibitable by non-radiolabelled bFGF in a dose-dependent manner (Fig. 3.5).

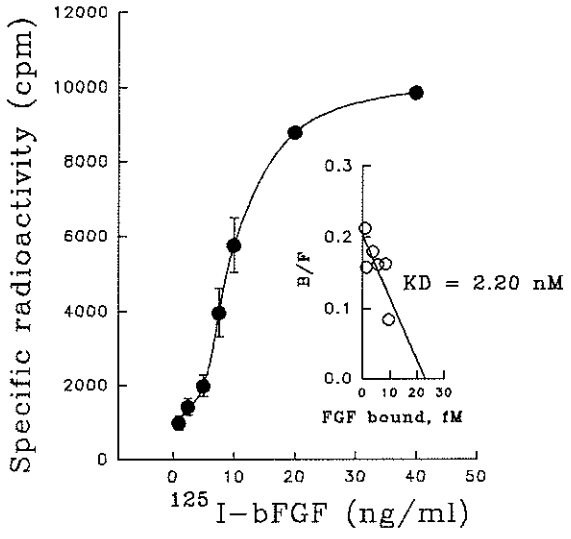
3.3.2 bFGF-saporin assay and internalization of bFGF by cardiomyocytes

The presence of functional FGFR in cardiomyocytes was investigated by examining the internalization of bFGF and bFGF-saporin in those myocytes. A preparation of isolated adult cardiac myocytes, consisting of approximately 80% viable (i.e. cylindrical, non-contracting) cells was incubated with bFGF alone, saporin alone and the bFGF-saporin complex. Cell viability was assessed after 0.5 and 1 hour of incubation. Viability did not change significantly in the presence of either bFGF or

a


125I-bFGF Binding (fM)
125I-bFGF (ng/ml)
B/F
KD=115 pM
KD = 1.14 nM
FGF bound, fM

b


Specific radioactivity (cpm)
125I-bFGF (ng/ml)
B/F
KD = 2.20 nM
FGF bound, fM

c

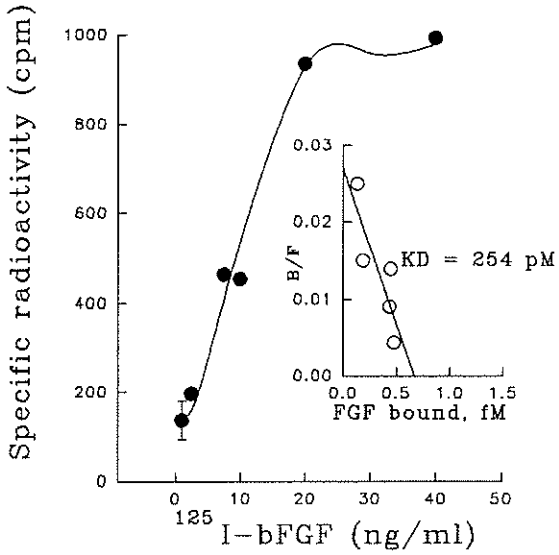

Specific radioactivity (cpm)
125I-bFGF (ng/ml)
B/F
KD = 254 pM
FGF bound, fM

Fig.3.4 Equilibrium binding of ^{125}I-bFGF to cardiac sarcolemmal preparations.

20 μg of cardiac SL protein was incubated with the indicated concentration of ^{125}I-bFGF for 90 min at 22°C. The binding was performed as described under "Materials and Methods". Background binding was determined by controls that contained the same amount ^{125}I-bFGF, but no membranes. The binding value resulting from subtracting the background was plotted as a function of the concentration of ^{125}I-bFGF added.

(a) Total specific binding (b) ^{125}I-bFGF released in the 2M NaCl, pH 7.5 wash ("low" affinity binding) (c) ^{125}I-bFGF released in the 2M NaCl, pH 4.0 wash ("high" affinity binding). Insets, Scatchard analysis of the data. Data are presented as mean ± SEM of quintuplet samples.

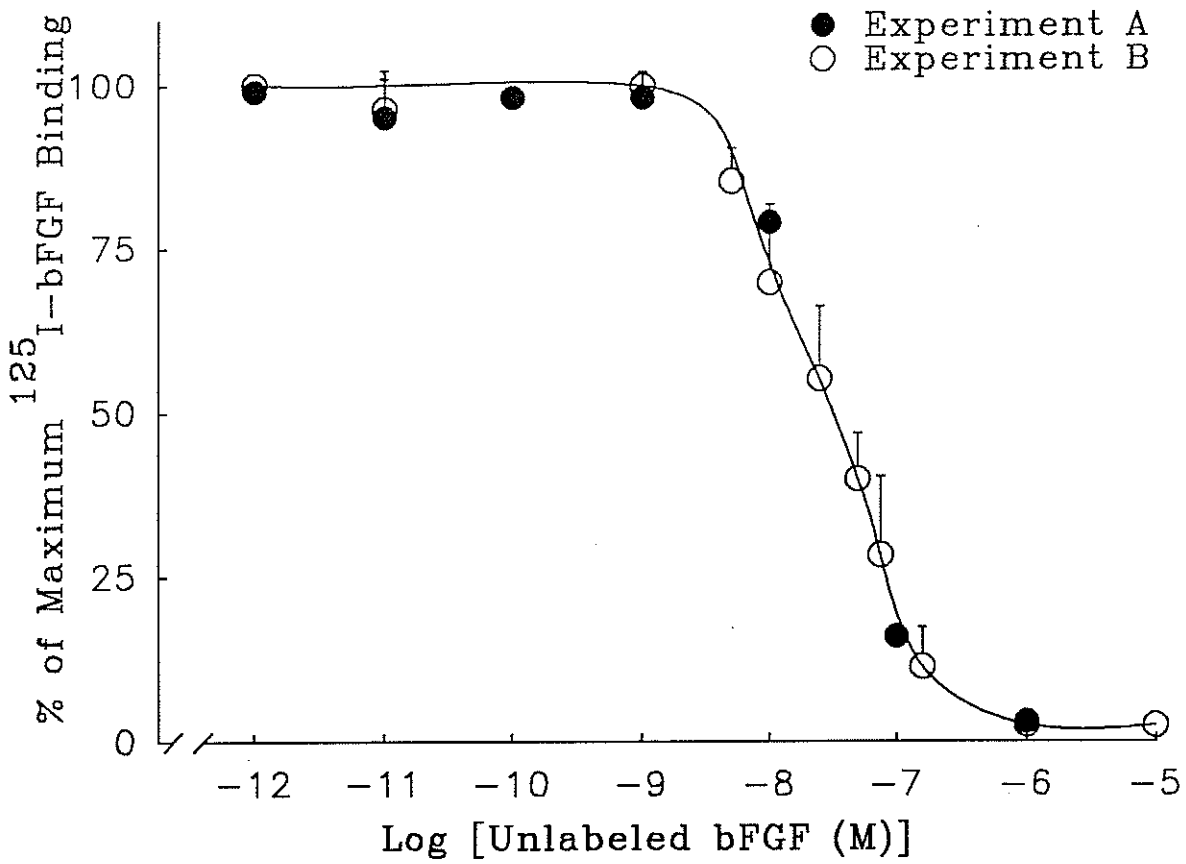


Fig.3.5 Competition of the binding of ^{125}I-bFGF to cardiac sarcolemmal membranes by unlabelled bFGF

Cardiac SL preparations (20 μg) were incubated for 90 min at 22°C in the presence of 85 pM ^{125}I-bFGF plus the indicated concentrations of unlabelled recombinant bFGF. The binding was performed and specific binding was culculated as described under "Materials and Mathods".

Data from two experiments (A ○ and B ●) are shown and are presented as mean ± SEM of triplication samples.

saporin (Fig.3.6): The percentage survival of cardiomyocytes was 79.4%, 90.4% and 75.1% in the presence of 1, 5, and 10 nM bFGF, respectively. Similarly, percentage survival in the presence of saporin (1, 5, and 10 nM) was 76.6%, 79.5% and 76.0%, respectively.

A significant decline in viability was observed in the presence of 5 and 10 nM of the bFGF-saporin conjugate after 1 hr incubation: percentage survival dropped to 50.3% and 36.2%, respectively, ($P<0.01$, compared to values obtained for PBS control, bFGF alone, saporin alone). There were no significant changes in myocyte survival at 30 min of incubation with 1, 5 and 10 nM FGF-saporin, FGF, saporin and PBS.

Internalization of ^{125}I-bFGF in proliferative or non-dividing myocytes was measured in the presence or absence of 10 μg/ml heparin. Heparin-resistant internalization of ^{125}I-bFGF was considered as internalized via the high affinity FGFR (Roghani and Moscatelli, 1992). The data, shown as the ratio of heparin-resistant over total internalization, are not directly comparable between the two phenotypes of myocytes, however they do indicate that ^{125}I-bFGF were internalized through high and low affinity receptors in both proliferative and non-dividing myocytes. Internalization through FGFR consists about 57.1 ± 4.5% of total internalized in proliferative cardiomyocytes and 47.0 ± 1.9% in non-dividing cardiomyocytes (Fig 3.7).

3.3.3 Expression of FGFR1 and N-syndecan genes

To investigate the gene expression for FGFR in cardiac myocyte, we examined

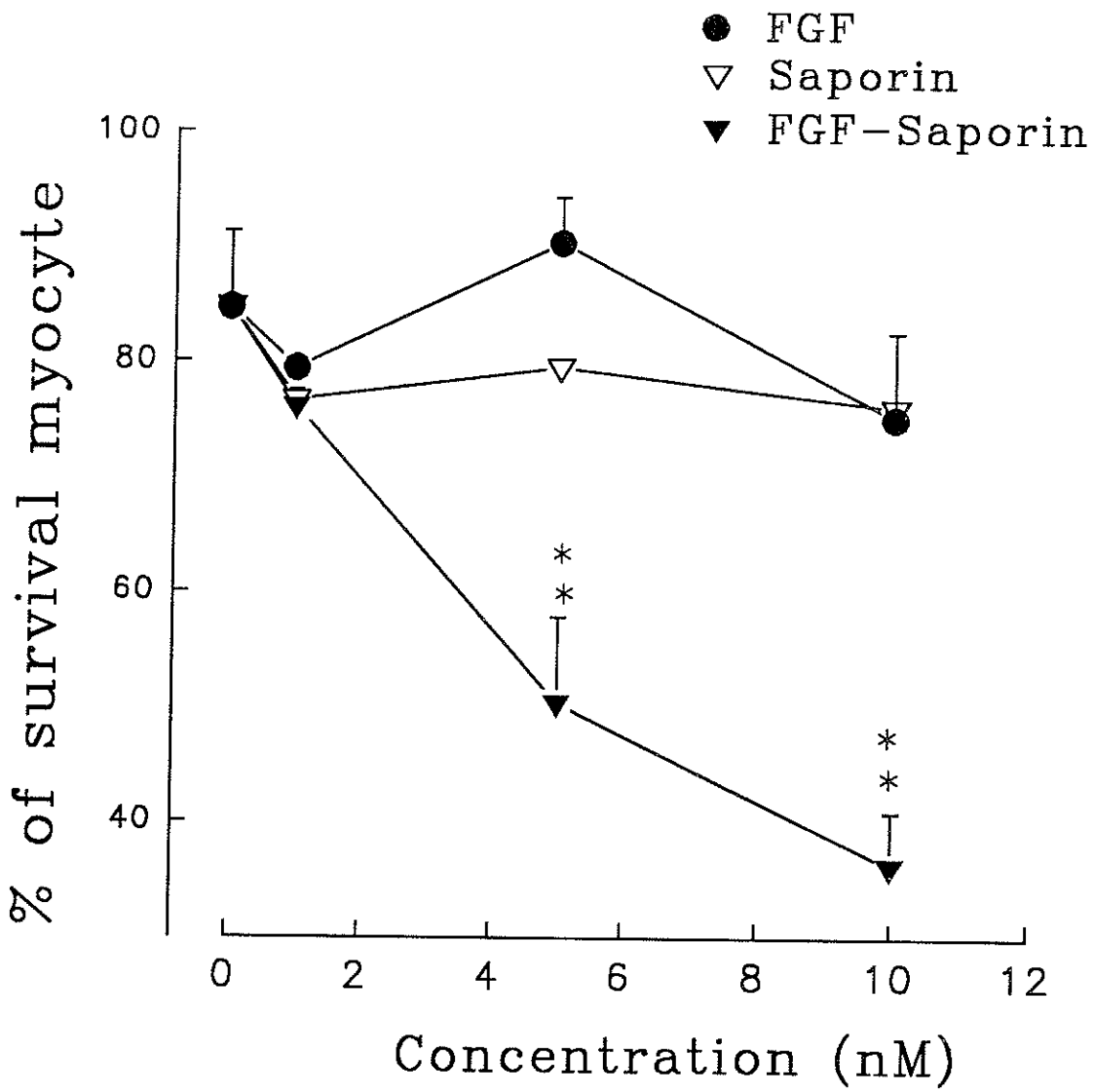


Fig.3.6 Effect of the mitotoxin bFGF-Saporin on the survival of isolated adult cardiac myocytes

Cells were treated with the indicated concentration of bFGF-Saporin (solid triangle), Saporin (hollowed triangle) and bFGF (solid circle). Data are presented as mean ± SEM of 4 distinct sets of experiments.

** $P<0.01$ FGF-Saporin group at indicated concentration vs control and FGF, Saporin groups at all the concentration points.

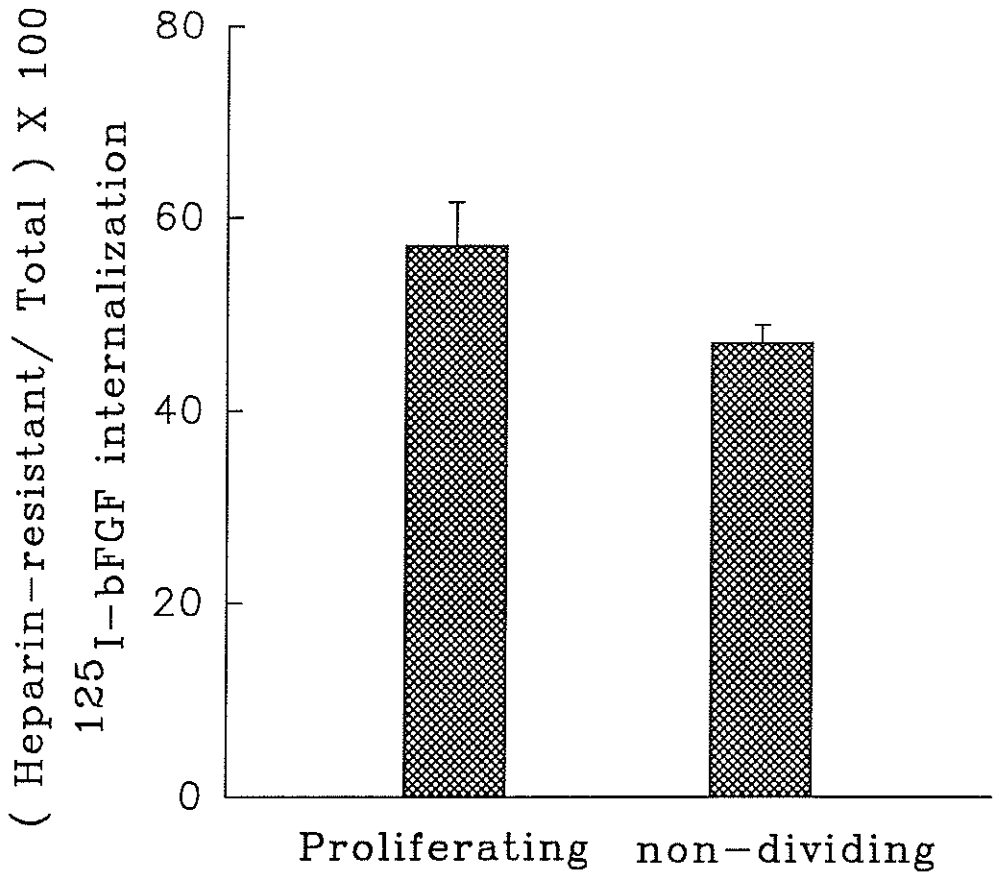


Fig.3.7 Internalization of ^{125}I-bFGF via the high affinity receptors in cultured myocytes.

Cardiomyocytes were incubated with 5 ng/ml ^{125}I-bFGF with or without 10 μg/ml heparin for 5 hr in 37°C. Heparin-resistant (in the presence of heparin) internalized ^{125}I-bFGF was measured in proliferative and post-mitotic myocytes in cultures and plotted as a percentage of total (in the absence of heparin) internalized bFGF.

the presence of mRNA for a high affinity FGFR, FGFR1 and a low affinity receptor, N-syndecan by Northen blot analysis. Total RNA was obtained from adult hearts as well as isolated adult cardiomyocytes and analyzed for presence of the mRNAs for FGFR1 (*flg*) and N-syndecan, by hybridization with corresponding rat cDNAs. Results are shown in Fig 3.8 a,b. Adult rat hearts consistently expressed the FGFR1 and N-syndecan mRNA, at 4.3 and 5.5 kb, respectively (Fig.3.8 a and b). Figure 3.9 shows the relative intensity of FGFR1 and N-syndecan mRNA signal estimated by densitometry of the correspondent autoradiographs. Levels of both mRNAs are similar between total heart and myocyte RNA.

Total RNA was also obtained from hearts at different developmental stages and probed for FGFR1 mRNA; relative intensity estimated by densitometry are shown in Fig.3.10: The expression of FGFR1 mRNA was highest in fetal hearts, steady state levels of FGFR1 declined during the first 2 weeks after birth but increased again at later stages of development. In adult rat hearts, levels of FGFR1 mRNA were comparable to those of fetal heart.

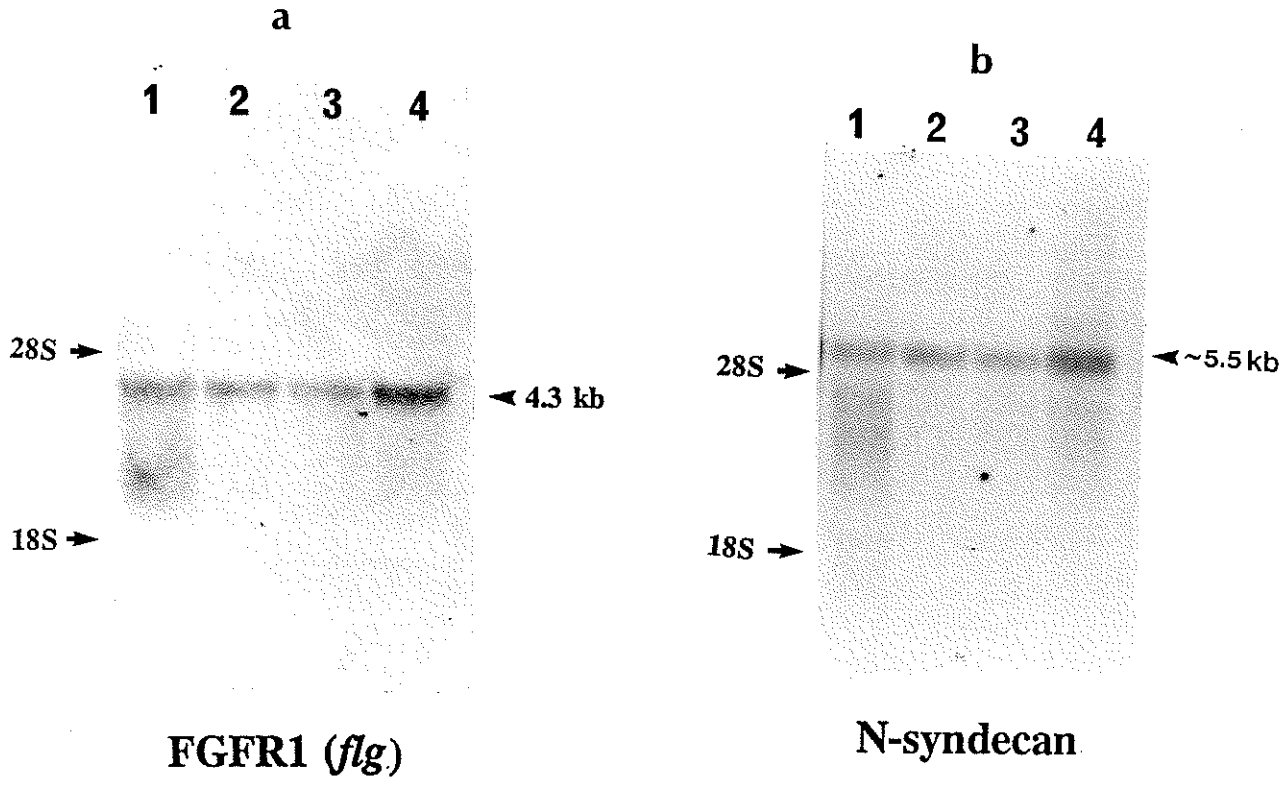


Fig.3.8 Northern blot analysis of total RNA for (a) FGFR1 (flg) and (b), N-syndecan.

Lane 1, 100 μg RNA from rat cardiac ventricles. Lane 2, 100 μg RNA from isolated cardiac myocytes (experiment #1), Lane 3, 100 μg RNA from isolated adult cardiac myocytes (experiment #2). Lane 4, 200 μg RNA from isolated adult cardiac myocytes (experiment #2).

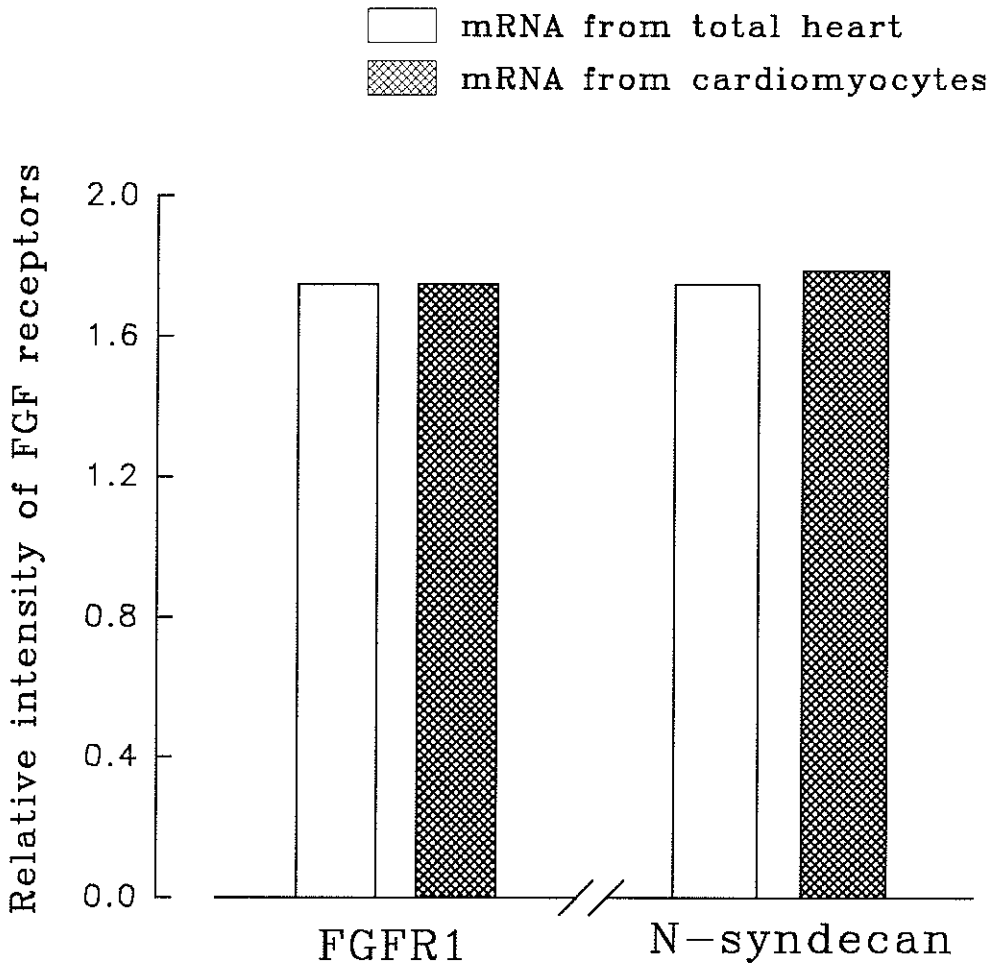


Fig.3.9 FGFR1 and N-syndecan levels in RNA obtained from total heart and isolated cardiomyocytes.

Total RNA from adult heart and isolated cardiomyocytes was analyzed by Northern blotting for FGFR1 and N-syndecan mRNA. The blot was also stipped in boiling distilled water for 3 times, 5 min each, and reprobed for GAPDH to correct for possible loading variations. Data from total heart and isolated cardiomyocytes were compared. Data from FGFR1 and N-syndecan are not comparable.

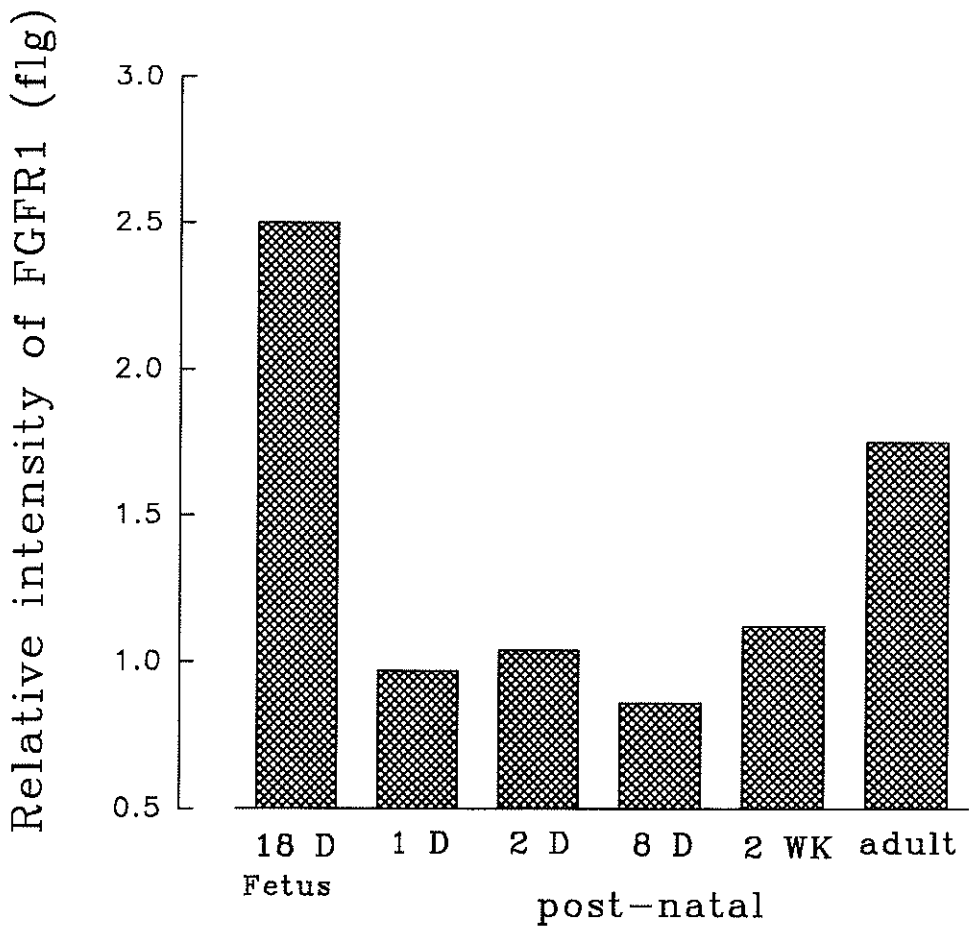


Fig. 3.10 FGFR1 (flg) levels in cardiac development.

Total RNA from 18-day fetuses, 1-day, 2-day, 8-day, 2-week-old and adult rat hearts was analyzed by Northern blotting for FGFR1 mRNA. The blot was also stripped and reprobed for GAPDH to correct for possible loading variations.

3.4 DISCUSSION

3.4.1 Identification of bFGF receptors in adult cardiac sarcolemmal membranes and cultured cardiomyocytes by affinity labelling

Radiolabelled ligand cross-linking to the cell surface or isolated membranes is a technique which has been used extensively to identify membrane growth factor receptors. Cross-linking of ^{125}I-bFGF to SL preparation was carried out according to the methods described by Burrus and Olwin (1989) with modifications. Cross-linking of ^{125}I-bFGF to cultured cardiac myocytes was performed at 4°C in order to label cell surface components and to limit possible internalization of growth factor receptor complexes. This method has revealed the presence of bFGF receptors migrating with an apparent molecular mass usually in the range of 105-165 kDa (Ledoux *et al.*, 1992). There exists a great variation in the size of FGF receptors cross-linked to different types of cells or cells from different species (Ledoux *et al.*, 1992). There is no previous information as to the size of bFGF receptors in cardiomyocytes; our affinity cross-linking data indicated the presence of a 134 kDa receptor in adult cardiac sarcolemmal membranes and cultured cardiac myocytes, and an additional 104 kDa binding protein in cultured cardiac myocytes, well within the size range of bFGF receptors. That the Mr. 134 and 104 species from cardiac myocytes represent bFGF receptors is supported by the fact that binding of ^{125}I-bFGF and cross-linking of these two complexes could be prevented by the addition of excess non-labelled bFGF.

The relationship between the two receptor species identified in cultured cardiomyocytes can be interpreted in a number of ways.

(1) It is possible that the lower Mr receptor species is a proteolytic breakdown product of the larger species. If that is the case, such a proteolytic event must have taken place before cell lysis, since proteinase inhibitors were used during all procedures.

(2) The two receptor species may represent differentially glycosylated forms of the same receptor. It is known that about 25-30 kDa of the bFGF receptor on baby hamster kidney (BHK) cells can be attributed to N-linked carbohydrates and that this exclusive N-linked glycosylation of the bFGF receptors are essential for bFGF binding (Feige and Baird, 1988). The 104-kDa FGF receptor might be a less glycosylated form of the larger receptor.

(3) The 104 kDa receptor species could represent a translational product of a truncated form of the mRNA for the 134 kDa receptor species. Numerous FGFR1 and FGFR2 variants, which differ in their extracellular and cytoplasmic domains, have been found resulting from alternative exon usage, different splicing and poly-adenylation (Partanen *et al.*, 1992). This possibility cannot be excluded, though we only detected one FGFR1 mRNA, the large form, by Northern blot analysis. Yazaki *et al.* (1993) reported detecting two isoforms of FGFR1 mRNA, designated as the short and long form (Fujita *et al.*, 1991; Johnson *et al.*, 1990; Reid *et al.*, 1990; Isacchi *et al.*, 1990; Itoh *et al.*, 1990), in Wistar rat heart tissue by polymerase chain reaction (PCR). Alternative splicing in the third Ig domain has been shown to be important for

determining ligand binding specificities (Werner *et al.*, 1992; Duan *et al.*, 1992; Johnson and William, 1993).

(4) The two FGF binding receptors may be the gene products of different receptor tyrosine kinase (RTK) FGFR genes. Five distinct gene products of which have been reported so far, encoding FGFRs sharing 56-92% amino acid sequence identity and similar overall structures (Jaye *et al.*, 1992). However, only the FGFR1 mRNA has been detected in young rat heart (Engelmann *et al.*, 1993; Speir *et al.*, 1992).

5). The two complexes are structurally unrelated. Beside RTK FGFR, there may exist an unrelated high affinity FGFR. Recently, a cysteine-rich FGF receptor that is distinct from RTK FGFRs has been identified in chick embryos and other vertebrates (Olwin *et al.*, 1991; Burrus *et al.*, 1992; Burrus and Olwin, 1989). Perderiset *et al.* (1992) reported another 150 kDa high affinity FGF receptor purified form adult bovine brain membrane, which does not cross-react with FGFR1- or FGFR2- specific antibodies.

It is not likely that the two FGF receptor isoforms and the regulation associated with myocyte phenotypes result from non-muscle cells, since our cultures contain more that 90% myocytes (confirmed by immunolocalization of striated muscle myosin), and cross-linking of ^{125}I-bFGF to cardiac fibroblasts cultured under the same conditions as those of myocytes did not produce any change in the FGF receptor isoforms (data not shown).

Two cross-linked cell surface FGF receptors displaying a 20-30 kDa difference have also been found in other cells. For example, a rat mammary cell line expressed ^{125}I-bFGF cross-linked surface bFGF receptor at 160 and 140 kDa (Fernig *et al.*, 1990). Cross-linking ^{125}I-bFGF to neuronal cultures labelled a major membrane protein of 135 kDa and a minor protein of 85 kDa (Walicke *et al.*, 1989). Cross-linking of ^{125}I-bFGF or ^{125}I-aFGF to their receptors on BHK-21 cells has identified receptors with molecular weights of 145 kDa and 125 kDa (Neufeld and Gospodarowicz, 1985; 1986). Two purified rat brain bFGF receptors are 140 and 110 kDa (Imamura *et al.*, 1988). Cross-linking studies with acidic and basic ^{125}I-FGF and immunoprecipitaion with anti-FGFR1 antibodies revealed the existence of 150 and 130 kDa species in the A204 rhabdomyosarcoma cell line (Ruta *et al.*, 1989).

3.4.2 Equilibrium binding studies detect high and low affinity bFGF binding sites in adult cardiac sarcolemma

When binding of ^{125}I-bFGF to cardiac sarcolemma was examined as a function of ^{125}I-bFGF concentration, two classes of binding sites were found. For the low affinity binding sites, Kds of 1.14 nM and 2.2 nM (eluted with 2 M NaCl, pH 7.5) were obtained, which are within the previously reported range for low affinity bFGF receptors (Hearn, 1991). For the high affinity sites, Kds of 115 and 254 pM (eluted with 2M NaCl, pH 4.0) were obtained. Both are within the range for high affinity FGFR (Hearn, 1991). Though the Kds from two methods used to determine binding, i.e. total binding and specific elution, are not identical, considering the relatively

limited sample size and high non-specific binding, this discrepancy is within the anticipated range. Our findings contrast with those reported by Ross and Hale (1990), who only detected low affinity binding sites with a Kd of 6.9 nM in bovine cardiac sarcolemmal vesicles. This discrepancy may reflect species differences. Alternatively it might be caused by the experimental methods used to obtain the Kd values. In Ross and Hale's report (1990), a displacement curve was obtained by competing ^{125}I-bFGF binding to the SL with increased amounts of "cold" bFGF. The Kd was then calculated as described by DeBlasi *et al.* (1989). This calculation was based on the assumption that, when the same ligand is used as tracer and competitor (self-displacement), the labelled and cold ligands have the same affinity to the receptor (Munson, 1983). However, it has been suggested that the affinity of ^{125}I-bFGF to its receptor is higher than that of unlabelled bFGF (Neufeld and Gospodarowicz, 1986; 1985). The same phenomenon was observed in our studies, when the ability of unlabelled bFGF to displace ^{125}I-bFGF from the cardiac sarcolemma binding sites was analyzed (Fig 3.5). If the affinity of the labelled agonist remains unchanged, then at a concentration of unlabelled competing bFGF equal to the concentration of labelled bFGF, one would expect that a 50% displacement of the ^{125}I-bFGF from high affinity sites would occur. This was clearly not the case since less than 5% of the ^{125}I-bFGF that bound to the membrane was displaced when the sarcolemmal membranes were incubated in the presence of unlabelled FGF at a concentration equal to the concentration of the ^{125}I-FGF. The reason for this is not established but is in agreement with the notion that the affinity of ^{125}I-FGF to its receptor on cardiac

sarcolemma is higher than that of unlabelled bFGF. It is not likely that our findings represent a displacement of ^{125}I-bFGF from its low affinity binding sites, since only 85 pM ^{125}I-bFGF was included in each sample, a value below the Kd for FGFR detected in cardiac SL and no clear distinction was noticed between displacement from high affinity versus other, lower affinity sites.

Scatchard analysis also indicated that the Bmax for low and high affinity binding sites were 670 fM/mg and 170 fM/mg sarcolemmal protein, respectively. There is no report about the binding capacity of high affinity sites in cardiac sarcolemmal membranes. Our data showed that the capacity of the low affinity sites is approximately 4-fold that of the high affinity sites. This is in agreement with the description of the high affinity FGF binding site as "low capacity", and the low affinity sites as of "high capacity" (Klagsbrun, 1989). Bmax of low affinity sites is in disagreement of that reported by Ross and Hale (1990), which was 15.2 pM bFGF/mg vesicle protein. This discrepancy may reflect differences in species (rat versus bovine), methods of preparation of cardiac sarcolemma vesicle (Pitts, 1979 versus Slaughter *et al.*, 1983) and methods to determine Bmax (equilibrium binding and Scatchard analysis versus displacement experiment and calculation according to DeBlasi *et al.* 1989).

3.4.3 The mitotoxin bFGF-saporin kills isolated adult cardiac myocytes

The mitotoxin bFGF-saporin is a conjugate of saporin, a powerful ribosome-

inactivating protein from the plant Saponaria *Officinalis*, and bFGF (Lappi *et al.*, 1989; Prieto *et al.*, 1991). Saporin is a cytotoxin that has no effect on cells if added exogenously. However, it kills cells if it gains access to the cytoplasm. This can be achieved by accessing cell surface receptors, such as the one for bFGF. The bFGF-saporin conjugate binds to cell surface FGFR in the same way as bFGF, enters cytoplasm of target cells , inhibits protein synthesis, and elicits cell death (Lappi *et al.*, 1991). Its cytotoxicity is dependent upon the interaction with cell surface FGFR (Martineau *et al.*, 1991). Although bFGF and bFGF-saporin are internalized through both cell surface FGFR and HSPG (Roghani and Moscatelli, 1992; Reiland and Rapraeger, 1993), it has been shown that bFGF-saporin internalized through HSPG is not targeted to a compartment from which the saporin has cytoplasmic access and kills the cells (Reiland and Rapraeger, 1993). Therefore, only bFGF-saporin internalized through FGFR, in the presence of HSPG, can gain access to the cytoplasm, and kill the cell (Reiland and Rapraeger, 1993). It is also shown that the efficiency of the mitotoxin correlated with the number of receptors per cell (Lappi *et al.*, 1991). Therefore, the cytotoxicity of bFGF-saporin is a good indicator of the presence of cell surface FGFR.

Our data clearly demonstrated that treatment with bFGF-saporin significantly increased the death rate of cardiac myocytes isolated from adult ventricles, in a dose dependent manner. Under our experimental conditions, isolated cardiac myocytes remained viable for approximately 4-5 hours. The decrease in myocyte viability (as

assessed by cell hypercontraction and "rounding up"), is interpreted as a cytotoxic effect of bFGF-saporin; all the controls (PBS, bFGF or saporin at the same concentration) did not decrease the viability of the myocytes when tested in a similar fashion. Basic FGF at 5 nM increased the survival of cardiomyocytes slightly (90.4% compared to 79.4% in the absence of added bFGF). This is consistent with a report that bFGF protects cultured cardiac myocytes from injury caused by serum starvation (Kardami *et al.*, 1993).

3.4.4 Internalization of bFGF by cultured myocytes

Evidence on the involvement of both high and low affinity receptors in the internalization pathway of bFGF comes from two sets of experiments. Based on a kinetic analysis of internalization data from Chinese hamster lung fibroblasts, Gannoun-Zaki *et al.* (1991) concluded that at equilibrium and at concentrations close to the Kd value, bFGF is internalized via high affinity receptors as well as low affinity receptors. Roghani and Moscatelli (1992) demonstrated more directly that bFGF was internalized at a rapid rate by Chinese hamster ovary (CHO) cells that do not express significant numbers of high affinity receptors for bFGF. This internalization paralleled the interaction of bFGF with low affinity heparan sulfate binding sites on the cell surface and was completely blocked by the addition of 10 μg/ml heparin in those cells. CHO cells that had been transfected with cDNA encoding FGFR1 or FGFR2 and bovine aortic endothelial cells which naturally express high affinity and low affinity receptors for bFGF exhibited heparin-sensitive and heparin-resistant internalization of

bFGF (Roghani and Moscatelli, 1992). So heparin-resistant or heparin-sensitive internalization represent bFGF internalized though the high affinity or low affinity binding sites, respectively (Roghani and Moscatelli, 1992).

We examined the internalization of bFGF by cultured cardiac myocytes according to the methods described by Roghani and Moscatelli (1992). Heparin-resistant internalization was detected in both proliferative and non-mitotic cardiac myocytes in culture, representing approximately 57% and 47% of total internalized bFGF, respectively. This indicated that bFGF was internalized by high affinity FGFR sites in both instances and offers additional support to the notion that non-mitotic cardiomyocytes express functional FGFR.

3.4.5 Expression of FGFR1 and N-syndecan mRNA by cardiac myocytes

Other investigators (Engelmann *et al.*, 1993) have reported that the mRNA for FGFR1 could only be detected in fetal and neonatal heart, but was not detectable in the adult rat heart by Northern blot analysis. Only limited signal could be detected in adult cardiac myocytes by *in situ* hybridization (Speir *et al.*, 1992). Since, however, the ^{125}I-bFGF binding studies presented here clearly showed presence of high affinity FGF receptors in adult myocytes, we decided to re-examine this point. We increased the sensitivity of the approach by using 100-200 μg of total RNA (compared to 10-20 μg employed by others), and a method (random primer labeling) which produced radiolabelled probes with high specific activity (1-3 x 10^9 cpm/μg DNA). Northern

blot analysis using an FGFR1 rat cDNA probe clearly showed hybridization with a 4.3 kb transcript, consistent with the size reported for FGFR1 (Wanaka *et al.*, 1990; Kim *et al.*, 1993; Moore *et al.*, 1991), for both total heart and isolated myocyte RNA. Recently, Yazaki *et al.* (1993) reported expression of FGFR1 mRNA in Wistar rats, in agreement with our data.

Expression of members of the syndecan family (low affinity, HSPG, bFGF receptors) has been reported for the neonatal heart (Carey *et al.*, 1992). We therefore examined expression of N-syndecan in adult myocardium. A 5.5 kb transcript was detected, in the previously reported N-syndecan mRNA size range (Carey *et al.*, 1992)

To estimate the contribution of cardiomyocytes to the Northern blot signals we compared levels of expression in total heart versus that in isolated myocyte RNA. Similar levels of FGFR1 and N-syndecan mRNA were detected, indicating the cardiomyocytes contribute most of the cardiac FGF receptor signal.

3.4.6 Adult cardiac myocytes express bFGF receptors

The presence of high affinity FGF receptors in rat mature cardiac myocytes is supported by the following evidence: (1). Affinity cross-linking data indicated the presence of a 134 kDa ^{125}I-bFGF binding protein in adult cardiac sarcolemma membranes, which is within the size range for FGFR1 (Jaye *et al.*, 1992) and was specifically blocked by "cold" bFGF. (2). Analysis of binding of ^{125}I-bFGF to highly

purified cardiac sarcolemma revealed the presence of high affinity binding sites with a Kd of 100-200 pM; this binding was abolished in 2M NaCl, pH 4.0, as expected for high-affinity bFGF receptors (Moscatelli, 1987). (3). ^{125}I-bFGF was internalized through a heparin-resistant pathway in cultured non-dividing cardiomyocytes. It has been shown previously that heparin-resistant entry into cells is mediated by high affinity FGFR. (4). The FGFR-specific mitotoxin bFGF-saporin exhibited cytotoxicity on adult cardiac myocytes isolated from rat ventricles. (5). FGFR1 mRNA was detected from RNA obtained from both total ventricle and isolated myocytes of adult hearts.

The presence of low affinity FGF receptor in myocardium has been described (Ross and Hale, 1990; Ross *et al.*, 1993). Our data confirmed that the mRNA of N-syndecan, a bFGF-binding HSPG, is expressed by cardiomyocytes and that "low" affinity bFGF binding sites are present in rat cardiac SL. These sites are identified as low affinity FGF receptors based on the following: (1) They bind bFGF with a Kd within the range of that described for low affinity FGF receptor. (2) Basic FGF was released from those receptor sites by 2 M NaCl at neutral pH, a condition known to elute bFGF from its low affinity receptor (Moscatelli, 1987). (3) Detection of heparin-sensitive internalization of ^{125}I-bFGF in cultured myocytes. It has been shown that HSPG-mediated internalization is blocked by heparin. (4) The cytotoxic effect of FGF-saporin has been shown to require the collaboration of FGFR and HSPG.

The presence of bFGF receptors in adult differentiated myocytes would be consistent with a role in normal cardiac physiology. The nature of this role is as yet unknown. Basic FGF is a mitogen for immature cardiomyocytes, and is present in the heart throughout development (Kardami, 1993), however, adult cardiomyocytes do not divide (Zak, 1984). It is possible that bFGF has non-mitogenic roles in the heart, as shown for other tissues (Nozaki *et al.*, 1993). Recent evidence from our laboratory points to a potential protective role for bFGF against cardiomyocyte injury: Basic FGF reduced the H_2O_2- and serum starvation-induced myocyte injury in culture (Kardami *et al.*, 1993), and protected perfused adult rat hearts from ischemia-reperfusion damage (Padua *et al.*, 1993). Furthermore, localization of bFGF in the cytoplasmic face of cardiac gap junctions suggested a role in intercellular communication (Kardami *et al.*, 1991).

It has long been considered that adult cardiomyocytes have lost their ability for cell division irreversibly (Claycomb, 1983; 1991; Rumyantsev 1991). This would explain the apparent inability of an endogenous mitogen such as bFGF to stimulate mitosis in the adult myocardium. Accumulating evidence from several laboratories, however, has shown that adult cardiomyocytes are capable of bFGF-induced stimulation of DNA synthesis (Claycomb and Moses, 1988; Speir *et al.*, 1992) and even cell division (Anversa *et al.*, 1990b). It is possible that: (1) bFGF and/or FGFR local concentration/composition are inadequate for stimulation of mitosis, (2) local conditions (other growth-affecting polypeptides or hormones, metabolic states of the

myocyte, contact inhibition) block or cancel bFGF activity on cardiomyocytes. Our laboratory has provided evidence in support of both possibilities: We have shown previously that while levels of 18 kDa bFGF actually increase in the adult heart (Liu *et al.*, 1993), those of the 21.5-22 kDa bFGF diminish; a hyperplastic response therefore may require higher levels of the N-extended bFGF. In addition, we have provided some evidence here to the effect that non-mitotic cultured myocytes express a different ratio of the high to low Mr. FGFR compared to the corresponding ratio when in the proliferative state, and that thyroid hormone may play a role in modulating this ratio. These findings would be consistent with different roles for the different bFGF receptors (see also following section). Finally, we have shown that local factors (such as TGF-ß) or blood hormones (such as thyroid hormone) cancel the stimulatory effect of bFGF on cardiac myocytes in culture (Kardami, 1990). Detailed studies are required to establish the mechanism of these interactions, mechanism of FGF signal transduction and regulation of bFGF/FGFR expression in the heart.

3.4.7 Regulation of FGFR during development and by thyroid hormone

Cells or tissues could achieve selective responsiveness to FGF at different developmental stages by the preferential expression of specific forms of FGF receptors, differing in activity, selectivity and signal transduction mechanisms. Alternative splicing of FGFR1, producing different receptor isoforms, occurs in a tissue-specific fashion (Bernard *et al.*, 1991; Werner *et al.*, 1992). Tissue-specific or development-dependent expression of the different FGFR genes has also been documented (Givol

and Yayon, 1992; Sato *et al.*, 1992; Shi *et al.*, 1992). There are no previous reports on the regulation of expression of different FGFR forms in the myocardium. A major role for bFGF in early developmental stages may be to promote proliferation and prevent terminal differentiation. Based on data presented here we can speculate that this activity is associated with the 104 kDa FGFR, expressed in higher levels in proliferative compared to post-mitotic myocytes. Similarly, the 134 kDa receptor may be linked to bFGF activities related to differentiated function. Future studies should address expression of these FGFR in cardiac development *in vivo*, and whether their regulation is a consequence or a cause of differentiation.

Since thyroid hormone can modulate the activity as well as expression of bFGFs we conducted initial experiments on the effect of thyroid hormone on FGFR expression. Levels of the 104 kDa receptor were decreased significantly when thyroid hormone was added to post-mitotic cardiomyocyte cultures. Since thyroid hormone promotes maturation, this finding is consistent with the hypothesis that down-regulation of the 104 kDa FGFR accompanies the differentiated phenotype in the heart and explains the predominance of the 134 kDa FGFR in adult myocyte sarcolemma. It should be noted, however, that thyroid hormone did not decrease levels of the 104 kDa form when added to proliferative myocytes; in fact a slight (statistically insignificant) increase was observed. Different regulatory mechanisms (for instance different types of thyroid hormone nuclear receptors) could exist between proliferative and non-dividing myocytes.

We examined the expression of FGFR1 mRNA in rat heart, in development. Levels of FGFR1 mRNA were highest in the embryonic heart, declining immediately after birth, in agreement with Engelmann *et al.* (1993), but increasing after the third post-partum week. In contrast however with Engelmann *et al.* (1993), we detected near embryonic levels of FGFR1 mRNA in rat mature myocardium (around 14-16 weeks old.). This finding was further validated by the ^{125}I-bFGF binding studies, confirming the presence of FGFR in the adult heart. Reasons for the discrepancy between our findings and those of Engelmann for the adult heart are not clear. It is possible that they reflect differences in animal species and age, methods used to detect mRNA as well as the particular probes used. We used 14-16 week old SD rats and a rat FGFR1 cDNA probe (Wanaka *et al.*, 1990), while Engelmann *et al.* employed Wistar rats and a human FGFR1 probe (Dionne *et al.*, 1990).

3.5 CONCLUDING REMARKS

In conclusion, we have examined the expression of FGFR1 and N-sydecan mRNAs in rat myocardium and the interaction of ^{125}I-bFGF with plasma membranes of adult cardiomyocytes (purified sarcolemmal vesicles) as well as cultured proliferative or bi-nucleated (post-mitotic) rat cardiomyocytes. Our results demonstrated that high affinity as well as low affinity receptors for FGF are present in adult differentiated myocytes, which would be consistent with a role of bFGF in normal cardiac physiology. We also provide evidence to the effect that a 104 kDa FGF receptor protein was associated with the proliferative phenotype of cardiac

myocytes and down regulated in non-dividing myocytes. Thyroid hormone treatment of the non-dividing myocytes was shown to decrease this 104 kDa FGF receptor further. The 134 kDa FGF receptor was present throughout development and appeared not to be regulated by thyroid hormone. FGFR1 mRNA levels were developmentally regulated: high in fetal heart, declining during the first 2 week after birth but increasing again in the mature heart.

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