

# Differential Modulation of Cell Phenotype by Different Molecular Weight Forms of Basic Fibroblast Growth Factor: Possible Intracellular Signaling by the High Molecular Weight Forms

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**Abstract.** To study possible functional differences of the 18-kD and high molecular weight forms of basic fibroblast growth factor (bFGF), we have examined the effect of endogenous production of different bFGF forms on the phenotype of NIH 3T3 cells. Cells transfected with cDNAs coding for either 18-kD bFGF (18-kD bFGF) or all four molecular forms (18, 22, 22.5, 24 kD; wild type [WT] bFGF) exhibit increased migration and decreased FGF receptor number compared to parental cells. However, migration and FGF receptor number of cells transfected with a cDNA coding only for high molecular weight bFGF (22, 22.5, and 24 kD; HMW bFGF) were similar to that of parental cells transfected with vector alone. Cells expressing HMW, 18 kD, or WT bFGF grew to high saturation densities in 10% serum. However, only cells expressing HMW or WT bFGF grew in low serum. Cell surface or metabolic labeling of the different cell types followed by immunoprecipitation with anti-bFGF antibody showed primarily cell surface-associated 18-kD bFGF. In addition, when cells ex-

pressing exclusively HMW bFGF were transfected with a cDNA coding for 18-kD bFGF, migration was increased, bFGF receptors were down-regulated, and 18-kD bFGF was found on the cell surface. Cells expressing 18-kD bFGF transfected with a cDNA encoding FGF receptor-2 lacking the COOH-terminal domain (dominant negative bFGF receptor) exhibited a flat morphology and decreases in migration and saturation density. Cells expressing HMW bFGF transfected with the dominant negative bFGF receptor continued to grow to a high saturation density, proliferated in low serum, and exhibited no morphological changes. These results indicate that increased cell migration and FGF receptor down-regulation are mediated by the extracellular interaction of 18-kD bFGF with its cell surface receptor. Growth in low serum may be stimulated by the intracellular action of HMW bFGF through mechanisms independent of the presence of a cell surface receptor. Thus, the different molecular forms of bFGF may act through distinct but convergent pathways.

**B**ASIC fibroblast growth factor (bFGF)<sup>1</sup> is the prototype member of the heparin-binding growth factor family that currently includes eight other proteins (Basilico and Moscatelli, 1992; Miyamoto et al., 1993). bFGF is a potent mitogen for a variety of cell types, including endothelial cells and fibroblasts, and affects a number of additional cellular functions such as protease production, surface integrin levels, and cell migration (Basilico and

Moscatelli, 1992; Klein et al., 1993). Many cells produce bFGF both in vivo and in vitro. Four different tyrosine kinase plasma membrane receptors for FGF have been described and are found on many cell types (Jaye et al., 1992; Basilico and Moscatelli, 1992). Basic FGF, like acidic FGF (aFGF), does not contain a signal sequence (Abraham et al., 1986) and is not released via the classical secretory pathway (McNeil et al., 1989; Muthukrishnan et al., 1991; Mignatti et al., 1992). Moreover, the mechanism(s) for release of bFGF and aFGF remain(s) unknown. Different molecular forms of bFGF representing alternative translation products from a single mRNA have been described (Moscatelli et al., 1987b; Sommer, 1987; Florkiewicz and Sommer, 1989; Prats et al., 1989). The translation of the smallest molecular weight form (18 kD) of bFGF is initiated at an internal AUG codon, whereas the translation of the higher molecular

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; BS<sub>3</sub>, bis (sulfosuccinimidyl) suberate; G418, geneticin; HMW bFGF, high molecular weight bFGF; K-fgf, Kaposi's sarcoma FGF; WT, wild type.

weight bFGF (HMW bFGF; 22-24 kD) forms is initiated at CUG codons 5' to the AUG codon. In vitro most cells produce all forms of bFGF. HMW bFGF is preferentially localized in the nucleus, whereas 18-kD bFGF is found predominantly in the cytosol (Quarto et al., 1991a,b; Renko et al., 1991; Bugler et al., 1991; Florkiewicz et al., 1991). The nuclear localization of HMW bFGF is determined by the additional amino-terminal sequences not found in 18-kD bFGF as proteins containing the unique amino-terminal sequences of HMW bFGF fused to  $\beta$ -galactosidase or chloramphenicol acetyltransferase accumulate in the nucleus (Quarto et al., 1991a; Bugler et al., 1991). Fusion of 18-kD bFGF to pyruvate kinase does not direct the chimeric protein to the nucleus (Quarto, N., unpublished observation).

The existence of multiple forms of bFGF with different subcellular localization raises the question of whether these forms of bFGF have different mechanisms of action and specialized functions. We have addressed this question by creating transfected NIH 3T3 cell lines that express exclusively 18-kD bFGF, HMW bFGF, or all forms of bFGF (wild type [WT] bFGF) (Quarto et al., 1989, 1991b). Cells expressing increased levels of 18-kD or WT bFGF had growth properties of transformed cells, including increased saturation density and anchorage-independent growth. Cells expressing HMW bFGF were of two types; many clones were small and had impaired growth as though HMW bFGF expression inhibited cell division. These cells contained low levels of HMW bFGF and had only one copy of the integrated HMW bFGF cDNA. Clones that expressed higher levels of HMW bFGF and had multiple copies of integrated HMW bFGF cDNA grew rapidly and had the properties of transformed cells (Quarto et al., 1991b).

To characterize potential functional differences between 18-kD and HMW bFGF, we extended our analysis of cells expressing specific forms of bFGF. In this paper, we provide evidence for a differential modulation of the cell phenotype by endogenous production of either 18-kD or HMW bFGF and for different mechanisms of action. Cells transfected with cDNAs for either 18-kD or WT bFGF display enhanced migration, whereas cells transfected with HMW bFGF cDNA exhibit no changes in migration. Although cells expressing HMW or WT bFGF grow in low serum, cells expressing 18-kD bFGF do not. Expression of a cDNA encoding a dominant negative bFGF receptor reverses those phenotypic properties associated with 18-kD bFGF expression but not those associated with HMW bFGF. Thus, high levels of expression of HMW bFGF may affect cell proliferation by a mechanism independent of the presence of a cell surface receptor. These data provide evidence for potentially different functional mechanisms for HMW and 18-kD bFGF action.

## Materials and Methods

### Reagents

Recombinant human bFGF (18 kD) was a gift from Synergen, Inc. (Boulder, CO). HMW bFGF was purified from guinea pig brains as described by Moscatelli et al. (1987). Na<sup>125</sup>I (16.93 mCi/ $\mu$ g) was purchased from Amersham Corp. (Arlington Heights, IL), and <sup>35</sup>S-L-cysteine (1,220 Ci/mmol) and <sup>32</sup>P-orthophosphate (8,500–9,120 Ci/mmol) from Dupont Radiochemicals (Boston, MA). All other reagents were research grade.

## Cells

NIH 3T3 cells were transfected with the Zip-neo vectors containing either a 1.1-kb insert of a cDNA coding all bFGF forms (24, 22.5, 22, 18 kD; clones WTFGFc1, WTFGFc3, WTFGFc7, WTFGFc22), a cDNA coding only for the 24–22-kD bFGF (clones 365FGFcl, 365FGFcl2, 365FGFcl9, 365FGFcl1, 365FGFcl5), or a cDNA coding only for 18-kD bFGF (clones 43FGFcl8, 43FGFcl21, 43FGFcl31) as described (Quarto et al., 1989). The cells were grown in DME containing 10% FCS plus 500  $\mu$ g/ml Geneticin (G418; GIBCO BRL, Gaithersburg, MD) and were subcultured at a ratio of 1:10.

Secondary transfection of cells with 18-kD bFGF cDNA: NIH 3T3 cells transfected with the Zip-neo vector containing HMW bFGF cDNA were subsequently transfected with 18-kD bFGF cDNA inserted in the Zip-neo vector plus the pCEP4 vector containing a hygromycin resistance gene (kindly provided by Dr. C. Basilico, New York University Medical Center, New York, NY) at a ratio of 8:1. Hygromycin-resistant clones were selected in DME containing 10% FCS, 200  $\mu$ g/ml of hygromycin B (Calbiochem-Behring Corp., La Jolla, CA) and 250  $\mu$ g/ml of G418. Secondary transfectants were identified by Western blotting of cell extracts with anti-bFGF antibodies (clones: 365/43 NC1; 365/43 NC2; 365/43 NC12; 365/43 NC33).

Transfection with a dominant negative bFGF receptor cDNA: NIH 3T3 cells transfected with the Zip-neo vector containing either HMW, 18 kD, or WT bFGF cDNAs were cotransfected with pRK5 containing a 1.3-kb insert of a human *bek* (FGF receptor 2) cDNA that lacks the COOH-terminal tyrosine kinase domain (dominant negative bFGF receptor; kindly provided by Dr. J. Schlessinger, N.Y.U. Medical Center) and the pCEP4 vector. Hygromycin-resistant clones were selected in DME containing 10% FCS, 200  $\mu$ g/ml of hygromycin, and 250  $\mu$ g/ml of G418. Resistant clones were tested for high affinity bFGF receptors according to Moscatelli (1987) and by cross-linking to cell surface receptors with <sup>125</sup>I-bFGF. HMW bFGF clones: 365DNc2, 365DNc4, 365DNc5, 365DNc6, 365DNc7, 365DNc12, 365DNc14, 365DNc24, 365DNc28, 365DNc32, 365DNc38, 365DNc39, 365DNc42, 365DNc43, 365DNc46, 365DNc47, 365DNc51, 365DNc63, 365DNc71, 365DNc80; control clones transfected with the cDNA encoding the hygromycin-resistant gene alone: 365FGFHc3, 365FGFHc5, 365FGFHc8; 18-kD bFGF clones: 43DNc8, 43DNc10, 43DNc11, 43DNc30, 43DNc31, 43DNc33, 43DNc38, 43DNc62, 43DNc72, 43DNc73; control clones transfected with the cDNA encoding the hygromycin-resistant gene alone: 43FGFHc3, 43FGFHc8, 43FGFHc10; WT bFGF clones: WTDNc1, WTDNc2, WTDNc3, WTDNc4, WTDNc7; control clones transfected with the cDNA encoding the hygromycin-resistant gene alone: WTFGFHc3, WTFGFHc5, WTFGFHc10.

These clones were analyzed with comparable results in the experiments shown in the figures and were obtained from at least two independent transfections.

### Cell Migration Assay

Cell migration was assayed by the phagokinetic track assay (Albrecht-Buehler, 1977) as modified by Mignatti et al. (1991, 1992). Briefly, 20 cells/well were seeded on coverslips coated with 1% BSA (Sigma Chem. Co., St. Louis, MO) and colloidal gold and incubated at 37°C in 24-well plates.

The medium was replaced with fresh medium after 2 h. After overnight incubation at 37°C, the cells were fixed with 3.7% formaldehyde and track areas were measured with an image analyzer (model 982; Artek Systems Corp., Farmingdale, NY). The area units obtained with the image analyzer (pixels) were converted into  $\mu$ m<sup>2</sup> by interpolation with a calibration curve obtained by measuring the size of different squares of a hemocytometer. The data were plotted as a probability plot using Kaleidograph software run on an Apple Macintosh IIsi computer.

### Cell Proliferation Assay

20,000 cells were seeded in 3.5 cm culture dishes. After overnight incubation at 37°C, the cells were refed with DME containing either 10% or 1% FCS. The cells were counted with a Coulter counter (Coulter Electronics, Hialeah, FL) every day for 8 to 10 days.

### Growth in Soft Agar

10,000–100,000 cells were seeded in 6 cm dishes in DME containing 10% FCS plus 0.3% agar (2 ml) on a 0.6% (3 ml) agar underlay. The cells were refed twice a week with DME containing 10% FCS and 0.3% (1.5 ml) agar. Colonies were counted after a 3-wk incubation at 37°C.

## Metabolic Labeling and Immunoprecipitation

Cells were labeled with  $^{35}\text{S}$ -L-cysteine (250  $\mu\text{Ci}/\text{ml}$ ) for 30 min in cysteine-free minimal Eagle's medium (MEM) and chased with MEM containing 7% FCS and 250  $\mu\text{M}$  of L-cysteine. At the indicated times, the medium was collected, and the cells were washed twice with 20 mM Tris-HCl, 2 M NaCl, pH 4.0, to remove cell surface-associated bFGF. The cells were harvested with 20 mM Tris-HCl, pH 7.4, containing 2 M NaCl, 3 mM EDTA, 20 U/ml of Trasylol (Sigma Chem. Co.), 1 mM DTT (Sigma Chem. Co.), and 0.5  $\mu\text{g}/\text{ml}$  of leupeptin (Sigma Chem. Co.) (buffer 1), sonicated and kept on ice for 30 min. The NaCl concentration was adjusted to 0.5 M with lysis buffer (20 mM Tris-HCl, pH 7.4, 3 mM EDTA, 0.3% SDS, 1% Triton X-100, 1 mM DTT, 0.5  $\mu\text{g}/\text{ml}$  leupeptin, 20 U/ml of Trasylol, and 0.1% BSA).

Conditioned media, 2 M NaCl cell surface washes, and cell extracts were precleared with 100  $\mu\text{l}$  of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and immunoprecipitated with anti-bFGF antiserum (1/200 vol/vol). In some experiments the NaCl concentration of the samples was adjusted to 0.5 M NaCl with NaCl-free buffer 1, and the samples were incubated with 150  $\mu\text{l}$  of heparin-Sepharose beads for 2 h at 37°C. The heparin-Sepharose beads were washed twice with the same buffer and eluted with buffer 1 containing 2 M NaCl. The NaCl concentration was adjusted to below 0.5 M and immunoprecipitation was performed as indicated above. Sample volumes were normalized to protein concentration. After overnight incubation at 4°C, the beads were washed five times with lysis buffer, and the entire sample loaded in reducing sample buffer on 10 or 15% SDS-polyacrylamide gels. After electrophoresis the gels were dried and analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or by autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY).

## $^{125}\text{I}$ -bFGF Binding Experiments

Binding experiments for high or low affinity bFGF receptors were performed as described (Moscatelli, 1987). Cells in 35 or 60 mm dishes were incubated in duplicate with the indicated concentrations of  $^{125}\text{I}$ -bFGF at 4°C with or without recombinant bFGF. After a 2-h incubation, the cells were washed three times with ice-cold PBS and binding to high or low affinity receptors analyzed as described (Moscatelli, 1987). Cross-linking of  $^{125}\text{I}$ -bFGF receptors was performed as follows: After a 2-h incubation with  $^{125}\text{I}$ -bFGF, cells were washed three times with ice-cold PBS and incubated for 20 min at room temperature with 1 mM bis (sulfosuccinimidyl) suberate (BS<sub>3</sub>; Pierce Chem. Co., Rockford, IL). At the end of the incubation the cells were washed twice with PBS, lysed with sample buffer (0.01% Bromphenol blue, 1% SDS, and 20% glycerol in 50 mM Tris, pH 6.8). The proteins were separated on either 6 or 8% SDS-polyacrylamide gels under reducing conditions, and the iodinated proteins visualized by autoradiography. The samples were analyzed in duplicate.

## Tyrosine Phosphorylation

Cells incubated for 10 min with or without 100 ng/ml of bFGF were labeled for 3 h with  $^{32}\text{P}$ -orthophosphate (125  $\mu\text{Ci}/60$  mm dish) in labeling medium (25 mM Hepes, 125 mM NaCl, 4.8 mM KCl, 2.6 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 5.6 mM glucose, and 0.1% BSA, pH 7.4). The cells were washed once with cold  $\text{Ca}^{2+}$ -,  $\text{Mg}^{2+}$ -free PBS and extracted in lysis buffer (10 mM Tris-HCl, 50 mM NaF, 50 mM NaCl, 30 mM Na pyrophosphate, 5 mM EDTA, 100  $\mu\text{M}$  Na orthovanadate, 1% Triton X-100, and 1 mM PMSF, pH 7.4).

Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies coupled to Agarose (R & D Systems, Minneapolis, MN) or affinity-purified anti-phosphotyrosine antibodies bound to protein A-Sepharose (kindly donated by Dr. W. Burgess, Holland Laboratories, American Red Cross, Rockville, MD). After overnight incubation, the beads were washed six times in wash buffer, placed in reducing sample buffer, and electrophoresed in 8 or 10% SDS polyacrylamide gels. The gels were dried and exposed to XAR-5 film.

## Cell Surface Labeling and Immunoprecipitation

Subconfluent NIH 3T3 cells in 15 cm dishes (Falcon Plastics, Cochranville, PA) were washed with PBS and detached with 5 mM EDTA. The suspended cells were washed three times with PBS, and surface proteins were labeled with lactoperoxidase (158  $\mu\text{g}/\text{ml}$ ; Sigma Chem. Co.), 0.0038%  $\text{H}_2\text{O}_2$ , and Na  $^{125}\text{I}$  (1 mCi/ml). The cells were washed three times with DME containing 0.02%  $\text{NaN}_3$  and solubilized in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 0.5% Triton X-100 containing

leupeptin (10  $\mu\text{g}/\text{ml}$ ), pepstatin A (4  $\mu\text{g}/\text{ml}$ ), and aprotinin (50  $\mu\text{g}/\text{ml}$ ). The  $^{125}\text{I}$ -labeled cell lysates were clarified in an Eppendorf microfuge for 30 min and preabsorbed with normal rabbit IgG and protein A-Sepharose. To adjust for changes in cell number due to bFGF, the radiolabeled cell extracts were normalized to TCA precipitable radioactivity. Antibody to bFGF (1/150 dilution) was added to aliquots of the cell lysates and rotated for 1 h at 4°C. Protein A-Sepharose beads were added to each sample, and the samples rotated for 1 h at 4°C. The beads were washed five times with 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 50 mM Tris HCl, pH 7.4, boiled for 5 min in reducing SDS-PAGE sample buffer, and electrophoresed in 12% SDS-polyacrylamide gels. Dried gels were exposed to XAR-5 film or analyzed by PhosphorImager scanning.

## Results

### Migration Is Stimulated in NIH 3T3 Cells Expressing 18-kD bFGF but not in Cells Expressing HMW bFGF

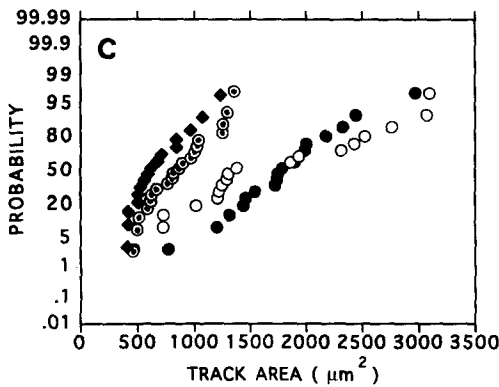
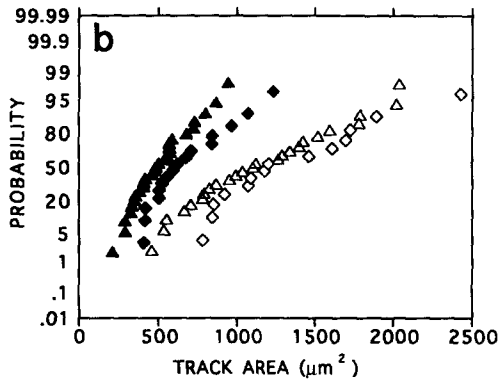
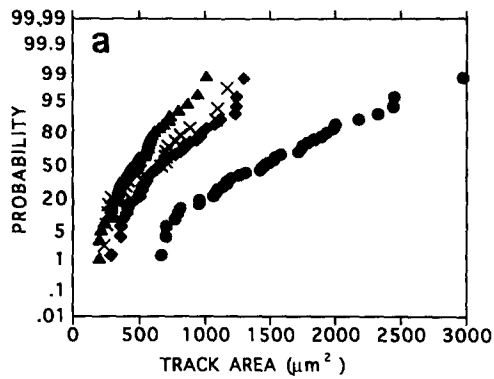
Because endogenous production of bFGF affects cell migration (Mignatti et al., 1991), we examined the migration of NIH 3T3 cells transfected with different species of bFGF. For these studies, we used the phagokinetic gold track assay as NIH 3T3 cells migrate in an FGF-dependent manner in this assay (Mignatti et al., 1991, 1992). As shown in Fig. 1 a, NIH 3T3 cells transfected with a cDNA encoding 18-kD bFGF have an increased capacity to migrate when compared with control cells transfected with vector alone. This result is consistent with data obtained previously with cells overexpressing WT bFGF (Mignatti et al., 1991). The cells produce approximately 84 ng 18-kD bFGF or 190 ng WT bFGF per mg of total cell protein as determined by ELISA (Bruner et al., 1993). In contrast, NIH 3T3 cells from two clones transfected with the HMW bFGF cDNA migrated to the same extent as control cells. These cells produce  $\sim$ 85 ng of HMW bFGF per mg of cell protein.

The migration of WT bFGF expressing cells has been shown to depend upon the extracellular concentration of bFGF (Mignatti et al., 1991). Therefore, we investigated whether addition of exogenous recombinant bFGF also increased the migration of NIH 3T3 cells transfected with HMW bFGF cDNA (Fig. 1 b). Addition of recombinant 18-kD bFGF increased the migration of cells transfected with the HMW bFGF cDNA (clone 365FGFc2) as well as that of control cells (Fig. 1 b). Thus, the lack of migration of cells expressing high amounts of HMW bFGF is not the result of the cells being refractory to the stimulation of exogenous bFGF. In contrast, the migration of cells transfected with the cDNA encoding 18-kD bFGF was not enhanced by exposure to exogenous bFGF (Fig. 1 c), probably because the migration of these cells is maximal under normal conditions (Mignatti et al., 1991, 1992).

HMW bFGF purified from guinea pig brain stimulated the migration of both control cells and cells expressing only HMW bFGF as efficiently as recombinant 18-kD bFGF (Table 1). Therefore, the low level of migration in NIH 3T3 cells expressing only HMW bFGF does not result from an inability to respond to extracellular bFGF or from HMW bFGF lacking migration-inducing activity on NIH 3T3 cells.

### Low Serum Is Permissive for The Growth of Cells Expressing HMW bFGF but not for Cells Expressing 18-kD bFGF

In the presence of 10% serum, cells expressing high levels of 18-kD, HMW, or WT bFGF grow to high saturation den-



**Figure 1.** Phagokinetic track areas formed by NIH 3T3 cells transfected with different bFGF cDNAs or with the vector alone. Cells were seeded onto colloidal gold-coated coverslips at a density of 20 cells/coverslip. Following 16-h incubation at 37°C, measurement of the phagokinetic track areas and elaboration of the results were performed as described in Materials and Methods. Each point shows the probability of finding a phagokinetic track with an area equal to or lower than the value indicated on the abscissa. The data were plotted using a probability scale on the ordinate. (a) Migration of cells expressing HMW bFGF. (▲) Cells expressing HMW bFGF, clone 365FGFc2, mean track area  $\pm$  SEM:  $481.1 \pm 28.8 \mu\text{m}^2$ , (X) clone 365FGFc1:  $609.4 \pm 64 \mu\text{m}^2$ ; (●) cells expressing 18-kD bFGF, clone 43FGFc31:  $1481.9 \pm 90.1 \mu\text{m}^2$ ; (◆) control cells, Zipneo:  $716.2 \pm 45.8 \mu\text{m}^2$ . (b) Migration of cells expressing HMW bFGF in the presence of exogenous bFGF. The migration of cells expressing HMW bFGF (clone 365FGFc2) or control cells (Zipneo) was measured either in the absence (▲, 365FGFc2; ◆, Zipneo) or the presence (Δ, 365FGFc2; Zipneo) of 20 ng/ml of bFGF. In the presence of bFGF, the mean track area of control cells was  $1339.8 \pm 128.7 \mu\text{m}^2$  (unstimulated  $679.6 \pm 61.8 \mu\text{m}^2$ ) and that of 365FGFc2 cells was  $1153.6 \pm 92 \mu\text{m}^2$  (unstimulated  $505.1 \pm 35.5 \mu\text{m}^2$ ). (c) Migration of cells expressing 18-kD bFGF in the

**Table I.** Effect of Guinea Pig HMW bFGF on the Migration of Either Cells Expressing HMW bFGF or Control Cells

Addition	Control cells	HMW bFGF expressing cells
	<i>Mean track area in <math>\mu\text{m}^2</math> + SEM</i>	
None	$721 \pm 64$	$460 \pm 52.6$
HMW bFGF (20 ng/ml)	$1236.9 \pm 70.9$	$1005 \pm 80.9$

Cells were plated on colloidal gold-coated coverslips and their migration was measured as described in Materials and Methods. The track areas ( $\mu\text{m}^2$ ) of at least 15 cells were measured for each sample.

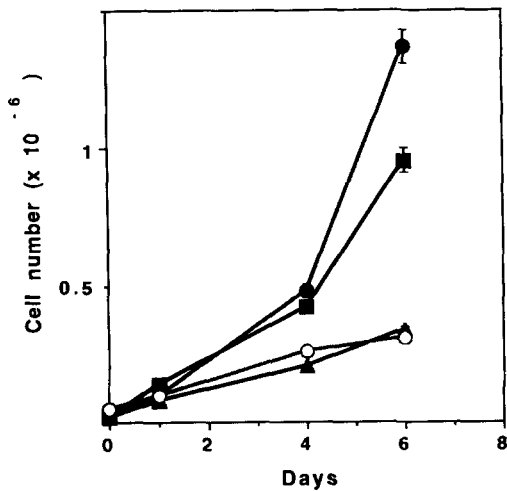
sities compared to control cells transfected only with vector (Quarto et al., 1989). However, in the presence of 1% FCS (Fig. 2), the growth of cells expressing HMW or WT bFGF was much higher than that of cells expressing only 18-kD bFGF. The growth of cells expressing 18-kD bFGF in 1% FCS was similar to that of the control cells transfected with vector alone. Similar results were obtained with two additional clones of each type isolated from an independent transfection (data not shown).

#### **FGF Receptors Are Down-modulated in Cells Expressing 18-kD bFGF but not in Cells Expressing HMW bFGF**

To investigate whether FGF receptors are modulated differently in NIH 3T3 cells expressing the different species of bFGF, the binding of  $^{125}\text{I}$ -bFGF to cells was examined. When the cells were incubated with saturating concentrations of  $^{125}\text{I}$ -bFGF, bFGF binding to high affinity receptors was decreased in cells expressing 18-kD bFGF compared to control cells (Fig. 3 a). In contrast, the binding to high affinity receptors in cells transfected with HMW bFGF was only slightly decreased. These differences were maintained for at least six days after plating. Cells expressing all forms of bFGF also displayed strongly reduced binding of  $^{125}\text{I}$ -bFGF to high affinity receptors as described previously (Moscatelli and Quarto, 1989). These results suggest that high affinity receptors for bFGF were down modulated in cells expressing 18-kD bFGF but not in cells expressing HMW bFGF.

To substantiate this hypothesis, bFGF receptors were characterized by chemical cross-linking to  $^{125}\text{I}$ -bFGF. Autoradiograms of the cross-linked cell extracts after SDS-PAGE showed a significant decrease in the amount of the  $\sim 130$ - $150$ -kD band corresponding to the bFGF receptor in cells expressing 18-kD bFGF (Fig. 3 b). However, in cells expressing only HMW bFGF, the intensity of the bFGF receptor band was only slightly diminished compared to control cells. Binding of  $^{125}\text{I}$ -bFGF was competed by in-

presence of exogenous bFGF or anti-bFGF antibodies. The migration of cells expressing only 18-kD bFGF (clone 43FGFc31) was measured either in the presence of (○;  $1717.1 \pm 205.0 \mu\text{m}^2$ ) or absence (◐;  $1802.3 \pm 198.4 \mu\text{m}^2$ ) of 20 ng/ml bFGF with (◑; mean track area  $\pm$  SEM:  $837.8 \pm 60.8 \mu\text{m}^2$ ) or without (●) affinity-purified anti-bFGF antibodies (20  $\mu\text{g}/\text{ml}$ ). Control cells (◆, Zipneo,  $679.6 \pm 61.8 \mu\text{m}^2$ ).



**Figure 2.** Growth of cells expressing HMW, 18 kD, or WT bFGF in 1% serum. Cell growth was monitored over the indicated time intervals in the presence of 1% FCS as described in Materials and Methods. Cells expressing HMW (●, clone 365FGFc2), 18-kD (▲, clone 43FGFc31), or WT bFGF (■, clone WTFGFc3). Control (○, Zipneo). This experiment was repeated three times with similar results. Each point represents the mean  $\pm$  SEM of duplicate samples.

incubating the cells with a 100-200-fold excess of unlabeled bFGF before cross-linking.

FGF receptor activation by exogenous bFGF was also investigated by immunoprecipitating  $^{32}\text{P}$ -orthophosphate-labeled cell extracts with antiphosphotyrosine antibodies. These results revealed that exogenous bFGF stimulated the phosphorylation of several proteins, including proteins at 130-150 and 90 kD, in both control and in cells expressing HMW bFGF, but not in cells expressing 18-kD bFGF (manuscript

in preparation). In cells expressing 18-kD bFGF, however, enhanced phosphorylation of the 130-150-kD band was observed without the addition of bFGF. On some occasions addition of recombinant bFGF to these cells decreased the level of protein phosphorylation. The reasons for this decrease are not understood.

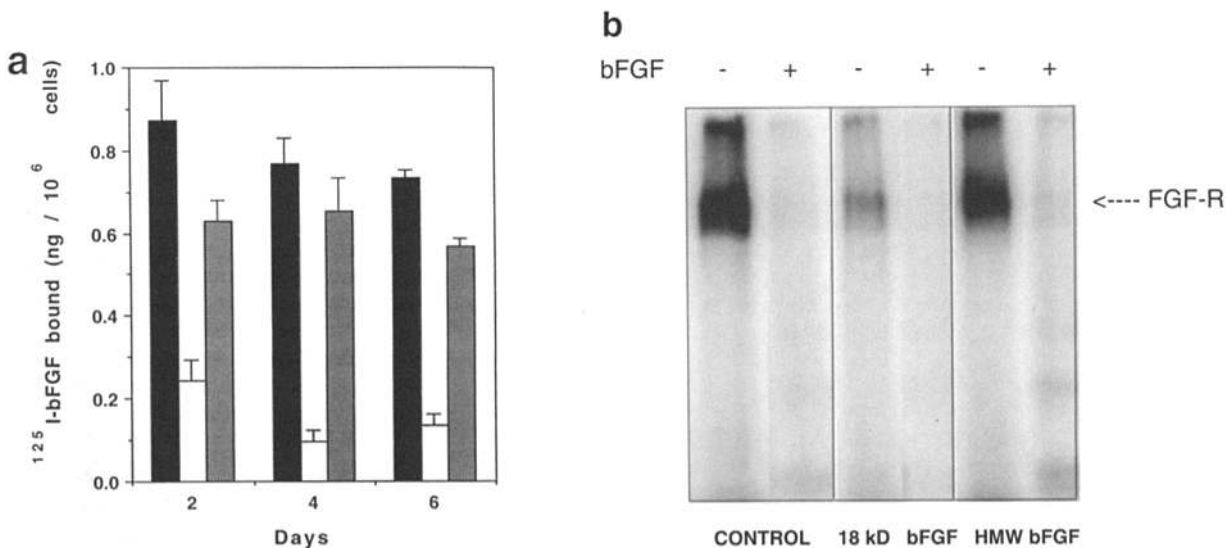
#### **Transfection of Cells Expressing Only HMW bFGF with 18-kD bFGF cDNA Results in Increased Migration and FGF Receptor Down-regulation**

To test the hypothesis that increased migration and receptor down-regulation were mediated by 18-kD bFGF, cells expressing only HMW bFGF were transfected with a cDNA encoding 18-kD bFGF and a cDNA containing a hygromycin resistance gene. The resultant cells that expressed both HMW and 18-kD bFGF were more refractile and spindle shaped than the parental cells that expressed only HMW bFGF (Fig. 4). These cells that expressed all forms of bFGF also resembled cells treated with recombinant 18-kD bFGF (data not shown).

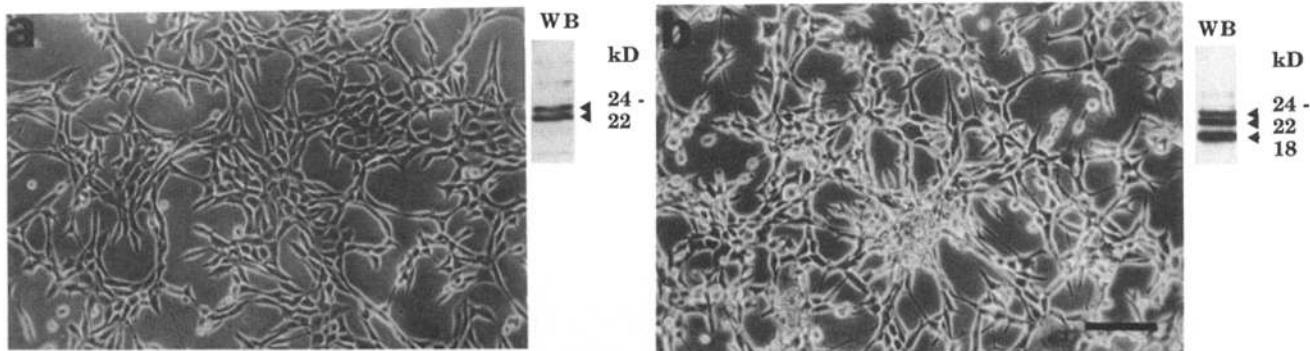
The doubly transfected cells displayed an increase in migration compared to control cells (Fig. 5). In addition, when cells expressing HMW bFGF transfected with the 18-kD bFGF cDNA were assayed for bFGF binding to high affinity receptors, a decrease in receptor number was observed (data not shown). However, the ability of cells expressing HMW bFGF to grow in the presence of 1% FCS was essentially unaffected by transfection with the 18-kD cDNA (Fig. 6).

#### **Cell Migration and Receptor Down-regulation Correlate With 18-kD bFGF Release**

The previous results indicating differences between cells expressing HMW or 18-kD bFGF are most easily explained by postulating the release of 18-kD bFGF but not HMW bFGF.



**Figure 3.** High affinity binding and cross-linking of  $^{125}\text{I}$ -bFGF to NIH 3T3 cells expressing HMW or 18-kD bFGF. (a) Binding of  $^{125}\text{I}$ -bFGF to cells. Binding experiments were performed as described in Materials and Methods. *Black bars*, control cells (Zipneo); *open bars*, cells expressing 18-kD bFGF (clone 43 FGFc31); *grey bars*, cells expressing HMW bFGF (clone 365FGFc2). The values represent the mean  $\pm$  SEM of duplicate samples. (b) Cross-linking of  $^{125}\text{I}$ -bFGF to cells. Control cells (Zipneo), cells expressing 18-kD (clone 43FGFc31), or HMW bFGF (clone 365FGFc2) were incubated with  $^{125}\text{I}$ -bFGF either in the absence (-) or presence (+) of unlabeled bFGF. Bound  $^{125}\text{I}$ -bFGF was cross-linked to cells as described in Materials and Methods and the samples analyzed by SDS-PAGE and autoradiography. The arrowhead indicates the position of the FGF receptor.



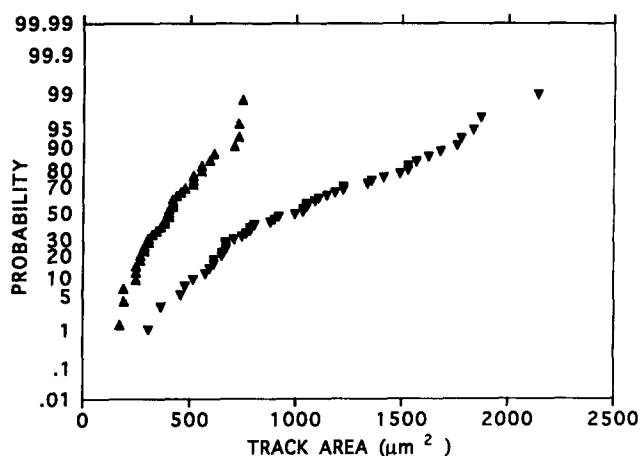
**Figure 4.** Morphological changes induced in cells expressing HMW bFGF by supertransfection with 18-kD bFGF cDNA. Cells were transfected as described in Materials and Methods; transfected cell clones were identified by Western blotting with anti-bFGF antibodies. (a) Cells expressing HMW bFGF (clone 365FGFHc3). (b) Cells supertransfected with 18-kD bFGF cDNA (clone 365/43NC33). The corresponding Western blots (WB) of cell extracts are shown in the right upper corner of each figure. Bar, 100  $\mu\text{m}$ .

However, bFGF has no secretion signal and is released inefficiently. In order to establish whether the phenotypic changes observed with cells expressing 18-kD bFGF were due to extracellular release of the growth factor, we tested the effects of protein G-purified or affinity-purified anti-bFGF IgG on migration and FGF receptor levels in those cells. In the presence of anti-bFGF antibodies, the migration of cells transfected with the 18-kD bFGF cDNA was reduced to control levels (Fig. 1 c). Previous experiments have shown that these antibodies also reduce the migration of cells expressing WT forms of bFGF (Mignatti et al., 1991). Because in this assay cell migration is mediated by the bFGF produced by the cells (Mignatti et al., 1991), this result demonstrated that 18-kD bFGF must be released in order to stimulate their migration. Treatment of the cells with protein G-purified or affinity-purified antibodies also increased the cell surface binding of bFGF to high affinity receptors (Fig. 7). This increase was 3.5-fold for cells expressing only 18-kD bFGF and sixfold for cells expressing WT bFGF. Similar results were obtained when receptor levels were measured by cross-linking of  $^{125}\text{I}$ -bFGF to cells treated with affinity-

purified antibody to bFGF (data not shown). Thus, downmodulation of bFGF receptors also may be due, at least in part, to the release of 18-kD bFGF and its interaction with the cell surface receptor.

We next assayed for cell surface-associated bFGF by immunoprecipitation of extracts from surface-labeled cells with anti-bFGF antibodies. As shown in Fig. 8, bFGF was only found on the surface of cells expressing either 18-kD bFGF or all forms of bFGF. Cells that expressed exclusively HMW bFGF exhibited only trace amounts of HMW bFGF and a degradation product migrating slightly slower than 18-kD bFGF. However, the total amount of HMW bFGF released was less than found in 18-kD bFGF producing cells. Immunoprecipitation of surface-labeled extracts of cells expressing HMW bFGF that were subsequently transfected with the 18-kD bFGF cDNA also revealed a band of 18-kD bFGF (Fig. 8).

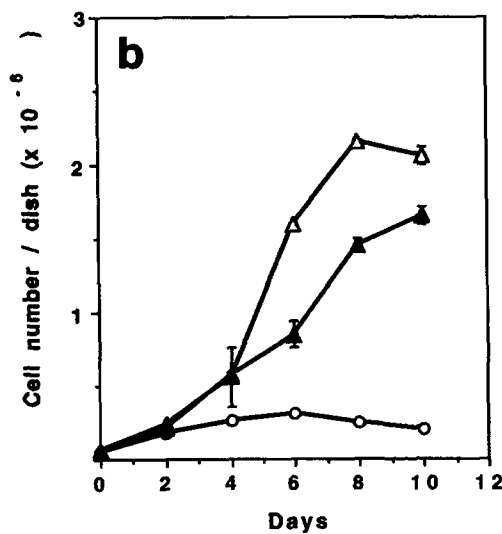
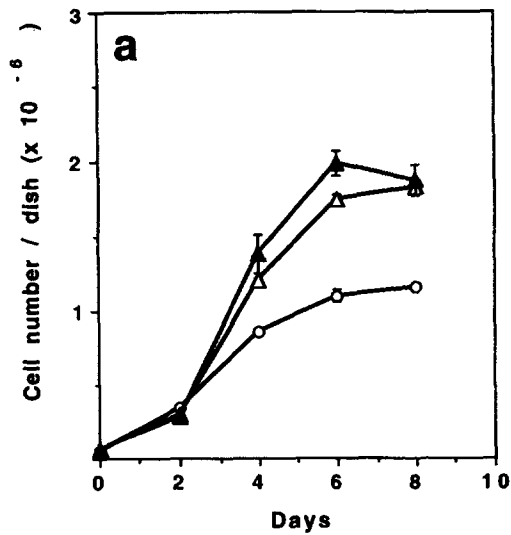
These results were confirmed by the analysis of the distribution of metabolically labeled bFGF. Cell extracts, culture media, and 2 M NaCl cell surface washes were immunoprecipitated with a specific anti-bFGF serum, and the immunoprecipitates were subjected to SDS-PAGE. bFGF was detected in the immunoprecipitates from the cell extracts of all transfected clones but not from the conditioned media (data not shown). In addition, 18-kD bFGF was present in the 2 M NaCl cell surface wash from cells transfected with 18-kD bFGF cDNA (clone 43FGFc31) or WT bFGF cDNA (clone WT FGFc3). No bFGF (either 18-kD or HMW bFGF) could be immunoprecipitated from the cell surface wash or from the medium of cells transfected with HMW bFGF cDNA (clone 365FGFc2) (data not shown). The difference in sensitivity between iodination (Fig. 8) and the metabolic labeling probably accounts for the inability to detect any surface-bound HMW bFGF by the latter approach. Thus, the changes in migration and bFGF receptor level of NIH 3T3 cells expressing different species of bFGF correlate with the release of 18-kD but not HMW bFGF on the cell surface.



**Figure 5.** Migration of cells expressing HMW bFGF transfected with 18-kD bFGF cDNA. Migration assays were carried out as described in Materials and Methods. Cells expressing HMW bFGF (clone 365FGFHc3: Mean Track Area  $\pm$  SEM =  $416 \pm 25 \mu\text{m}^2$ ) ( $\blacktriangle$ ); Cells expressing HMW bFGF transfected with 18-kD bFGF cDNA (clone 365/43nc33:  $1044.1 \pm 65.5 \mu\text{m}^2$ ) ( $\blacktriangledown$ ).

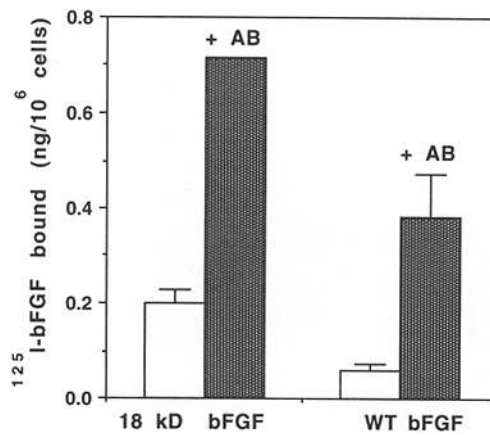
#### **Expression of a Dominant Negative bFGF Receptor Reverts the Phenotype of Cells Expressing 18-kD bFGF but not That of Cells Expressing HMW bFGF**

To determine whether the interaction of bFGF with its high affinity receptor was responsible for the phenotypic changes



**Figure 6.** Proliferation of cells expressing HMW bFGF transfected with 18-kD bFGF cDNA. The experiment was carried out as described in Materials and Methods. (a) Growth in 10% FCS; (b) growth in 1% FCS. Cells expressing HMW bFGF ( $\Delta$ , clone 365 FGF Hc3); 365FGFHc3 cells transfected with 18-kD bFGF cDNA ( $\blacktriangle$ , 365/43nc33), or Zipneo ( $\circ$ ). The data represent mean  $\pm$  SEM of duplicate samples.

exhibited by cells expressing the different forms of bFGF, NIH 3T3 cells expressing 18-kD, HMW, or WT bFGF were transfected with a cDNA encoding a dominant negative type 2 FGF receptor. Ueno et al. (1992) have shown that expression of a dominant negative receptor of this type inhibits signaling initiated by all FGF receptor types. Stable transfectants were isolated and were characterized by binding and cross-linking of  $^{125}\text{I}$ -bFGF to cell surface receptors. By quantitating the amount of  $^{125}\text{I}$ -bFGF bound to cells, as illustrated in Fig. 3 a, clones were obtained that bound up to 17 times more bFGF than did the control Zipneo cells (data not shown). The presence of the dominant negative FGF receptor was verified by the demonstration of a major band of 90 kD and multimeric complexes of 150-200 kD seen after

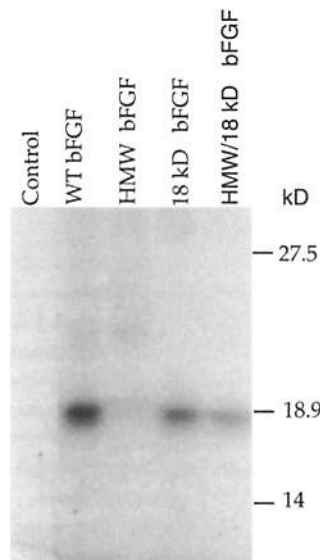


**Figure 7.** Effect of anti-bFGF antibodies on FGF receptor expression. Cells expressing 18-kD or WT bFGF were incubated for 24 h with or without 100  $\mu\text{g}/\text{ml}$  of protein-G-purified anti-bFGF antibody (AB) or 100  $\mu\text{g}/\text{ml}$  of non-immune antibody. Binding of  $^{125}\text{I}$ -bFGF to its receptor was determined as described in Materials and Methods. Open bars, non-immune antibody; filled bars, anti-bFGF antibody.

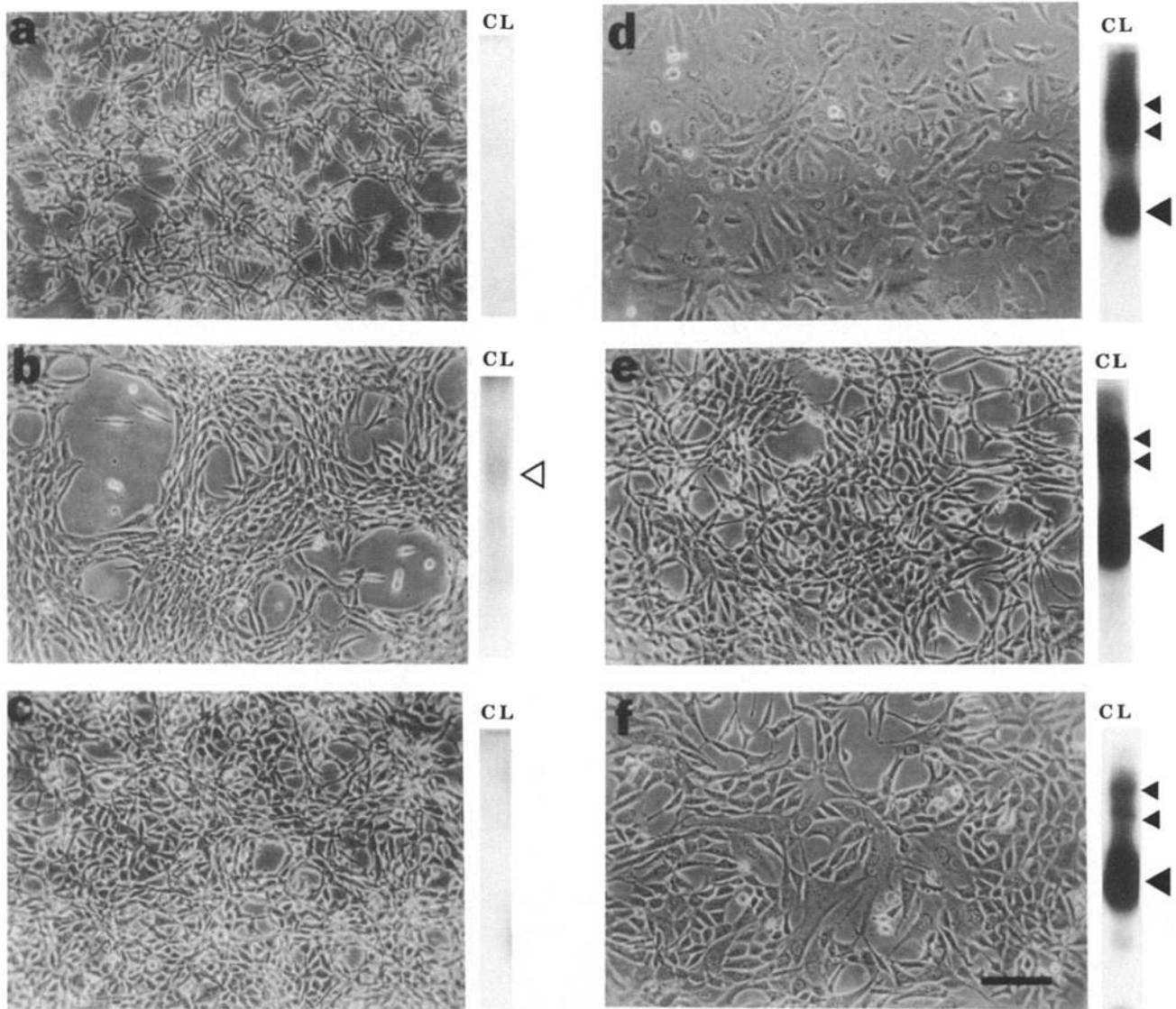
incubation of the cells with  $^{125}\text{I}$ -bFGF and cross-linking with  $\text{BS}_3$  (Fig. 9, insets).

Cells expressing 18-kD or WT bFGF and high levels of the dominant negative receptor cDNA lost their spindle shape and displayed a flat morphology (Fig. 9, compare images a to d and c to f). In contrast, cells co-expressing HMW bFGF and the dominant negative receptor exhibited no morphological change despite their high expression of the dominant negative bFGF receptor (Fig. 9, compare image b to e).

Cells expressing 18-kD or WT bFGF plus the dominant negative bFGF receptor were also characterized for migration. These cells showed decreased motility in phagokinetic assays (Fig. 10) compared to cells expressing only 18 kD or WT bFGF. Thus, expression of the dominant negative receptor prevented the signaling necessary for the stimulation of cell migration induced by 18-kD bFGF.



**Figure 8.**  $^{125}\text{I}$ -labeling of cell surface-associated bFGF.  $^{125}\text{I}$ -labeling of cells, immunoprecipitation, SDS-PAGE, and PhosphorImager analysis were carried out as described in Materials and Methods. Cells expressing 18-kD bFGF (clone 43FGFHc8), HMW bFGF (clone 365FGFHc3), WT bFGF (clone WT FGFc3), or cells expressing HMW bFGF transfected with a cDNA encoding 18-kD bFGF (HMW/18-kD bFGF; clone 365/43NC33) were immunoprecipitated with anti-bFGF antibodies. Control, cells transfected with the vector alone (Zipneo).



**Figure 9.** Morphological changes induced by transfection with a dominant negative FGF receptor cDNA. Cells expressing 18-kD bFGF, HMW bFGF, or WT bFGF were transfected with a dominant negative FGF receptor type 2 cDNA. Cells expressing high levels of the dominant negative receptor were isolated as described in Materials and Methods. Cells transfected with the cDNA encoding the dominant negative receptor (clones *d*, 43 DNc1; *e*, 365DNC28; *f*, WTDNc2). Control cells (*a*, 43FGFHc8; *b*, 365FGFHc3; *c*, WTFGFHc31). Autoradiograms of extracts from cells cross-linked (CL) with  $^{125}\text{I}$ -bFGF and subjected to SDS-PAGE as described in Materials and Methods are shown in the right upper corner of each figure. *Open arrow*, cross-linked  $^{125}\text{I}$ -bFGF to endogenous receptors in cells expressing HMW bFGF; *large black arrow*, major cross-linked band of 90 kD in cells expressing the dominant negative FGF receptor; *small black arrows*, multimeric complexes of 150-200 kD in cells expressing the dominant negative bFGF receptor. Bar, 100  $\mu\text{m}$ .

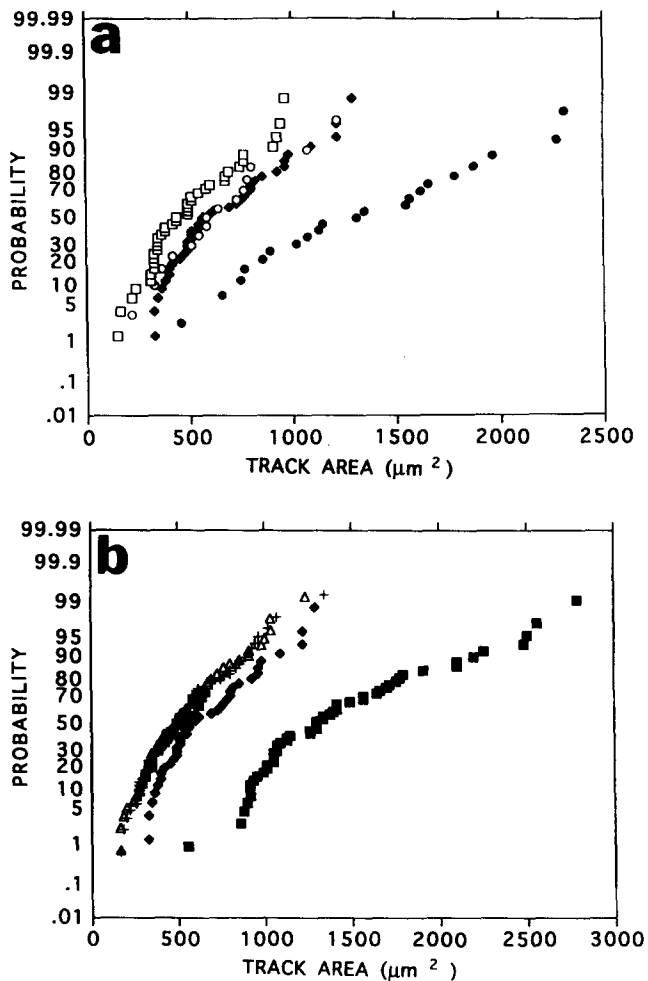
### ***HMW bFGF Modulates Cell Growth by a Mechanism Independent of Its Cell Surface Receptor***

In the presence of 10% FCS cells expressing the dominant negative FGF receptor and 18-kD bFGF grew to saturation densities that were lower than those of cells that did not express the dominant negative receptor (Fig. 11 *a*). These saturation densities were comparable to that of control cells transfected with the vector alone. In contrast, cells that expressed both HMW bFGF and the dominant negative FGF receptor grew to a saturation density comparable to that of cells that did not express the dominant negative receptor (Fig. 11 *b*).

In the presence of 1% FCS, cells transfected with 18-kD bFGF and the dominant negative FGF receptor, as well as cells transfected with the vector alone, grew slowly (Fig. 11 *c*). In contrast, cells expressing HMW bFGF and the dominant negative FGF receptor grew rapidly (Fig. 11 *d*, and data not shown). As shown above, transfection of cells expressing HMW bFGF with 18-kD bFGF cDNA did not affect their growth rate or saturation density in the presence of either 10 or 1% FCS (Fig. 6).

In addition, expression of the dominant negative receptor significantly inhibited the soft agar growth only of cells expressing exclusively 18-kD bFGF (Table II). The cloning efficiency of cells expressing only 18-kD bFGF was reduced

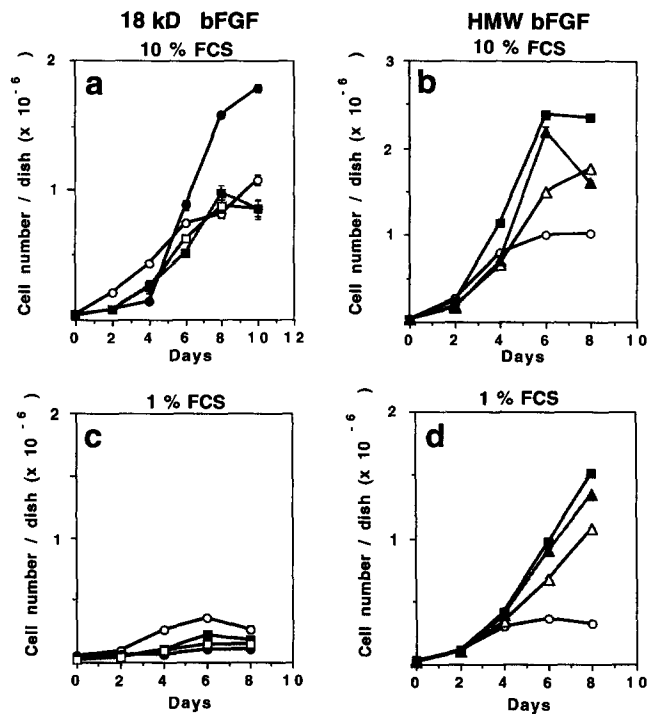




**Figure 10.** Migration of cells expressing 18-kD bFGF or WT bFGF plus the dominant negative FGF receptor. Cells were isolated as described in Materials and Methods and their migration measured as described in the legend to Fig. 1. (a) Track areas of cells expressing 18-kD bFGF and the dominant negative FGF receptor ( $\square$ , clone 43DNc11: mean track area  $\pm$  SEM =  $494.2 \pm 35.0 \mu\text{m}^2$ ; and  $\circ$ , clone 43DNc38:  $582.4 \pm 69.8 \mu\text{m}^2$ ), and cells expressing only 18-kD bFGF cells ( $\blacklozenge$ , clone 43FGFHc8:  $1332.9 \pm 115.4 \mu\text{m}^2$ ). Cells transfected with the vector alone ( $\blacklozenge$ , Zipneo: mean track area  $\pm$  SEM =  $666.4 \pm 42.5$ ). (b) Cells expressing WT bFGF and the dominant negative FGF receptor ( $\triangle$ , clone WTDNc2:  $491.4 \pm 27.6 \mu\text{m}^2$ ;  $+$ , WTDNc4:  $509.6 \pm 28.6 \mu\text{m}^2$ ), and cells expressing only WTbFGF ( $\blacksquare$ , clone WT FGFHc5:  $1413 \pm 67.2 \mu\text{m}^2$ ). Cells transfected with the vector alone ( $\blacklozenge$ , Zipneo:  $666.4 \pm 42.5 \mu\text{m}^2$ ).

from 2.1 to 0.2% in cells expressing both 18-kD bFGF and the dominant negative bFGF receptor. The cloning efficiency for cells expressing WT bFGF was 2.8%. This value decreased to 0.87% for cells expressing WT bFGF plus the dominant negative bFGF receptor. The cloning efficiencies of cells that expressed only HMW bFGF and that of cells expressing both HMW bFGF and the dominant negative receptor were not significantly different (2%). With control Zipneo cells, only a few colonies were detected (0.06% cloning efficiency).

These results indicate that HMW bFGF, but not 18-kD bFGF, stimulates cell growth by a mechanism independent of its cell surface receptor.



**Figure 11.** Growth curves of cells expressing 18-kD or HMW bFGF and a dominant negative FGF receptor. Cells were isolated and growth experiments performed as described in Materials and Methods. (a and c) Cells expressing 18-kD bFGF transfected with the dominant negative FGF receptor ( $\blacksquare$ , clone 43DNc11;  $\square$ , 43DNc31); cells transfected with 18-kD bFGF cDNA ( $\bullet$ , 43FGFHc3). Control cells ( $\circ$ , Zipneo). (b and d) Cells expressing HMW bFGF transfected with the dominant negative FGF receptor ( $\blacksquare$ , clone 365DNc5;  $\blacktriangle$ , 365DNc63); non-transfected cells ( $\triangle$ , 365FGFHc3). Control cells ( $\circ$ , Zipneo). (a and b) 10% FCS; (c and d) 1% FCS. The data presented represent the mean  $\pm$  SEM of duplicate samples and are representative of three experiments.

## Discussion

The data reported in this paper show that the 18-kD and HMW forms of bFGF may mediate certain cell functions through separate mechanisms of action. This conclusion is based on the following observations: (a) Cells expressing 18-kD bFGF have high motility and surface-associated 18-kD bFGF. In contrast, cells expressing HMW bFGF have low motility and virtually no surface-associated bFGF. (b) bFGF

**Table II. Growth in Soft Agar**

Cells	Control	DN receptor transfected
		% $\pm$ SEM
18 kD	2.06 $\pm$ 0.05	0.21 $\pm$ 0.03
WT bFGF	2.85 $\pm$ 0.15	0.87 $\pm$ 0.10
HMW bFGF	2.04 $\pm$ 0.22	2.03 $\pm$ 0.26

Cells expressing 18-kD bFGF, WT bFGF, or HMW bFGF transfected with the dominant negative bFGF receptor (18-kD bFGF: clone 43 DNc11; WT bFGF: clone WTDNc2; HMW bFGF: clone 365DNc2) or control cells (18-kD bFGF: clone 43FGFHc10; WT bFGF: WTFGFHc3; HMW bFGF: 365FGFHc3) were plated at a density of 100,000 cells in 60 mm dishes in 2 ml of 0.3% plating agar on a 0.6% agar underlay. After three weeks, the number of colonies in each plate was counted. The data are expressed as a percent  $\pm$  SEM of the total cells plated.

receptors are down-regulated in cells expressing 18-kD bFGF, but not in cells expressing HMW bFGF. (c) Cells expressing HMW bFGF have a reduced serum requirement for growth, whereas cells expressing 18-kD bFGF proliferate poorly in low serum. (d) Transfection of cells expressing HMW bFGF with 18-kD bFGF cDNA results in increased motility but has no effect on cell growth in low serum. (e) Expression of a dominant negative FGF receptor inhibits the migration and suppresses the growth in soft agar as well as the saturation density of cells expressing 18-kD bFGF, but has no effect on the growth of cells expressing HMW bFGF.

Measurement of the extracellular levels of 18 kD and HMW bFGF revealed significantly more 18-kD bFGF than HMW bFGF. We interpret these results to indicate that the extracellular release of 18-kD bFGF down-regulates receptors, increases motility, and stimulates growth via interaction with plasma membrane receptors. We propose that in the cells we have examined, HMW bFGF is not released in amounts sufficient to affect migration or bFGF receptor levels, but enhances growth by an intracellular mechanism. It is important to note that the responses we have observed are probably dependent upon the absolute expression levels of each of the individual forms of bFGF. As reported by Quarto et al. (1991b), transfection by the HMW bFGF cDNA we have used results in many clones of cells whose growth is inhibited and in a few clones of cells whose growth is rapid.

Among the transfected clones, those clones that grow rapidly have multiple copies of integrated HMW bFGF cDNA, whereas the poorly growing cells usually have only one copy. The mechanism of growth inhibition induced by low levels of HMW bFGF expression is not understood at this time. Our studies have characterized those cells which grow rapidly. No inhibition of cell growth was observed in cells expressing low levels of 18-kD bFGF (Quarto et al., 1991b). These cells grew in a manner similar to cells expressing high levels of 18-kD bFGF. However, the observation that even low expression of HMW bFGF results in impaired growth, whereas low expression of 18-kD bFGF does not, implies that HMW bFGF yields a phenotype different than that of 18-kD bFGF.

The absolute level of expression of 18-kD bFGF may also be critical for the results we have observed. Contrary to our results, others have reported the growth in low or no serum of cells transformed by 18-kD bFGF (Sasada et al., 1988; Neufeld et al., 1988). However, the cDNA used in those experiments began at or close to the AUG initiation codon specifying 18-kD bFGF and did not contain the 5' sequences required for HMW bFGF expression. Prats et al. (1992) have reported that these additional 5' sequences attenuate 18-kD bFGF translation. Therefore, the expression levels of 18-kD bFGF in the cells described by Sasada et al. (1988) and Neufeld et al. (1988) were probably higher than those achieved by our cells. At these higher levels of expression, the serum requirement we observed might be masked.

Cells expressing HMW bFGF are less motile than cells expressing 18-kD bFGF. However, exposure of these cells to exogenous HMW bFGF stimulates cell migration as efficiently as exposure to 18-kD bFGF. This indicates that the low motility of cells expressing HMW bFGF is not because HMW bFGF lacks migration-stimulating activity. Rather, the poor motility and normal levels of receptor in cells expressing HMW bFGF result from the extracellular concen-

trations of bFGF insufficient to trigger receptor signaling. In addition, expression of a dominant negative FGF receptor reduces the motility of cells expressing 18-kD bFGF, indicating that increased cell migration requires the externalization of 18-kD bFGF and its interaction with cell surface receptors.

Expression of a dominant negative FGF receptor affects neither the growth in 1% FCS nor the anchorage-dependent growth of cells producing HMW bFGF. These data indicate that HMW bFGF can modulate cell proliferation by an intracellular mechanism independent of cell surface receptors. In the light of our present knowledge, the mechanism(s) by which intracellular HMW bFGF modulates cell proliferation is not clear. A number of hypotheses can be considered. First, several data suggest that HMW bFGF can be translocated into the nucleus to exert its mitogenic activity as has been described for the Schwannoma-derived growth factor (Kimura, 1993). The transport of exogenous 18-kD bFGF and aFGF to the nucleus, and their interaction with nuclear receptors or binding molecules have been described in several systems (Baldin et al., 1990; Bouche et al., 1987; Imamura et al., 1991; Zhan et al., 1993; Wiedlocha et al., 1994). Other proteins and cytokines, including interleukin-1, are also translocated into the nucleus and may interact with nuclear receptors (Curtis et al., 1990; Weitzmann and Savage, 1992). However, a receptor for bFGF localized in the nuclear membrane and processed by the pathway used by the plasma membrane receptors would not have its ligand-binding domain exposed to the nucleoplasm. Thus, the mechanism by which HMW bFGF might interact with this receptor is not obvious. Other molecules involved in signal transduction, including phosphoinositides and protein kinase C, are present in the nuclear membrane, suggesting the possibility of additional signaling systems in the nucleus (Divecha et al., 1991, 1993). Second, HMW bFGF may act as a transcription factor. This hypothesis is supported by the finding that 18-kD bFGF can modulate gene transcription in a cell-free system (Nakanishi et al., 1992), although the concentrations at which bFGF exhibits this activity are high. Also, bFGF lacks a consensus sequence for a classical transcription factor, and it is, therefore, unlikely that HMW bFGF directly promotes gene transcription. HMW bFGF might act indirectly by forming complexes with either transcription factors or growth suppressor genes, or HMW bFGF may interfere with the activity of molecules involved in growth control, such as cyclins or phosphatases. Third, HMW bFGF may interact with a cytoplasmic form of its membrane receptor. Overexpression of a cytoplasmic truncated form of type 1 FGF receptor results in cell transformation (Yan et al., 1993). Therefore, soluble cytoplasmic receptors may be functional, although the mechanism(s) by which these receptors transduce a signal is(are) unknown. The identification of proteins that specifically interact with HMW bFGF may help to elucidate the mechanism of transformation by HMW bFGF.

In conclusion, our results show that 18-kD and HMW bFGF can have distinct mechanisms of action. The former modulates cell motility and proliferation through the interaction with its cell surface receptors. The latter can act as a mitogen and an inducer of anchorage-independent growth through an intracellular mechanism of action but has essentially no effect on cell migration. Although additional roles

for the different molecular weight forms of bFGF cannot be ruled out, these conclusions indicate a spatial and functional bipolarity in the action of bFGFs; the cell membranes as the site of action for 18-kD bFGF, and an intracellular, perhaps nuclear, site for HMW bFGF.

The authors thank Izabela Ortonowski for expert technical assistance, Dr. J. Schlessinger (Department of Pharmacology, New York University Medical Center) for the pRKS vector, Dr. C. Basilico (Department of Microbiology, New York University Medical Center) for the pCep4 vector, and Dr. W. Burgess (Department of Molecular Biology, American Red Cross, Rockville, MD) for antiphosphotyrosine antibody. We also thank Dr. T. Morimoto for helpful discussions and Dr. D. Moscatelli for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (No. CA34282) to D. B. Rifkin, the Italian Association for Cancer Research (AIRC) to P. Mignatti, and the International Agency for Cancer Research (WHO) to A. Bikfalvi. S. Klein was supported by NIH grant (No. 5T32GM07238-19) and a Berlex student fellowship. G. Pintucci was a recipient of a fellowship from the Regional Council of Abruzzo (Italy) and the Commission of the European Communities (CEE/Abruzzo).

Received for publication 20 October 1994 and in revised form 22 December 1994.

## References

- Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjeirriid, D. Gospodarowicz, and J. C. Fiddes. 1986. Nucleotide sequence of a bovine clone encoding the angiogenic protein basic fibroblast growth factor. *Science (Wash. DC)*. 233:545-548.
- Baldin, V., A. M. Roman, I. Bosc-Bierne, F. Amalric, and G. Bouche. 1990. Translocation of bFGF to the nucleus is G<sub>1</sub> phase cell cycle specific in bovine aortic endothelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1511-1517.
- Basilico, C., and D. Moscatelli. 1992. The FGF family of growth factors and oncogenes. *Adv. Cancer Res.* 59:115-165.
- Bouche, G., N. Gas, H. Prats, V. Baldin, J. P. Tauber, J. Teissie, and F. Amalric. 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G<sub>0</sub>-G<sub>1</sub> transition. *Proc. Natl. Acad. Sci. USA.* 84:6770-6774.
- Brunner, G., H. Nguyen, J. Gabrilove, D. B. Rifkin, and E. Wilson. 1993. Basic fibroblast growth factor expression in human bone marrow cells. *Blood.* 81:631-638.
- Bugler, B., F. Amalric, and H. Prats. 1991. Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol. Cell. Biol.* 11:573-577.
- Curtis, B. M., M. B. Widmer, P. de Roos, and E. E. Qvarnstrom. 1990. IL-1 and its receptor are translocated to the nucleus. *J. Immunol.* 144:1295-1303.
- Divecha, N., H. Banfic, and R. F. Irvine. 1991. The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-1) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3207-3214.
- Divecha, N., H. Banfic, and R. F. Irvine. 1993. Inositides and the nucleus and inositides in the nucleus. *Cell.* 74:405-407.
- Florkiewicz, R. Z., and A. Sommer. 1989. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc. Natl. Acad. Sci. USA.* 86:3978-3981.
- Florkiewicz, R. Z., A. Baird, and A. M. Gonzalez. 1991. Multiple forms of bFGF: differential nuclear and cell surface localization. *Growth Factors.* 4:265-275.
- Imamura, T., K. Engleka, X. Zhan, Y. Tokita, R. Forough, D. Roeder, A. Jackson, J. A. Maier, T. Hla, and T. Maciag. 1991. Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. *Science (Wash. DC)*. 249:1567-1570.
- Jaye, M., J. Schlessinger, and C. Dionne. 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochem. Biophys. Acta.* 1135:185-199.
- Kimura, H. 1993. Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity. *Proc. Natl. Acad. Sci. USA.* 90:2165-2169.
- Klein, S., F. G. Giancotti, M. Presta, S. M. Albelda, C. A. Buck, and D. B. Rifkin. 1993. Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell.* 4:973-982.
- McNeil, P. L., L. Muthukrishnan, E. Warder, and P. A. D'Amore. 1989. Growth factors are released by mechanically wounded endothelial cells. *J. Cell Biol.* 109:811-822.
- Mignatti, P., T. Morimoto, and D. B. Rifkin. 1991. Basic fibroblast growth factor released by single isolated cells stimulates their migration in an autocrine manner. *Proc. Natl. Acad. Sci. USA.* 88:11007-11011.
- Mignatti, P., T. Morimoto, and D. B. Rifkin. 1992. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J. Cell. Physiol.* 151:81-93.
- Miyamoto, M., K. Naruo, C. Seko, S. Matsumoto, T. Kondo, and T. Kurokawa. 1993. Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Mol. Cell. Biol.* 13:4251-4259.
- Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell. Physiol.* 131:123-130.
- Moscatelli, D., and N. Quarto. 1989. Transformation of NIH 3T3 cells with basic fibroblast growth factor or the hst/k-fgf oncogene causes downregulation of the fibroblast growth factor receptor: reversal of morphological transformation and restoration of receptor number by suramin. *J. Cell Biol.* 109:2519-2527.
- Moscatelli, D., M. Presta, and D. B. Rifkin. 1986. Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc. Natl. Acad. Sci. USA.* 83:2091-2095.
- Moscatelli, D., J. Joseph-Silverstein, R. Manejias, and D. B. Rifkin. 1987. Mr 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 84:5778-5782.
- Muthukrishnan, L., E. Warder, and P. L. McNeil. 1991. Basic fibroblast growth factor is efficiently released from cytosolic storage sites through plasma membrane disruptions of endothelial cells. *J. Cell. Physiol.* 148:1-16.
- Nakanishi, Y., K. Kihara, K. Mizuno, Y. Masamune, Y. Yoshtake, and K. Nishikawa. 1992. Direct effect of basic fibroblast growth factor on gene transcription in a cell-free system. *Proc. Natl. Acad. Sci. USA.* 89:5216-5220.
- Neufeld, G., R. Mitchell, P. Ponte, and D. Gospodarowicz. 1988. Expression of human basic fibroblast growth factor cDNA in baby hamster kidney-derived cells results in autonomous cell growth. *J. Cell Biol.* 106:1385-1394.
- Prats, A.-C., S. Vagner, H. Prats, and F. Amalric. 1992. Cis-acting elements involved in the alternative translation initiation process of human basic fibroblast growth factor mRNA. *Mol. Cell. Biol.* 12:4796-4805.
- Prats, H., M. Kaghad, A. C. Prats, M. Klagsbrun, J. M. Lelias, P. Liauzun, P. Chalou, J. P. Tauber, F. Amalric, J. A. Smith, and D. Caput. 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc. Natl. Acad. Sci. USA.* 86:1836-1840.
- Quarto, N., D. Talarico, A. Sommer, R. Florkiewicz, C. Basilico, and D. B. Rifkin. 1989. Transformation by basic fibroblast growth factor requires high levels of expression: comparison with transformation by hsf/K-fgf. *Oncogene Res.* 5:101-110.
- Quarto, N., F. P. Finger, and D. B. Rifkin. 1991a. The NH<sub>2</sub>-terminal extension of high molecular weight bFGF is a nuclear targeting signal. *J. Cell Physiol.* 147:311-318.
- Quarto, N., D. Talarico, R. Florkiewicz, and D. B. Rifkin. 1991b. Selective expression of high molecular weight basic fibroblast growth factor confers a unique phenotype to NIH 3T3 cells. *Cell Regul.* 2:699-708.
- Renko, M., N. Quarto, T. Morimoto, and D. B. Rifkin. 1991. Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell. Physiol.* 144:108-114.
- Sasada, R., T. Kurokawa, M. Iwane, and K. Igarashi. 1988. Transformation of mouse Balb/c 3T3 cells with human basic fibroblast growth factor cDNA. *Mol. Cell. Biol.* 8:588-594.
- Sommer, A., M. T. Brewer, R. C. Thompson, D. Moscatelli, M. Presta, and D. B. Rifkin. 1987. A form of human fibroblast growth factor with an extended aminoterminal. *Biochem. Biophys. Res. Commun.* 144:543-550.
- Taylor, W. R., A. H. Greenberg, E. A. Turley, and J. A. Wright. 1993. Cell motility, invasion, and malignancy induced by overexpression of K-FGF or bFGF. *Exp. Cell Res.* 204:295-301.
- Ueno, H., M. Gunn, K. Dell, A. Tseng, and L. Williams. 1992. A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. *J. Biol. Chem.* 267:1470-1476.
- Weitzmann, M. N., and N. Savage. 1992. Nuclear internalization and DNA binding activities of interleukin-1, interleukin-1 receptor and interleukin-1/receptor complexes. *Biochem. Biophys. Res. Commun.* 187:1166-1171.
- Wiedlocha, A., P. O. Falnes, I. H. Madhus, K. Sandvig, and S. Olsnes. 1994. Dual mode of signal transduction by externally added acidic fibroblast growth factor. *Cell.* 76:1039-1051.
- Yan, G., F. Wang, Y. Fukabori, D. Sussman, J. Hou, and W. L. McKeehan. 1992. Expression and transforming activity of a variant of the heparin-binding fibroblast growth factor receptor (fg) gene resulting from splicing of an alpha exon at an alternate 3'-acceptor site. *Biochem. Biophys. Res. Commun.* 183:423-430.
- Zhan, X., X. Hu, R. Friesel, and T. Maciag. 1993. Long-term growth factor exposure and differential tyrosine phosphorylation are required for DNA synthesis in Balb/c 3T3 cells. *J. Biol. Chem.* 268:9612-9619.