

Enhancement of Fibronectin Fibrillogenesis and Bone Formation by Basic Fibroblast Growth Factor via Protein Kinase C-Dependent Pathway in Rat Osteoblasts

Chih-Hsin Tang, Rong-Sen Yang, Tsang-Hai Huang, Shing-Hwa Liu, and Wen-Mei Fu

Departments of Pharmacology (C.-H.T., W.-M.F.), Orthopedics (R.-S.Y., T.-H.H.), and Toxicology (S.-H.L.), College of Medicine, National Taiwan University, Taipei, Taiwan

Received January 22, 2004; accepted June 10, 2004

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Fibronectin (Fn) is involved in early stages of bone formation and basic fibroblast growth factor (bFGF) is an important factor regulating osteogenesis. We here found that bFGF enhanced extracellular assembly from either endogenously released or exogenously applied soluble Fn in primary cultured osteoblasts. bFGF increased protein levels of Fn using Western blotting analysis. Protein kinase C (PKC) inhibitors such as H7, 3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)]maleimide (Bisindolylmaleimide IX), methanesulfonate (Ro 318220), or 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole (Gö 6976) antagonized the increase of Fn protein by bFGF. Treatment of osteoblasts with bFGF increased membrane translocation of various isoforms of PKC, including α , β , ϵ , and δ . However, treatment with antisense of various PKC isoforms demonstrated that α and β isozymes play important roles in the enhancement action of bFGF on Fn assembly. Down-regulation

of PKC by prolonged treatment with 1 μ M 12-O-tetradecanoylphorbol-13 acetate for 24 h inhibited the potentiating action of bFGF. It has been reported that $\alpha 5 \beta 1$ integrin is related to Fn fibrillogenesis, and immunocytochemistry showed that bFGF treatment increased the clustering of $\alpha 5$ integrins. Flow cytometry analysis demonstrated that bFGF increased cell surface expression of $\alpha 5$ and $\beta 1$ integrins and PKC inhibitors antagonized the increase by bFGF. Local administration of bFGF into the metaphysis of the tibia via the implantation of a needle cannula significantly increased the protein levels of Fn in the area of trabecular spongiosa, which was inhibited by coadministration of PKC inhibitors. Furthermore, local injection of bFGF increased the bone volume of secondary spongiosa in tibia, which was significantly antagonized by PKC inhibitors. These results suggest that bFGF increased bone formation and Fn fibrillogenesis both in vitro and in vivo via PKC-dependent pathway.

The cell functions, including cell adhesion, migration, proliferation, and differentiation, are regulated by the intimate interaction of extracellular matrix (ECM) and cells. Fibronectin (Fn), a unique dimeric glycoprotein, is one of the major ECM components. It is composed of two similar but nonidentical subunits with molecular weights of $\sim 250,000$ (Mosher, et al., 1992). Fn has been proven to be distributed throughout many tissues as in insoluble form as well as in the serum as in soluble form. The insoluble form of Fn may mediate various kinds of physiological events during embryogenesis, angiogenesis, thrombosis, inflammation, and wound healing

(Hay, 1991). It has been demonstrated that Fn is formed in the early phase of osteogenesis (Weiss and Reddi, 1981) and is maintained within mineralized matrix (Grzesik and Robey, 1994). Fn is the earliest bone matrix protein synthesized by osteoblast and precedes collagen synthesis in developing bone (Cowles et al., 1998). With regarding to the bone metabolism, Fn is closely related to the mineralization of bone matrix, induction of bone cell differentiation, and the survival of bone cells, although the precise function of Fn in bone is not definitive (Moursi et al., 1997).

The process of assembling extracellular Fn matrix network is closely related to the integrin receptors on the cell surface. Integrins are a family of dimeric transmembrane receptors

This work was supported by grants from National Science Council. C.-H.T. and R.-S.Y. contributed equally to this work.

ABBREVIATIONS: ECM, extracellular matrix; Fn, fibronectin; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; TPA, 12-O-tetradecanoylphorbol-13 acetate; U73122, 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; D609, tricyclodecan-9-yl-xanthogenate; Ro 318220, 3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)]maleimide (Bisindolylmaleimide IX), methanesulfonate; Gö 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole; FGFs, fibroblast growth factors; GF109203X, 3-[1-[3-(dimethylaminopropyl)-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione monohydrochloride; bFGF, basic fibroblast growth factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene difluoride; PKC, protein kinase C; BMD, bone mineral density; BMC, bone mineral content; PI-PLC, phosphoinositide-specific phospholipase C; PC-PLC, phosphatidylcholine-specific phospholipase C.

containing α and β chains (Wu et al., 1993; Dzamba et al., 1994). Different combinations of α and β chains form different kinds of cell transmembrane receptors. They will bind various kinds of ECM molecules. Previous studies have shown that human and rat osteoblasts express $\alpha 2$, $\alpha 5$, $\alpha 3$, $\alpha \nu$, and $\beta 1$ chains. The $\alpha 2\beta 1$ receptor binds collagen and laminin, $\alpha 5\beta 1$ receptor binds Fn, and $\alpha \nu\beta 5$ contains receptors for vitronectin (Wayner et al., 1991; Clover et al., 1992). The integrins provide a site for cell attachment and cell interactions that may induce reorganization of cytoskeleton and influence many cellular physiological events, including proliferation, differentiation, survival, and migration (Rosales et al., 1995; Lauffenburger and Horwitz, 1996). Furthermore, integrins are involved in the signal transduction of translating the strain in the organic matrix to the biochemical signals in the bone cells (Juliano and Haskill, 1993). However, the role of cytokines in the cell-matrix interactions in osteoblasts has not been extensively studied.

Fibroblast growth factors (FGFs) are a family of polypeptides that are important factors controlling cell proliferation, differentiation, and survival in cells. bFGF is a 16.5-kDa heparin binding growth factor that influences the proliferation and differentiation of various cell types in vitro (Gospodarowicz et al., 1987). The skeleton is an important target tissue for FGFs; these factors are involved in bone development, growth, remodeling, and repair (Rodan et al., 1989). Mutation in the genes for human FGF receptor 1 (FGFR1), FGFR2, and FGFR3 causes a variety of disorders in the development of the skeleton (Ornitz, 2000). The cellular actions of FGFs are known to be mediated by interactions with FGFRs, a family of tyrosine kinase receptors. Binding of bFGF to its receptor results in autophosphorylation of the receptor (Ullrich and Schlessinger, 1990), activation, and ultimately the induction of transcription regulatory proteins (Mohammadi et al., 1991; Hall et al., 1991). bFGF is a constituent of the bone matrix, and cultured osteoblasts produce bFGF (Baylink et al., 1993; Hurley et al., 1994). In addition, it is well known that bFGF expression is increased during the repair of bone fracture (Bolander, 1992). PKC isozymes of α , β , ϵ , and δ have been identified in osteoblasts (Yang et al., 2002). It has recently been reported that PKC pathway plays a central role in the bFGF-stimulated expression and transactivation activity of Runx2, a key transcription factor in osteoblast differentiation (Kim et al., 2000). bFGF may thus play an important role in bone remodeling and fracture healing. Both stimulatory and inhibitory effects of exogenous bFGF on collagen synthesis have been reported in osteoblastic cell cultures (McCarthy et al., 1989). Little is known about the effects of bFGF on Fn assembly. We here investigated the regulatory action of bFGF on Fn fibrillogenesis and bone formation. The results suggest that PKC is involved in the regulatory action of bFGF.

Materials and Methods

Materials. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), 12-*O*-tetradecanoylphorbol-13 acetate (TPA), and trichloroacetaldehyde were obtained from Sigma-Aldrich (St. Louis, MO). Genistein, herbimycin A, U73122, D609, Ro 318220, Gö 6976, and GF109203X were from Calbiochem (San Diego, CA). bFGF and soluble human Fn were purchased from Invitrogen (Carlsbad, CA).

Primary Osteoblast Cultures. Primary osteoblastic cells were obtained from the calvaria of the 18-day-old fetal rats. In brief, the

pregnant rats were anesthetized using intraperitoneal injection of trichloroacetaldehyde (400 mg/kg). The calvaria of fetal rats were then dissected from fetal rats with aseptic technique. The soft tissues were removed under dissecting microscope. The calvaria were divided into small pieces and were treated with 0.1% type I collagenase (Sigma Chemical, St. Louis, MO) solution for 10 min at 37°C. The next two 20-min sequential collagenase digestions were then pooled and filtered through 70 μ m nylon filters (Falcon; BD Biosciences, San Jose, CA). The cells were grown on the plastic cell culture dishes in 95% air/5% CO₂ with Dulbecco's modified Ca²⁺-free Eagle's medium (Invitrogen) which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (pH adjusted to 7.6). The cell medium was changed twice a week. The characteristics of osteoblasts were confirmed by morphology and the expression of alkaline phosphatase.

Immunocytochemistry. Osteoblasts were grown on glass coverslips. Cultures were rinsed once with phosphate-buffered saline (PBS), and fixed for 15 min at room temperature in phosphate buffer containing 4% paraformaldehyde. Cells were then rinsed three times with PBS. After blocking with 4% BSA for 15 min, cells were incubated with rabbit anti-rat Fn (1:100; Invitrogen) for 1 h at room temperature. Cells were then washed again and labeled with FITC-conjugated goat anti-rabbit IgG (1:150; Leinco Tec. Inc., St. Louis, MO) for 1 h. Finally, cells were washed, mounted, and examined with the use of a Zeiss confocal microscope (LSM 410) as soon as possible. The mean fluorescence under 10 to 15 cells (approximately three to five fields per culture) was measured with the use of the Zeiss confocal microscope (LSM 410). The focus of the *z*-axis was on the substratum of the monolayer cells. The value for contrast and offset adjustment of confocal microscope was fixed so that the variation of the relative fluorescence of control experiments is rather small. When the $\alpha 5$ integrin was examined, the cells were fixed with acetone for 30 s. After fixation, cells were washed with PBS and incubated with 4% BSA for 1 h. Cells were then incubated with rabbit anti-rat $\alpha 5$ (1:500; Chemicon, Temecula, CA) for 3 h and FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature.

To observe Fn assembly apart from Fn synthesis by rat osteoblasts, human soluble Fn (30 μ g/ml) was added to the cultures for overnight. Rat osteoblasts also used the exogenous human soluble Fn to form fibrillar Fn underneath the cells. After washout of residual soluble Fn, fixation, and blocking with 4% BSA for 15 min, cells were incubated for 1 h at room temperature with mouse anti-human Fn (1:50; BD Transduction Laboratories, Lexington, KY), which does not recognize endogenously released rat Fn. Cells were then washed again and labeled with FITC-conjugated goat anti-mouse IgG (1:150; Jackson ImmunoResearch, West Grove, PA) for 1 h. Finally, cells were washed, mounted, and examined with a Zeiss fluorescence microscope.

Quantification of Extracellular Immobilized Fn by ELISA.

The level of extracellular immobilized Fn was also determined by an enzyme-linked immunosorbent assay (ELISA). After treatment with bFGF at 37°C, the cells were washed twice with PBS and fixed at room temperature with 1% paraformaldehyde for 30 min. After washing with PBS, they were then blocked with 1% BSA in PBS for 15 min before being incubated successively with rabbit anti-rat Fn antibody (1:150) for 1 h and horseradish peroxidase-labeled anti-rabbit antibody (1:1000) for 30 min. After each incubation, the cells were washed two times with PBS. *O*-Phenylenediamine dihydrochloride substrate [0.4 mg/ml in phosphate-citrate buffer, pH 5.0, 24.3 mM citric acid, 51.4 mM Na₂HPO₄ · 12 H₂O, and 12% H₂O₂ (v/v)] was then applied to the cells for 30 min, and 3 M sulfuric acid added to stop the reaction. The absorbance was measured at 450 nm by an ELISA reader (Bio-Tek, Winooski, VT). Each assay was performed in triplicate. In pretreatment experiments, cells were incubated with various kinds of inhibitors before addition of bFGF.

Oligonucleotide Transfection. Osteoblasts were grown to confluence on 24-well dishes for quantification of extracellular Fn by ELISA. The complete medium was replaced with Opti-MEM (Invitrogen) containing the antisense phosphorothioate oligonucleotides (5 μ g/ml) that had been preincubated with Lipofectin (10 μ l/ml; Invitrogen) for 30 min. The cells were washed after 24 h of incubation at 37°C and washed before the addition of medium containing bFGF. All antisense oligonucleotides were synthesized and HPLC-purified by MDBio, Inc., (Taipei, Taiwan).

Sequences are as follows: PKC- α , AAAACGTCAGCCATG; PKC- β , AAGATGGCTGACCCGGCTCGC; PKC- δ , GTGCCATGATGGAGC-CTTTT; PKC- ϵ , TTGAACACTACCATG (Lu et al., 1998; Tinsley et al., 2004).

Flow Cytometric Analysis. Osteoblasts were plated in six-well (35-mm) dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with rabbit anti-rat $\alpha 5$ or $\beta 1$ integrin antibody (1:100; Chemicon, Temecula, CA) for 1 h at 4°C. Cells were then washed again and incubated with FITC-conjugated secondary IgG for 45 min and analyzed by flow cytometry using FACScalibur (CellQuest software; BD Biosciences).

Western Blotting Analysis. Osteoblasts were plated on six-well (35-mm) dishes. Cells were incubated with bFGF for different time intervals as indicated under *Results* and then washed with PBS, lysed for 30 min at 4°C with radioimmunoprecipitation assay buffer (200 μ l/per well; composition, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 1 mM sodium fluoride, 50 mM sodium orthovanadate, 5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin, pH 7.5). After centrifuging for 15 min at 10,000g the soluble fraction was used to run the Western blotting. Equal protein (30 and 80 μ g for the measurement of Fn and PKC, respectively) was applied in each lane, and electrophoresis was performed under denaturing conditions on a 7.5% polyacrylamide-SDS gel and transferred to an Immobilon polyvinylidene difluoride (PVDF) membranes at 4°C overnight. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-rat antibodies against Fn (1:1500) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). For normalization purposes, the same blot was also probed with mouse anti-rat α -tubulin antibody (1:1000; Oncogene Science, Cambridge, MA).

For the study of PKC translocation, cells were rinsed with PBS and suspended in homogenization buffer (20 mM Tris-HCl, 5 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μ g/ml leupeptin, pH 7.5) and then sonicated on ice. The lysates were separated into cytosolic and pellet fractions by centrifugation at 40,000g for 45 min. Membrane-bound PKC was extracted from the pellet with 1% Triton X-100 in the above-mentioned buffer for 20 min at 4°C. The resultant suspension was centrifuged at 100,000g for 30 min, and the supernatant was used as the membrane fraction of cellular PKC. Equal amounts of each protein from cytosolic and membrane fractions were separated by 7.5% polyacrylamide-SDS gel and then electrotransferred to PVDF membranes. The washed membranes were incubated overnight at room temperature with mouse monoclonal antibodies against various isoforms of PKC (1:1000; BD Transduction Laboratories). After washing with PBS, the blots were incubated for 1 h at room temperature with sheep anti-mouse peroxidase-conjugated secondary antibody (1:2000).

Protein Content of Fn in the Tibia of Young Rats. Male Sprague-Dawley rats weighing 76 to 90 g were used. Implantation of a cannula (22 gauge) was done from the posterolateral side into the proximal tibial metaphysis in both limbs of rats anesthetized with

trichloroacetaldehyde. The cannula had its outer end in the subcutaneous tissue. bFGF (300 ng/ml, 10 μ l) was percutaneously injected into the proximal tibia through the cannula (once per day) for 5 consecutive days. The same volume of vehicle was injected into the contralateral side for comparison. Rats were then sacrificed, and tibiae were frozen in liquid nitrogen. The regional trabecular spongia and bone marrow (as shown in Fig. 8A) were removed, homogenized, and sonicated in lysis radioimmunoprecipitation assay buffer. After centrifugation for 15 min at 10,000g, the supernatant was then obtained for Western blotting analysis. For immunohistochemical staining, tibiae were fixed, decalcified, and embedded in paraffin. Serial sections (5 μ m) were cut longitudinally, and endogenous peroxidase activity was inactivated by treatment with 3% H₂O₂ in methanol for 20 min. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C. The sections were detected by avidin-biotin-peroxidase detection system (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma Chemical, St. Louis, MO).

Measurement of Bone Mineral Density and Bone Volume. Local injection of bFGF into tibia of young Sprague-Dawley rats for 7 consecutive days through cannula was done as described above. On the day 14, the rats were sacrificed, and tibiae were also removed and cleaned of soft tissue. Bone mineral density (BMD) and bone mineral content (BMC) of the tibia were measured with the use of a dual-energy X-ray absorptiometer (XR-26; Norland, Fort Atkinson, WI). The mode adapted to the measurements of small subjects was adopted. A coefficient of variation of 0.7% was calculated from daily measurements of BMD on a lumbar phantom for more than 1 year. The whole tibiae were scanned, and BMD and BMC were measured by absorptiometer. At the end of the program, the tibia was fixed with 4% ice-cold paraformaldehyde for 48 h. The tibia was then decalcified in 0.5 N hydrochloric acid, dehydrated in an ascending series of ethanol solution and acetone, and embedded in paraffin. Serial sections (5 μ m) were cut longitudinally and stained with Mayer's hematoxylin-eosin solution. Images of the growth plate and

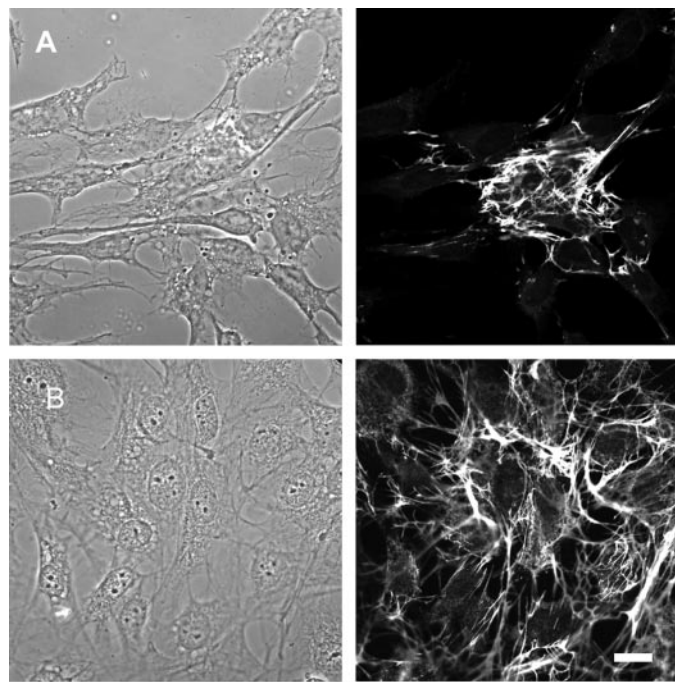


Fig. 1. Increase of Fn fibrillogenesis by bFGF in cultured rat osteoblasts. Fn network, which was shown by immunofluorescence, formed underneath the cultured osteoblasts. Compared with control (A), treatment with bFGF (30 ng/ml) for 24 h increased Fn fibrillogenesis in cultured osteoblasts (B). Phase-contrast images are shown at left. Bar, 10 μ m.

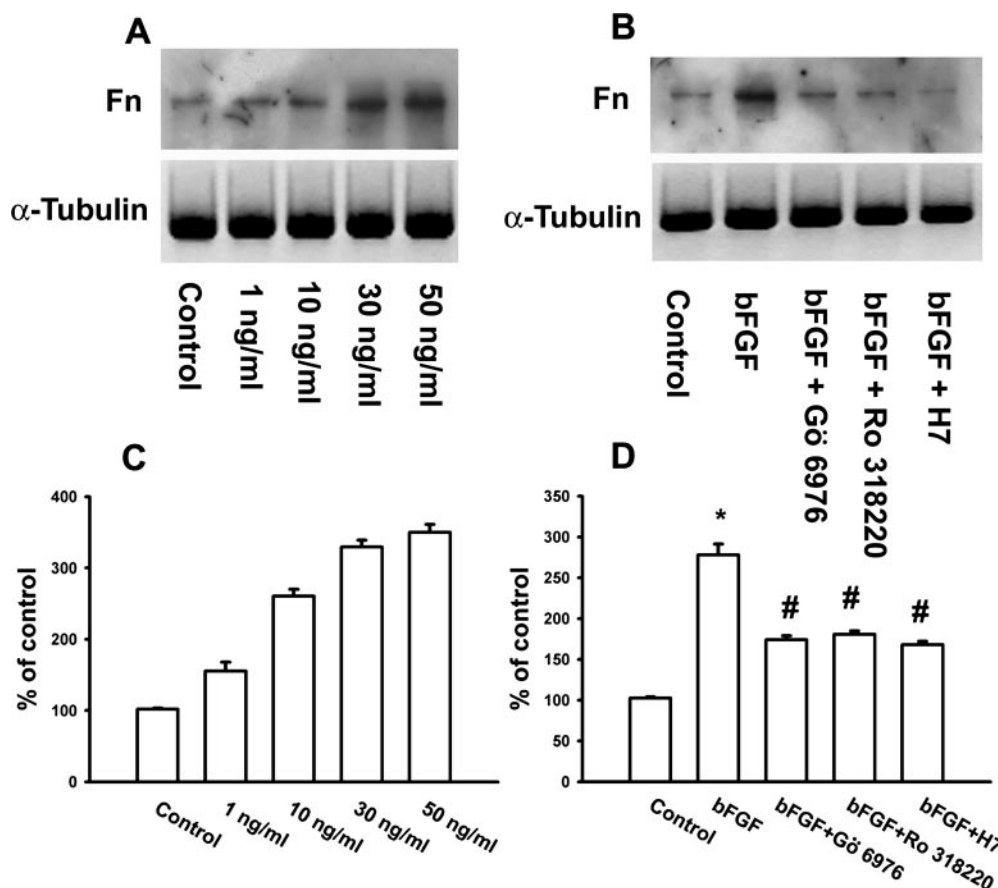


Fig. 2. Up-regulation of protein levels of Fn by bFGF through PKC-dependent pathway. A, osteoblast cultures were treated for 24 h with different concentrations of bFGF. The cultures were washed with ice-cold PBS, and protein samples for Western blotting analysis were collected by the direct addition of lysis buffer to cultures without trypsin digestion. Compared with control, bFGF dose-dependently increased the protein levels of Fn. B, PKC inhibitors Gö 6976 (0.1 μ M), Ro 318220 (1 μ M), and H7 (10 μ M) antagonized the increase of Fn by bFGF when examined at a concentration of 30 ng/ml. The quantitative data are shown in C and D. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with bFGF-treated group.

proximal tibia were photographed using an IX70 microscope (Olympus, Tokyo, Japan). Measurement of bone volume was performed on the secondary spongiosa, which is located 1.0 to 3.0 mm distal to the epiphyseal growth plate and is characterized by a network of larger trabeculae. Bone volume was calculated using image analysis software (Image Pro Plus 3.0) and expressed as percentage of bone area. All measurements were done in a single-blind fashion. All protocols complied with institutional guidelines and were approved by the Animal Care Committee of Medical College, National Taiwan University.

Statistics. The values given are means \pm S.E.M. The significance of difference between the experimental group and control was assessed by Student's t test. The difference is significant if the p value is less than 0.05.

Results

bFGF Enhanced Fn Fibrillogenesis in Cultured Osteoblasts. The fibrillogenesis from the endogenously released Fn by the primary cultured rat osteoblasts was studied using immunocytochemistry. Osteoblasts from days 3~5 were changed to serum-free medium and incubated with bFGF (30 ng/ml) for 24 h. Immunostaining of Fn was examined in 4% formaldehyde-fixed and nonpermeabilized cells. The mean immunofluorescence intensity underneath a cell group of 10 to 15 cells was measured using confocal microscope. As shown in Fig. 1A, osteoblasts are able to form Fn network underneath the cell using endogenously released Fn. Extracellular assembly of Fn fibril increased in response to 24-h treatment of bFGF (Fig. 1B). The mean fluorescence intensity underneath 10 to 15 cells was 42.5 ± 3.3 and 84.1 ± 4.5 ($n = 26-33$; n represents the field number) for control and

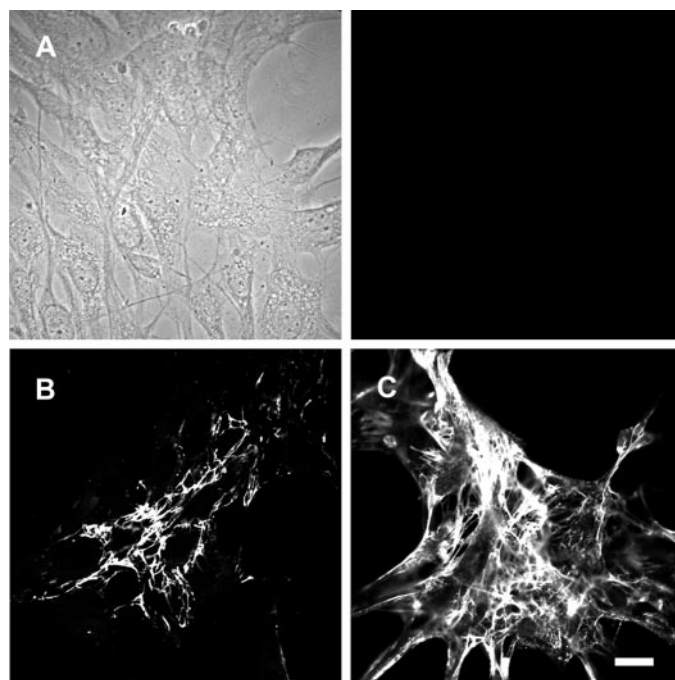


Fig. 3. bFGF increased extracellular Fn assembly from exogenously-applied soluble Fn. Culture medium of osteoblasts was replaced by serum-free medium containing soluble human Fn (30 μ g/ml) in the presence or absence of bFGF (30 ng/ml). Immunocytochemistry was performed 24 h later using mouse anti-human Fn antibody, which does not recognize endogenously-released Fn from rat osteoblasts (A). Compared with control (B), treatment with bFGF markedly enhanced extracellular assembly of Fn (C). Bar, 10 μ m.

bFGF-treated cells, respectively. These results suggest that bFGF increased Fn fibrillogenesis in cultured rat osteoblasts. Western blotting was used to examine the effect of bFGF on the protein levels of Fn. Osteoblasts from days 3~5 were changed to serum-free culture medium and treated with bFGF for 24 h. The cultures were then washed with ice-cold PBS, and protein samples were collected by the addition of lysis buffer without trypsin digestion. The result from Western blotting may contain both soluble cytosolic Fn and extracellular immobilized Fn. As shown in Fig. 2A, bFGF concentration-dependently increased protein levels of Fn 24 h after incubation with cells. H7 (10 μ M), a nonspecific kinase inhibitor that also targets PKC, as well as PKC inhibitor Ro 318220 (1 μ M), antagonized the increase of Fn protein level by bFGF. In addition, Gö 6976 (0.1 μ M), which is an inhibitor of classic PKC isoforms (Gschwendt et al., 1996), also antagonized the increase of Fn protein level by bFGF when examined at a concentration of 30 ng/ml (Fig. 2B). These results indicate that bFGF may increase Fn fibrillogenesis via PKC-dependent pathway.

Regulation of Fn Assembly from Exogenous Origin by bFGF. Increase of Fn fibril formation by bFGF may result from the effect on Fn synthesis and/or extracellular Fn assembly. To simply look at the effect of bFGF on Fn assembly apart from Fn synthesis, soluble human Fn (30 μ g/ml) was applied into cultures for 24 h in the presence or absence of bFGF (30 ng/ml). The cells are still able to use exogenous soluble Fn to form Fn fibril even if the cells are attached to the dishes. The cultures were then washed with plain culture medium to remove the remaining soluble form of human Fn, and immunocytochemistry of immobilized Fn was performed using mouse anti-human Fn monoclonal antibody, which does not recognize endogenously released rat Fn. The cultured osteoblasts showed no significant immunoreactive staining without addition of exogenous soluble human Fn (Fig. 3A), even in the presence of bFGF (30 ng/ml) (data not shown). Taking advantage of this antibody specificity, we are able to observe the assembly of Fn matrix by the application of exogenous soluble human Fn. Compared with control, bFGF (30 ng/ml) markedly enhanced the formation of Fn

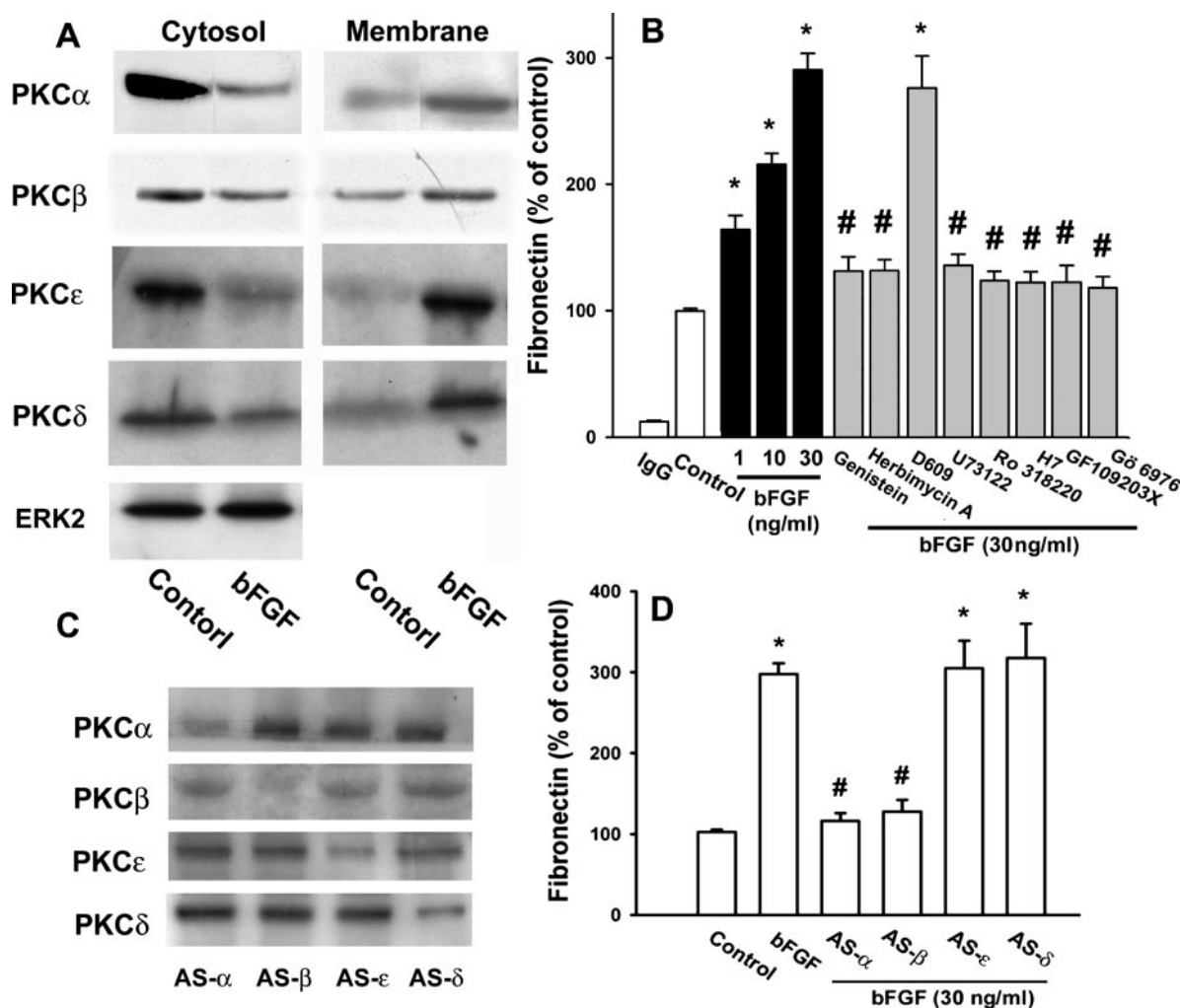


Fig. 4. Involvement of various PKC isoforms in the potentiation of Fn expression by bFGF. Treatment of osteoblasts with bFGF (30 ng/ml) for 30 min decreased cytosolic and increased membrane translocation of PKC isoforms, including α , β , ϵ , and δ (A). Expression of extracellular Fn was measured by ELISA in this experiment. Pretreatment with the tyrosine kinase inhibitors genistein (30 μ M) and herbimycin A (3 μ M), the PI-PLC inhibitor U73122 (3 μ M), and the PKC inhibitors Ro 318220 (1 μ M), H7 (10 μ M), GF109203X (1 μ M), and Gö 6976 (0.1 μ M), but not PC-PLC inhibitor D609 (30 μ M), antagonized the potentiation of Fn expression by bFGF (B). Treatment of osteoblast with antisense (AS) directed against different isoforms of PKC for 24 h, specifically reduced cell content of respective PKC isoforms (C). The antisense of PKC α and β but not of ϵ and δ inhibited the enhancement of bFGF on Fn expression (D). Results are expressed as the mean \pm S.E.M. of three independent experiments performed in triplicate. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with bFGF-treated group.

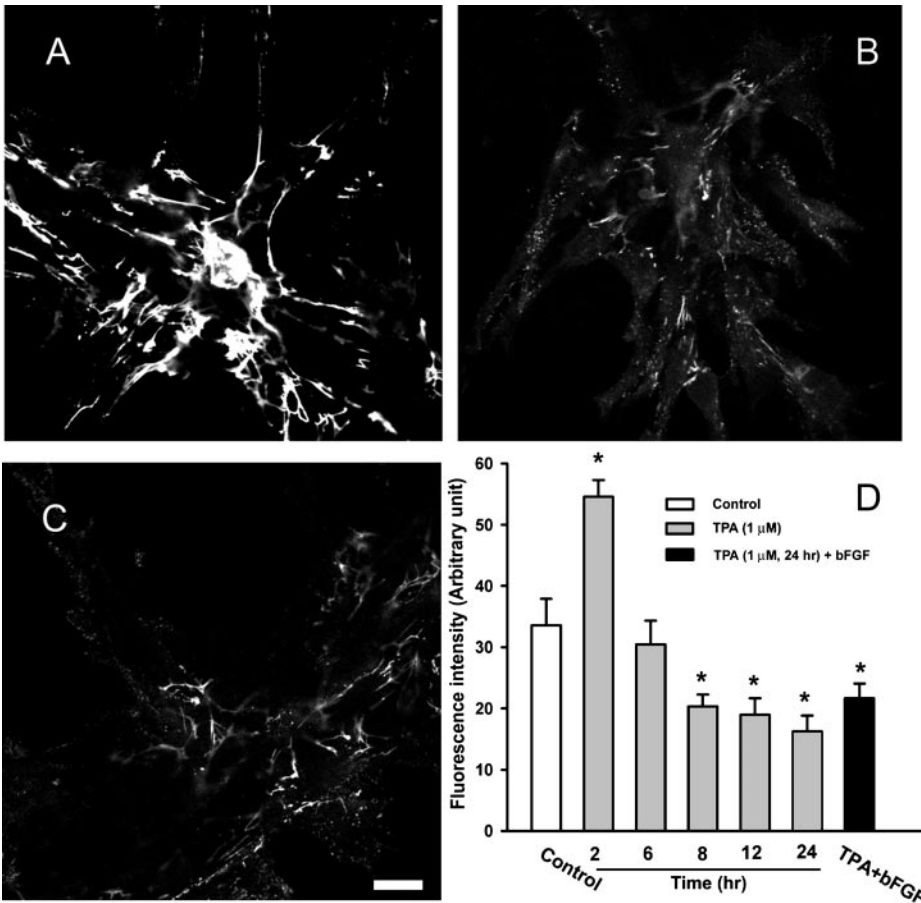


Fig. 5. Down-regulation of PKC antagonizes the enhancement of Fn assembly by bFGF. Osteoblasts were incubated with 1 μ M TPA for 24 h to down-regulate PKC. Culture medium of osteoblasts was then replaced with serum-free medium containing soluble human Fn (30 μ g/ml) in the presence or absence of bFGF (30 ng/ml). Immunocytochemistry was performed 24 h later using mouse anti-human Fn antibody, which does not recognize endogenously released Fn from rat osteoblasts. Compared with control (A), prolonged treatment with TPA markedly inhibited extracellular assembly of Fn (B). Down-regulation of PKC by long-term treatment of 1 μ M TPA markedly inhibited the potentiating action of bFGF on Fn assembly (C). The time-dependent biphasic action of TPA on Fn assembly and the inhibition of bFGF action by prolonged treatment with 1 μ M TPA were shown in (D). Data are presented as mean \pm S.E.M. (n). *, $p < 0.05$ compared with control. Bar, 10 μ m. $n = 17$ to 25.

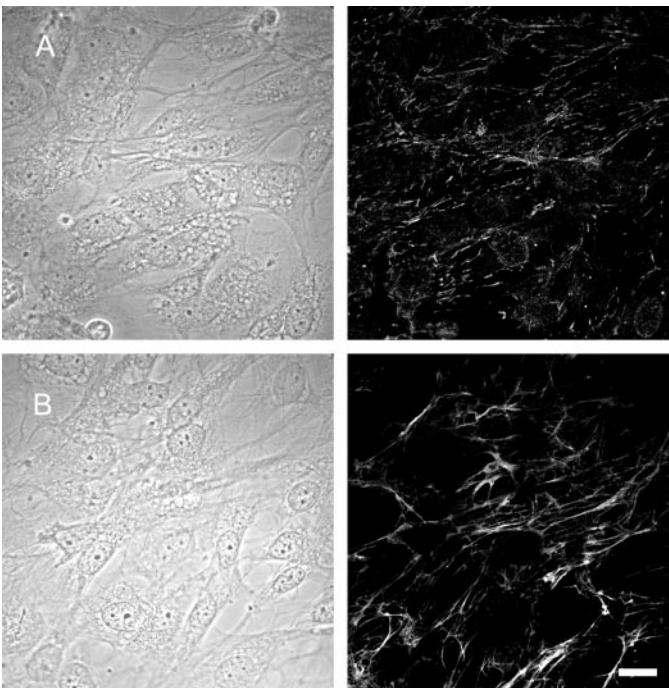


Fig. 6. Effects of bFGF on the clustering of $\alpha 5$ integrins. Osteoblast cultures were treated with bFGF (30 ng/ml) for 24 h. Immunocytochemistry was performed and fluorescent images were obtained from confocal microscope. Compared with control (A), treatment with bFGF markedly enhanced the clustering of $\alpha 5$ integrins (B). Bar, 10 μ m.

fibril from exogenously-applied soluble Fn (Fig. 3, B and C). The mean fluorescence intensity underneath 10~15 cells was 23.5 ± 2.8 and 48.6 ± 5.6 ($n = 16-21$) for the control and bFGF-treated group, respectively.

bFGF Increased Fn Fibrillogenesis in a PKC-Dependent Pathway. We then investigated which isoform of PKC is involved in the stimulatory effect of bFGF. PKC isoforms, including α , β , ϵ , and δ , have been identified in osteoblasts (Yang et al., 2002). Incubation of osteoblasts with bFGF (30 ng/ml) for 30 min increased membrane translocation of PKC isoforms, including α , β , ϵ , and δ (Fig. 4). To examine the intracellular signaling pathway of bFGF, ELISA was used to detect extracellular immobilized Fn. Pretreatment of osteoblasts with the tyrosine kinase inhibitors genistein (30 μ M) and herbimycin A (3 μ M), the PI-PLC inhibitor U73122 (3 μ M), but not the PC-PLC inhibitor D609 (30 μ M), antagonized the potentiating effect of bFGF (Fig. 4B). In addition, the PKC inhibitors such as Ro 318220, H7, GF109203X, and Gö 6976 antagonized the increase of Fn fibrillogenesis by bFGF (Fig. 4B). To examine which PKC isoforms are involved in the potentiation of Fn fibrillogenesis by bFGF, isoform-specific antisense oligonucleotides were used. As shown in Fig. 4C, treatment of antisense against PKC α , β , ϵ , and δ for 24 h selectively decreased the specific isoform and left the other isoforms unaffected in cell lysate. It was demonstrated that antisense of PKC isoforms α and β isoforms but not ϵ and δ antagonized the potentiating action of bFGF in Fn fibrillogenesis. The PKC-dependent effects of bFGF on the assembly of Fn in osteoblasts were further examined by the down-

regulation of PKC. We have shown previously that treatment with high concentration of TPA ($1 \mu\text{M}$) for 24 h is able to down-regulate these isoforms of PKC (Yang et al., 2002). Exogenous soluble human Fn ($30 \mu\text{g/ml}$) was then applied to the cultures for the assembly of Fn network by osteoblasts in the absence or presence of bFGF (30 ng/ml). As shown in Fig. 5, prolonged treatment with high concentration of TPA to down-regulate PKC exerted an inhibitory effect on the assembly of exogenous Fn underneath the cells (Fig. 5B). The potentiation of Fn fibrillogenesis by bFGF was also antagonized by PKC down-regulation (Fig. 5, C and D), suggesting that PKC is involved in the potentiating action of bFGF. Because PKC activation by TPA enhanced Fn fibrillogenesis (Yang et al., 2002), the time-dependent effect of high concentration ($1 \mu\text{M}$) of TPA was shown in Fig. 5D. Incubation with $1 \mu\text{M}$ TPA within 2 h increased extracellular Fn assembly, whereas the incubation time longer than 8 h decreased the assembly.

Effect of bFGF on the Distribution of Integrin. The assembly of extracellular Fn matrix underneath the cells may be related to integrins (Wu et al., 1993). Integrins are a family of heterodimeric transmembrane receptors that contain α and β subunits. The different combination of α and β chains forms different receptors for the various kinds of ECM molecules. $\alpha 5 \beta 1$ integrin is a specific receptor for Fn. We have previously found that application of GRGDS ($50 \mu\text{g/ml}$)

or triflavin ($2.8 \mu\text{M}$), an Arg-Gly-Asp (RGD)-dependent disintegrin (Huang et al., 1991), inhibited Fn assembly and the enhancing effect of BMP-4, indicating that RGD motif is involved in Fn fibrillogenesis (Tang et al., 2003). We further used immunocytochemistry to visualize the localization of integrins. The $\alpha 5$ staining for the control shows a punctate pattern (Fig. 6A). However, treatment with bFGF for 24 h greatly enhanced the clustering of $\alpha 5$ integrin, and the cells often show a staining of fibrillar pattern (Fig. 6B). We then used flow cytometry to investigate the effect of bFGF on the cell surface expression of integrins. As shown in Fig. 7, incubation with bFGF for 24 h significantly enhanced the fluorescence intensity of $\alpha 5$ and $\beta 1$ integrins (Fig. 7, A and B). The increase of cell surface expression of integrins by bFGF was antagonized by PKC inhibitors Gö 6976, Ro 318220, and H7 (Fig. 7, C and D). However, PKC inhibitor alone slightly reduced the expression of integrin, suggesting that a basal activity of PKC is involved in the regulation of integrin activity in cultured osteoblasts (Fig. 7, C and D).

bFGF Enhanced Fn Formation and Bone Volume of Tibia in Young Rat. Trabecular bone is composed of a lattice or network of branching bone spicules. The spaces between the bone spicules contain bone marrow. bFGF (300 ng/ml , $10 \mu\text{l}$, once per day) was locally administered into tibia for 5 consecutive days via an implantation of a needle cannula (22 gauge) in young rats weighing 76 to 90 g (Fig. 8A).

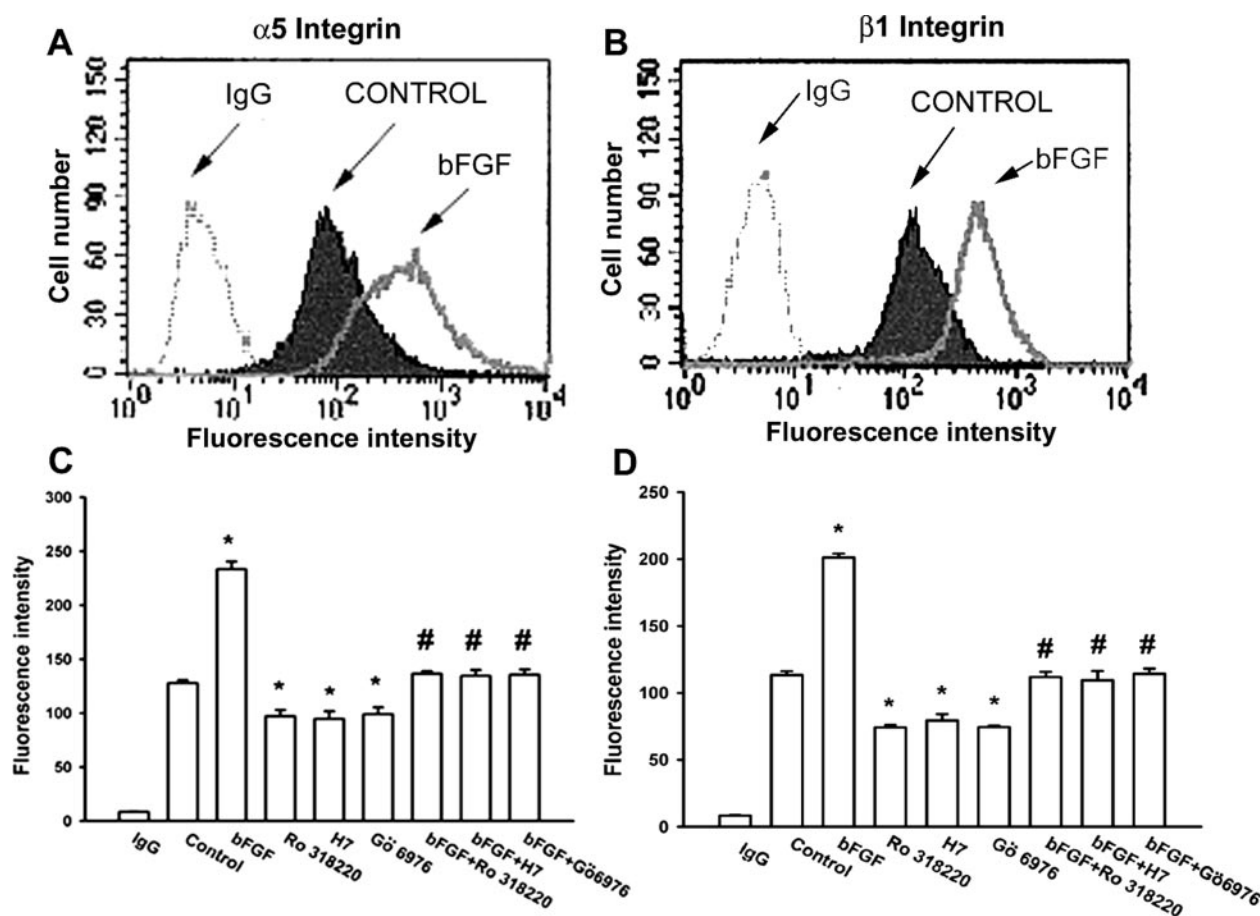


Fig. 7. Increase of the cell surface expression of $\alpha 5$ and $\beta 1$ integrins by bFGF using flow cytometric analysis. Compared with control, treatment with bFGF (30 ng/ml) for 24 h significantly enhanced the fluorescence intensity of $\alpha 5$ and $\beta 1$ integrins (A and B). PKC inhibitors Gö 6976 ($0.1 \mu\text{M}$), Ro 318220 ($1 \mu\text{M}$), and H7 ($10 \mu\text{M}$) antagonized the increase of cell surface expression of $\alpha 5$ (C) and $\beta 1$ (D) integrins by bFGF. Data are presented as mean \pm S.E.M. ($n = 4$). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with bFGF-treated group.

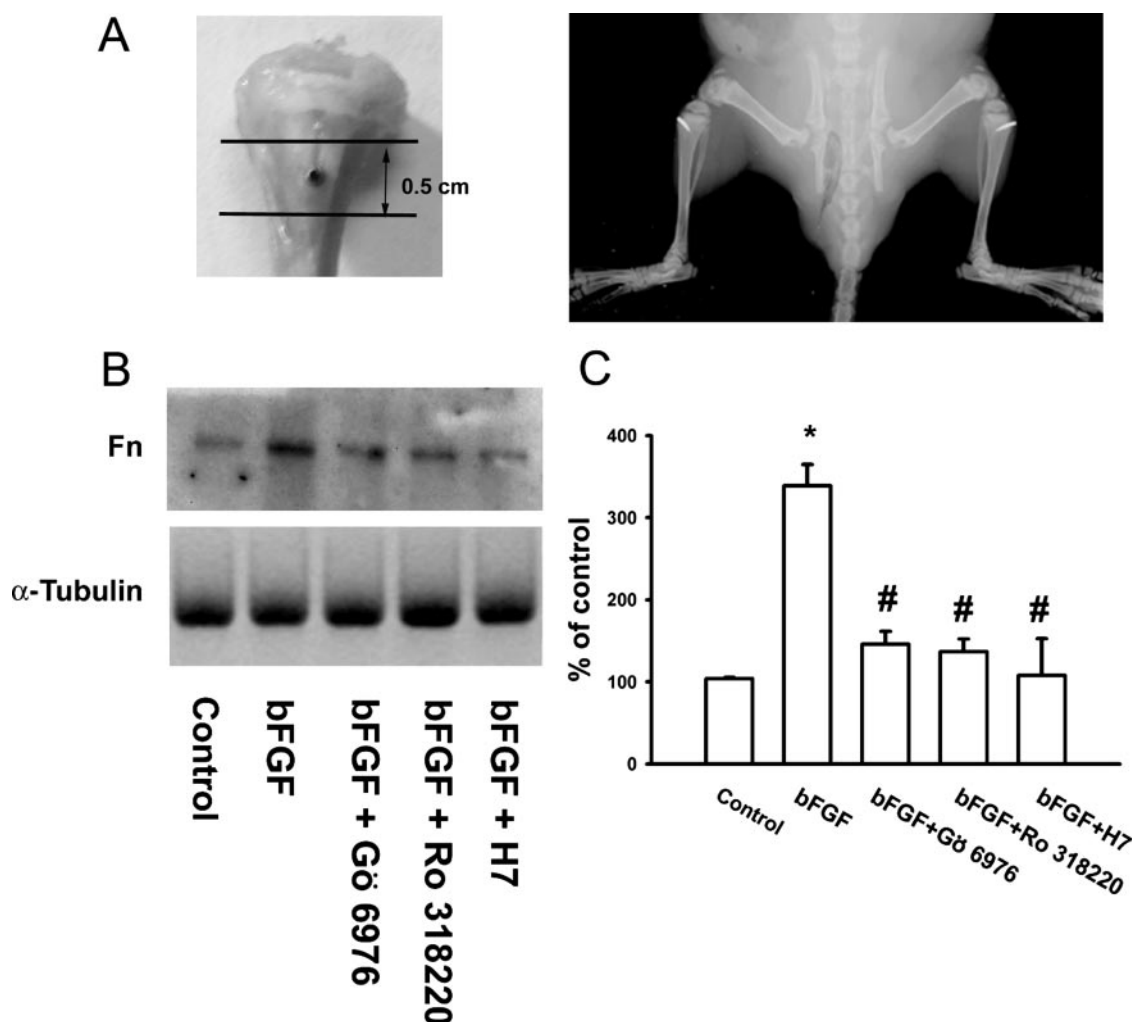


Fig. 8. bFGF increased Fn formation in tibia metaphysis of young rats. A, implantation of cannula (22 gauge) was done from the posterolateral side into the proximal tibial metaphysis in limbs of young rats. The cannula had its outer end in the subcutaneous tissue (as shown by X-ray image, right). bFGF (300 ng/ml, 10 μ l, once per day) was locally administered into tibia through the cannula for 5 consecutive days. The two solid lines indicated the area from which protein was extracted for Western blotting analysis on day 6 (left). Note that bFGF increased Fn protein level, and coadministration with PKC inhibitors Gö 6976 (10 pmol), Ro 318220 (100 pmol), or H7 (1 nmol) antagonized the potentiating effect of bFGF (B). The quantitative data are shown in C ($n = 6$). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with bFGF-treated group.

The protein from trabecular bone and bone marrow surrounding the injection site (as shown in Fig. 8A, left) was then isolated on day 6 for Western blotting analysis of Fn. Local injection of bFGF significantly increased the protein level of Fn. Coadministration of PKC inhibitors such as Gö 6976 (10 pmol), Ro 318220 (100 pmol), or H7 (1 nmol) antagonized the increase of Fn by bFGF (Fig. 8, B and C). We further examined the long-term effect of bFGF on the bone formation by local injection of bFGF into tibia for 7 consecutive days, and the rats were sacrificed later on day 14. The vehicle was injected into contralateral side for comparison. Compared with the vehicle-injected side (Fig. 9A; arrow shows the hole of the injection site), bFGF significantly increased bone volume of the secondary spongiosa (Fig. 9B). Trabecular bone in the secondary spongiosa increased by 72.5% after local administration of bFGF. The immunohistochemistry also showed that Fn predominantly localized around trabecular bone, and bFGF increased the staining of Fn (Fig. 9, C and D). In addition, BMD and BMC increased after long-term application of bFGF (Table 1). Coadministration of PKC inhibitors H7, Ro 318220, or GF109203X inhibited

the potentiating action of bFGF (Table 1). Furthermore, H7, Ro 318220, or GF109203X alone slightly decreased the bone volume, suggesting that basal PKC activity is involved in the regulation of bone formation (Table 1).

Discussion

bFGF has been demonstrated to be able to increase the amount of bone formation (Gonzales et al., 1990), to enhance the bone formation during fracture healing (Kawaguchi et al., 1994), and to stimulate proliferation of osteoblastic cells (Rodan et al., 1987). In the present study, immunocytochemistry was used to investigate the Fn fibrillogenesis from either endogenously released or exogenously applied Fn. We previously demonstrated that rat osteoblasts can synthesize and secrete Fn to form fibril matrix network underneath the cells (Yang et al., 2002). The Fn network is an important factor for the differentiation, expression of physiological function, and survival of osteoblasts (Globus et al., 1998). In this study, we further identify Fn as a target protein for bFGF signaling pathway in cultured osteoblasts. We also show that

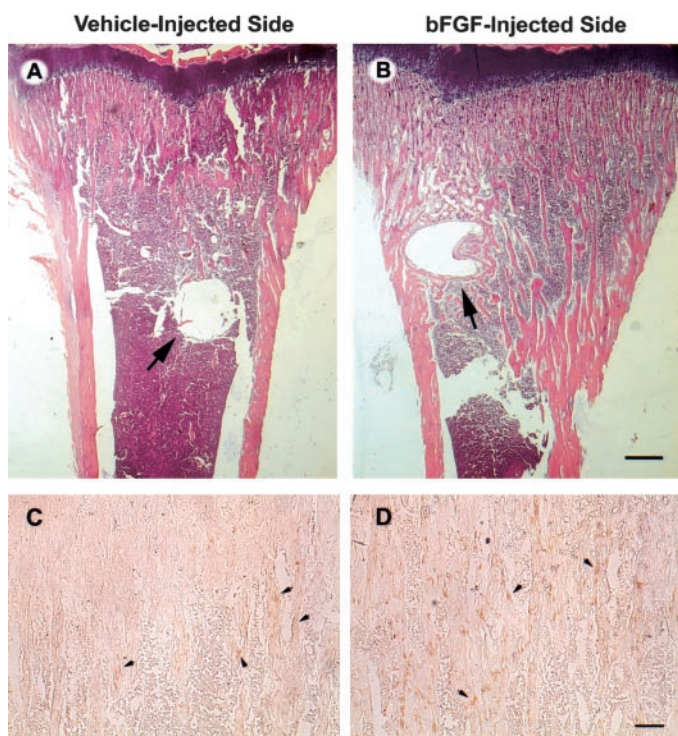


Fig. 9. bFGF increased bone volume and Fn immunostaining in tibia metaphysis of rats. bFGF (300 ng/ml, 10 μ l, once per day) was locally administered into tibia through the needle cannula (as shown by arrow) in the proximal tibia for 1 week. Vehicle was injected into the contralateral side for comparison. Rats were sacrificed and the tibiae were used for the analysis of bone volume 7 days after the last injection. Compared with vehicle-treated side (A), long-term treatment with bFGF markedly increased bone volume (B). Immunostaining showed that Fn predominantly localized around the trabecular bone (arrowhead) (C) and bFGF increased the staining of Fn (D). Bar, 0.5 mm (A and B) and 100 μ m (C and D).

potentiation of Fn fibrillogenesis by bFGF requires an activation of PKC signaling pathway.

bFGF stimulated Fn fibrillogenesis using endogenous or exogenous origin of Fn in a concentration-dependent manner. Furthermore, bFGF increased the protein levels of Fn as demonstrated by Western blotting analysis. Several isoforms of PKC exist in primary cultured osteoblasts, including α , β , ϵ , and δ (Yang et al., 2002). All of these PKC isoforms possess a phorbol ester-binding site and are capable of being activated by TPA. These PKC isoforms are down-regulated in osteoblasts in response to prolonged treatment of high con-

centration of TPA (Yang et al., 2002). PKC inhibitors such as H7, Ro 318220, GF109203X, and Gö 6976 antagonized the potentiating actions of bFGF. The membrane translocation of PKC isoforms, including α , β , ϵ , and δ , was increased by bFGF in a manner similar to that of TPA. However, prolonged treatment with a high concentration of TPA caused a down-regulation of PKC and inhibited Fn fibrillogenesis (Yang et al., 2002). The enhancement of Fn fibrillogenesis by bFGF was inhibited by PKC down-regulation, suggesting that bFGF acts in a PKC-dependent pathway. It has been reported that bFGF stimulates PLC and results in the activation of PKC and the formation of IP₃ in MC3T3-E1 cells (Kim et al., 2000, Suzuki et al., 2000). The PI-PLC inhibitor U73122, but not the PC-PLC inhibitor D609, inhibited the increase of Fn expression by bFGF, suggesting that the PI-PLC pathway is involved in PKC activation by bFGF. In addition, genistein and herbimycin A also inhibit the potentiating action of bFGF. These results indicated that both PKC and tyrosine kinase activity are required for the effect of bFGF. It has also been found that bFGF increases N-cadherin expression in human calvaria osteoblasts via activation of PKC pathways (Debiais et al., 2001). We here show that bFGF is able to increase membrane translocation of various PKC isoforms, including α , β , ϵ , and δ in primary osteoblastic cultures. We have previously demonstrated that PKC increases and PKA inhibits the Fn fibrillogenesis (Yang et al., 2002). Here, we further define a ligand that can increase Fn formation via PKC activation. Treatment with antisense oligonucleotides directed against PKC isoforms α and β but not PKC ϵ and δ antagonized the potentiating action of bFGF in the Fn expression, indicating that α and β isoforms are much more important to mediate the action of bFGF in osteoblasts. bFGF has been reported to enhance TGF β gene expression in osteoblastic cells (Noda and Vogel, 1989). However, we previously found that TGF β inhibited Fn assembly (Tang et al., 2003), suggesting that enhancement of Fn fibrillogenesis by bFGF does not result from the release of TGF β .

Direct osteoblast interactions with the extracellular matrix are mediated by a selective group of integrin receptors, including α 5 β 1, α 3 β 1, α v β 3, and α 4 β 1 (Clover et al., 1992; Grzesik and Robey, 1994). α 5 β 1 integrin, a specific Fn receptor, mediates critical interactions between osteoblasts and Fn required for both bone morphogenesis and osteoblast differentiation (Moursi et al., 1997). Interfering with interactions between Fn and integrin Fn receptors in immature fetal

TABLE 1

Effect of bFGF and PKC inhibitors on the BMD, BMC, and bone volume in tibia

bFGF (300 ng/ml, 10 μ l, once per day) and PKC inhibitor Ro 318220 (100 pmol), H7 (1 nmol), or GF109203X (100 pmol) were locally administered into tibia through the needle cannula in the proximal tibia for 1 week. Vehicle was injected into the contralateral side for comparison. Rats were sacrificed, and the tibiae were used for analysis of 7 days after the last injection. $n = 8-12$

	BMD	BMC	BV/TV
	$g/cm^2/1TD$	g	%
Control	0.090 ± 0.002	0.081 ± 0.002	8.9 ± 0.6
bFGF	0.102 ± 0.004^a	0.111 ± 0.01^a	18.2 ± 1.8^a
H7	0.087 ± 0.004	0.073 ± 0.005	5.8 ± 0.4^a
Ro 318220	0.087 ± 0.005	0.073 ± 0.006	6.9 ± 0.7^a
GF109203X	0.086 ± 0.004	0.072 ± 0.004	5.9 ± 0.6^a
bFGF + H7	0.093 ± 0.004^b	0.085 ± 0.007^b	9.3 ± 0.8^b
bFGF + Ro318220	0.093 ± 0.006^b	0.091 ± 0.006^b	8.1 ± 1.2^b
bFGF + GF109203X	0.092 ± 0.005^b	0.087 ± 0.006^b	8.8 ± 0.8^b

BV/TV, bone volume/tissue volume.

^a $P < 0.05$: compared with control groups.

^b $P < 0.05$: compared with bFGF-treated groups.

rat calvarial osteoblasts suppressed formation of mineralized nodules in vitro and delayed expression of tissue-specific genes, including osteocalcin (Moursi et al., 1997). Enhancement of surface expression of $\alpha 5$ and $\beta 1$ integrins by bFGF is correlated to the increase of Fn assembly by bFGF. Increased surface expression of $\alpha 5$ and $\beta 1$ integrin by bFGF was antagonized by PKC inhibitors H7, Ro 318220, and Gö 6976. On the other hand, prolonged treatment with PKC inhibitor alone for 24 h slightly reduced the expression of both $\alpha 5$ and $\beta 1$ integrin, suggesting that a basal PKC activity is involved in regulating integrin function in cultured osteoblasts.

Several studies have shown that gene for bFGF is expressed in early embryonic development and have suggested that the growth factor may play an important role in tissue growth and differentiation (Kimelman et al., 1988; Gonzales et al., 1990). Using local injection of bFGF into tibia for 5 consecutive days, we have demonstrated that local administration of bFGF increased the protein level of Fn and bone volume in young rats. The present results suggest that bFGF plays an important role in the developing bone as well. Previous studies have shown that bFGF is a more potent mitogen for fibroblast and preosteoblasts than for differentiated osteoblast (McCarthy et al., 1989). The increase of bone formation may be also partially mediated by the increase of proliferation and survival of osteoblasts (Shimoaka et al., 2002); bFGF also increased differentiation marker of osteocalcin (Xiao et al., 2002). Local injection of H7, Ro 318220, or GF109203X alone slightly reduced the bone volume and markedly inhibited the potentiating action of bFGF, indicating that PKC activity plays an important role in the regulation of bone formation.

In conclusion, the present study demonstrated that bFGF increased Fn fibrillogenesis and the cell surface expression of $\alpha 5$ and $\beta 1$ integrins via a PKC-dependent pathway. Long-term administration of bFGF into tibia of young rats also increased the protein level of Fn and bone volume of secondary spongiosa. bFGF may thus play an important role in bone formation via the regulation of Fn matrix network in developing bones.

References

- Baylink DJ, Finkleman RD, and Mohan S (1993) Growth factors to stimulate bone formation. *J Bone Miner Res* **8**:S565–S572.
- Bolander ME (1992) Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med* **200**:165–170.
- Clover JF, Dodds RA, and Gowen M (1992) Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. *J Cell Sci* **103**:267–271.
- Cowles EA, DeRome ME, Pastizzo G, Brailey LL, and Gronowicz GA (1998) Mineralization and the expression of matrix proteins during in vivo bone development. *Calcif Tissue Int* **62**:74–82.
- Debiais F, Lemonnier J, Hay E, Delannoy P, Caverzasio J, and Marie PJ (2001) Fibroblast growth factor-2 (FGF-2) increases N-cadherin expression through protein kinase C and Src-kinase pathways in human calvaria osteoblasts. *J Cell Biochem* **81**:68–81.
- Dzamba BJ, Bultmann H, Akiyama SK, and Peters DM (1994) Substrate-specific binding of the amino terminus of fibronectin to an integrin complex in focal adhesions. *J Biol Chem* **269**:19646–19652.
- Globus RK, Doty SB, Lull JC, Holmuhamedov E, Humphries MJ, and Damsky CH (1998) Fibronectin is a survival factor for differentiated osteoblasts. *J Cell Sci* **111**:1385–1393.
- Gonzales AM, Buscaglia M, and Ong M (1990) Distribution of basic fibroblast growth factor in the 18-day rat fetus: localization in the basement membrane of diverse tissues. *J Cell Biol* **110**:753–765.
- Gospodarowicz D, Ferrera N, Schweigerer L, and Newfeld G (1987) Structural characterization and biological functions of fibroblast growth factor. *Endocr Rev* **8**:95–114.
- Grzesik WJ and Robey PG (1994) Bone matrix RGD-glycoproteins: immunolocalization and their interaction with human primary osteoblastic bone cells in vitro. *J Bone Miner Res* **9**:487–496.
- Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, and Johannes FJ (1996) Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase C isoenzymes. *FEBS Lett* **392**:77–80.
- Hall SH, Berthelton MC, Avallet O, and Sacz JM (1991) Regulation of c-fos, c-jun, jun-B and c-myc messenger ribonucleic acids by gonadotropin and growth factors in cultured pig Leydig cell. *Endocrinology* **129**:1243–1249.
- Hay ED (1991) *Cell Biology of Extracellular Matrix*, 2nd ed. Plenum Press, New York.
- Huang TF, Sheu JR, Teng CM, Chen SW, and Liu CS (1991) Triflavin, an antiplatelet Arg-Gly-Asp-containing peptide, is a specific antagonist of platelet membrane glycoprotein IIb/IIIa complex. *J Biochem (Tokyo)* **109**:328–334.
- Hurley MM, Abreu C, Gronowicz G, Kawaguchi H, and Lorenzo J (1994) Expression and regulation of basic fibroblast growth factor mRNA levels in mouse osteoblastic MC3T3–E1 cells. *J Biol Chem* **269**:9392–9396.
- Juliano RL and Haskill S (1993) Signal transduction from the extracellular matrix. *J Cell Biol* **120**:577–585.
- Kawaguchi H, Kurokawa T, Hanada K, Hiayama Y, Tamura M, Ogata E, and Matsumoto T (1994) Stimulation of fracture repair by recombinant human basic fibroblast growth factor in normal and streptozotocin-diabetic rats. *Endocrinology* **135**:774–781.
- Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, and Ryoo HM (2000) The protein kinase C pathway plays a central role in the fibroblast growth factor stimulated expression and transactivation activity of Runx2. *J Biol Chem* **275**:319–326.
- Kimelman DJ, Abraham JA, Haaparanta T, Palisi TM, and Kirschner MW (1988) The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. *Science (Wash DC)* **242**:1053–1056.
- Lauffenburger DA and Horwitz AF (1996) Cell migration: a physically integrated molecular process. *Cell* **84**:359–369.
- Lu D, Yang H, Lenox RH, and Raizada MK (1998) Regulation of angiotensin II-induced neuromodulation by MARCKS in brain neurons. *J Cell Biol* **142**:217–227.
- McCarthy TL, Centrella M, and Canalis E (1989) Effects of fibroblast growth factors on deoxyribonucleic acid and collagen synthesis in rat parietal bone cells. *Endocrinology* **125**:2118–2126.
- Mohammadi M, Honegger AM, Rotin D, Fisher R, Bellot F, Li W, Dionne CA, Jaye M, Rubinstein M, and Schlessinger J (1991) A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol Cell Biol* **11**:5068–5078.
- Mosher DF, Sottile J, Wu C, and McDonald JA (1992) Assembly of extracellular matrix. *Curr Opin Cell Biol* **4**:810–818.
- Moursi AM, Globus RK, and Damsky CH (1997) Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. *J Cell Sci* **110**:2187–2196.
- Noda M and Vogel R (1989) Fibroblast growth factor enhances type beta 1 transforming growth factor gene expression in osteoblast-like cells. *J Cell Biol* **109**:2529–2535.
- Ornitz DM (2000) Fibroblast growth factors, chondrogenesis and related clinical disorders, in *Skeletal Growth Factors* (Canalis E, ed) pp 197–209. Lippincott Williams & Wilkins, Philadelphia.
- Rodan SB, Wesolowski G, Thomas K, and Rodan GA (1987) Growth stimulation of rat calvaria osteoblastic cells by acidic fibroblast growth factor. *Endocrinology* **121**:1917–1923.
- Rodan SB, Wesolowski G, Thomas KA, Yoon K, and Rodan GA (1989) Effects of acidic and basic fibroblast growth factors on osteoblastic cells. *Connect Tissue Res* **20**:283–288.
- Rosales C, O'Brien V, Kornberg L, and Juliano R (1995) Signal transduction by cell adhesion receptors. *Biochim Biophys Acta* **1242**:77–98.
- Shimoaka T, Ogasawara T, Yonamine A, Chikazu D, Kawano H, Nakamura K, Itoh N, and Kawaguchi H (2002) Regulation of osteoblast, chondrocyte and osteoclast functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. *J Biol Chem* **277**:7493–7500.
- Suxuki A, Palmer G, Bonjour JP, and Caverzasio J (2000) Stimulation of sodium-dependent phosphate transport and signaling mechanisms induced by basic fibroblast growth factor in MC3T3–E1 osteoblast-like cells. *J Bone Miner Res* **15**:95–102.
- Tang CH, Yang RS, Liou HC, and Fu WM (2003) Enhancement of fibronectin synthesis and fibrillogenesis by BMP-4 in cultured rat osteoblast. *J Bone Miner Res* **18**:502–511.
- Tinsley JH, Teasdale NR, and Yuan SY (2004) Involvement of PKCdelta and PKD in pulmonary microvascular endothelial cell hyperpermeability. *Am J Physiol* **286**:C105–C111.
- Ullrich A and Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**:203–212.
- Wayner EA, Orlando RA, and Cheresch DA (1991) Integrins $\alpha v \beta 3$ and $\alpha v \beta 5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J Cell Biol* **113**:919–929.
- Weiss RE and Reddi AH (1981) Appearance of fibronectin during the differentiation of cartilage, bone and bone marrow. *J Cell Biol* **88**:630–636.
- Wu C, Bauer JS, Juliano RL, and McDonald JA (1993) The alpha 5 beta 1 integrin fibronectin receptor, but not the alpha 5 cytoplasmic domain, functions in an early and essential step in Fn matrix assembly. *J Biol Chem* **268**:21883–21888.
- Xiao G, Jiang D, Gopalakrishnan R, and Franceschi RT (2002) Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. *J Biol Chem* (2002) **277**:36181–36187.
- Yang RS, Tang CH, Ling QD, Liu SH, and Fu WM (2002) Regulation of fibronectin fibrillogenesis by protein kinases in cultured rat osteoblasts. *Mol Pharmacol* **61**:1163–1173.

Address correspondence to: Fu Wen-Mei, Department of Pharmacology, College of Medicine, National Taiwan University, 1, Sec. 1, Jen-Ai Road, Taipei, Taiwan. E-mail: wenmei@ha.mc.ntu.edu.tw