Prostaglandin E₂ Stimulates Fibronectin Expression through EP₁ Receptor, Phospholipase C, Protein Kinase C α and c-Src Pathway in Primary Cultured Rat Osteoblasts

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Running title: EP₁-dependent increase of fibronectin expression by PGE₂.

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Fibronectin (Fn) is involved in early stages of bone formation and prostaglandin E (PGE) is an important factor regulating osteogenesis. Here we found that PGE₂ enhanced extracellular Fn assembly in rat primary osteoblasts, as shown by immunofluorescence staining and enzyme-linked immunosorbent assay. PGE₂ also increased the protein levels of Fn using Western blotting analysis. Using pharmacological inhibitors or activators or genetic inhibition by EP receptor antisense oligonucleotides (AS-ODN) revealed that EP₁ receptor but not other PGE receptors is involved in PGE₂-mediated up-regulation of Fn. At the mechanistic level, Ca²⁺ chelater (BATA-AM), phosphatidylinositol-phospholipase C (PI-PLC) inhibitor (U73122) or Src inhibitor (PP2) attenuated the PGE₂-induced Fn expression. Protein kinase C (PKC) inhibitor (GF109203X) also inhibited the potentiating action of PGE₂. Furthermore, treatment with AS-ODN of various PKC isoforms including α, β, ε, and δ demonstrated that α isozyme plays an important role in the enhancement action of PGE₂ on Fn assembly. Flow cytometry and RT-PCR showed that PGE₂, 17-phenyl trinor PGE₂ (EP₁/EP₃ agonist) increased the surface expression and mRNA level of α5 or β1 integrins. Fn promoter activity was enhanced by PGE₂ and 17-phenyl trinor PGE₂ in cells transfected with pGL2F1900-Luc. Cotransfection with dominant negative mutants of PKCα or c-Src inhibited the potentiating action of PGE₂ on Fn promoter activity. Local administration of PGE₂ or 17-phenyl trinor PGE₂ into the metaphysis of the tibia via the implantation of a needle cannula significantly increased the Fn and α5β1 integrin immuostaining and bone volume of secondary spongiosa in tibia. Taken together, our results provided evidence that PGE₂ increased Fn and promotes bone formation in rat osteoblasts via the EP₁/PLC/PKCα/c-Src signaling pathway.
The extracellular matrix (ECM) provides positional and environmental information that is essential for tissue function. The ECMs produced by osteoblasts are complex and consist of several different classes of molecules that may regulate the modeling and remodeling of bone. The ECMs also serve as a reservoir for growth factors, including members of the prostaglandins (PGEs) and fibroblast growth factor superfamily (1, 2). Acting either alone or together, these components of the ECM produced by osteoblasts may subsequently regulate the cell adhesion, migration, proliferation, differentiation, survival, as well as the rate of bone formation.

Fibronectin (Fn) is an extracellular matrix component that is also present as a soluble protein in plasma and other body fluids (3). The matrix form of Fn is believed to support cell adhesion, migration during embryogenesis, tumor growth, wound healing, angiogenesis and inflammation (4). Assembly of soluble Fn into matrix is a multi-step process under cellular control (5). Among the membrane components implicated in Fn matrix assembly, integrins have been demonstrated to have a central role (6). Integrins, composed of α and β subunits, are a family of transmembrane receptors mediating adhesion to both ECM and cell surface molecules (7, 8). The specific adhesion depends on the interaction between the cell-binding domain of Fn and cell surface integrin receptors. However, the mechanisms regarding how integrins modulate Fn assembly are not well understood. Transfection of α5 integrin and expression of α5β1-integrin by Chinese hamster ovary (CHO) cells results in a large increase in Fn assembly, whereas α5-deficient CHO B2 cells failed to assemble plasma Fn into ECM (9, 10). Osteoblast differentiation is an essential part of bone formation, because active osteoblasts should be recruited at the site of osteoclastic bone resorption to compensate the continuous loss of bone matrix and maintain structural integrity of skeletal system. The biology of this process is also of considerable interest when applying therapies to promote bone repair after injury or during disease processes. Furthermore, integrins are involved in the signal transduction of translating the strain in the organic matrix to the biochemical signals in the bone cells (11). However, the role of cytokine in the cell-matrix interactions in osteoblasts has not been extensively studied.

PGEs are considered important local factors that modulate bone metabolism through their effects on osteoblastic cells and osteoclasts (12). PGE\(_2\) is a major eicosanoid produced by osteoblasts. To explain the diverse effects of PGE\(_2\), the presence of multiple receptors for PGE\(_2\) in osteoblasts was postulated. Recent cloning of four subtypes of PGE receptor has made it possible to analyze the PGE receptor subtypes (EP\(_1\)-EP\(_4\)) on osteoblasts (13, 14). EP\(_1\) is coupled to Ca\(^{2+}\) mobilization, EP\(_2\) and EP\(_4\) activate adenylate cyclase, whereas EP\(_3\) inhibits adenylate cyclase (15-17). An EP\(_1\) agonist stimulated cell growth, whereas an EP\(_4\) agonist reduced cell growth and increased alkaline phosphatase activity in MC3T3-E1 osteoblast-like cells (18). These studies indicate that osteoblasts express multiple subtypes of the PGE receptor and that each subtype might be
linked to different actions of PGE₂.

The distribution of Fn in areas of skeletogenesis suggests that it may be involved in early stages of bone formation (19). However, the effect of PGE₂ on Fn fibrillogenesis in osteoblasts is mostly unknown. We here found that PGE₂ enhanced Fn fibrillogenesis of osteoblasts by increasing the synthesis and assembly of Fn. Furthermore, the increase of clustering of α5 and β1 integrins is involved in the action mechanism of PGE₂. EP₁ receptor, PI-PLC, PKCα and c-Src-dependent pathways may be involved in the increase of osteoblast Fn expression and bone formation by PGE₂.

EXPERIMENTAL PROCEDURES

Materials:
Mouse monoclonal antibody for PKCα was purchased from BD Transduction Laboratories (Lexington, KY). Mouse monoclonal antibody for α-tubulin was purchased from Oncogene Science (Cambridge, MA). Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for fibronectin, phosphotyrosine residues (PY20) and c-Src were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for α5, β1, α5β1 integrin and type I collagen were purchased from Chemicon (Temecula, CA). PGE₂, 17-phenyl trinor PGE₂, butaprost, sulprostone, 11-deoxy PGE₁ and SC 19220 were purchased from Cayman Chemical (Ann Arbor, MI). U73122, U73343, D609 and GF109203X were purchased from Calbiochem (San Diego, CA). Avidin-biotin-peroxidase detection system was purchased from Vector (Burlingham, CA). The fibronectin promoter construct (pGL2F1900-Luc) was a gift from Dr. I.S. Kim (Kyungpook National University, Korea). The PKCα dominant negative mutant was a gift from Dr. V. Martin (Louis Pasteur de Strasbourg University, France). The c-Src dominant negative mutant was a gift from Dr. S. Parsons (University of Virginia Health System, Charlottesville, VA). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Primary osteoblast cultures

Primary osteoblastic cells were prepared by the method as previously described (20). The calvaria of fetal rats were dissected from fetal rats and divided into small pieces then treated with 0.1% type I collagenase solution for 10 minutes at 37°C. The next two 20 minute sequential collagenase digestions were then pooled and filtered through 70 μm nylon filters (Falcon, BD BioSciences, San Jose, CA). The cells were grown on the plastic cell culture dishes in 95 % air-5 % CO₂ with α-MEM (Gibco, Grand Island, NY) which was supplemented with 20 mM HEPES and 10 % heat-inactivated FCS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (pH adjusted to 7.6). 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 (1:1000) for 1 hr 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 hr. Finally, cells were washed, mounted and examined with Zeiss confocal microscope (LSM 410) as soon as possible. The mean fluorescence under 10-15 cells (3~5 fields per culture) was measured using Zeiss confocal microscope. 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.

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 PGE2 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, the cultures were then blocked with 1% BSA in PBS for 15 min before being incubated sequentially with rabbit anti-rat Fn antibody (1:150) for 1 hr 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 NaHPO4·12 H2O; 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, Burlington, VA). Each assay was performed in triplicate.

Oligonucleotide (ODN) transfection

Osteoblasts were cultured to confluence, the complete medium was replaced with OPTI-MEM (Invitrogen) containing the antisense phosphorothioate oligonucleotides (5 μg/ml) that had been preincubated with Lipofectamine 2000 (10 μl/ml) (LF2000; Invitrogen) for 30 min. The cells were washed after 24 h of incubation at 37°C and washed prior to the addition of medium containing PGE2. All antisense ODNs were synthesized and HPLC purified by MDBio, Inc., (Taipei, Taiwan).

Sequences are as follows,
EP 1 antisense-ODN (AS-ODN): CTGCAGTTTCATTTCTCC; missense-ODN (MM-ODN): CGACAATTGAATTCATCT
EP 2 AS-ODN: GCCTGGAGTCATTGA; MM-ODN: CGCGTGAGTCTATGA
EP 3 AS-ODN: ACACGCCGGCCATAGTGG; MM-ODN: AGACCCCGCCGAGAGTGT
EP 4 AS-ODN: GACTCCGGGGATGGA; MM-ODN:GACCTCGGGAGTGAG.
(21, 22)
PKC-α AS-ODN: AAAACGTCAGCCATG
PKC-β AS-ODN: AAGATGGCTGACCCGGCTGC
PKC-δ AS-ODN: GTGCCATGATGGAGCTTTT
PKC-ε AS-ODN: TTGAACACTACCATG (23).

**mRNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from osteoblasts using a TRizol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers:

- EP₁ (336 base pairs (bp))
  - CGCAGGGTTCACGCACACGA and CACTGTGCGCCGAACCTACGC
- EP₂ (369 bp)
  - CCAGGTCATCCTATTTGC and GCTCCGAAGCTGCATGCGAA
- EP₃ (537 bp)
  - GCCGGGAGAGCAAACGCAAAAA and ACACCAGGGCTTTGATGGTCGCCAGG
- EP₄ (423 bp)
  - TTCCGCTCGTGGTGCGAGTGTTC and GAGGTGGTGTCTGCTTGGGTCAG
- GAPDH (452 bp)
  - ACCACAGTCCATGCCATCAC and TCCACCACCTGTGCTGTA. (21, 22)

Each PCR cycle was carried out for 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

**Immunoprecipitation and Western Blot Analysis**

The cellular lysates were prepared as described previously (20). Equal amounts of protein were incubated with specific antibody immobilized onto protein A/G-Sepharose for 12 h at 4°C with gentle rotation. Beads were washed extensively with lysis buffer, boiled, and microcentrifuged. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinylidifluoride (PVDF) membranes. 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) or c-Src (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) 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). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Determination of cytosolic Ca²⁺ with fluo-3-AM**

Fluo-3-AM (Fluo3-acetoxymethyl ester) was used to measure cytosolic free Ca²⁺. Cells were incubated 60 minutes in the dark at room temperature with fluo-3-AM (4 μM) and the cells were then washed and cytosolic Ca²⁺ was measured by FACS Calibur (Cell
Quest software, BD Biosciences). Excitation and emission wavelengths were 488 and 530 nm respectively.

Quantification of integrin expression

Osteoblasts were plated in 6-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 α5 or β1 integrin antibody (1:100) for 1 hr at 4°C. Cells were then washed again and incubated with FITC-conjugated secondary IgG for 45 min and analyzed by flow cytometry using FACS Calibur.

Transfection and reporter gene assay

Osteoblasts were cotransfected with 1 µg Fn promoter plasmid and 1 µg β-galactosidase expression vector. Osteoblasts were grown to 60% confluent in 12-well plates and were transfected on the following day by LF2000, premix DNA with OPTI-MEM, and LF2000 with OPTI-MEM, respectively, for 5 min. The mixture was then incubated for 25 min at room temperature and added to each well. After 24-h incubation, transfection was complete, and cells were incubated with the indicated agents. After 24 hr incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 µl of reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30s. Aliquots of cell lysates (10 µl) containing equal amounts of protein (10–20 µg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The luciferase activity value was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. In experiments using dominant-negative mutants, cells were cotransfected with reporter (0.5 µg) and β-galactosidase (0.25 µg) and either the PKCα or c-Src mutant or the empty vector (1.0 µg).

Measurement of bone mineral density (BMD) and bone volume

The local injection of young rats was prepared by the method as previously described (23). Male Sprague-Dawley rats weighing 73-88 gm were used. Implantation of a cannula (22G) was done from the posteriolateral side into the proximal tibial metaphysis in both limbs of rats anesthetized with trichloroacetaldehyde. The cannula had its outer end in the subcutaneous tissue. PGE$_2$ or 17-phenyl trinor PGE$_2$ (30 µM, 10 µl) was percutaneously injected into the proximal tibia through the cannula (once/day) for 7 consecutive days. The same volume of vehicle was injected into the contralateral side for comparison. On the Day-14, the rats were sacrificed and tibiae were also removed, cleaned of soft tissue. BMD and BMC of the tibia were measured with a dual-energy X-ray absorptiometer (DEXA, 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 decalcified, and embedded in paraffin. Serial sections (5 μm) 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 non-specific binding, followed by incubation with rabbit anti-rat Fn, α5β1 integrin and type I collagen antibody (1:300) overnight at 4°C. The sections were detected by avidin-biotin-peroxidase detection system and diaminobenzidine. For measurement of bone volume, the sections were stained with Mayer’s hematoxylin-eosin solution. Images of the growth plate and proximal tibia were photographed using Olympus microscope IX70. Measurement of bone volume was performed on the secondary spongiosa, which is located 1.0 to 3.0 mm distal to epiphyseal growth plate and characterized by a network of larger trabeculae. Bone volume was calculated using image analysis software (Image-pro plus 3.0) and expressed as % of bone area. All measurements were done in a single-blind fashion.

All protocols complied with institutional guidelines and were approved by Animal Care Committee of Medical College, National Taiwan University.

Statistics
The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student’s t test. The difference is significant if the p value is <0.05.

RESULT

**PGE₂ enhanced Fn fibrillogenesis in cultured osteoblasts**

The fibrillogenesis from the endogenously released Fn by the primary cultured rat osteoblasts was studied using immunocytochemistry. Day-3~5 osteoblasts were changed to serum-free medium and incubated with PGE₂ (3 μM) for 24 hr. Immunostaining of Fn was examined in 4% paraformaldehyde-fixed and non-permeabilized cells. The mean immunofluorescence intensity underneath a cell group of 10-15 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. Fn fibril formation increased in response to the treatment of PGE₂ for 24 hr (Fig. 1B). The quantitative data showed a dose-dependent increase of fluorescence intensity (Fig. 1C). We also used ELISA to detect extracellular immobilized Fn. PGE₂ also increased Fn expression in a concentration-dependent manner (Fig. 1D). Western blotting was used to examine the effect of PGE₂ on the protein levels of Fn. Day-3~5 osteoblasts were changed to serum-free culture medium and treated with PGE₂ for 24 hr. The cultures were then washed with 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. 1E and F (PGE\(_2\) at 3 \(\mu\)M), PGE\(_2\) increased protein levels of Fn in a concentration and time-dependent manner.

**Involvement of EP\(_1\) Receptors in PGE\(_2\)-Mediated increase of Fn formation**

PGEs exert their effects through interaction with specific EP\(_{1-4}\) receptors (14). To investigate the role of EP\(_{1-4}\) subtype receptors in PGE\(_2\)-mediated increase of Fn formation, we assessed the distribution of these EP subtype receptors in rat primary osteoblasts by RT-PCR analysis. The mRNAs of EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\) subtype receptors could be detected in primary rat osteoblasts (Fig. 2A). Upon PGE\(_2\) treatment for 6 h, the mRNA level of EP\(_1\) subtype receptor was evidently increased, whereas other subtypes EP receptor mRNA remained unchanged (Fig. 2A). We next examined which EP subtype receptors are involved in the PGE\(_2\)-mediated increase of Fn formation, specific inhibition of EP\(_1\) receptor expression was accomplished with AS-ODN. It was found that EP\(_1\) receptor-specific AS-ODN but not other EP receptor’s AS-ODN or MM-ODN significantly blocked PGE\(_2\)-mediated increase of Fn formation in primary rat osteoblasts (Fig. 2B). To determine the role of EP\(_1\) receptor-dependent signaling in regulation of Fn expression in osteoblasts, the cells were treated with EP\(_{1-4}\) specific agonists and then examined the expression level of Fn. Of these agonists tested, only EP\(_1\)/EP\(_3\)-selective receptor agonist, 17-phenyl trinor PGE\(_2\) (3 \(\mu\)M), significantly increased the protein level of Fn (Fig. 3A). In contrast, butaprost (EP\(_2\) agonist; 10 \(\mu\)M), sulprostone (EP\(_3\) agonist; 10 \(\mu\)M) and 11-deoxy PGE\(_1\) (EP\(_2\)/EP\(_4\) selective agonist; 10 \(\mu\)M) failed to up-regulate Fn expression. In addition, treatment of EP\(_1\) receptor antagonist SC19220 (10 \(\mu\)M) effectively antagonized the potentiating effect of PGE\(_2\) on Fn expression (Fig. 3A). It has been reported that sulprostone also acts on the rat EP\(_1\) receptor (26). We then examined the concentration-dependent effect of sulprostone on the expression of Fn. Treatment of osteoblast with sulprostone did not increase protein level of Fn unless at a higher concentration of 20 \(\mu\)M. Pretreatment of osteoblasts with EP\(_1\) AS-ODN but not EP\(_3\) AS-ODN antagonized the potentiating action of 20 \(\mu\)M sulprostone (Fig. 3B). From results shown above using pharmacological treatment or genetic inhibition clearly demonstrated a critical role of EP\(_1\) receptor in the PGE\(_2\)-mediated increase of Fn formation. It has been reported that activation of EP\(_1\) augments intracellular calcium mobilization, which is related to downstream signals (15). We then investigated the effect of chelating intracellular Ca\(^{2+}\) on the potentiating action of PGE\(_2\) on Fn expression. Pretreatment with BAPTA-AM (0.1-10 \(\mu\)M) for 30 min significantly abrogated PGE\(_2\)-induced Fn formation (Fig. 3C). The quantitative data are shown in the lower panels. Flow cytometry was used to investigate the effect of PGE\(_2\) on the change of intracellular Ca\(^{2+}\) concentration. As shown in Fig. 3D, incubation with PGE\(_2\) (3 \(\mu\)M), 17-phenyl trinor PGE\(_2\) (3 \(\mu\)M) and sulprostone (20 \(\mu\)M) enhanced the fluorescence intensity of fluo-3. However, sulprostone at 10 \(\mu\)M only slightly increased the intracellular Ca\(^{2+}\) concentration.
ELISA detection also showed that pretreatment of osteoblasts with the EP\(_1\) AS-ODN, SC19220 and BATA-AM but not AS-ODN of EP\(_2\)-EP\(_4\) or any MM-ODN antagonized the potentiating effect of PGE\(_2\) (Fig. 3E).

The signaling pathways of PI-PLC, PKC and c-Src are involved in the potentiating action of PGE\(_2\)

To study the intracellular signaling pathway involved in PGE\(_2\)-induced Fn expression, osteoblasts were pretreated for 30 min with PI-PLC inhibitor, U73122 (1 and 3 \(\mu\)M). It was found that U73122 but not inactive analogue of U73122, U73343 (30 \(\mu\)M) or PC-PLC inhibitor D609 (30 \(\mu\)M) antagonized the potentiating effect of PGE\(_2\). Furthermore, U73122, U73343 and D609 had no effect on the basal level of Fn expression (Fig. 4). The quantitative data are shown in the lower panels. Because PGE\(_2\)-induced Fn expression was inhibited by U73122, indicating the involvement of PI-PLC pathway, which increases diacylglycerol levels leading to the activation of PKC. The PKC inhibitor GF109203X was thus used to examine whether PKC is involved in the action of PGE\(_2\). Pretreatment with GF109203X (1-10 \(\mu\)M) concentration-dependently inhibited the enhancement effect of PGE\(_2\) (Fig. 5A). PKC isozymes, including \(\alpha\), \(\beta\), \(\varepsilon\) and \(\delta\), have been identified in osteoblasts (27). To examine which PKC isoforms are involved in the potentiating of Fn fibrillogenesis by PGE\(_2\), isoform-specific AS-ODN was used (23). It was demonstrated that treatment with AS-ODN of PKC isoform \(\alpha\) but not \(\beta\), \(\varepsilon\) and \(\delta\) antagonized the potentiating action of PGE\(_2\) using ELISA analysis (Fig. 5B). We also directly measured the PKC\(\alpha\) translocation in response to PGE\(_2\). Incubation of osteoblasts with PGE\(_2\) (3 \(\mu\)M) for 10 or 15 min increased membrane translocation of PKC\(\alpha\). Pretreatment of osteoblasts for 30 min with SC19220 (10 \(\mu\)M) or U73122 (3 \(\mu\)M) markedly attenuated the PGE\(_2\)-induced PKC\(\alpha\) translocation (Fig. 5C). We then investigated the role of Src in mediating PGE\(_2\)-induced Fn expression using the specific Src inhibitor PP2. As shown in Fig. 6A, PGE\(_2\)-induced Fn expression was markedly attenuated by pretreatment of cells for 30 min with PP2 (1-10 \(\mu\)M) in a concentration-dependent manner. To directly confirm the crucial role of Src in Fn expression, we measured the level of Src phosphorylation in response to PGE\(_2\). As shown in Fig. 6B, treatment of osteoblasts with PGE\(_2\) (3 \(\mu\)M) for 15 min increased c-Src activity, as assessed by immunoblotting samples for phosphotyrosine immunoprecipitated from lysates using c-Src (Fig. 6B). To determine the relationship among EP\(_1\) receptor, PLC, PKC and Src in the PGE\(_2\)-mediated signaling pathway, we found that pretreatment of cells for 30 min with SC19220 (10 \(\mu\)M), U73122 (3 \(\mu\)M), GF109203X (10 \(\mu\)M) and PP2 (10 \(\mu\)M) markedly inhibited the PGE\(_2\)-induced c-Src activity (Fig. 6B). ELISA measurements also showed that pretreatment of osteoblasts with the U73122 (3 \(\mu\)M), GF109203X (10 \(\mu\)M) and PP2 (10 \(\mu\)M) but not U73343 (30 \(\mu\)M) or D609 (30 \(\mu\)M) antagonized the Fn up-regulation effect of PGE\(_2\) (Fig. 6C). Based on these results, it appears that PGE\(_2\) acts through EP\(_1\) receptor, PLC, PKC and c-Src-dependent signaling pathway to enhance Fn fibrillogenesis in osteoblasts.
Effect of PGE\textsubscript{2} on the Distribution of Integrin

The assembly of extracellular Fn matrix underneath the cells may be related to integrins (9). Integrins are a family of heterodimeric transmembrane receptors containing \(\alpha\) and \(\beta\) subunits. The different combination of \(\alpha\) and \(\beta\) chains forms different receptors for various kinds of ECM molecules. \(\alpha\)5\(\beta\)1 integrin is a specific receptor for Fn. Flow cytometry was used to investigate the effect of PGE\textsubscript{2} on the cell surface expression of integrins. As shown in Fig. 7A, incubation with PGE\textsubscript{2} (3 \(\mu\)M) for 24 h significantly enhanced the fluorescence intensity of \(\alpha\)5 and \(\beta\)1 integrins. The increase of cell surface expression of integrins by PGE\textsubscript{2} was antagonized by SC19220 (10 \(\mu\)M), U73122 (3 \(\mu\)M), GF109203X (10 \(\mu\)M) and PP2 (10 \(\mu\)M) (Fig. 8A). In cotransfection experiments, the increase of Fn promoter activity by PGE\textsubscript{2} was inhibited by EP\textsubscript{1} AS-ODN, but not by AS-ODN of EP\textsubscript{2}-EP\textsubscript{4} (Fig. 8B). Increase of Fn promoter activity by PGE\textsubscript{2} was also inhibited by the dominant negative mutants of PKC\(\alpha\) and c-Src (Fig. 8C). Taken together, these data suggest that the activation of EP\textsubscript{1}/PI-PLC/PKC\(\alpha\)/c-Src pathway is required for the increase of Fn by PGE\textsubscript{2} in rat osteoblasts.

PGE\textsubscript{2} 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. PGE\textsubscript{2} and 17-phenyl trinor PGE\textsubscript{2} (30 \(\mu\)M, 10 \(\mu\)l, once per day) was locally administered into tibia for 7 consecutive days via an implantation of a needle cannula (22 gauge) in young rats weighing 73 to 88 g 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), PGE\textsubscript{2} and 17-phenyl trinor PGE\textsubscript{2} significantly increased bone volume of the secondary spongiosa (Fig. 9A). Trabecular bone in the secondary spongiosa increased by 91.3 and 81.7 \% after local administration of PGE\textsubscript{2} and 17-phenyl trinor PGE\textsubscript{2}. The immunohistochemistry also showed that Fn and \(\alpha\)5\(\beta\)1 integrin predominantly localized around trabecular bone.
Long-term administration of PGE$_2$ and 17-phenyl trinor PGE$_2$ increased the staining of Fn, $\alpha_5\beta_1$ integrin as well as type I collagen (Fig. 9B, C, D). In addition, BMD and BMC increased after application of PGE$_2$ and 17-phenyl trinor PGE$_2$ (Table 1).

**DISCUSSION**

PGEs are among the most potent regulators of bone cell function (28). It is generally accepted that prostaglandins are mediators in bone metabolism. Among various prostaglandins, PGE$_2$ is the most important in bone formation and resorption. In a recent study, Mo et al (29) demonstrated that PGE$_2$ treatment increases trabecular bone mass in rats. Extensive studies have demonstrated that PGE$_2$ has both anabolic and catabolic effects on osteoblasts. The results from this study provide evidence that PGE$_2$ also regulates Fn fibrillogenesis in cultured rat osteoblasts. In the present study, immunocytochemistry, ELISA and Western blotting analysis were used to investigate the effect of PGE$_2$ on Fn assembly. The Fn network is an important factor for the differentiation, expression of physiological function, as well as survival of osteoblasts (30). Here we further identify Fn as a target protein for PGE$_2$ signaling pathway that regulates cell survival and differentiation. We also show that potentiation of Fn fibrilogenesis by PGE$_2$ requires an activation of EP$_1$ receptor, PI-PLC, PKC$_\alpha$ and c-Src signaling pathway.

PGE$_2$ stimulated Fn fibrillogenesis in a concentration-dependent manner detected by immunocytochemistry and ELISA. Furthermore, PGE$_2$ increased the protein levels of Fn as demonstrated by Western blotting analysis. PGEs, acting through different cell surface receptors on osteoblastic cells, stimulate bone remodeling by promoting both anabolic and catabolic responses, the relative responses being dependent on the target cell population and the concentration of PGE$_2$. However, we demonstrate that the EP$_1$ but not other EP receptors was required for PGE$_2$-induced Fn formation. Treatment with butaprost (EP$_2$ agonist), sulprostone (EP$_3$ agonist) and 11-deoxy PGE$_1$ (EP$_2$/EP$_4$ selective agonist) failed to up-regulate Fn expression (Fig. 3A). Furthermore, we could not inhibit PGE$_2$-induced Fn up-regulation by EP$_2$, EP$_3$ and EP$_4$ receptor-specific antisense oligonucleotides (Fig. 2B). It has been reported that sulprostone also acts on the rat EP$_1$ receptor (26). Here we found that sulprostone did not increase Fn expression unless at a high concentration of 20 $\mu$M. Pretreatment of osteoblasts with EP$_1$ AS-ODN but not EP$_3$ AS-ODN antagonized the increase of Fn by 20 $\mu$M sulprostone. These results indicate that sulprostone also activates EP$_1$ receptor at higher concentrations in osteoblasts, which is consistent with the result of VEGF-C expression in lung cells (31). EP$_1$ receptor antagonist significantly suppressed PGE$_2$-induced Fn formation, suggesting that EP$_1$ receptor-dependent pathway is involved in Fn up-regulation by PGE$_2$. EP$_1$ receptor is coupled to $Ca^{2+}$ mobilization (15) and the intracellular free calcium chelator (BAPTA/AM) antagonized the up-regulation of Fn by PGE$_2$. In addition, PGE$_2$ and 17-phenyl trinor PGE$_2$ also increase fluorescence intensity of fluo-3. The increase of [$Ca^{2+}$], may be attributable to the activation of PGE$_2$ through EP$_1$ receptor.

Several isoforms of PKC exist in primary
cultured osteoblasts, including α, β, ε, and δ (27). Treatment with antisense oligonucleotides directed against PKC α isoform but not PKC β, ε and δ antagonized the potentiating action of PGE₂ in Fn expression, indicating that α isoform is much more important to mediate the action of PGE₂ in osteoblasts. We demonstrated that the PKC inhibitors GF109203X antagonized the PGE₂-mediated potentiation of Fn expression in a dose-dependent manner, suggesting that PKC activation is an obligatory event in PGE₂-induced Fn expression in these cells. This was further confirmed by the result that the dominant negative mutant of PKCα inhibited the enhancement of Fn promoter activity by PGE₂. PKC is activated by the physiological activator, diacylglycerol, which can be generated either directly, by the action of PLC, or indirectly, by a pathway involving the production of phosphatidic acid by PLD, followed by a dephosphorylation reaction catalyzed by phosphatidate phosphohydrolase. The PLC involved in the production of diacylglycerol is PI-PLC, or PC-PLC (32, 33). The PI-PLC inhibitor, U73122, inhibited PGE₂-induced Fn expression, whereas, the PC-PLC inhibitor D609 and the inactive U73122 analogue, U73343, did not affect the action of PGE₂.

The cytoplasmic protein tyrosine kinase c-Src was found to be activated by PGE₂ in osteoblastic cells (34). These effects were inhibited by GF109203X, indicating the involvement of PKC-dependent c-Src activation in PGE₂-mediated Fn induction. In addition to gene expression, a similar signal pathway has also been reported in the development of ischemic preconditioning in the conscious rabbit, which involved PKCε-dependent Src and Lck activation (35), in the G protein-coupled receptors regulating N-methyl-D-aspartic acid receptor in CA1 pyramidal neurons, which involved PKC-dependent c-Src activation (36), and in the cellular response to oxidative stress, which involved PKCδ-dependent c-Abl activation (37). Taken together, our results provided evidence that PGE₂ up-regulates Fn in rat osteoblasts via the EP₁/PI-PLC/PKCα/c-Src signaling pathway.

Direct osteoblast interactions with the extracellular matrix are mediated by a selective group of integrin receptors including α5β1, α3β1, αvβ3 and α4β1 (38, 39). α5β1 integrin, a specific Fn receptor, mediates critical interactions between osteoblasts and Fn required for both bone morphogenesis and osteoblast differentiation (19). Interfering with interactions between Fn and integrin Fn receptors in immature fetal rat calvarial osteoblasts suppressed formation of mineralized nodules in vitro and delayed expression of tissue-specific genes, including osteocalcin (19). The finding that enhancement of surface expression of α5 and β1 integrins by PGE₂ correlated to the increase of Fn assembly by PGE₂. Increase of the surface expression of α5 and β1 integrin by PGE₂ was also antagonized by SC19220, U73122, GF109203X and PP2, suggesting that the regulation of α5 and β1 integrin expression is parallel to the increase of Fn assembly.

PGEs are considered important local factors that modulate bone metabolism through their effects on osteoblastic cells and osteoclasts...
The skeleton is an important target tissue for PGE$_2$, which is involved in bone development, growth, remodeling, and repair (40). Using local injection of PGE$_2$ and 17-phenyl trinor PGE$_2$ into tibia for 7 consecutive days, we have demonstrated that local administration of PGE$_2$ and 17-phenyl trinor PGE$_2$ increased the bone volume and immunostaining of Fn, $\alpha_5\beta_1$ integrin as well as type I collagen in young rats. The present results suggest that PGE$_2$ plays an important role in the developing bone as well. The increase of bone formation may be also partially mediated by the increase of proliferation and survival of osteoblasts, since PGE$_2$ also increased differentiation marker of bone sialoprotein (41). Local injection of PGE$_2$ and 17-phenyl trinor PGE$_2$ also increased BMD and BMC in young rats, indicating that PGE$_2$ plays an important role in the regulation of bone formation via EP$_1$ receptor. We injected high concentration of drugs in small volume in the in vivo studies. Therefore, the action of EP$_1$ agonist on the other EP receptors can not be excluded.

In conclusion, the signaling pathway involved in PGE$_2$-induced Fn expression in rat osteoblasts has been explored. PGE$_2$ increases $\alpha_5$, $\beta_1$ integrins and Fn expression by binding to EP$_1$ receptor, activation of phospholipase C, PKCa and c-Src. Local administration of PGE$_2$ and EP$_1$ agonist increases Fn and promotes bone formation in rat.

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FOOTNOTES
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Abbreviations: ECM, extracellular matrix; PGE, prostaglandins; Fn, fibronectin; AS, Antisense; MM, missense; ODN, oligonucleotide; BMD, bone mineral density; BMC, bone mineral content; PI-PLC, phosphatidylinositol-phospholipase C; PKC, protein kinase C. enzyme-linked immunosorbent assay, ELISA.

FIGURE LEGENDS
Fig 1. Increase of Fn fibrillogenesis by PGE2 in cultured rat osteoblasts. Fn network, which was shown by immunofluorescence, formed underneath the cultured osteoblasts. Compared with control (A), treatment with PGE2 (3 μM) for 24 hr increased Fn fibrillogenesis in cultured osteoblasts (B). Phase-contrast images are shown in the left panels. Bar=10 μm. The mean fluorescence under 10–15 cells was measured using a Zeiss confocal microscope. Note that treatment with PGE2 (0.3-10 μM) for 24 hr increased Fn fibrillogenesis. The quantitative data are shown in (C) (n=18-25). Expression of extracellular Fn was also measured by ELISA. Treatment with PGE2 (0.3-10 μM) for 24 hr increased Fn expression in a concentration-dependent manner (D). Osteoblast cultures were treated with different concentrations of PGE2 for 24 h. The cultures were then washed with 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, PGE2 (3 μM) increased the protein levels of Fn in a concentration- (E) and time (F)-dependent manner. Data are presented as mean ± SE. *, p<0.05 as compared with control (n=3).

Fig 2. Up-regulation of Fn expression by PGE2 acting through EP1 receptor in primary rat osteoblast. Total RNA was extracted from primary rat osteoblastic cells, and subjected to RT-PCR for EP1, EP2, EP3, and EP4 mRNAs using the respective primers. Note that primary rat osteoblasts express EP1-EP4 receptor mRNA and EP1 mRNA increased in response to
PGE$_2$ (3 μM) application for 6 hr (A). Osteoblasts were transfected with EP receptor antisense oligonucleotides (AS-ODN) or missense oligonucleotides (MM-ODN) for 24 hr followed by incubation with PGE$_2$ for 6 hr and 24 hr to analyze the mRNA and protein expression, respectively. Total protein and RNA were isolated and the expression of Fn and EP receptors were analyzed by Western blotting (WB) and RT-PCR (RT) (B). Results are representative of at least three independent experiments.

Fig 3. EP$_1$ and Ca$^{2+}$ are involved in PGE$_2$-mediated increase of Fn expression.

(A) Osteoblast cultures were treated with PGE$_2$ (3 μM), 17-phenyl trinor PGE$_2$ (3 μM), butaprost (10 μM), sulprostone (10 μM), 11-deoxy PGE$_1$ (10 μM), and SC 19220 (10 μM) for 24 h. Cells were lysed for the immunoblotting of Fn or α-tubulin. (B) Cells were transfected with antisense oligonucleotides (AS-ODN) for 24 hr followed by incubation with sulprostone for 24 hr to analyze the protein level of Fn by Western blotting. Note that sulprostone did not increase Fn expression unless at a higher concentration of 20 μM, which is inhibited by EP$_1$ AS-ODN. (C) Cells were pretreated for 30 min with intracellular free calcium chelator, BAPTA/AM (0.1-10 μM) and then stimulated with PGE$_2$ (3 μM) for 24hr. Cells were then lysed and the protein samples were obtained for Western blotting analysis. The quantitative data were shown in the lower panels (n=3). (D) Cells were detached and labeled with Fluo-3-AM and then the change of [Ca$^{2+}$]$_i$ was analysed on flow cytometer. The arrow indicates the point at which drugs were applied to the cells. The data points represent the means ± S.E.M of at least three independent experiments. (E) Treatment with PGE$_2$ (3 μM), 17-phenyl trinor PGE$_2$ (0.3-3 μM) increased extracellular Fn expression. Osteoblasts were then transfected with antisense oligonucleotides (AS-ODN), missense oligonucleotides (MM-ODN) for 24 hr or treated with SC19220 (10 μM) or BATA-AM (10 μM) for 30 min followed by incubation with PGE$_2$ for 24 hr to analyze the extracellular Fn by ELISA. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *: p<0.05 as compared with control. #: p < 0.05 as compared with PGE$_2$-treated group.

Fig 4. Involvement of PI-PLC in the potentiating action of PGE$_2$ on Fn expression.

Osteoblasts were pretreated with U73122 (1, 3 μM), U73343 (30 μM) and D609 (μM) for 30 min followed by stimulation with PGE$_2$ for 24 hr, and Fn expression was determined by immunoblotting with an antibody specific for Fn. The lower panel shows the results of three independent experiments (mean ± S.E.M.). *: p<0.05 as compared with control. #: p < 0.05 as compared with PGE$_2$-treated group.

Fig 5. PKCα isoform is involved in the potentiation of Fn expression by PGE$_2$.

(A) Osteoblasts were pretreated with PKC inhibitor GF109203X (1-10 μM) for 30 min
followed by stimulation with PGE$_2$ for 24 hr, and Fn expression was determined by immuno blotting with an antibody specific for Fn. The quantitative data are shown in the lower panel (B). Osteoblasts were transfected with AS-ODN directed against different isoforms of PKC for 48 hr followed by incubation with PGE$_2$ for 24 hr and then subjected to the analysis of the extracellular Fn by ELISA. (C) Treatment of osteoblasts cells with PGE$_2$ (3 µM) for 10 or 15 min decreased cytosolic and increased membrane translocation of PKCa. Cells were incubated with SC19220 (10 µM) or U73122 (3 µM) for 30 min followed by stimulation with PGE$_2$ for 15 min, and cell lysates were then immunoblotted with an antibody specific for PKCa. Results are expressed as the mean ± S.E.M. of three independent experiments. *: p<0.05 as compared with control. #: p < 0.05 as compared with PGE$_2$-treated group.

Fig 6. c-Src is involved in PGE$_2$-induced Fn expression.
(A) Osteoblasts were pretreated with Src inhibitor PP2 (1-10 µM) for 30 min followed by stimulation with PGE$_2$ for 24 hr, and Fn expression was determined by immuno blotting with an antibody specific for Fn. The quantitative data are shown in the lower panel. (B) Osteoblasts were incubated with SC19220 (10 µM), U73122 (3 µM), GF109203X (10 µM) and PP2 (10 µM) for 30 min followed by stimulation with PGE$_2$ for 15 min, and cell lysates were then immunoprecipitated (IP) with an antibody specific for c-Src. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted (WB) with anti-phosphotyrosine (PY) (C). Osteoblasts were preincubated with SC19220 (10 µM), U73122 (3 µM), GF109203X (10 µM) and PP2 (10 µM) for 30 min followed by incubation with PGE$_2$ for 24 hr to analyze the extracellular Fn by ELISA. Results are expressed as the mean ± S.E.M. of three independent experiments. *: p<0.05 as compared with control. #: p < 0.05 as compared with PGE$_2$-treated group.

Fig 7. Increase of the cell surface expression of α5 and β1 integrins by PGE$_2$.
Compared with control, treatment with PGE$_2$ (3 µM) for 24 hr significantly enhanced the fluorescence intensity of α5 and β1 integrins using flow cytometric analysis (A). Osteoblasts were pretreated with SC19220 (10 µM), U73122 (3 µM), GF109203X (10 µM) and PP2 (10 µM) for 30 min followed by incubation with PGE$_2$ for 24 hr and the cell surface expression of integrins was analyzed by flow cytometry. The quantitative data are shown in (B). Data are presented as mean ± S.E.M. (n=4). Osteoblasts were transfected with EP receptor AS-ODN (C) for 24 hr or treated with U73122 (3 µM), GF109203X (10 µM) and PP2 (10 µM) (D) for 30 min followed by incubation with PGE$_2$ for 6 hr and the mRNA for α5 and β1 integrins were analyzed by RT-PCR. Results are representative of at least three independent experiments. *: p < 0.05 as compared with control. #: p < 0.05 as compared with PGE$_2$-treated group.
Fig 8. PI-PLC-PKC-c-Src signaling pathway mediated the increase of Fn promoter activity by PGE$_2$.

The Fn promoter activity was evaluated by transfection with the pGL2F1900 luciferase expression vector as described in “Experimental procedures”. Osteoblasts were pretreated with vehicle, SC19220 (10 μM), U73122 (3 μM), GF109203X (10 μM) and PP2 (10 μM) for 30 min before incubation for 24 hr with PGE$_2$ (3 μM) (A). Cells were co-transfected with pGL2F1900 and AS-ODN of EP$_1$-EP$_4$ (B), or the PKCα, c-Src mutant, or the respective empty vector (C), and then treated for 24 hr with PGE$_2$ (3 μM). Luciferase activity was then measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E.M for three independent experiments performed in triplicate. *: p<0.05 as compared with control. #: p < 0.05 as compared with PGE$_2$-treated group.

Fig 9. PGE$_2$ increased bone volume and immuostaining of Fn, α5β1 integrin and type I collagen in tibia metaphysis of rats.

PGE$_2$ or 17-phenyl trinor PGE$_2$ (30 μM, 10 μl, once/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, chronic treatment with PGE$_2$ or 17-phenyl trinor PGE$_2$ markedly increased bone volume (A). Immunostaining showed that Fn predominantly localized around the trabecular bone (arrowhead) and PGE$_2$ or 17-phenyl trinor PGE$_2$ increased the staining of Fn (B), α5β1 integrin (C) and type I collagen (D). Bar = 0.5 mm (A) and 100 μm (B,C,D).
Figure 1
Figure 2

A

| PGE2 | - | + |
|------|---|---|
| EP-1 (336 bp) | | |
| EP-2 (369 bp) | | |
| EP-3 (537 bp) | | |
| EP-4 (423 bp) | | |
| GAPDH (452bp) | | |

B

|          | - | + | + |
|----------|---|---|---|
| PGE2     | - | + | + |
| EP1-AS-ODN | - | - | - |
| EP1-MM-ODN | - | - | + |
| GAPDH    | RT |
| EP-1     | RT |
| EP-2     | RT |
| Fn       | WB |
| α-tubulin | WB |
|          | - | + | + |
| PGE2     | - | + | + |
| EP3-AS-ODN | - | - | - |
| EP3-MM-ODN | - | - | + |
| GAPDH    | RT |
| EP-3     | RT |
| EP-4     | RT |
| Fn       | WB |
| α-tubulin | WB |

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Figure 3

A

B

C

D

E
Figure 4

[Image of a Western blot experiment showing protein expression levels of Fn and α-tubulin under different conditions: Control (Con), PGE2, UT3122 1 μM, UT3122 3 μM, UT3343, D609, and UT3122, UT3343, D609. The graph below the blot shows the percentage of control for each condition, with asterisks (*) indicating significant differences from control.]

% of control

- Control
- PGE2
- UT3122 1 μM
- UT3122 3 μM
- UT3343
- D609

Bars are labeled with asterisks (*) and a hash (#) to indicate significance.
Figure 6

A

PGE2

| 0  | 1  | 3  | 10 | 10 (µM) |
|----|----|----|----|---------|
| PP2 Control | PGE2 | | | |
| Fn | | | | |
| α-tubulin | | | | |

% of control

B

IP: c-Src

PGE2

Con PGE2 SC19220 UT1220 GF109203X PP2 SC19220 UT1220 GF109203X PP2

WB:PY

c-Src-P

WB:c-Src

c-Src

C

Fibronectin (% of control)

Control PGE2 U73122 U73343 D699 GF109203X PP2

PGE2
Figure 7

A) α5 Integrin

B) β1 Integrin

C) PGE2

D) α5

β1

GAPDH
Figure 8

(A) SC19220
(B) U73122
(C) GF109203X
(D) PP2

Fibronectin luciferase activity (% of control)

Control
PGE2
AS-EP1
AS-EP2
AS-EP3
AS-EP4

* PGE2
# SC19220

Vector
PKCα
c-Src

* PGE2
# SC19220

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Figure 9
Table 1. Effect of PGE$_2$ and 17-phenyl trinor PGE$_2$ on the bone mineral density, bone mineral content and bone volume in tibia

|                  | BMD (g/cm$^2$) | BMC (g)    | BV/TV (%) |
|------------------|----------------|------------|-----------|
| Control          | 0.092±0.003    | 0.083±0.003| 9.3±1.2   |
| PGE$_2$          | 0.105±0.005$^a$| 0.116±0.009$^a$| 17.8±2.2$^a$|
| 17-phenyl trinor PGE$_2$ | 0.103±0.003$^a$| 0.111±0.005$^a$| 16.9±1.9$^a$|

PGE$_2$ and 17-phenyl trinor PGE$_2$ (30 μM, 10 μl, once/day) 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 7 days after the last injection.

BMD, bone mineral density; BMC, bone mineral content; BV/TV, bone volume/tissue volume.

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

n=9-11
Prostaglandin E2 stimulates fibronectin expression through EP1 receptor, phospholipase C, protein kinase C α and c-Src pathway in primary cultured rat osteoblasts
Chih-Hsin Tang, Rong-Sen Yang and Wen-Mei Fu

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