THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 19, pp. 17133–17143, May 13, 2011

N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) Promotes Osteoblast Differentiation via the *N*-Formyl Peptide Receptor 1-mediated Signaling Pathway in Human Mesenchymal Stem Cells from Bone Marrow*[S]

Received for publication, October 25, 2010, and in revised form, February 7, 2011 Published, JBC Papers in Press, March 3, 2011, DOI 10.1074/jbc.M110.197772

Min Kyoung Shin[‡1], Young Hoon Jang[‡1], Hyun Jung Yoo[‡1], Dong Woo Kang[‡], Mi Hee Park[‡], Mi Kyoung Kim[‡], Ju Hyun Song[‡], Sang Doo Kim[§], Gyesik Min[¶], Hyung Keun You[‖], Kang-Yell Choi[**], Yoe-Sik Bae[§], and Do Sik Min[‡2]**

*From the [‡]Department of Molecular Biology, College of Natural Science, Pusan National University, 30 Jangjeon Dong, Geumjeong gu, Busan 609-735, the [§]Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, the [¶]Department of Pharmaceutical Engineering, Gyeongnam National University of Science and Technology, Jinju 660-758, the [‖]Department of Periodontology, College of Dentistry, Wonkwang University, Iksan 570-740, and the **Translational Research Center for Protein Function Control and Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seodaemun-gu, Seoul 120-749, Korea*

Binding of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) to its specific cell surface receptor, *N*-formyl peptide receptor (FPR), triggers different cascades of biochemical events, eventually leading to cellular activation. However, the physiological role of fMLP and FPR during differentiation of mesenchymal stem cells is unknown. In this study, we attempted to determine whether fMLP regulates differentiation of mesenchymal stem cells derived from bone marrow. Analysis by quantitative-PCR and flow cytometry showed significantly increased expression of FPR1, but not FPR2 and FPR3, during osteoblastic differentiation. fMLP, a specific ligand of FPR1, promotes osteoblastic commitment and suppresses adipogenic commitment under differentiation conditions. Remarkably, fMLP-stimulated osteogenesis is associated with increased expression of osteogenic markers and mineralization, which were blocked by cyclosporine H, a selective FPR1 antagonist. In addition, fMLP inhibited expression of peroxisome proliferator-activated receptor-γ1, a major regulator of adipocytic differentiation. fMLP-stimulated osteogenic differentiation was mediated via FPR1-phospholipase C/phospholipase D-Ca^{2+}-calmodulin-dependent kinase II-ERK-CREB signaling pathways. Finally, fMLP promoted bone formation in zebrafish and rabbits, suggesting its physiological relevance *in vivo*. Collectively, our findings provide novel insight into the functional role of fMLP in bone biology, with important implications for its potential use as a therapeutic agent for treatment of bone-related disorders.

Mesenchymal stem cells (MSCs)[3] derived from bone marrow have the potential for differentiation into several different cell types, including osteoblasts, chondrocytes, adipocytes, and myocytes in response to stimulation by multiple environmental factors (1–6). Regulation of stem cell fate down these various lineages is essential for tissue development, homeostasis, and repair (1, 6). Recent studies have revealed critical transcription factors involved in the commitment of different MSC-derived lineages (7). For example, osteoblastic differentiation is controlled by Runx2, whereas PPAR-γ is involved in adipose commitment.

Continuous remodeling of bone occurs throughout life by a tightly coupled process involving absorption by osteoclasts and formation of osteoblasts. Dysregulation of this coupled remodeling leads to diseases such as osteoporosis (8, 9). Osteoporosis is a degenerative disease of the skeleton that occurs due to alteration of bone turnover homeostasis and is characterized by fragile bones and increased susceptibility to bone fractures (10). Decreased bone synthesis because of reduced osteoblastic activity, which occurs in parallel with increased adipocyte formation, is a mechanism that can lead to this degenerative disorder (11). MSCs can be derived from bone marrow, manipulated in culture, and administered back to donor individuals; therefore, MSCs are of great promise in regeneration medicine, providing potential tools for use in therapeutic intervention in diseases related to impaired function of osteoblasts (12–14). However, the mechanistic pathways that drive differentiation of MSCs along the osteoblast lineage are not completely understood. Therefore, elucidation of molecular mechanisms underlying osteogenesis is not only important to our understanding

* This work was supported by National Research Foundation Grant KRF-2008-313-C00698 funded by the Korean Government, Translational Research Center for Protein Function Control Grant NSF 2009-0092960, South Korea, and by Financial Supporting Project of Long Term Overseas Dispatch of the PNU Tenure-track Faculty, 2009.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 82-51-510-3682; Fax: 82-51-513-9258; E-mail: minds@pusan.ac.kr.

[3] The abbreviations used are as follows: MSC, mesenchymal stem cells; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FPR, *N*-formyl peptide receptor; ALP, alkaline phosphatase; PLC, phospholipase C; PLD, phospholipase D; CysH, cyclosporine H; PPAR-γ, peroxisome proliferator-activated receptor-γ; CREB, cAMP-response element-binding; Q-RT-PCR, quantitative RT-PCR; PTX, pertussis toxin; OM, osteogenic medium; h, human; MFI, mean fluorescence intensity; F, forward; R, reverse; hpf, h postfertilization; dpf, days postfertilization; GM, growth medium; SRE, serum-response element; CRE, cAMP-response element; ADL, apodize dark light; LWD, long working distance.

of bone development, but it may also advance strategies for bone repair. Targeting of therapeutic molecules to bone for enhancement of bone forming activity of osteoblast precursors may aid in treatment of bone disease.

For the proper action of MSCs in differentiation into a certain type of cell in a specific location, MSCs should migrate to the site of injury. Recruitment of MSCs to damaged tissues is a promising approach to *in situ* tissue regeneration. Formyl peptide receptor (FPR), a chemoattractant receptor belonging to the G protein-coupled receptor family (15), binds to *N*-formyl peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), which is derived either from bacterial proteins (16) or from endogenous mitochondrial proteins, and is released as a result of cell death or severe dysfunction (17). Expression of FPR in phagocytic leukocytes and nonphagocytic cells, such as fibroblasts, has been reported (18). Activation of FPR receptor-mediated signal transduction pathways is known to be responsible for various biochemical responses that contribute to the physiological defense against bacterial infection and cell disruption in the regulation of myeloid cell lineage, such as neutrophils (19). We (20) and Viswanathan *et al.* (21) have recently reported on the expression of functional FPR in human bone marrow-derived MSCs, which has been suggested as playing potential roles in mediation of adhesion and migration of stem cells to sites of injury for tissue repair. However, it is unknown whether FPR1 and its ligand, fMLP, regulate differentiation of MSCs. Thus, this study was undertaken in an effort to determine whether fMLP influences human MSC commitment along osteoblastic and adipocytic lineages.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified essential medium (DMEM), fetal bovine serum, trypsin-EDTA, and antibiotic/antimycotic solution were purchased from Invitrogen. Anti-FPR1, anti-FPR2, and anti-FPR3 mouse monoclonal antibodies and phycoerythrin-conjugated anti-mouse IgG were purchased from R&D Systems, Inc. (Minneapolis, MN). Isotype mouse IgG was used as a negative control antibody. Anti-PLCβ1 antibody was purchased from Santa Cruz Biotechnology. Previously described procedures were used for generation of the polyclonal anti-PLD antibody that recognizes both PLD1 and PLD2 (22). The secondary antibody was FITC-conjugated goat anti-mouse IgG (BD Biosciences). fMLP, cyclosporine H (CysH), and 5-fluoro-2-indolyl des-clorohalopemide hydrochloride hydrate were purchased from Sigma. Pertussis toxin (PTX), U73122, U73343, KN-92, KN-93, and U0126 were purchased from Calbiochem. A 21-nucleotide sequence siRNA corresponding to the human FPR1 sequence (5′-AGAAAUUGGUAUUGCAGUGUU-3′), PLCβ1 (5′-GGAGACGGAGCUGUUAGAU-3′), and PLD1 (5′-AAGGUGGGACGACAAUGAGCA-3′) were purchased from Dharmacon Research (Lafayette, CO). Antibodies to CREB, phospho-CREB, ERK, or phospho-ERK were purchased from Cell Signaling, Inc. (Beverly, MA). Horseradish peroxidase-conjugated antibodies to rabbit IgG were purchased from Kirkegaard & Perry, Inc. (Gaithersburg, MD).

Culture of hMSCs—Human (h) MSCs were purchased from Cambrex BioScience (Walkersville, MD) and cultured in Dulbecco's modified Eagle's media (low glucose) containing 20% fetal bovine serum (FBS) (Invitrogen). hMSCs were used for the experiments until six passages. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

Osteoblastic Differentiation Assay— hMSCs were cultured to confluence in the growth medium (20% FBS in DMEM). Osteoblastic differentiation was induced by exposure of confluent hMSCs to osteogenic medium (10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerolphosphate, and 50 μM ascorbic acid in α-minimum essential medium), and extracellular matrix calcification was visualized by Alizarin Red-S staining. Briefly, cells were washed twice with PBS and fixed with 95% ethanol at 4 °C for 30 min. Fixed cells were incubated with 4% Alizarin Red-S (Sigma) for 15 min with shaking. To minimize nonspecific staining, cells were rinsed five times with deionized water and once with PBS for 20 min. Osteoblastic differentiation was quantified by measurement of the area of Alizarin Red staining and photographed using a digital camera equipped with Nikon TS100 inverted microcopy (Nikon, Japan) using 10×/0.25 ph1 ADL or LWD 40×/0.55 ph1 ADL and density using Scion imaging software (Scion Corp., Frederick, MD). Mineralization, an osteogenic marker, was also evaluated by calcein staining for indication of osteoblastic differentiation. For assays, the culture was supplemented with 1 μg/ml of calcein AM (Sigma C1359) during the observation period. An image analyzer (Axiovert 200 M, Zeiss, Germany) with EC PLAN-NEOFLUAR 10×/0.3 ph1 and AxioVision designed especially for fluorescence microscopy was used for visualization of fluorescence of incorporated calcein.

Adipogenic Differentiation Assay—MSCs were cultured to confluence in the growth medium and then treated with adipogenic differentiation medium (10% FBS, 0.1 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, 10 μg/ml insulin, and 200 μM, indomethacin). As described previously, accumulation of intracellular triglyceride droplets was visualized by Oil Red O staining (23), and phase contrast images were photographed using a digital camera equipped with Nikon inverted microcopy (Nikon, Japan) using 10×/0.25 ph1 ADL or LWD 40×/0.55 ph1 ADL. The number of cells committed to the adipogenic lineage was quantified by counting the number of cells having numerous cytoplasmic lipid-filled vacuoles in three randomly selected visual fields in the same well.

Flow Cytometric Analysis— hMSCs undifferentiated or differentiated into osteoblasts for 12 days were incubated with monoclonal antibody against FPR1, FPR2, or FPR3 for 30 min at 4 °C, followed by washing with FACS buffer (PBS containing 5% FBS, 0.1% sodium azide), and then incubated with FITC-conjugated anti-mouse IgG for 20 min at 4 °C. Samples were acquired on an FACSCalibur™ flow cytometer, and CellQuest Pro software (BD Biosciences) was used for data analysis.

Western Blot Analysis—Cells were lysed with lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 200 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethylsulfonyl fluoride) and analyzed by immunoblot. Enhanced chemiluminescence was used for detection of immunoreactive bands (Amersham Biosciences).

Phospholipase C Activity Assay—MSCs were seeded into 35-mm dishes at 2×10^5 cells per dish and grown for 1 day.

Cells were labeled with *myo*-[2-^{3}H]inositol (3 μCi/ml) in inositol-free DMEM for 24 h. Labeled cells were subsequently washed and pretreated with 20 mM LiCl for 15 min in DMEM containing 20 mM Hepes, pH 7.2, and 1 mg/ml bovine serum albumin. Stimulation was initiated by addition of fMLP and terminated by addition of ice-cold 5% $HClO_4$. Following incubation of the reaction mixture for 30 min in an ice bath, cell extracts were centrifuged, diluted with distilled water, and applied to a Bio-Rad Dowex AG 1-X8 anion exchange column. The column was initially washed with 10 ml of 60 mM ammonium formate containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with a solution containing 1 mM ammonium formate and 0.1 M formic acid.

Phospholipase D Activity Assay—PLD activity was assessed by measurement of formation of [^{3}H]phosphatidylbutanol, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. MSCs in 6-well plates were serum-starved in the presence of 2 μCi of [^{3}H]myristic acid/ml. After overnight starvation, the cells were washed three times with 5 ml of PBS and pre-equilibrated in serum-free DMEM for 1 h. For the final 10 min of preincubation, 0.3% butan-1-ol was incubated. At the end of preincubation, cells were treated with fMLP. Previously described procedures were used for extraction and characterization of lipids by thin layer chromatography (24).

Alkaline Phosphatase Assay—For measurement of alkaline phosphatase (ALP) activity of the cell layer, cells were rinsed twice with Hanks' balanced salt solution and then incubated with 5 mM *p*-nitrophenyl phosphate in 50 mM glycine and 1 mM $MgCl_2$, pH 10.5, at 37 °C for 20 min. Absorbance of *p*-nitrophenol produced by ALP activity was monitored at 405 nm using a microplate spectrophotometer.

Transient Transfections and Luciferase Reporter Assay—MSCs were plated in 12-well plates at 1×10^5 cells/well and grown to 50–60% confluence. Plasmid mixtures containing luciferase reporter plasmids were transfected by Lipofectamine Plus, according to the manufacturer's instructions. Following 24 h of transfection, the medium was changed, and cells were treated with inhibitors, as indicated. Cells were lysed, followed by measurement of reporter activity using the Dual-Luciferase assay system (Promega). For transfection efficiency, relative luciferase activity was obtained by normalization of firefly luciferase activity against the *Renilla* luciferase activity of the internal control.

RNA Isolation and Quantitative Real Time PCR (q-PCR)—Total RNA was isolated by the TRIzol method (Sigma). First strand cDNA was synthesized using AMV-RTase (Promega, Madison, WI). Real time q-PCR was conducted with cDNA using the Quantitect SYBR Green PCR kit (Qiagen) and a Rotor-Gene RG-3000A apparatus (Corbett Research). All data were normalized with GAPDH gene expression values. Oligonucleotide primer sequences were as follows (F, forward primer; R, reverse primer): FPR1-F, 5′-GGCATCATCCGGTTCATCATT-3′, and FPR1-R, 5′-AGGGCACTTGTCACATCCACT-3′; FPR2-F, 5′-GTCGGACCTTGGATTCTTGCT-3′, and FPR2-R, 5′-CTTTTTGTGGATCTTGGCTGCA-3′; FPR3-F, 5′-CGCACAGTCAACACCATCTG-3′, and FPR-R, 5′-AGCTGTTAAAAAAGGCCAAG-3′; GAPDH-F, 5′-GTGGTCTCCTCTGACTTCAAC-3′, and GAPDH-R, 5′-TCTCTTCCTCTTGTGCTCTTG-3′; Runx2-F, 5′-CCAGATGGGACTGTGGTTACC-3′, and Runx2-R, 5′-ACTTGGTGCAGAGTTCAGGG-3′; PPARγ-F, 5′-TCTCTCCGTAATGGAAGACC-3′, and PPARγ-R, 5′-GCATTATGAGACATCCCCAC-3′; and Zebrafish RUNX2-F, 5′-CTCAGTGAACTGGAGCGCAT-3′, and Zebrafish RUNX2-R, 5′-AGTGAGGAGAAGGCAGGGAA-3′.

Bone Formation in Zebrafish—Zebrafish (*Danio rerio*) embryos were collected from spontaneous spawnings of the AB line and reared at 28.5 °C. Three days postfertilization (~1 day after hatching), feeding of the commercial powder for baby fish was started. Zebrafish embryos were stimulated with fMLP from 8 to 48 h postfertilization (hpf), washed, and raised at 28.5 °C. At 5 days postfertilization (dpf), 20 zebrafish were stained with Alizarin Red for examination of the effect of fMLP on bone formation. Zebrafish were fixed in 4% paraformaldehyde for 12–18 h. Following fixation, fish were depigmented overnight in 1% KOH containing 3% H_2O_2 and immersed for 2 h in 0.5% KOH containing 0.05% Alizarin Red, pH 4.2. Alizarin-stained fish were then cleared again in 1% KOH and observed under a microscope.

Bone Formation in Rabbit—Four rabbits were used for this experiment. After preliminary breeding, they were divided into two groups according to the design of the experiment; control and 4 weeks. Rabbits were in good health prior to the experiment and were bred in an excluded environment. All animal experiments were approved by the guidelines of the Institutional Animal Care and Use Committee of Wonkwang University. Rabbits were subjected to general anesthesia by intramuscular injections with 0.2 ml/kg of xylazine HCl (Rompun®, Bayer in Korea) and 0.3 ml/kg of ketamine HCl (Ketalar®, Yuhan Co., Korea). Lidocaine containing epinephrine was administered subcutaneously for local anesthesia. Full thickness flap was performed for exposure of the cranium and a trephine bur with a diameter of 4.8 mm was used to make bony defects. For improvement of manipulation, fMLP was added to hyaluronic acid gel as a carrier. Fifty μM of fMLP was administered with hyaluronic acid for the experimental group, and hyaluronic acid was added only to the control group. The total volume administered to each defect was 10 μl. At 4 weeks postsurgery, animals were induced by general anesthesia and sacrificed with CO_2 inhalation for histological examination. After dyeing with Masson trichrome, sections were observed under a microscope. Peak-Pro software (BaromKorea, Korea) was used for quantification of bone formation. Bone gain was quantified by a relative percentage of regenerated bone compared with the area of surgically created bone defect under ×20 magnification.

Statistics—Results are expressed as mean ± S.D. of the number of determinations indicated. Statistical significance of differences was determined by analysis of variance. Significance was accepted with $p < 0.05$.

RESULTS

Expression of Functional FPR1 Is Significantly Increased during Osteoblastic Differentiation of Human MSCs—To determine whether expression of FPR family members is regulated during osteoblastic differentiation, hMSCs were differentiated into osteoblasts, and an analysis of FPR expression was per-

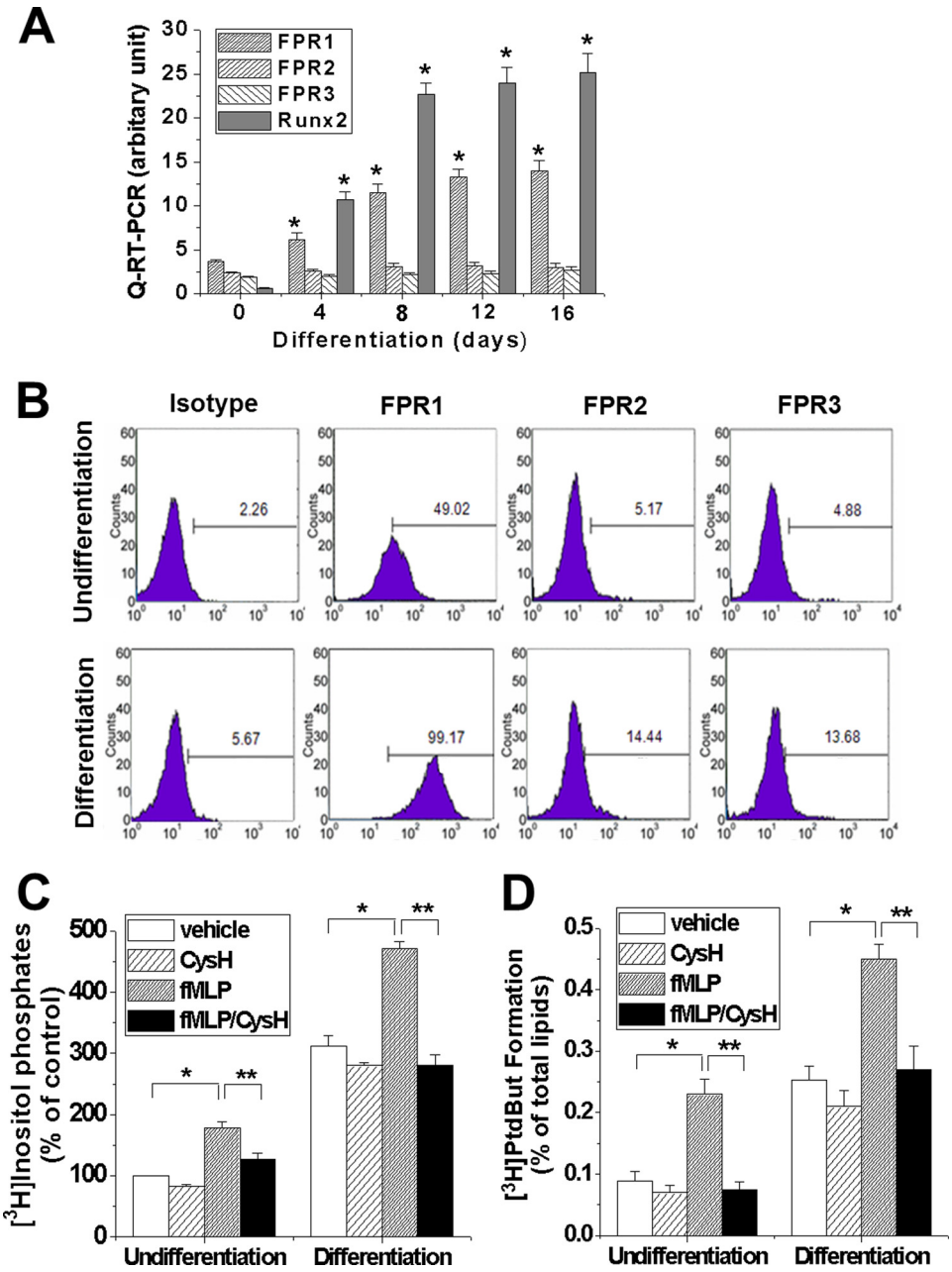

FIGURE 1. **Expression of functional FPR1 is significantly increased during osteoblast differentiation of human MSCs.** *A,* osteoblast differentiation of hMSCs was induced in the presence of osteogenic media for the indicated number of days. Expression levels of FPRs and Runx2 were determined by analysis of Q-RT-PCR. Levels of FPRs and Runx2 were normalized to GAPDH. *, $p < 0.05$ *versus* undifferentiation. *B,* flow cytometric analyses of expression of FPR1, FPR2, and FPR3 at the protein level. Surface expression of FPR1, FPR2, and FPR3 from undifferentiated or differentiated hMSCs into osteoblasts for 12 days was determined by FACS analysis. Results are representative of at least three independent experiments. *C,* MSCs were cultured in growth media or differentiated into osteoblasts for 12 days, labeled with [^{3}H]myoinositol, pretreated with or without CysH (1 μM) for 30 min, and treated with or without 1 μM fMLP for 1 h; PLC activity was then measured. *D,* under the same conditions as those of *C*, MSCs were labeled with [^{3}H]myristic acid, pretreated with or without CysH (1 μM) for 30 min, and treated with 1 μM fMLP for 1 h; PLD activity was then measured. Values represent the mean ± S.D. of three independent experiments. * and **, $p < 0.05$.

formed. As shown in Fig. 1*A*, expression of FPR1, but not FPR2 and FPR3, was significantly increased in a time-dependent manner during osteoblastic differentiation of MSCs, as analyzed by q-PCR. Expression of Runx-2, a major transcription factor in osteoblast differentiation, also increased during the indicated time course of osteoblast differentiation. To further examine the protein level of FPR family members during osteoblast differentiation, we performed immunostaining of MSCs followed by flow cytometric analyses. Without permeabilization, the cells were immunostained with monoclonal antibodies against FPR1, FPR2, or FPR3. In immunostaining of undifferentiated cells, mean fluorescence intensity (MFI) for FPR1 was 49.02, whereas MFI for FPR2 and FPR3 were 5.17 and 4.88, respectively (Fig. 1*B*). However, immunostaining of the isotype antibody resulted in background staining with a MFI of 2.26. In MSCs differentiated into osteoblasts for 12 days, MFI for FPR1 was 99.17, whereas MFI for FPR2 and FPR3 were 14.44 and 13.68, respectively. Immunostaining of the isotype antibody resulted in background staining with an MFI of 5.67 (Fig. 1*B*). These results indicate that the expression level of FPR1 protein,

but not those of FPR2 and FPR3, is significantly increased during osteoblastic differentiation, suggesting that FPR1 may play a role in osteoblast differentiation. In the FPR signaling pathway, binding of fMLP, a specific ligand of FPR1, to FPR1 leads to a number of downstream cellular events, including activation of PLC and PLD (19). To determine whether FPR in MSCs is a functional response to fMLP stimulation during osteogenic differentiation, we measured the activities of PLC and PLD before or after osteoblastic differentiation. Cells were labeled with [^{3}H]inositol or [^{3}H]myristic acid and stimulated with 1 μM fMLP. As shown in Fig. 1, *C* and *D*, fMLP induced an increase in formation of inositol phosphates and phosphatidylbutanol in both undifferentiated and differentiated cells. In differentiated cells, both basal and fMLP-stimulated PLC or PLD activity showed a significant increase, compared with undifferentiated cells. In addition, fMLP-induced PLC or PLD activation was significantly attenuated by pretreatment with CysH, a potent and selective FPR1 antagonist (25, 26). Taken together, these data indicate that expression of functional FPR, particularly FPR1, is significantly increased during osteogenic differentiation of MSCs.

fMLP Promotes Osteoblastic Differentiation of MSCs via FPR1—To investigate the question of whether fMLP affects osteoblastic differentiation, osteoblastic differentiation of MSCs was induced by exposure of cells to OM in the presence of different concentrations of fMLP. As shown in Fig. 2*A* (*left panel*), continuous treatment with fMLP resulted in a dose-dependent increase of mineralization, which was induced by osteoblastic differentiation for 14 days, as visualized by Alizarin Red-S staining. However, fMLP itself at various concentrations did not induce extracellular mineralization of MSCs in cultures maintained in growth medium (GM). Fig. 2*A*, *right panel,* shows the quantitation of Alizarin Red-S staining. For determination of whether continuous treatment of MSCs with fMLP is essential for osteoblastic differentiation, cells were pulse-chased with 1 μM fMLP in OM for 3 days and washed; cells were then cultured in the absence of fMLP during the indicated time course of osteogenesis. Pulse treatment of MSCs with fMLP resulted in a significant increase of mineral deposition, compared with that of MSCs cultured in OM in the absence of fMLP (Fig. 2*B*). These results suggest that exposure of MSCs to fMLP during early stages of differentiation is essential for fMLP-stimulated osteoblastic differentiation. To further examine the question of whether fMLP stimulates osteoblast differentiation of MSCs, cells were cultured in OM containing calcein for 14 days, and mineralization was detected by the calcein assay. Fluorescent spots, indicative of calcein uptake into newly mineralized extracellular matrix, showed a significant increase upon application of 1 μM fMLP to the cell culture (supplemental Fig. 1*A*). However, fMLP-stimulated mineralization was suppressed by pretreatment with CysH, suggesting selective mediation of fMLP-stimulated osteoblast differentiation of MSCs via FPR1. Because activity of ALP, an early marker of osteoblastic differentiation, plays an important role in both osteoblastic differentiation and eventual mineralization processes, we next measured ALP activity in these cells. ALP activity in MSCs increased by exposure to OM for 5 days, and treatment of cells with 1 μM fMLP resulted in further promotion of ALP activity (Fig. 2*C*). However, fMLP-stimulated ALP activity was inhibited by pretreatment with CysH. fMLP-stimulated ALP activity is consistent with increased mineralization in fMLP-treated cells during osteoblastic differentiation (Fig. 2*C*). We attempted to further determine whether fMLP-stimulated osteogenesis is mediated via FPR1. Analysis by Alizarin Red-S staining and the ALP assay showed that knockdown of FPR1 using siRNA resulted in a marked reduction of fMLP-stimulated osteoblastic differentiation of MSCs (Fig. 2*D*). Expression of FPR1 by transfection with siRNA for FPR1 was analyzed by q-PCR (Fig. 2*D*). In addition, quantitation of Alizarin Red-S staining was performed (Fig. 2*D*). Gene silencing of FPR1 also inhibited osteogenesis at the basal level, compared with control siRNA. These data suggest an important role for FPR1 in osteogenesis. The N-terminal fragment of annexin-1, Anx-1(2–26), is an endogenous host-derived agonist of FPR1 (27). Thus, we examined the effect of annexin-1 on osteogenesis of MSCs. Alizarin Red-S staining and the ALP assay showed that Anx-1(2–26) significantly promoted osteoblastic differentiation of MSCs in the presence of OM (Fig. 2*E*), and Anex-1-stimulated osteogenesis was suppressed by pretreatment with CysH. Anex-1 alone did not affect osteoblastic differentiation in the presence of GM (data not shown). In addition, we observed that knockdown of FPR1 inhibited expression of Runx2 under OM-induced conditions, as analyzed by q-PCR (Fig. 2*F*). Taken together, these results suggest that promotion of osteoblastic differentiation of MSCs in response to fMLP is selectively mediated via FPR1.

fMLP Stimulates Expression of Osteoblastic Markers—Osteoblast differentiation of MSCs occurs along with an increase in expression of Runx2, a master transcription factor in osteogenesis. Cyclooxygenase 2 (COX-2) is an important inducer of osteoblastic differentiation in bone biology (28). To further confirm the pro-osteogenic effect of fMLP, we examined expression of osteoblast marker genes. Expression levels of Runx2 and COX2 increased after exposure of MSCs to OM for 14 days (Fig. 3*A*). Compared with nontreated cells, treatment of cells with OM along with fMLP promoted an increase in expression levels of Runx2 and COX2 (Fig. 3*A*). In the presence of GM, fMLP alone did not induce an increase in the expression levels of osteogenic markers in MSCs (data not shown). To further examine fMLP-stimulated expression of osteomarker genes, MSCs were transfected with promoters of osteogenic marker genes and treated with fMLP. fMLP induced a significant increase in the promoter activities of Runx2, ALP, osteocalcin, and bone sialoprotein (Fig. 3*B*). These results are consistent with the observation that fMLP stimulates extracellular mineralization of MSCs exposed to OM, suggesting that fMLP promotes osteoblastic differentiation through increase of the expression levels of osteogenic markers.

fMLP Suppresses Adipogenic Differentiation of MSCs—Osteoblasts and adipocytes are differentiated from bone marrow-derived MSCs, and reciprocal regulation of their differentiation occurs through mechanisms that are largely unknown. To explore the question of whether fMLP affects differentiation of MSCs to adipocytes, adipogenic differentiation of MSCs was induced by exposure of cells to adipogenic differentiation medium in the presence of fMLP, and accumulation of intra-

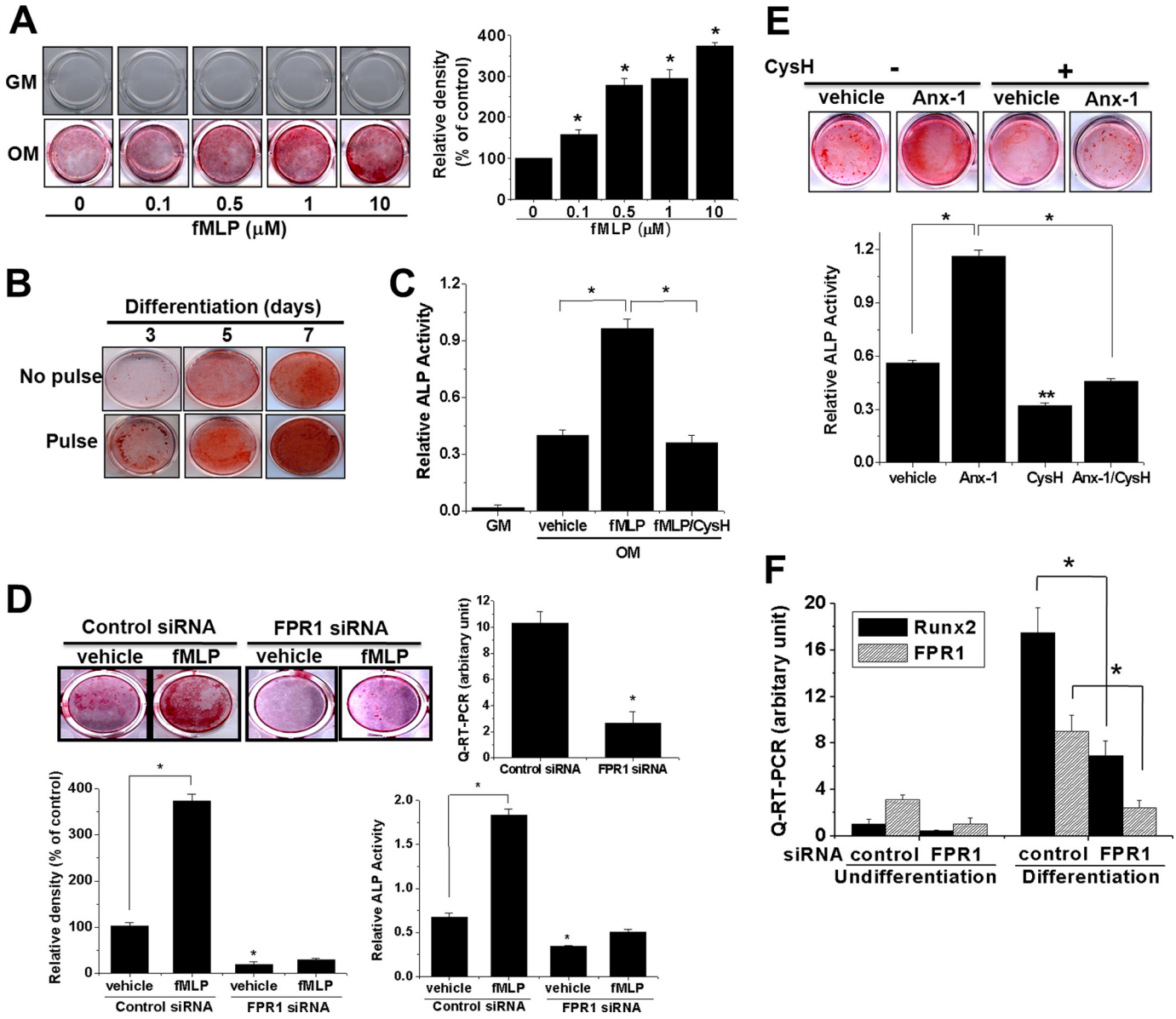

FIGURE 2. **fMLP promotes osteoblast differentiation of MSCs via FPR1.** *A,* hMSCs were cultured in GM or OM in the presence of the indicated concentrations of fMLP. After 14 days, matrix mineralization was determined by Alizarin Red-S staining and photographed using a digital camera equipped with a Nikon TS100 inverted microscope (Nikon, Japan). Relative density of each well in the presence of OM was quantified using the Scion image program, and results from triplicate determinations are shown as means ± S.D. *, $p < 0.05$ *versus* nontreatment. *B,* during osteoblast differentiation, MSCs were pulse-treated with fMLP (1 μM). After 3 days, cells were washed and cultured in the absence of fMLP during the indicated time course of *in vitro* osteogenesis; matrix mineralization was then assessed by Alizarin Red-S staining and photographed with a digital camera. Data are representative of three experiments. *C,* MSCs were cultured in OM for 5 days, pretreated with or without CysH (1 μM), and stimulated with 1 μM of fMLP. ALP activity was then measured. Stem cells were transfected with siRNA for control or FPR1 and treated with or without fMLP (1 μM) in osteogenic media for 7 days. Alizarin Red-S staining was then conducted. Expression of FRP1 was analyzed by q-PCR, and the level of FPR1 was normalized to GAPDH. *, $p < 0.05$ *versus* control siRNA. Relative density of each well was quantified using the Scion image program. ALP assays were then conducted. *, $p < 0.05$ *versus* vehicle in control siRNA. Results from triplicate determination are shown as means ± S.D. *E,* MSCs were cultured in OM in the presence of 1 μM Anx-1(2–26), pretreated with or without CysH (1 μM). After 10 days, matrix mineralization was determined by Alizarin Red-S staining and photographed with a digital camera. ALP assays were conducted. *, $p < 0.05$; **, $p < 0.05$ *versus* vehicle. *F,* MSCs were transfected with siRNA for control and FPR1 in undifferentiated and differentiated conditions. Expression of FPR1 and Runx2 was measured by q-PCR. *, $p < 0.05$. Data represent the mean ± S.D. of four independent experiments.

cellular triglyceride droplets was visualized by Oil Red O staining. Exposure of MSCs to adipogenic differentiation medium for 16 days resulted in differentiation into adipocytes (Fig. 4*A*). However, fMLP treatment resulted in a dose-dependent reduction of lipid droplet accumulation (Fig. 4*A*). The number of Oil Red O-positive cells was quantitated. In addition, fMLP inhibited expression of PPARγ, a major transcription factor of adipogenesis (Fig. 4*B*). Therefore, it is suggested that fMLP has a potent anti-adipogenic activity and might be involved in negative regulation of adipocyte differentiation of MSCs.

fMLP-stimulated Osteogenesis of MSCs Is Mediated by the FPR1-PLC/PLD-CaMKII-ERK Signaling Pathway—To further comprehend the mechanism through which fMLP promotes osteogenesis of MSCs, cells were pretreated with inhibitors of downstream targets of fMLP-mediated signaling pathways, and mineral deposition was detected by Alizarin Red-S staining

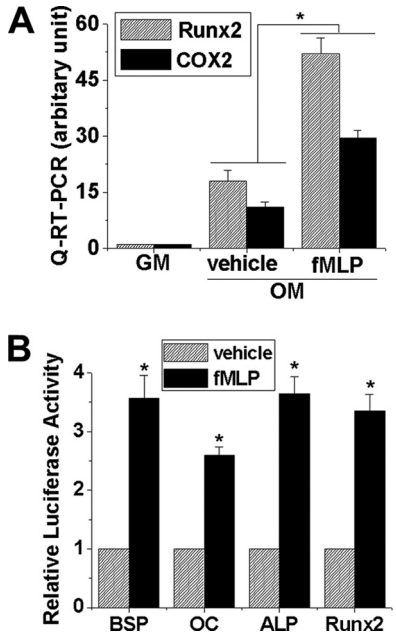

FIGURE 3. **fMLP increases expression of osteogenic marker genes.** *A,* MSCs were cultured in osteogenic media in the absence or presence of 1 μM fMLP for 14 days. Expression of Runx2 and COX2 was analyzed by Q-RT-PCR. *, $p < 0.05$. *B,* MSCs were transfected with the indicated reporter constructs and treated with fMLP (1 μM) for 36 h, and luciferase activity was subsequently measured. A *Renilla* luciferase control vector was co-transfected for normalization of transfection efficiency. Values are represented as the mean ± S.D. of five independent experiments. Promoter gene constructs. *, $p < 0.05$. *BSP,* bone sialoprotein; *OC,* osteocalcin.

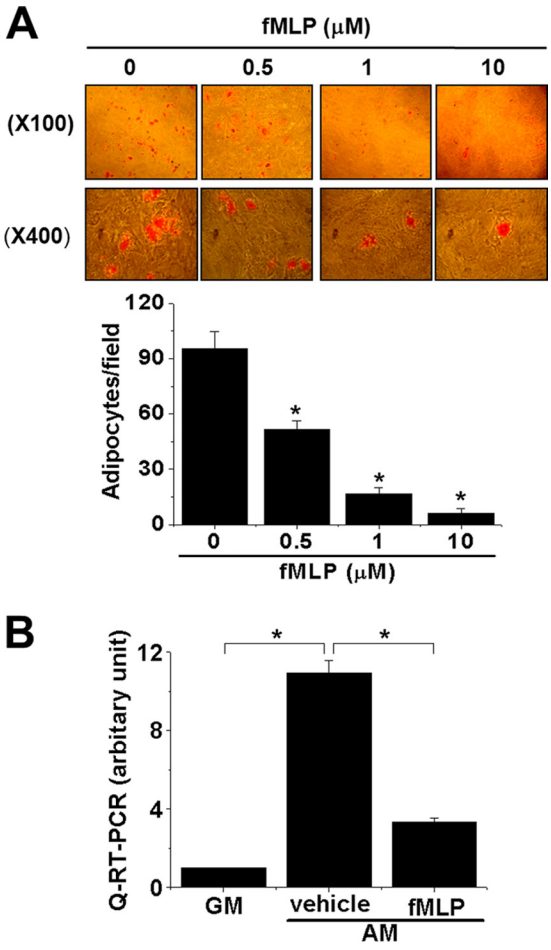

FIGURE 4. **fMLP suppresses adipocyte differentiation of hMSCs.** *A,* MSCs were cultured in adipoinductive media (*AM*) in the absence or presence of fMLP (1 μM). After 16 days, lipid droplets were visualized by Oil Red O staining, and phase contrast images were photographed using a digital camera equipped with a Nikon TS100 inverted microscope (Nikon, Japan) using 10×/0.25 ph1 ADL or LWD 40×/0.55 ph1 ADL (×100, ×400) (*upper panel*). The number of Oil Red O-positive cells was quantitated (*lower panel*). *, $p < 0.05$ *versus* nontreatment. *B,* MSCs were cultured in GM or adipoinductive media with or without 1 μM fMLP over a period of 16 days. Expression of PPAR-γ was analyzed by Q-RT-PCR. The level of PPAR-γ was normalized to GAPDH. *, $p < 0.05$. Values are represented as the mean ± S.D. of three independent experiments.

(Fig. 5*A*). Culture of MSCs in the presence of OM for 10 days resulted in stimulation of osteogenesis by fMLP. We treated DMSO as a vehicle control, and the dose of effective inhibitors was selected as described in many reports. Pretreatment with CysH inhibited fMLP-stimulated osteoblast differentiation, indicating that stimulation of osteogenesis in response to fMLP is mediated via FPR1. In fact, FPR is functionally coupled to PTX-sensitive G protein (29). After binding to fMLP, FPR1 transmits signals to heterotrimeric G proteins, which rapidly dissociate into α and $\beta\gamma$ subunits, resulting in activation of PLC (30). We investigated the effect of PTX, which specifically blocks coupling of G protein-coupled receptors to G_i on fMLP-induced signaling. Pretreatment with PTX resulted in significant inhibition of fMLP-stimulated osteogenesis. Activation of PLC, an effector molecule of fMLP-coupled G protein, generates second messengers and diacylglycerol. Although inositol 1,4,5-trisphosphate regulates the mobilization of Ca^{2+} from intracellular stores, diacylglycerol activates protein kinase C. We found that pretreatment with PLC inhibitor, U7312, but not its inactive analog, U73343, resulted in dramatic suppression of fMLP-stimulated osteoblastic differentiation (Fig. 5). We have recently reported that stimulation of MSCs with fMLP causes an increase of $[Ca^{2+}]_i$ via a PTX-sensitive pathway (20). Many of the cellular effects of Ca^{2+} are mediated by the Ca^{2+}-binding protein calmodulin (CaM), which is known to bind to and activate numerous targets, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII has been known to play a critical role in osteoblastic differentiation (30). KN93, an inhibitor of CaMKII but not its inactive analog, KN92, significantly abolished fMLP-stimulated osteogenesis (Fig. 5). These results suggest that fMLP might stimulate osteogenesis via FPR1/PTX-sensitive G protein/PLC/CaMKII in MSCs. MAPK is known to be regulated by CaMKII and is also important for osteoblastic differentiation (31). Pretreatment with inhibitor of MEK (U0126), upstream of MAPK, blocked mineralization stimulated by fMLP. PLD is another intracellular effector in the fMLP receptor signaling cascade (15). In addition, 5-fluoro-2-indolyl des-clorohalopemide hydrochloride hydrate, a PLD inhibitor, essentially abrogated fMLP-stimulated osteoblast differentiation (Fig. 5*A*).

In addition, to exclude the possibility that pharmacological inhibitors themselves have pleiotropic effects on cellular metabolism, which would render them nonspecific, we used siRNA specific for diminishing the individual signaling components in the presence of fMLP treatment. As shown in Fig. 5*B*, analysis by the ALP assay showed that knockdown of PLCβ1 and PLD1 suppressed fMLP-stimulated osteoblastic differentiation. Expression of the principal signaling compo-

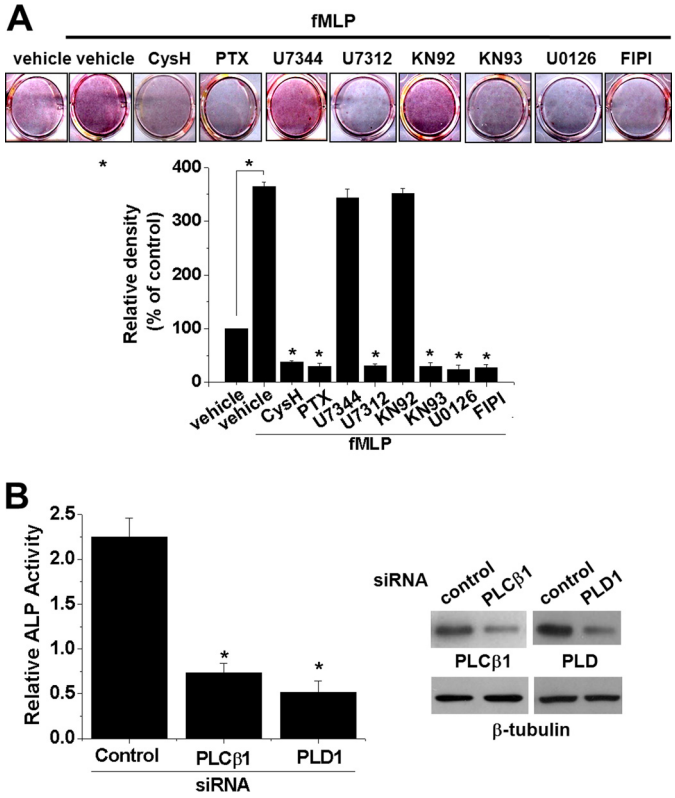

FIGURE 5. **fMLP-stimulated osteogenesis of MSCs is mediated by FPR1-PLC/PLD-CaMKII-ERK signaling pathways.** *A, top panel*, MSCs were pretreated with or without the indicated inhibitors (1 μM CysH or PTX; 10 μM U7312, U7344, KN92, KN93, U0126; or 5-fluoro-2-indolyl des-clorohalopemide hydrochloride hydrate (*FIPI*)) and cultured in OM in the presence of 1 μM fMLP for 10 days. Media were changed every 2 days for the duration of the culture. Matrix mineralization was assessed by Alizarin Red-S staining and photographed using a digital camera equipped with a Nikon TS 100 inverted microscope (Nikon, Japan). Data are representative of three experiments. *Bottom panel*, relative density of each well was quantified using a Scion image program, and results from triplicate determination are shown as means ± S.D. *, $p < 0.05$ *versus* vehicle in the presence of OM. *B,* MSCs were transfected with siRNA for PLCβ1 and PLD1 and then cultured in osteogenic media with fMLP for 7 days. ALP activity was then measured. *, $p < 0.05$ *versus* control siRNA, Expression of PLCβ1 and PLD1 was analyzed by Western blot using the indicated antibody.

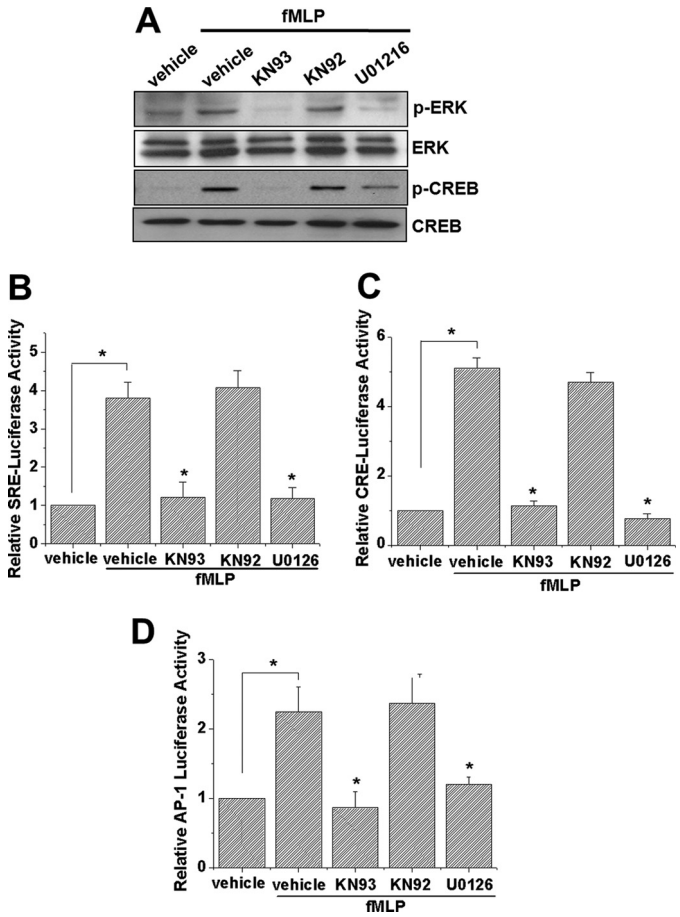

FIGURE 6. **CaMKII is involved in fMLP-induced ERK and CREB signaling pathways.** *A,* MSCs cultured in OM were starved for 24 h and preincubated with 10 μM KN92, KN93, or U0126 for 30 min. Cells were then treated with fMLP (1 μM) for 20 min, and phosphorylation of ERK or CREB (Ser-133) was determined by Western blot analysis. Blots are representative of three experiments. *B,* MSCs were transfected with an SRE-luciferase (*B*), CRE-luciferase (*C*), or AP-1 luciferase (*D*) reporter construct, pretreated with the indicated inhibitors for 30 min, and treated with fMLP for 12 h. Reporter activity was then measured. Data are representative of the mean ± S.D. of three independent experiments. *, $p < 0.05$.

nents is shown, as analyzed by Western blot. Taken together, these results suggest that fMLP accelerates osteogenesis of MSCs via a FPR1-PLC/PLD-CaMKII-ERK-mediated signaling pathway.

CaMKII Is Involved in fMLP-induced ERK and CREB Signaling Pathways—We examined the effects of CaMKII on the two signaling pathways, ERK and CREB, which are known to be regulated by CaMKII and important for osteoblastic differentiation (31). As shown in Fig. 6*A*, KN93 and U0126, but not KN92, suppressed fMLP-induced ERK activation and phosphorylation of CREB (Ser-133), indicating involvement of CaMKII in fMLP-induced ERK or CREB activation in MSCs. In addition, fMLP increased transactivation of the serum-response element (SRE), a downstream target of ERK, and pretreatment of KN93 and U0126, but not KN92, inhibited fMLP-stimulated transactivation of SRE (Fig. 6*B*). These results demonstrate the critical role of CaMKII in activation of the ERK/SRE signaling pathway by fMLP. We also investigated the effect of pharmacological inhibition on CRE transactivation, which is known to be a downstream target of CREB. Both KN93 and U0126, but not KN92, induced significant inhibition of CRE transactivation (Fig. 6*C*). These results demonstrate that CaMKII and ERK are critical kinases in osteoblasts. Regulation of the expression of c-*jun*, an immediate response gene, by two important regulatory response elements, CRE and SRE, has been demonstrated (32, 33). Thus, we examined the question of whether fMLP indeed affects AP-1 transactivation. Transactivation of AP-1 in MSCs was increased by treatment with fMLP and inhibited by pretreatment with KN93 and U0126 but not KN92 (Fig. 6*D*). Taken together, our results demonstrate that CaMKII activation by fMLP regulates the activity of both ERK and CREB, which in turn affects transactivation of SRE and CRE. This leads to AP-1 transactivation and ultimately influences osteoblastic differentiation.

fMLP Increases Osteogenesis in Vivo—For a better understanding of bone formation by fMLP, we examined the effect of fMLP on bone formation of zebrafish, *D. rerio,* which is known to be a good model system for the study of bone development (34). Most of the regulatory factors or pathways that control bone formation are highly conserved in vertebrates, including

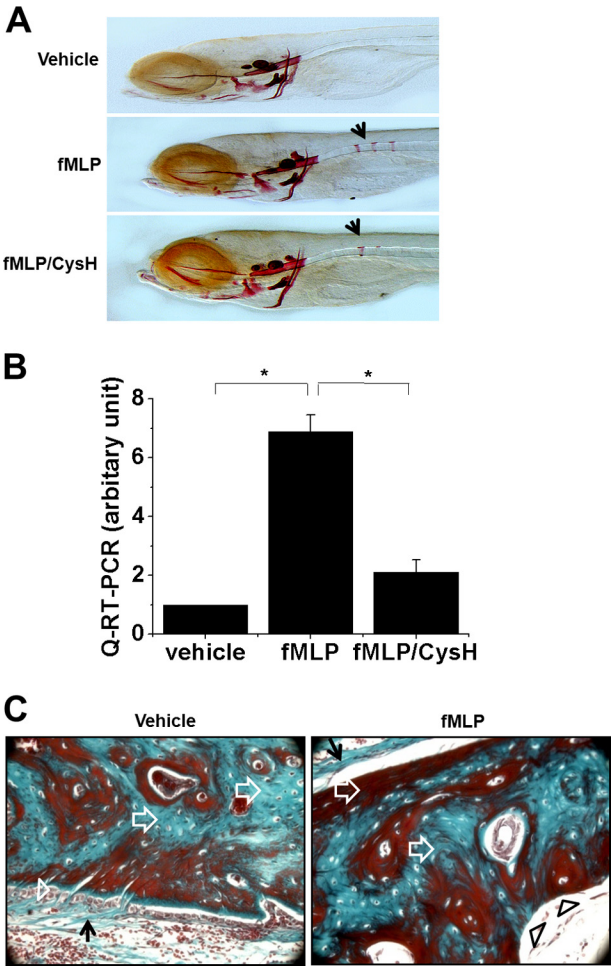

FIGURE 7. **fMLP promotes bone formation *in vivo*.** *A,* Alizarin Red-S staining of whole zebrafish embryos revealed enhanced skeletal ossification in fMLP-treated embryos. Fertilized eggs were transferred to 24-well plates and treated with or without fMLP (1 μM) at 8 hpf (70% epiboly) in a 28 °C incubator. After 40 h (48 hpf), embryos were washed and raised in normal egg water. At 5 dpf, zebrafish were stained with Alizarin Red-S, and lateral skeleton views were photographed. *B,* zebrafish were raised and treated with or without fMLP (1 μM) or CysH (1 μM) under the same conditions. Using Q-RT-PCR analysis, expression of Runx2 was examined from young zebrafish. The level of Runx2 was normalized to GAPDH. *, $p < 0.05$. *C,* histological findings of the effect of fMLP in rabbit calvarial defects at 4 weeks. The most significant area of newly formed bone was woven bone (*white arrow*), rather than lamellar bone, and bone marrow spaces were not wider in the vehicle-treated group (×400). An abundance of osteoblasts (*white arrowhead*) was observed along the surface of bone, and connective tissue (*black arrow*), like periosteum, was not observed around bone in the vehicle group. Lamella bone (*white arrow*) was observed in newly formed bone, and bone remodeling was more progressed in the fMLP-treated group than in the vehicle group. Formation of bone marrow spaces (*black arrowhead*) has widely progressed, and connective tissue (*black arrow*) covering bone was dense, like periosteum, in the fMLP-treated group (×400).

zebrafish (34). Twenty zebrafish embryos were treated with fMLP from 8 to 48 hpf, a time at which regulators of bone formation, such as Runx2, are expressed during skeletal development of zebrafish (35). *In vivo* bone development was visualized in whole embryos (5 dpf) with Alizarin Red (Fig. 7*A*). fMLP enhanced lateral skeletal development, whereas pretreatment with CysH inhibited fMLP-stimulated bone development. Extensive skeletal development was evident at 5 dpf in over 80% of fMLP-treated zebrafish. In addition, compared with non-treated larvae, treatment with fMLP resulted in increased expression of Runx2; however, pretreatment with CysH resulted in significant suppression of fMLP-stimulated Runx2 expression (Fig. 7*B*), suggesting that fMLP-induced osteogenesis is mediated via FPR1 in zebrafish. Moreover, we attempted to determine whether fMLP affects bone formation in a mammalian system. Rabbits were subjected to a calvarial defect. The healing process was fair in all animals, and none of the wounds showed severe inflammation. At 4 weeks, most areas of newly formed bone were woven bone, rather than lamellar bone, and bone marrow spaces were not wider in the calvaria of the vehicle-treated rabbit. An abundance of osteoblasts was observed along the surface of bone, and connective tissue, like periosteum, was not observed around bone in the calvaria of vehicle-treated rabbits (Fig. 7*C, left panel*). In fMLP-treated calvaria, the quality of newly formed bone was more developed compared with that of the vehicle (Fig. 7*C, right panel*). The pattern of lamellar bone was shown in areas of newly formed bone, and bone remodeling was more progressed in fMLP-treated calvaria than in vehicle-treated calvaria. Bone marrow spaces were widely formed, and connective tissue covering bone was dense, like periosteum, in fMLP-treated calvaria. Mean bone formation within the surgically created defect in rabbit calvaria was 36.88 ± 16.79% in the vehicle group and 57.28 ± 25.76% in the fMLP-treated group, as measured by histomorphometry, indicating increased bone formation by fMLP in a mammalian system. Taken together, it is suggested that fMLP has a beneficial effect in bone formation from zebrafish and rabbits, suggesting its physiological relevance *in vivo*.

DISCUSSION

This study provides evidence to suggest regulation of lineage-specific differentiation of MSCs by fMLP in favor of osteoblastic differentiation and against adipogenic differentiation. MSCs can be easily isolated from bone marrow and rapidly expanded in culture; therefore, the cells have been regarded as an important therapeutic tool for clinical application in the field of damaged tissue remodeling (36). FPR is an important chemoattractant receptor, and expression of FPR has mainly been identified in phagocytic cells, including monocytes, neutrophils, and dendritic cells. We (20) and Viswanathan *et al.* (21) have recently reported that MSCs functionally express FPR and show positive chemotaxis toward *N*-formyl peptide, which might play an important role in the repopulation of stem cells. A potential role for FPR on the surface of MSCs in sensing microenvironmental signals and the regulation of MSC homing, migration, tissue remodeling, and engraftment have been suggested (20, 21). However, the role of fMLP, a selective ligand of FPR1 in differentiation of MSCs, has not been studied. In this study, we demonstrate a novel role for fMLP or FRP1 in osteogenic/adipogenic differentiation of MSCs. fMLP promotes osteoblastic differentiation of MSCs and a selective antagonist, and knockdown of FPR1 inhibited fMLP-stimulated osteoblast differentiation, suggesting an important role for FPR1 in osteogenesis. fMLP stimulates expression of osteogenic genes and mineralization in MSCs. Annexin-1, a family of phospholipid-binding proteins, has reportedly been found in many tissues, including lung, bone marrow, and intestine (37). We and our collaborators have recently reported that Anx-1(2–26), an

endogenous FPR1 agonist, induced chemotactic migration of MSCs (18). In this study, we found that Anx-1(2–26), like fMLP, promoted osteoblastic differentiation of MSCs via FPR1. Thus, we propose a new aspect of annexin 1 as a regulator of migration and osteogenesis of MSCs.

Pulse treatment of MSCs with fMLP for the initial 3 days was sufficient for fMLP-stimulated osteoblastic differentiation, suggesting that exposure of cells with fMLP during the early stages of osteogenic differentiation is essential for pro-osteogenic activity of fMLP. fMLP stimulates osteogenesis of MSCs via the FPR1/PTX-sensitive G protein/PLC (PLD)/CaMKII/ERK/CREB signaling cascade. Treatment of osteoblasts in culture with cAMP analogs is known to inhibit these cells from deposition of mineral salts in their extracellular matrix. cAMP is a G protein-coupled signaling component of several GPCRs. However, because G_i-coupled GPCR, such as FPR, inhibits cAMP production, an effect of cAMP on fMLP-induced response can be ruled out.

Although fMLP itself did not induce osteoblastic differentiation of MSCs in the presence of nonosteogenic media, skeletal development in fMLP-treated zebrafish was evidently enhanced at 5 dpf, and CysH inhibited fMLP-induced bone formation in zebrafish. In addition, using rabbits subjected to calvarial defects, we also found that fMLP was beneficial in mammalian bone formation. The quality of newly formed bone at 4 weeks was better in the fMLP-treated group than in the control group. Lamellar bone in the fMLP-treated group showed better formation than that in the control, and the progress of bone remodeling was rapid so that bone marrow cut into lamellar bone. Thus, it is suggested that stem cell niches could be important factors for fMLP itself in induction of bone formation *in vivo*. Because MSCs are the progenitor cells for both osteoblasts and adipocytes, differentiation of MSCs into adipocytes, rather than osteoblasts, may be responsible for reduction in the number of osteoblastic cells in osteoporosis and aging (38). Moreover, the volume of adipose tissue in bone increases with age in normal subjects and is substantially elevated in age-related osteoporosis (39). These results suggest that bone loss in age-related osteoporosis is at least in part caused by a shift from the osteoblastic to the adipocytic pathway of MSC differentiation. We also found that fMLP suppressed differentiation of bone marrow-derived monocytes into osteoclasts (data not shown), suggesting the possibility of an anti-osteoporosis effect of fMLP. It has been suggested that the shift of MSC differentiation from an adipocytic to an osteoblastic lineage may be helpful for cell therapy of bone disease (40). Regulation of osteoblast and adipocyte differentiation is often reciprocal. Therefore, it is interesting to note that fMLP promotes osteogenesis through increased expression of osteogenic marker genes and suppression of adipocyte differentiation by inhibiting expression of PPARγ. Our studies imply that deciphering the action mechanisms of bioactive small molecules such as fMLP could provide new insight into MSC biology. In particular, further dissection of the molecular mechanisms underlying the effects of fMLP in lineage-specific differentiation could aid in the advancement of our current understanding of osteogenesis. Use of MSCs for clinical applications associated with bone loss is also being contemplated (6, 12). A better understanding of osteogenic inductive pathways *in vivo* will be required for advancement of MSCs as tools to promote bone regeneration. Strategies employing bone morphogenetic proteins have been used successfully for regeneration of bone in animals and humans (41, 42). However, the high cost and supraphysiological doses of bone morphogenetic proteins necessary for achievement of osteoinductive activity illustrate the need for additional strategies for stimulation of osteoblast differentiation and bone formation *in vivo*. Hence, complementary and/or more cost-effective strategies for clinical modulation of bone formation are needed. fMLP could be one such candidate molecule.

Despite their potential for clinical use in regenerative medicine and tissue engineering, bone formation by hMSCs is poor. Findings from this study showing that fMLP enhances osteoblast differentiation via an FPR1-mediated signaling pathway and results in bone formation in an *in vivo* model demonstrate the possibility that systemic or local application of hMSCs to patients together with fMLP would potentially be useful for restoration of bone-forming capacity in bone diseases. Collectively, our findings, which define a novel role for fMLP and FPR1 in osteogenic signaling of MSCs, will shed light on understanding of their molecular mechanisms in bone biology, with important implications for development of novel strategies for the promotion of bone healing. Further studies with animal models will allow us to better describe the pre-osteogenic and anti-adipogenic properties of fMLP.

REFERENCES

1. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) *Science* **284,** 143–147
2. Caplan, A. I. (1994) *Clin. Plast. Surg.* **21,** 429–435
3. Jaiswal, N., Haynesworth, S. E., Caplan, A. I., and Bruder, S. P. (1997) *J. Cell Biochem.* **64,** 295–312
4. Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O., and Pittenger, M. F. (1998) *Tissue Eng.* **4,** 415–428
5. Jaiswal, R. K., Jaiswal, N., Bruder, S. P., Mbalaviele, G., Marshak, D. R., and Pittenger, M. F. (2000) *J. Biol. Chem.* **275,** 9645–9652
6. Prockop, D. J. (1997) *Science* **276,** 71–74
7. Harada, S., and Rodan, G. A. (2003) *Nature* **423,** 349–355
8. Fulfaro, F., Casuccio, A., Ticozzi, C., and Ripamonti, C. (1998) *Pain* **78,** 157–169
9. Gangji, V., and Appelboom, T. (1999) *Clin. Rheumatol.* **18,** 266–267
10. Riggs, B. L., and Melton, L. J., 3rd (1992) *N. Engl. J. Med.* **327,** 620–627
11. Cummings, S. R., and Melton, L. J. (2002) *Lancet* **359,** 1761–1767
12. Horwitz, E. M., Gordon, P. L., Koo, W. K., Marx, J. C., Neel, M. D., McNall, R. Y., Muul, L., and Hofmann, T. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 8932–8937
13. Pittenger, M. F., and Martin, B. J. (2004) *Circ. Res.* **95,** 9–20
14. Dezawa, M., Ishikawa, H., Itokazu, Y., Yoshihara, T., Hoshino, M., Takeda, S., Ide, C., and Nabeshima, Y. (2005) *Science* **309,** 314–317
15. Le, Y., Murphy, P. M., and Wang, J. M. (2002) *Trends Immunol.* **23,** 541–548
16. Marasco, W. A., Phan, S. H., Krutzsch, H., Showell, H. J., Feltner, D. E., Nairn, R., Becker, E. L., and Ward, P. A. (1984) *J. Biol. Chem.* **259,** 5430–5439
17. Carp, H. (1982) *J. Exp. Med.* **155,** 264–275
18. VanCompernolle, S. E., Clark, K. L., Rummel, K. A., and Todd, S. C. (2003) *J. Immunol.* **171,** 2050–2056
19. Selvatici, R., Falzarano, S., Mollica, A., and Spisani, S. (2006) *Eur. J. Pharmacol.* **534,** 1–11
20. Kim, M. K., Min do, S., Park, Y. J., Kim, J. H., Ryu, S. H., and Bae, Y. S. (2007)

FEBS Lett. **581,** 1917–1922
21. Viswanathan, A., Painter, R. G., Lanson, N. A., Jr., and Wang, G. (2007) *Stem Cells* **25,** 1263–1269
22. Min, D. S., Kwon, T. K., Park, W. S., Chang, J. S., Park, S. K., Ahn, B. H., Ryoo, Z. Y., Lee, Y. H., Lee, Y. S., Rhie, D. J., Yoon, S. H., Hahn, S. J., Kim, M. S., and Jo, Y. H. (2001) *Carcinogenesis* **22,** 1641–1647
23. Novikoff, A. B., Novikoff, P. M., Rosen, O. M., and Rubin, C. S. (1980) *J. Cell Biol.* **87,** 180–196
24. Min, D. S., Ahn, B. H., Rhie, D. J., Yoon, S. H., Hahn, S. J., Kim, M. S., and Jo, Y. H. (2001) *Neuropharmacology* **41,** 384–391
25. Wenzel-Seifert, K., Grünbaum, L., and Seifert, R. (1991) *J. Immunol.* **147,** 1940–1946
26. Wenzel-Seifert, K., and Seifert, R. (1993) *J. Immunol.* **150,** 4591–4599
27. La, M., D'Amico, M., Bandiera, S., Di Filippo, C., Oliani, S. M., Gavins, F. N., Flower, R. J., and Perretti, M. (2001) *FASEB J.* **15,** 2247–2256
28. Zhang, X., Schwarz, E. M., Young, D. A., Puzas, J. E., Rosier, R. N., and O'Keefe, R. J. (2002) *J. Clin. Invest.* **109,** 1405–1415
29. Le, Y., Oppenheim, J. J., and Wang, J. M. (2001) *Cytokine Growth Factor Rev.* **12,** 91–105
30. Zayzafoon, M. (2006) *J. Cell. Biochem.* **97,** 56–70
31. Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) *Mol. Cell. Biol.* **14,** 6107–6116
32. Perez-Albuerne, E. D., Schatteman, G., Sanders, L. K., and Nathans, D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11960–11964
33. Shaywitz, A. J., and Greenberg, M. E. (1999) *Annu. Rev. Biochem.* **68,** 821–861
34. Du, S. J., Frenkel, V., Kindschi, G., and Zohar, Y. (2001) *Dev. Biol.* **238,** 239–246
35. Flores, M. V., Lam, E. Y., Crosier, P., and Crosier, K. (2006) *Dev. Dyn.* **235,** 3166–3176
36. Grove, J. E., Bruscia, E., and Krause, D. S. (2004) *Stem Cells* **22,** 487–500
37. Christmas, P., Callaway, J., Fallon, J., Jones, J., and Haigler, H. T. (1991) *J. Biol. Chem.* **266,** 2499–2507
38. Chan, G. K., and Duque, G. (2002) *Gerontology* **48,** 62–71
39. Meunier, P., Aaron, J., Edouard, C., and Vignon, G. (1971) *Clin. Orthop. Relat. Res.* **80,** 147–154
40. Nuttall, M. E., and Gimble, J. M. (2000) *Bone* **27,** 177–184
41. Fleet, J. C., Cashman, K., Cox, K., and Rosen, V. (1996) *Endocrinology* **137,** 4605–4610
42. Johnson, E. E., and Urist, M. R. (2000) *Clin. Orthop. Relat. Res.* **371,** 61–74