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Regulation of mRNA Expression of Matrix Extracellular Phosphoglycoprotein (MEPE)/
Osteoblast/Osteocyte Factor 45 (OF45) by Fibroblast Growth Factor 2 in Cultures of Rat
Bone Marrow-derived Osteoblastic Cells

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Abstract

Matrix extracellular phosphoglycoprotein (MEPE)/ osteoblast/osteocyte factor 45 (OF45) is a recently isolated RGD-containing matrix protein that acts as the tumor-derived phosphaturic factor in oncogenic hypophosphatemic osteomalacia. It is also highly expressed by osteoblasts and osteocytes. We examined the regulation of MEPE/OF45 mRNA expression in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells incubated with dexamethasone, β -glycerophosphate and ascorbic acid. The level of MEPE/OF45 mRNA in these cells was down-regulated by the addition of fibroblast growth factor 2 (FGF2) for 48 h. These effects were observed in a dose-dependent manner between 2 and 10 ng/ml. FGF2 also reduced the expression of osteocalcin mRNA in these cells. In contrast, bone sialoprotein mRNA expression was increased by FGF2, while α 1(I) procollagen mRNA expression was not altered. Additionally, neither Runx-2 and osterix mRNA expression nor cell proliferation were affected by the addition of FGF2 in these high-density cultures, indicating that regulation by FGF2 may not be dependent on these transcription factors or on the proliferation of cells. Experiments using actinomycin D indicated that FGF2 decreased the stability of the MEPE/OF45 mRNA. Moreover, inhibition of a specific

mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase kinase (MEK) by PD098059 blocked FGF2-regulated MEPE/OF45 expressions, indicating that this regulation requires the MAPK pathway. These results suggest that MEPE/OF45 gene is one of the targets of FGF2 and may play an important role during bone formation and calcification.

Keywords: MEPE/OF45; FGF2; rat bone marrow stromal cells; osteoblast.

Introduction

Bone formation and remodeling are complex processes that involve the recruitment of osteoprogenitor cells, their proliferation, and terminal differentiation into osteoblasts, resulting in the secretion of abundant bone matrix proteins that coordinate the mineralization process. Elucidation of the molecular mechanisms controlling function and differentiation of osteoblasts has been one of the major subjects in bone biology. Extracellular signals including hormones, growth factors, cytokines, and extracellular matrix components, as well as their intracellular mediators, regulate cell differentiation or expression of phenotypes by modulating the activities of transcription factors involved in the expression of their respective target genes. The major component of the bone matrix protein of these cells is type I collagen. Additionally, non-collagenous matrix proteins have been identified that influence the processes of bone formation, remodeling and repair. The non-collagenous matrix includes proteins modified to contain Gla amino acid residues, proteins that are highly phosphorylated with an RGD-motif, glycoproteins, and proteoglycans. Examples include osteocalcin, osteopontin, bone sialoprotein (BSP), matrix glutamic acid protein, osteonectin, biglycan, and so on. The exact functions of

many of these proteins have not yet been delineated, although, for most, targeted deletion of these genes in mice provide evidence of a role in regulating mineralization events in bone remodeling (1).

Recently, matrix phosphoglycoprotein (MEPE) was isolated as the tumor-derived phosphaturic factor in oncogenic hypophosphatemic osteomalacia and was found to be highly expressed in osteoblasts and osteocytes (2). Petersen, et al. (3), also identified a cDNA for a serine/glycine-rich secreted peptide that contained numerous potential phosphorylation sites and one RGD sequence motif. The sequence was bone specific and coded for a matrix protein that they named osteoblast/osteocyte factor 45 (OF45). Similarities in nucleotide sequence between MEPE and OF45 revealed that MEPE and OF45 are the same protein. MEPE/OF45 is highly expressed in the tibia shaft and metaphysis as well as in osteoblasts from induced bone marrow stromal cells, calvaria, and the UMR106 osteoblastic cell line (3). Immunohistochemical studies indicated that abundant MEPE/OF45 protein is produced in osteocytes in the rat tibia (3). Furthermore, targeted disruption of the MEPE/OF45 gene in mice results in increased bone formation and bone mass (4). Although MEPE/OF45 thus appears to be a new

member of the bone matrix protein family, and, perhaps, is involved in regulation of bone metabolisms *in vivo*, regulation the expression of this gene in osteoblasts had not yet been investigated.

Fibroblast growth factor 2 (FGF2 or basic FGF) is a heparin-binding growth factor that occurs as several isoforms: An 18 kD cytoplasmic isoform and four larger molecular weight nuclear isoforms (22, 22.5, 24 and 34 kD). FGF2 has pleiotropic roles in many cell types and tissues; it is mitogenic, angiogenic and a survival factor that is involved in cell migration, cell differentiation, and in a variety of developmental processes in a large range of cells and tissues including bone (5). FGF2 is not essential for embryonic development as null mice for this growth factor are viable and fertile although they exhibit abnormalities of neuronal differentiation. Osteoblasts synthesize FGF2 and store it in a bioactive form in the extracellular matrix of bone (6). Additionally, in primates, local exposure to FGF2 has been shown to increase bone formation in an experimental long-bone defect (7). The FGF family of molecules transduces signals to the cytoplasm via a family of transmembrane receptors with tyrosine kinase activity, and four distinct FGF receptors (FGFRs: FGFR1-4) were identified. Mutations in FGFRs

have been linked to a number of human autosomal dominant skeletal disorders including craniosynostosis. Mutation in the FGFR1 gene is associated with Pfeiffer's syndrome, which is one of the classic craniosynostotic disorders. Moreover, mutations in FGFR2 and FGFR3 are responsible for genetic disorders involving bone development, the Jackson-Weiss syndrome, Crouzon syndromes, and thanatophoric dysplasia. Analysis of FGFR3-deficient mice has revealed prolonged bone growth showing that FGFR3 is a negative regulator of bone growth (8) and thus providing further evidence that FGF and its signaling is involved in the developmental process of skeletogenesis.

In this report, we examined the effects of FGF2 on the regulation of MEPE/OF45 gene expression in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells incubated in the presence of dexamethasone, β -glycerophosphate and ascorbic acid. We now report that MEPE/OF45 mRNA expression in these cells was down-regulated by the addition of FGF2. We also show that suppression of MEPE/OF45 mRNA expression by FGF2 may require the activation of the mitogen activated protein kinase (MAPK) signaling pathway in these cells.

Results

FGF2 down-regulates MEPE/OF45 mRNA expression in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells

Rat bone marrow stromal cells have been reported to differentiate into several types of cells including osteoblasts, adipocytes, and so on, depending on the specific culture conditions. To have these stromal cells differentiate into osteoblastic cells, cultures were incubated in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate and 50 μ g/ml of ascorbic acid for 2 weeks. These culture conditions were previously shown to give cells that expressed numerous mRNAs characteristic of osteoblasts including bone matrix proteins such as BSP, osteocalcin and osteopontin. We also detected these mRNAs, as well as a high level of alkaline phosphatase activity and positive staining with Von Kossa's stain (data not shown); indicating that these cells differentiated into osteoblastic cells in culture. Then, we examined the effects of a 48 h treatment with FGF2 at concentrations from 2 to 10 ng/ml on the expression of MEPE/OF45 mRNA in these cells. RT-PCR analysis of total RNA isolated from these cells revealed the presence of transcripts of MEPE/OF45, and that this MEPE/OF45

mRNA level was dramatically and dose-dependently decreased by the addition of FGF2, while α 1(I) procollagen mRNA expression was not altered in a presence of 15% fetal bovine serum (FBS) (Fig. 1A). Osteocalcin mRNA levels also decreased with the addition of FGF2. In contrast, FGF2 enhanced BSP mRNA expression (Fig. 1A). As an internal control, mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found not to be altered by the addition of FGF2. To confirm the specific regulation of expression of the transcript by FGF2, we performed quantitative real-time RT-PCR analysis. The level of MEPE/OF45 mRNA was decreased to 30% by FGF2 within 48 h compared to vehicle controls (Fig. 1B). FGF2 also reduced the MEPE/OF45 mRNA level in a condition of serum starvation that the presence of 0.3% FBS in cultures (data not shown), suggesting that the effect of FBS or many growth factor containing FBS did not mediate the response of FGF2 in our study.

To further examine the specific regulation of expression of these transcripts by FGF2, we performed analyses of time dependent changes in these mRNAs in response to the addition of FGF2. Time dependent changes in MEPE/OF45, osteocalcin and BSP revealed that their expression was regulated after 24 h then remained constant up to 48 h

by RT-PCR analysis (Fig. 1C) and quantitative real-time RT-PCR analysis (Fig. 1D).

Since it has been shown that FGF2 stimulates cell growth and has mitogenic activity in several types of cells including osteoblasts in culture, we examined the effect of FGF2 on viable cell numbers and incorporation of 5-bromo-2'-deoxyuridine (BrdU) in these high-density cultures after 2 weeks in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate and 50 μ g/ml of ascorbic acid. We were unable to detect any increase in viable cells after the addition of FGF2 for 24 or 48 h as detected by the MTT method (Fig. 2A). Also, FGF2 at concentrations from 2 to 10 ng/ml did not change the incorporation of BrdU by the cell proliferation assay (Fig. 2B). Furthermore, the addition of FGF2 did not alter cellular morphologies in these cultures (Fig. 2C).

In Runx-2 or osterix null mice, differentiation of osteoblasts is arrested in both endochondral and intramembranous calcification of bone (9, 10), indicating that Runx-2 and osterix are transcription factors required for osteoblastic differentiation and bone formation. To elucidate the regulation of Runx-2 and osterix mRNA expression by FGF2 in our high-density cultures of osteoblasts, RT-PCR analysis was performed. Neither Runx-2 nor osterix mRNA levels changed with the addition of 10 ng/ml of FGF2 for 6 or

12 h in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate and 50 μ g/ml of ascorbic acid (Fig. 3).

We next investigated if MEPE/OF45 mRNA expression and its regulation by FGF2 in these cells depend on culture time. MEPE/OF45 mRNA expression in response to the presence of dexamethasone, β -glycerophosphate and ascorbic acid was examined over 3 weeks and was found to be increased after 2 weeks and to remained high at 3 weeks in culture. Osteocalcin mRNA expression was also up regulated over the 3 weeks in a similar manner. Moreover, mRNA expression of Runx2 and osterix also increased over the 3 weeks of culture (Fig. 4A). By Northern blot analysis, FGF2 down-regulated MEPE/OF45 mRNA expression in the cells after both one (undifferentiated osteoblastic cells) (lane 2 vs. lane 1, Fig. 4B) and two weeks in cultures (lane 4 vs. lane 3, Fig. 4B). In contrast, MEPE/OF45 mRNA expression was not altered by FGF2 in highly differentiated osteoblastic cells of three weeks in cultures (lane 6 vs. lane 5, Fig. 4B).

Moreover, MC3T3-E1 cells of murine calvaria-derived osteoblastic cell line, FGF2 also repressed the level of MEPE/OF45 mRNA expression cultured for 48 h by both quantitative real-time RT-PCR analysis (Fig. 5A) and RT-PCR analysis (Fig. 5B),

indicating that this regulation occurs across species. These results also indicated that FGF2 regulates MEPE/OF45 mRNA expression in not only bone marrow derived osteoblastic cells but also calvaria-derived osteoblastic cells.

Involvement of RNA degradation, protein synthesis, the MAPK signaling pathway in the regulation of MEPE/OF45 mRNA expression by FGF2 in osteoblastic cell derived high-density cultures of primary rat bone marrow stromal cells

To clarify whether FGF2 affected at the level of gene transcription or RNA degradation of MEPE/OF45 mRNA, assay of RNA degradation using inhibitor of RNA transcription was performed. Although the treatment effective dose of actinomycin D (0.1 µg/ml) to block RNA synthesis in the cultures, the amount of MEPE/OF45 mRNA in addition with FGF2 was also reduced compared to that of vehicle by quantitative real-time RT-PCR analysis, indicating that FGF2 might not reduce transcription of MEPE/OF45 gene but decrease RNA stability of MEPE/OF45 mRNA (Fig. 6A). Next, to examine the potential role of protein synthesis in the down-regulation of MEPE/OF45 mRNA expression by FGF2, we used 10 µg/ml of cycloheximide to block *de novo*

synthesis of protein. After the addition of FGF2 with cycloheximide, the level of MEPE/OF45 mRNA expression was reduced by the same amounts as occurred with FGF2 without cycloheximide by quantitative real-time RT-PCR analysis (Fig. 6B), indicating that the regulation of MEPE/OF45 mRNA level by FGF2 does not depend upon new protein synthesis.

The MAPK signaling pathway was previously reported to mediate changes in the expression of several mRNAs in response to certain growth factors in osteoblast-like cells. To address the involvement of MAPK in the mechanism by which FGF2 reduces MEPE/OF45 mRNA expression in these cells, we used three MAPK inhibitors; PD98059 to block MEK, U0126 to block activation of extracellular signal-regulated kinase (ERK) 1/2, and SB203580 to block p38 MAPK activity. With regard to the concentration of each inhibitor, we examined MAPK responsive gene expressions by quantitative real-time RT-PCR analyses to estimate each effective dose in our cultures (data not shown) and used in our previous study (11). After the addition of 50 μ M PD98059, the down-regulation of MEPE/OF45 mRNA expression by FGF2 was not observed by RT-PCR analysis (Fig. 6C) and quantitative real-time PCR analysis (Fig. 6D). Also, the

MEPE/OF45 mRNA level did not suppress in the addition of 100 μ M PD98059 (data not shown). However, the addition of 10 μ M SB203580 and 20 μ M U0126 in the absence of FGF2 decreased steady-state MEPE/OF45 mRNA levels. This decrease in MEPE/OF45 mRNA levels was blocked by the co-addition of FGF2 (Fig. 6C).

Discussion

The bone marrow stromal cell population contains mesenchymal stem cells and several *in vitro* studies indicated that under appropriate conditions, common pluripotential stem cells might give rise to the osteogenic, chondrogenic, adipogenic, and stromal cell lineages (12). Rat bone marrow stromal cells cultured in the presence of dexamethasone, β -glycerophosphate and ascorbic acid have been shown to express numerous markers of the osteoblast, including alkaline phosphatase, BSP, osteocalcin and osteopontin (13), and to form mineralized bone-like nodules (14). These observations, led us to use stromal cells cultured under the reported conditions for 2 weeks. These cultures contained functional osteoblasts as defined by the capability to facilitate hydroxyapatite mineral formation. MEPE/OF45 mRNA was detected at very low levels in rat bone marrow stromal cell culture without dexamethasone, β -glycerophosphate and ascorbic acid (data not shown) but was increased by the addition of these differentiation agents. The induction continued over time in our culture with these agents, indicating that MEPE/OF45 mRNA expression increased with each increment of differentiation in the osteoblast lineage. Peterson, et al. (3), also showed that MEPE/OF45 mRNA was barely detectable under basal culture conditions in UMR106 cells, but was greatly increased

when cells were maintained at confluence in the presence of dexamethasone. They have shown that specific immunostaining with antibodies to MEPE/OF45 was detected in osteocytic cells on both trabecular and cortical bone surfaces, indicating the presence of MEPE/OF45 expression in highly differentiated osteoblasts in normal adult bone tissues. Moreover, cells of the hematopoietic lineage contained in the marrow compartment were negative for MEPE/OF45 staining. Finally, with the finding that MEPE/OF45 mRNA expression increases with cellular progression along the osteoblast lineage, the totality of evidence indicates that MEPE/OF45 is a specific marker of the osteoblast.

MEPE/OF45 gene null mice exhibited increased bone formation and bone mass, as well as an increased osteoblast cell number and activity, while osteoclastogenesis and bone resorption was unaffected, demonstrating that MEPE/OF45 plays an inhibitory role in bone formation (4). Also, osteocalcin null mice have been reported to have an increased bone formation (15). One interpretation of these observations is that MEPE/OF45 and osteocalcin function *in vivo* as a negative regulator of osteoblastic activity. FGF2 was found to have inhibitory effects on both MEPE/OF45 and osteocalcin mRNA expression in our study which opens the possibility that FGF2 may be a positive

regulator of osteoblast activity in bone tissue. Moreover, both bone matrix proteins may play a similar role during matrix mineralization and bone formation, processes regulated by FGF2.

In general, the amount of mRNA is regulated by either the rate of mRNA transcription or mRNA stability. We showed that FGF2 suppresses MEPE/OF45, that is a specific target of FGF regulation, and that FGF2 produced divergent effects on mRNA expression of bone matrix proteins such as osteocalcin and BSP. Shimizu-Sasaki, et al. identified an FGF response element (FRE) in the BSP promoter containing the core AP-1 like sequence GGTGAGAA (16). In the present work, induction of BSP mRNA expression by FGF2 might involve this AP-1 like sequence in rat BSP promoter. Instead, Boudreaux and Towler showed that an AP-1 like site in the osteocalcin promoter (GCACTCA) is necessary for the stimulation of osteocalcin expression and its promoter activity by FGF2 (17). However, we observed that FGF2 decreases MEPE/OF45 mRNA levels predominantly by decreasing MEPE/OF45 mRNA stability and this message stability appears to be mediated through a protein synthesis-independent mechanism. Recently, Tessner et al. (18), showed that FGF2 treatment altered cyclooxygenase-2

mRNA stability in an intestinal cell line I407. These findings suggested that the FGF2-responsiveness of MEPE/OF45 gene might not be mediated by the responsive elements in its gene promoter and its transcription.

FGF2 has been shown to stimulate proliferation of cells including osteoblasts and bone marrow stromal cells and, as a consequence, it was suggested that the regulation of bone matrix protein synthesis by FGF2 was direct due to increased cell replication (6). Disruption of the FGF2 gene in mice results in decreased osteoblast replication, decreased mineralized nodule formation in bone marrow stromal cell cultures, and decreased new bone formation *in vivo* (19). In this study, since osteoblastic cell derived high-density cultures were over-confluent; FGF2 might not stimulate proliferation of cells with contact inhibition. Furthermore, regulation of MEPE/OF45, BSP and osteocalcin mRNA expression by FGF2 might not depend upon cell proliferation in these cultures.

Although *in vivo* studies have clearly established an essential role for FGF2 in bone formation, the mechanism of FGF2 action in osteoblasts is not well understood and appears to be complex, involving multiple signaling pathways and factors. The cellular

actions of the FGFs are known to be mediated by interactions with FGFRs; FGFs binding to FGFRs induce intrinsic activation of multiple signal transduction pathways including Raf, MAPK, MEK/ERK, p38 MAP kinases and protein kinase C (6). Since members of the MAPK family of ERK1/2 and p38 MAP kinases have been implicated in both cell proliferation and differentiation in osteoblasts, it was likely that these kinases were also involved in FGF2 signaling (6). We showed that activation of the MEK/ERK pathway involves the down-regulation of MEPE/OF45 mRNA expression by FGF2, suggesting that FGF2 regulates MEK/ERK activity in these cultures. Shimizu-Sasaki, et al. (16) also showed that induction of BSP mRNA expression by FGF2 in osteoblasts mediated the MEK/ERK branch of the MAPK pathway.

MEPE/OF45 mRNA down-regulated by FGF2 might mediate neither Runx2 nor osterix transcription factor related osteoblastic differentiation in these cells. In contrast to our results, Tsuji and Noda (20) showed that FGF2 transiently suppressed Runx-2 expression in ROS17/2.8 cells derived from a rat osteosarcoma. In this study, FGF2 also down-regulated MEPE/OF45 mRNA expression in the cells in undifferentiated (culture for 1 week) rat bone marrow-derived osteoblastic cells and

MC3T3-E1 cells of murine calvaria-derived osteoblastic cell line (21). In contrast, FGF2 did not alter the expression of MEPE/OF45 mRNA in highly differentiated rat bone marrow-derived osteoblastic cells (culture for 3 weeks). These differences in response may simply reflect experimental differences (i.e., different cell types) or may be explained if FGF2 action might depend on the stage of osteoblast differentiation. Further studies will be required to understand this regulatory mechanism.

Finally, there is evidence for a hormone/enzyme/extracellular matrix protein cascade involving fibroblastic growth factor 23 (FGF23), a phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), and MEPE/OF45 that regulates systemic phosphate homeostasis and mineralization (22). FGF 23, with significant homology to proteins within FGF family, have identified as an abundantly expressed gene in a tumor-induced osteomalasia (TIO). Several observations indicated that FGF23 is a phosphaturic hormone and is causing the hypophosphatemia and that FGF23 binds to FGFR2C, which is expressed in osteoblasts (23). Also, MEPE/OF45 was isolated from a TIO tumor and thought to be a candidate for phosphatonin (22). At present, it is not clear whether FGF23 regulates MEPE/OF45 production in osteoblasts.

The current findings that FGF2 regulates MEPE mRNA expression suggest FGF23 may regulate MEPE/OF45 expression using FGF receptors in osteoblasts. Indeed, MEPE/OF45 expression has been shown to highly correlate with increased FGF23 in parathyroidectomized Hyp mice (24).

In conclusion, MEPE/OF45 is down-regulated by the addition of FGF2 in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells. The results of this study indicate that the MEPE/OF45 regulated by FGF2 has putative physiological significance for cellular functions in osteoblasts and the physiological role of this regulatory process may be important in the control of matrix calcification of cells in bone tissue.

Materials and Methods

Cell cultures

Rat bone marrow stromal cells were prepared according to the method of Maniatopoulos, et al. (14), with some modifications (11). In brief, femora were aseptically removed from male Wistar rats (6 weeks old) after sacrifice under ether anesthesia. The adherent soft tissues were removed, and the bone was washed five times with phosphate buffered saline. After both ends of the bone were removed, the bone marrow cavity was flushed out with 6 ml of alpha modified essential medium (α -MEM; Invitrogen, Carlsbad, CA.) supplemented with 15% (v/v) fetal bovine serum (FBS) (HyClone, UT, USA) and 60 μ g/ml of kanamycin (Nacalai tesque, Kyoto, Japan). The cells were separated by repeated aspiration through a 21-gage needle, and collected by centrifugation of 1,000g for 3 min at 4°C. The cells were resuspended and then inoculated into 24-well plates or 25 cm² cell culture flasks (Corning, New York, NY) at a cell density of 1.5 X 10⁶ cells/cm². The cells were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂. After culture for 24 h, the entire medium and floating cells were removed by aspiration and fresh medium added that was supplemented with

10^{-8} M dexamethasone (Wako, Osaka, Japan), 1 mM β -glycerophosphate (Wako, Osaka, Japan), and 50 μ g/ml of ascorbic acid (Wako, Osaka, Japan). Cultures were maintained for several weeks in this medium with changes every 2 days, and were used for the following experiments. This study was carried out in accordance with the guidelines on the care and use of laboratory animals issued by Hokkaido University. MC3T3-E1 cells (21) were purchased from RIKEN Cell Bank (Tsukuba, Japan), and were cultured in α -MEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO₂ in air.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. Complementary DNA was synthesized with Omniscript reverse transcriptase (Qiagen) using a (dT)₁₅ primer (1 μ M) according to the manufacturer's instructions. Amplification by PCR of MEPE/OF45, BSP, osteocalcin and α 1(I) procollagen cDNAs was performed for 25 cycles under the following conditions: 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min followed by a final

heating at 72°C for 7 min. Runx-2 and osterix cDNAs were amplified with a PCR profile of 94°C for 30 sec, 45°C for 30 sec and 72°C for 1 min for 30 cycles followed by heating at 72°C for 7 min. GAPDH cDNA was amplified for 23 cycles under the following conditions: 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min. The primer sequences used for PCR amplification were designed based on cDNA sequences of mRNA for rat MEPE/OF45 (3) (GenBank NM_024142, 1,116-1,426 nt), murine MEPE/OF45 (25) (GenBank NM_053172, 939-1,231 nt), rat BSP (26) (GenBank NM_012587, 288-733 nt), rat osteocalcin (27) (GenBank NM_013414, 138-471 nt), rat α 1(I) procollagen (28) (GenBank Z78279, 5,406-5,613 nt), murine Runx2 (29) (GenBank NM_009820, 1,407-1,715 nt), rat osterix (10) (GenBank AY177399, 340-726 bp), and rat GAPDH (30) (GenBank NM_017008, 34-333 nt). The primer sequences used were 5'-GCAAAGGCCAGTCTTCTCAC-3' and 5'-CTGCTGCGTCCTCTCTTTCT-3' for rat MEPE/OF45, generating a 311 bp fragment; 5'-ACTATCCACAAGTGGCCTCG-3' and 5'-CCGCTGTGACATCCCTTTAT-3' for murine MEPE/OF45, generating a 293 bp fragment; 5'-ATGGAGATGGCGATAGTTTCG-3' and 5'-TGCTGTTGTTTCCTTCTGCAC-3' for BSP, generating a 446 bp fragment; 5'-

TGACAAAGCCTTCATGTCCA -3' and 5'- ACTTTATTTTGGAGCAGCTG -3' for osteocalcin, generating a 334 bp fragment; 5'-AGGAGAGAGTGCCAACTCCA-3' and 5'-CCACCCCAGGGATAAAAAC-3' for α 1(I) procollagen, generating a 208 bp fragment, 5'-CCCAGCCACCTTTACCTACA-3' and 5'-TCAGCGTCAACACCATCATT-3' for Runx2, generating a 309 bp fragment, 5'-AGCTCTTCTGACTGTCTGCC-3' and 5'-CACC ACTCCC YTCYAGYTGC-3' for osterix, generating a 387 bp fragment (Y=C plus T), 5'-GTGAAGGTCGGTGTC AACG-3' and 5'-GGTGAAGACGCCAGTAGACTC-3' for GAPDH, generating a 300 bp fragment. For subsequent use, amplified PCR products were subcloned into the pGEM[®]-T Vector (Promega, Madison, WI) using the pGEM[®]-T Vector System II and the sequences of the subcloned cDNAs were checked by a DNA sequencer (Prism 310, Applied Biosystems, Foster city, CA). All the primers were synthesized by Hokkaido System Science (Sapporo, Japan). Amplified products were visualized by agarose gel electrophoresis after staining with ethidium bromide. PCR experiments were performed using samples from at least three different cell preparations and the results were confirmed by triplicate PCR experiments on each of the three cell

samples.

Quantitative real-time RT-PCR

Quantifications of MEPE/OF45, BSP and osteocalcin mRNA were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described previously (31). One μg of each RNA sample was subjected to reverse transcription using the omniscript RT kit (Qiagen) and RNase-free DNase Set (Qiagen) in a total volume of 20 μl . Then, 2.5 μl of the reaction mixture were incubated with the double-stranded DNA dye SYBR Green I (Qiagen) in a total volume of 25 μl . The primers used for detection were as described in above. All reactions were run in with a hot start pre-incubation step of 10 min at 95°C, following by cycles of 15 sec at 95°C, 1 min at 60°C. The amount of template was quantified using the comparative cycle threshold method as outlined in the manufacturer's technical bulletin. Measured mRNA levels were normalized to the mRNA copies of GAPDH. We performed these experiments using samples from at least three different cell preparations and quantification of mRNA was confirmed using the same cell sample at least in triplicate.

Preparation of cDNA probes and Northern analysis

To construct DNA probes for detection of MEPE/OF45 mRNA, amplified PCR products were subcloned into the pGEM[®]-T Vector and we confirmed the sequence of DNA fragments inserted in the vector by DNA sequencer. To generate DNA probe, plasmid was linearized with EcoRI and then labeled with [α -³²P]dCTP using a random primer DNA labeling kit (Takara Biomedicals, Shiga, Japan). The total RNAs were isolated from the cells by Isogen, and then equal amounts of total RNA (15 μ g) were electrophoresed in 1% agarose gel containing 2.2 M formaldehyde then transferred to Hybond-N⁺ nylon membranes (Amersham). The membranes were prehybridized at 65°C for 30 min in the rapid hybridization buffer (Amersham). Then hybridization was carried out for 2 h at 65°C in the same buffer with [α -³²P]-labeled DNA probe. Subsequently, the blots were washed in low-stringency wash solution at room temperature for 10 min and twice in high-stringency wash solution (0.1 X SSC containing 0.1% SDS) as described previously (32). Filter was exposed to x-ray film using intensifying screens (Du Pont-New England Nuclear) at -80°C for several days. Equal loading of RNA in each lane was checked by ethidium bromide staining.

MTT cell viability assay and cell proliferation assay

MTT assay is based on the ability of viable mitochondria to convert MTT [3-(4,5-dimethyl-thiazoyl-2-yl) 2, 5-diphenyltetrazolium bromide], a water-soluble tetrazolium salt, into a water-insoluble formazan precipitate. This conversion is catalyzed by a cellular mitochondrial dehydrogenase. Because the rate of this reaction is proportional to the number of surviving cells, the MTT assay is widely used to quantify viable cells. To observe the effect of FGF2 on cell viability, primary rat bone marrow stromal cells (cell density of 1.5×10^6 cells/cm²) were cultured with 10^{-8} M dexamethasone, 1 mM β -glycerophosphate and 50 μ g/ml of ascorbic acid for 2 weeks in 24-well plates, and then were exposed to 10 ng/ml of FGF2 for 24 or 48 h. Cells were washed and then incubated with 100 μ l of MTT at 37°C for 30 min. The reagent was removed, and 100 μ l of isopropanol/HCl (400 μ l of HCl plus 100 ml of isopropanol) was added to each well. The solution was mixed and the absorbance of each well was determined at 570 nm using a micro plate reader (Tosoh, Shinnanyou, Japan).

The rate of cell proliferation was measured by incorporation of BrdU with the cell proliferation assay kit (Amersham), according to the manufacturer's instructions.

Briefly, cells in a 24-well cell culture plate were added with 2-10 ng/ml of FGF2 or vehicle for 24 h, BrdU was then added and the cells were incubated for a further 2 h. Incorporated BrdU was determined by a specific monoclonal antibody and a peroxidase-conjugated secondary antibody. Incubation with chromogen peroxidase substrate yielded a soluble green dye, readable as absorbance at 450 nm on a micro plate reader. All MTT assays and cell proliferation assay were conducted in triplicate on each of at least three different cell preparations.

Reagents

Recombinant human FGF2 was purchased from Progen Biotechnik (Heidelberg, Germany). PD98059 (2'-amino-3'-methoxyflavone), dimethyl sulphoxide (DMSO), cycloheximide, actinomycin D and MTT were purchased from Sigma. U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-amino-phenylthio)butadiene] and SB203580 [4-(4'-fluorophenyl)-2-(4'-methyl-sulfinylphenyl)-5-(4'pyridyl)imidazole] were purchased from Promega.

Statistical analysis

All experiments were repeated three to four times and representative results are presented. The data are reported as the mean \pm SD, and were analyzed by the Student's t-test, where values of $p < 0.05$ were considered significant.

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Figure Legends

Fig. 1. Dose and time dependent changes in mRNA expressions of MEPE/OF45, BSP, osteocalcin and $\alpha 1(I)$ procollagen in osteoblastic cells derived high-density cultures of primary rat bone marrow stromal cells with the addition of FGF2.

Rat bone marrow stromal cells were plated at 1.5×10^6 cells/cm² in 25 cm² cell culture flasks in presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate, and 50 μ g/ml of ascorbic acid. After 2 weeks, the medium was changed and either 2-10 ng/ml of FGF2 or vehicle was added in α -MEM supplemented with 15% FBS (A and B), after which cells were cultured for a further 48 h. After 2 weeks, the medium was changed and 10 ng/ml of FGF2 was added in α -MEM supplemented with 15% FBS, after which the cells were cultured for a further 0-48 h (C and D). Total cellular RNA was isolated, and then RT-PCR analysis and quantitative real-time PCR analysis were performed as described in Materials and Methods. PCR was carried out with primers for rat MEPE/OF45, BSP, osteocalcin and $\alpha 1(I)$ procollagen and GAPDH cDNA. The PCR products were separated on 2% agarose gels containing ethidium bromide, and then observed on an ultraviolet transilluminator. The data shown represent one of three independent experiments with

similar results. The levels of MEPE/OF45 mRNA were analyzed by quantitative real-time PCR analysis (B and D). Fluorescent signals were detected by a sequence detection system and then each mRNA levels were normalized to the mRNA copies of GAPDH. Other experimental conditions were as described in Materials and Methods.

*significant difference from vehicle (without FGF2), $p < 0.05$.

Fig. 2. Cell viability, cell proliferation and cell morphology of osteoblastic cells derived high-density cultures of primary rat bone marrow stromal cells with the addition of FGF2. Rat bone marrow stromal cells were plated at 1.5×10^6 cells/cm² in 24 well plates in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate, and 50 μ g/ml of ascorbic acid. After 2 weeks, the medium was changed and either 10 ng/ml of FGF2 (+) or vehicle (-) was added in the presence of 15% FBS, after which the cells were cultured for a further 24 or 48 h. (A) Cell viabilities were measured by the MTT method as described in Materials and Methods. (B) The cells were labeled with BrdU for 2 h. The rate of proliferation of the cells is expressed as a ratio of that of the vehicle. No significant difference from vehicle as a control ($p > 0.05$). (C) Photomicrographs of cells,

magnification X 20.

Fig. 3. Effect of FGF2 on the mRNA expression of Runx-2 and osterix in osteoblastic cells derived high-density cultures of primary rat bone marrow stromal cells.

Rat bone marrow stromal cells were plated at 1.5×10^6 cells/cm² in 25 cm² cell culture flasks in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate, and 50 μ g/ml of ascorbic acid. After 2 weeks, the medium was changed and 10 ng/ml of FGF2 (+) or vehicle (-) was added, after which the cells were cultured for a further 0-12 h. Then total cellular RNA was isolated, and RT-PCR analysis was performed as described in Materials and Methods. The data shown represent one of three independent experiments with similar results.

Fig. 4. Time dependent change of MEPE/OF45, osteocalcin, Runx2 and osterix mRNA expression and regulation of MEPE/OF45 mRNA expression by FGF2 in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells.

Rat bone marrow stromal cells were plated at 1.5×10^6 cells/cm² in 25 cm² cell culture

flasks in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate, and 50 μ g/ml of ascorbic acid. (A) After which, the cells were cultured for 1, 2 and 3 weeks, total cellular RNA was isolated, and RT-PCR analysis was performed as described in Materials and Methods. (B) After which, the cells were cultured for 1, 2 or 3 weeks, the medium was changed and 10 ng/ml of FGF2 (+) or vehicle (-) was added, after which the cells were cultured for a further 48 h. Then total cellular RNA was isolated, and Northern blot analysis was performed with 32 P-labeled MEPE/OF45 cDNA as a probe as described in Materials and Methods (upper). Equal loading in lanes was checked by 28S and 18S ribosomal RNA (lower). The data shown represent one of three independent experiments with similar results.

Fig.5. Regulation of MEPE/OF45 mRNA expressions in MC3T3-E1 cells by FGF2.

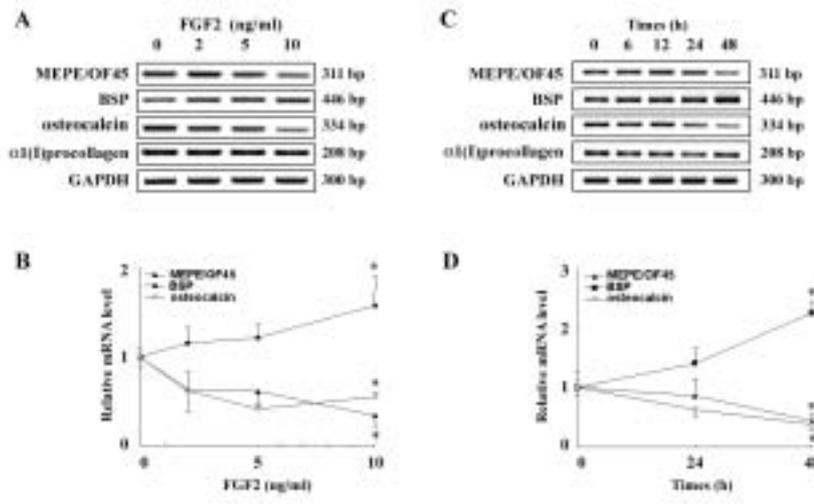
MC3T3-E1 cells were plated at 1.5×10^5 cells/cm² in 100 mm culture dish. After cells reached confluence, the medium was changed and 10 ng/ml of FGF2 (+) or vehicle (-) was added, after which cells were cultured for a further 48 h. Total cellular RNA was isolated, and then the level of MEPE/OF45 mRNA was analyzed using primers of murine

MEPE/OF45 sequences by quantitative real-time PCR analysis (A) and RT-PCR analysis (B). Other experimental conditions were as described in Materials and Methods. The data shown represent one of three independent experiments with similar results. *significant difference from vehicle (without FGF2), $p < 0.01$.

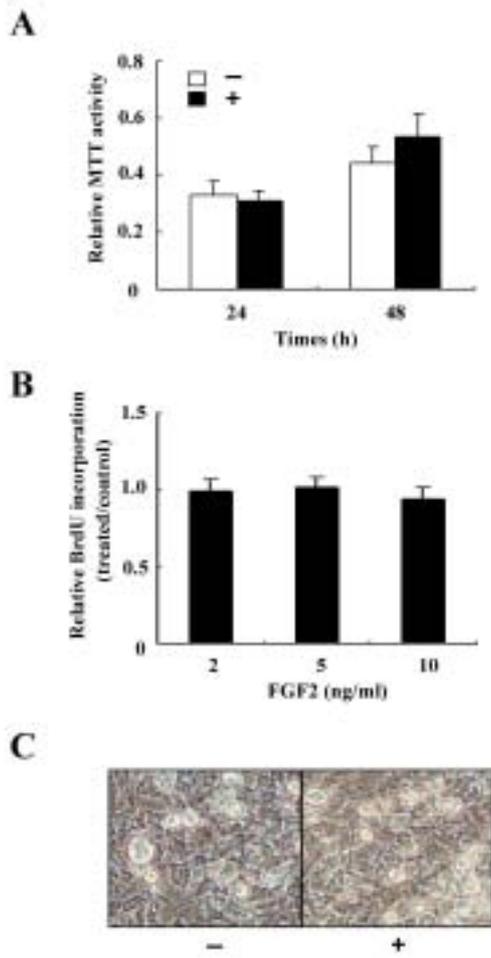
Fig. 6. Effects of Actinomycin D, cycloheximide and MAPK inhibitors on FGF2-regulated MEPE/OF45 mRNA expression in osteoblastic cells derived from high-density cultures of primary rat bone marrow stromal cells.

Rat bone marrow stromal cells were plated at 1.5×10^6 cells/cm² in 25 cm² cell culture flasks in the presence of 10^{-8} M dexamethasone, 1 mM β -glycerophosphate, and 50 μ g/ml of ascorbic acid. After 2 weeks, the cells were cultured for 48 h with 10 ng/ml of FGF2 in the presence or absence of 0.1 μ g/ml of actinomycin D, 10 μ g/ml of cycloheximide, 50 μ M PD98059, 20 μ M U0126, or 10 μ M SB203580. Then, total cellular RNA was isolated, and quantitative real-time PCR analysis (A, B and D) or RT-PCR analysis (C) was performed as described in Materials and Methods. These data were evaluated from one of three independent experiments with similar results. *significant difference from vehicle

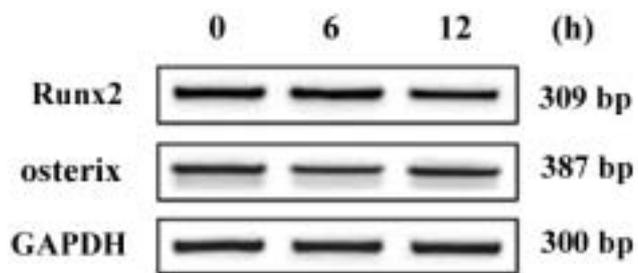
(without FGF2), $p < 0.05$.



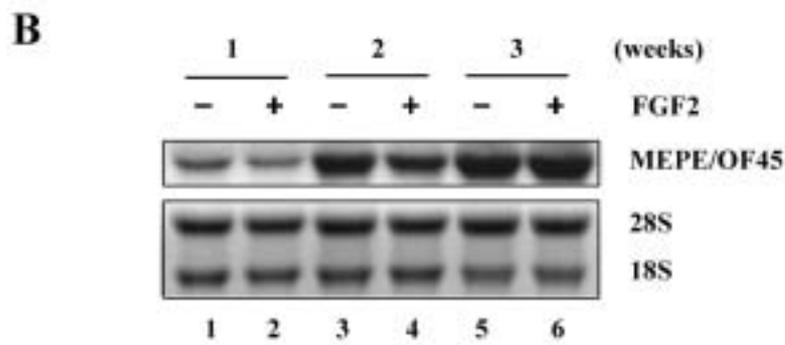
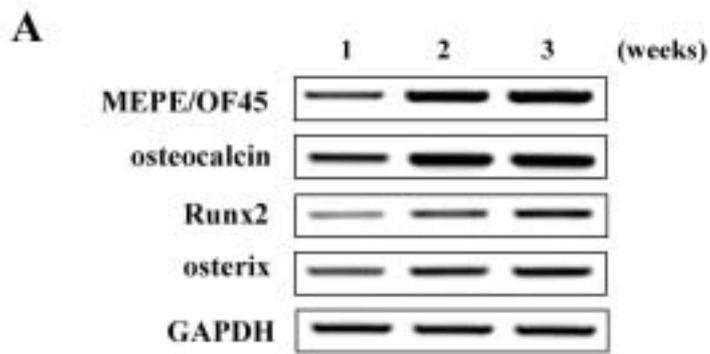
Zhang et al,
Fig. 1



Zhang et al.,
Fig. 2

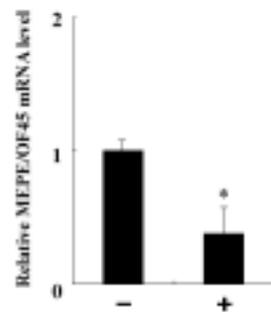


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Fig. 3

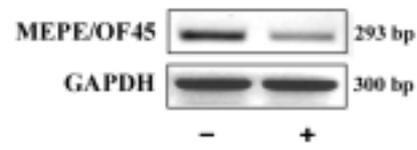


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Fig. 4

A

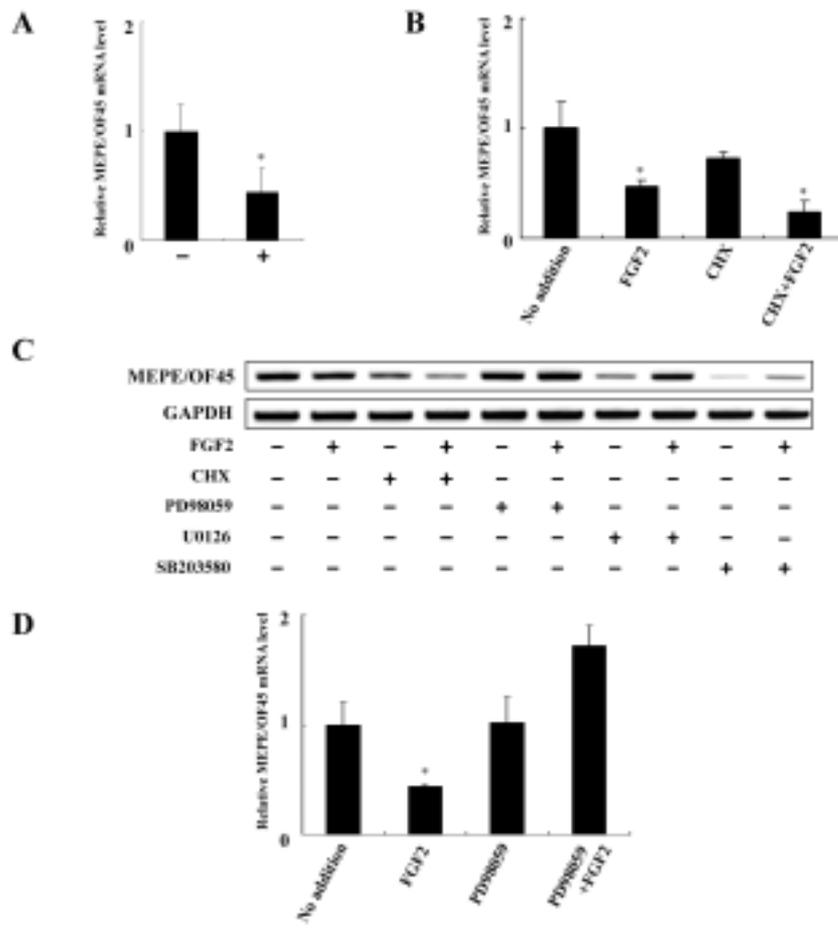


B



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Fig. 5



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Fig. 6

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