Regulatory effects of fibroblast growth factor-8 and tumor necrosis factor-α on osteoblast marker expression induced by bone morphogenetic protein-2.

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Abbreviations:  
ALK, activin receptor-like kinase  
ActRII, activin type-II receptor  
ALP, alkaline phosphatase  
BMP, bone morphogenetic protein  
BMPRII, BMP type-II receptor  
ERK, extracellular signal-regulated kinase  
FGF, fibroblast growth factor  
FGFR, FGF receptor  
MAPK, mitogen-activated protein kinase  
OA, osteoarthritis  
RA, rheumatoid arthritis  
RANK, receptor activator of NFκB  
Runx2, runt-related transcription factor 2  
SAPK/JNK, stress-activated protein kinase / c-Jun NH2-terminal kinase  
TGF-β1, transforming growth factor-β  
TNF, tumor necrosis factor  
TNFR, TNF receptor
**Abstract**

BMP induces osteoblast differentiation, whereas a key proinflammatory cytokine, TNF-α, causes inflammatory bone damage shown in rheumatoid arthritis. FGF molecules are known to be involved in bone homeostasis. However, the effects of FGF-8 on osteoblast differentiation and the interaction between FGF-8, BMPs and TNF-α have yet to be clarified. Here we investigated the effects of FGF-8 in relation to TNF-α actions on BMP-2-induced osteoblast marker expression using myoblast cell line C2C12, osteoblast precursor cell line MC3T3-E1 and rat calvarial osteoblasts. It was revealed that FGF-8 inhibited BMP-2-induced expression of osteoblast differentiation markers, including Runx2, osteocalcin, alkaline phosphatase, type-1 collagen and osterix, in a concentration-dependent manner. The inhibitory effects of FGF-8 on BMP-induced osteoblast differentiation and Smad1/5/8 activation were enhanced in the presence of TNF-α action. FGF-8 also inhibited BMP-2-induced expression of Wnt5a, which activates a non-canonical Wnt signaling pathway. FGF-8 had no significant influence on the expression levels of TNF receptors, while FGF-8 suppressed the expression of inhibitory Smad6 and Smad7, suggesting a possible feedback activity through FGF to BMP receptor (BMPR) signaling. Of note, inhibition of ERK activity and FGF receptor (FGFR)-dependent protein kinase, but not JNK or NFκB pathway, suppressed the FGF-8 actions on BMP-induced osteoblast differentiation. FGF-8 was revealed to suppress BMP-induced osteoblast differentiation through the
ERK pathway and the effects were enhanced by TNF-α. Given the finding that FGF-8 expression was increased in synovial tissues of rheumatoid arthritis, the functional interaction between FGFR and BMPR signaling may be involved in the development process of inflammatory bone damage.
Introduction

Bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily, have various activities including cell proliferation, differentiation [1] and endocrine regulation [2]. BMP activity is critical for the differentiation of mesenchymal stem cells into chondrocytes and/or osteoblasts [3].

Cooperative activities of osteoblasts and osteoclasts are crucial for maintenance of bone remodeling. Disruption of the functional balance between these cells leads to impaired bone formation and abnormal bone loss [4]. In rheumatoid arthritis (RA), increased levels and activities of proinflammatory cytokines such as tumor necrosis factor (TNF)-α result in joint and/or bone destruction. A paradigm shift in the treatment of RA has been brought about by the clinical application of TNF-α inhibitors [5, 6]. TNF-α is known to be a stimulator of osteoclast differentiation. NFκB, one of main signal pathways activated by TNF-α, is essential for RANK- and cytokine-mediated osteoclastogenesis [7]. Moreover, TNF-α not only activates osteoclasts but also inhibits osteoblast maturation [4].

The fibroblast growth factor (FGF) family is composed of 25 related proteins that are involved in many biological processes including cell growth, migration, differentiation and survival. Mutations of FGF receptors cause several genetic skeletal dysmorphic syndromes, suggesting that FGF signaling is essential for bone metabolism [8, 9]. FGF-2, -8 and -18 have been implicated as pertinent contributing factors in bone and cartilage homeostasis [10]. In the
process of embryonic development, BMP and FGF signals interact to regulate cell differentiation in several organs. For instance, FGF-2 enhances the BMP-4 signal by suppressing the expression of a BMP antagonist, noggin, during skull development [11].

FGF-8 was originally cloned from a conditioned medium of an androgen-dependent mouse mammary carcinoma cell line [12]. In embryonic development, FGF-8 is known to regulate gastrulation and other steps of development [13]. In neurological development, FGF-8 is involved in the isthmic organizing activity and stabilizes or changes the expression of transcription factors in the mid/hindbrain region [14]. It was also reported that FGF-8 plays a role as one of key regulators of cardiovascular development [15]. FGF-8 has been shown to be a key regulator for limb development and cranial formation [16]. FGF-8 is expressed in the dorsal costal cartilage, periosteum, ribs, osteoblast compartment of calvarial bone, mandible, cortical bone, and growth plates of developing long bones in later embryonic development [17, 18].

However, the detailed mechanism underlying the action of FGF-8 in osteoblast differentiation remains to be elucidated. Previous reports indicated different actions of FGF-8 in the proliferation and differentiation phases of osteoblasts [19]. Of interest, FGF-8 was reported to enhance degradation of the extracellular matrix in osteoarthritis (OA) model rats [20]. There are also some reports suggesting involvement of FGFs, BMPs and inflammatory cytokines in both RA and OA [21, 22]. However, the interaction between FGF-8 and BMPs in the process of osteoblast differentiation and the relationship
between FGF-8 and proinflammatory cytokines in BMP-induced osteogenesis have not been determined.

In the present study, we investigated the functional crosstalk between FGF-8 and TNF-α receptor signaling, which acts against BMP-2-induced osteoblast differentiation. The results obtained in this study may provide a new clue for understanding and preventing the progress of inflammatory bone damages.
Methods

Reagents and supplies

Recombinant human BMP-2 and mouse FGF-8b were purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant human TNF-α was obtained from PeproTech EC Ltd. (London, UK). The MEK inhibitor U0126 was from Promega Corp. (Madison, WI), JNK inhibitor SP600125 was from Biomol Lab., Inc. (Plymouth Meeting, PA), inhibitor of the tyrosine kinase activity FGF receptor SU5402, and NFκB activation inhibitor IV were from Calbiochem (San Diego, CA). Total RNAs purified from the synovium resected from RA and normal patients were purchased from OriGene Technologies, Inc. (Rockville, MD).

Cell culture

The mouse myoblast cell line C2C12 and osteoblast precursor cell line MC3T3-E1 were cultured in high-glucose DMEM and MEM Alpha, respectively, at 37°C under a humid atmosphere with 5% CO₂. Each medium was supplemented with 10% FCS and penicillin-streptomycin solution. Rat calvarial osteoblast cells, dissociated from Sprague-Dawley rat embryos, were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and cultured in high-glucose DMEM supplemented with 10% FCS and growth SingleQuots™ kit (Lonza Walkersville) following the manufacturer’s protocol.

RNA extraction and quantitative real-time PCR analysis
To prepare total cellular RNA, cells were cultured in a 12-well plate and treated with the indicated concentrations of TNF-α and BMP-2 in combination with FGF-8, U0126, SP600125, SU5402 or NFκB inhibitor in serum-free DMEM. After 24- or 48-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). The extracted RNA (1.0 µg) was subjected to RT reaction using the First-Strand cDNA synthesis system® (Invitrogen Corp.). Primer pairs were selected from different exons of the corresponding genes as follows: Wnt5a, 1210-1230 and 1365-1385 (from NM_009524); and FGF-8, 46-66 and 159-179 (from U36223). Primer pairs for osteocalcin, osterix, alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), type-1 collagen (collagen-1), Id-1, TNF receptor 1 (TNFR1) and TNFR2, BMP type-I and type-II receptors, Smads and a house-keeping gene ribosomal protein L19 (RPL19) were selected as we reported [23, 24] (Table 1). Real-time PCR was performed using the StepOnePlus® real-time PCR system (Applied Biosystems, Foster City, CA) under optimized annealing conditions, according to the manufacturer’s protocol with the following profile: 40 cycles each at 95°C for 3 sec and 58-62°C for 30 sec. Ct values were calculated using StepOnePlus™ system software (Applied Biosystems). The relative expression of each mRNA was calculated by the ΔCt Method, in which ΔCt is the value obtained by subtracting the Ct value of RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to RLP19 mRNA was expressed as $2^{-\Delta C_t}$. Data are expressed as the ratio of target mRNA to RPL19 mRNA.
Western immunoblot analysis

After preculture, the medium was replaced with fresh serum-free medium, and after 16-h culture, cells were treated with the indicated concentrations of BMP-2, TNF-α and FGF-8. Following stimulation with growth factors for 15 to 60 min, the cells were solubilized in 100 µl RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) containing 1 mM Na₃VO₄, 1 mM sodium fluoride, 2% sodium dodecyl sulfate, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-phospho-Smad1/5/8, anti-Smad1, anti-phospho- and total-ERK1/2, anti-phospho- and total-P38, anti-phospho- and total-SAPK/JNK, anti-phospho- and total-NFκB-p65, anti-phospho-FGFRs (Cell Signaling Technology, Inc., Beverly, MA), anti-FGFR3 and FGFR4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-actin antibodies (Sigma-Aldrich Co. Ltd., MO). The relative integrated density of each protein band was digitized by NIH image J 1.34s or by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE).

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The differences between groups were analyzed for statistical significance using ANOVA with Fisher’s PLSD test or unpaired t-test, when appropriate, to determine differences (JMP 9.0 software package, SAS Institute Inc., Cary, NC, USA). P values < 0.05 were accepted as statistically significant.
Results

We first investigated the effects of FGF-8 on BMP-2-induced osteoblast differentiation of myoblastic C2C12 cells. After 48-h culture, BMP-2 (100 ng/ml) significantly increased mRNA levels of osteoblast markers, including osteocalcin, osterix, ALP, Runx2 and collagen-1. FGF-8 (10-100 ng/ml) treatment inhibited the expression of these osteoblast markers induced by BMP-2 in a concentration-responsive manner (Fig. 1A). BMP-2 also induced mRNA expression of Id-1, a target gene of BMP receptor (BMPR) signaling, and FGF-8 inhibited the expression of Id-1 for 24- to 48-h culture (Fig. 1A).

We next investigated the effects of FGF-8 in combination with TNF-α actions on BMP-2-induced osteoblast differentiation. TNF-α (10 ng/ml) inhibited the BMP-2 (100 ng/ml)-induced expression of osteoblast markers. Of note, the inhibitory effects of TNF-α (10 ng/ml) on BMP-2-induced expression of osteoblast differentiation markers such as osteocalcin and ALP were enhanced under the condition of co-treatment with FGF-8 (10-100 ng/ml) (Fig. 1B). To investigate the effects of FGF-8 in the process of osteoblast differentiation, we performed experiments using osteoblastic MC3T3-E1 cells, which exhibit osteoblastic differentiation in later stages than the stage of C2C12 cells and are capable of calcification in vitro [26], and using rat primary calvarial osteoblasts. In accordance with the effects on C2C12 cells, FGF-8 (10-100 ng/ml) inhibited BMP-2 (100 ng/ml)-induced ALP mRNA expression in MC3T3-E1 cells (Fig. 1C) and in rat primary osteoblasts (Fig. 1D) concentration-dependently. TNF-α (10 ng/ml) also potently suppressed BMP-2 (100 ng/ml)-induced ALP
expression and, of interest, the effects of TNF-\(\alpha\) were enhanced by co-treatment with FGF-8 (10-100 ng/ml) in MC3T3-E1 cells (Fig. 1C) as well as in rat osteoblasts (Fig. 1D).

The impact of FGF-8 on BMP-2 receptor signaling was then studied using C2C12 and MC3T3-E1 cells. As shown in Western blots (Fig. 2A), BMP-2 (100 ng/ml) readily stimulated Smad1/5/8 phosphorylation in these two cell lines. BMP-induced Smad phosphorylation was suppressed by co-treatments with TNF-\(\alpha\) (100 ng/ml) and FGF-8 (100 ng/ml), and the combined treatments with these reagents additively suppressed the phosphorylation. To quantify the effects of TNF-\(\alpha\) and FGF-8 on BMPR signaling activity, Id-1 mRNA level was quantified. As shown in Fig. 2B, FGF-8 treatment (10 to 100 ng/ml) augmented the inhibitory effect of TNF-\(\alpha\) (10 ng/ml) on BMP-2 (100 ng/ml)-induced Id-1 expression. In addition, the mRNA level of BMP-2-induced non-canonical Wnt signaling ligand Wnt5a, which is also known to be involved in signaling induced by BMPs [27], was also suppressed by FGF-8 (100 ng/ml) in combination with TNF-\(\alpha\) (10 ng/ml) in C2C12 cells (Fig. 2C).

To elucidate the mechanism by which FGF-8 inhibited BMP-2-induced osteoblast differentiation, the effects of FGF-8 on expression levels of BMP-2 receptors, including activin receptor-like kinase (ALK)-2, -3 and BMPRII, and inhibitory Smads, Smad6 and Smad7, were examined. As shown in Fig. 2D, the mRNA levels of BMP-2 receptor subtypes including ALK-2 and BMPRII were not changed by treatment with FGF-8 (100 ng/ml) or BMP-2 (100 ng/ml), though FGF-8 tended to increase the expression level of ALK-3 (\(p=0.0826\)).
Interestingly, as shown in Fig. 2E, FGF-8 treatment (100 ng/ml) significantly reduced inhibitory Smad6/7 mRNA levels in the presence of BMP-2 (100 ng/ml), suggesting a possible feedback system of FGF-8 to BMPR signaling. Treatment with FGF-8 or TNF-α alone had no effect on the expression levels of Smad6/7. The inhibitory effects of FGF-8 (100 ng/ml) on Smad6/7 expression were preserved regardless of TNF-α (10 ng/ml) action (Fig. 2E). Of note, FGF-8 (100 ng/ml) also inhibited BMP-2-induced expression of inhibitory Smad6/7 in rat primary osteoblasts (Fig 2E).

We further examined the intracellular signaling of FGF-8 for antagonizing BMP-induced osteoblast differentiation. As shown in Fig. 3A, phosphorylation of FGFR was readily induced by FGF-8 (100 ng/ml) but not by BMP-2 (100 ng/ml) stimulation. Mitogen-activated protein kinase (MAPK) is known as a major downstream pathway of FGF-8. It was found that FGF-8 (100 ng/ml) potently stimulated phosphorylation of ERK1/ERK2 and SAPK/JNK pathways but not the P38 or NFκB pathway. TNF-α (100 ng/ml) activated phosphorylation of SAPK/JNK and NFκB, but not ERK, pathways. FGF-8 (100 ng/ml)-induced ERK phosphorylation was not influenced by co-treatment with BMP-2 (100 ng/ml). The expression levels of TNFR1 and TNFR2 were not affected either by TNF-α (10 ng/ml) or FGF-8 (100 ng/ml) (Fig. 3B). In addition, to determine the involvement of endogenous FGFs in the interaction of TNF-α and BMP-2 for the osteoblastic process, an FGF receptor-dependent protein kinase inhibitor, SU5402, was utilized. Although SU5402 was originally reported as an inhibitor of the tyrosine kinase domain of FGFR1 [28], it was also
reported that SU5402 inhibits the phosphorylation of FGFR2 and FGFR3 [29]. Treatment with SU5402 alone had no effect on the expression of osteoblast differentiation markers. As shown in Fig. 3C, SU5402 (3 μM) had no influence on the TNF-α (10 ng/ml) inhibition of BMP-2 (100 ng/ml)-induced osteoblast differentiation, suggesting that endogenous FGF activity is not functionally involved in the inhibitory actions of TNF-α on BMP-induced osteoblast differentiation in C2C12 cells.

To clarify the signaling pathway that is intracellularly linked to FGF-8 inhibition of BMP-induced osteoblast differentiation, the effects of FGFR-related signal inhibition were examined using chemical inhibitors for FGFR, ERK, SAPK/JNK and NFκB. Treatments with inhibitors alone had no significant effect on the expression levels of osteocalcin and collagen-1 mRNAs (Fig. 3D and 3E). SU5402 (3 μM) significantly reversed the inhibitory effects of FGF-8 (100 ng/ml) on the expression of osteocalcin (Fig. 3D) and collagen-1 (Fig. 3E) mRNA induced by BMP-2 in C2C12 cells. U0126 (10 μM), a highly selective inhibitor of MEK1/MEK2, partially but significantly reversed the inhibitory effects of FGF-8 (100 ng/ml) on BMP-2 (100 ng/ml)-induced expression of osteocalcin (Fig. 3D) and collagen-1 (Fig. 3E). The SAPK/JNK inhibitor SP600125 (3 μM) and NFκB inhibitor (3 μM) failed to change the effects of FGF-8 (100 ng/ml) on BMP-2 (100 ng/ml)-induced osteoblastic differentiation. These findings indicated that FGF-8 elicits inhibitory effects on BMP-2-induced osteoblast differentiation mainly thorough ERK activation via FGFR activation.
Discussion

In the present study, a novel role of FGF-8 and the interaction between FGF-8 and TNF-α in the BMP-2-induced process of osteoblastic marker expression was revealed (Fig. 4). It was clarified that FGF-8-induced ERK activation was a key for suppressing BMP-2-induced osteoblast differentiation in the process of osteoblast differentiation. In addition, the present results showing that FGF-8 suppressed BMP-induced Smad6 and Smad7 expression and tended to enhance the expression of ALK-3 indicated the possible existence of a feedback activity of FGF-8 to BMPR signaling in the process of osteoblast differentiation.

Functional roles of FGF activity in osteoblast differentiation have not been fully clarified. Among the FGF ligands, FGF-8 has been shown to induce cancer cell proliferation and tumor growth in cell culture [30]. FGF-8 can bind to three distinct tyrosine kinase receptors, FGFR2-IIIc, FGFR3-IIIc and FGFR4 [31]. We previously reported that FGF-8 facilitates cell proliferation by upregulating endogenous estrogenic actions as well as by suppressing BMPR signaling in estrogen receptor-expressing breast cancer cells [32]. The interaction between FGF-8 and BMPs was also revealed to regulate ovarian steroidogenesis through oocyte-granulosa cell communication [33].

As for the effects of FGF-8 on osteoblast differentiation, Lin et al. demonstrated the existence of FGF-8 in rat primary osteoblastic cells [18]. In that study, it was found that FGF-8 potently stimulated the proliferation of osteoblastic cells, while it inhibited the formation of mineralized bone nodules in
long-term cultures of osteoblasts [18]. Uchii et al. also reported high expression levels of FGF-8 in hyperplastic synovial cells and fibroblasts in osteoarthritis model mice [20]. They also demonstrated that FGF-8 injection into knee joints enhanced degradation of the extracellular matrix in rats and that this effect was reversed by treatment with an anti-FGF-8 antibody [20]. Valta et al. showed the stage-dependent effects of FGF-8 on osteoblast differentiation by using mouse bone marrow cultures for different time periods [19]. In that study, continuous exposure of mouse bone marrow cells to FGF-8b, throughout the proliferation and differentiation phases, inhibited bone nodule formation, whereas FGF-8b stimulation, only in the proliferation phase, increased calcium contents of mouse osteoblast culture [19]. These differences might be attributed to the differentiation stages of osteoblastic cells and different profiles of FGF receptors at various stages of differentiation. In accordance with Valta’s results, the results of the present study also showed that continuous stimulation with FGF-8 inhibited osteoblast differentiation induced by BMP-2. In the present study, it was of note that FGF-8 inhibited BMP-2-induced osteoblast differentiation not only in myoblast C2C12 but also in a later-stage osteoblast differentiated MC3T3 and in rat calvarial osteoblasts, suggesting that FGF-8 is functionally involved in the wide-range process of osteoblast differentiation.

Wnt/β-catenin pathway and BMP-2 signaling cooperatively regulate the expression of osteoprotegerin [34]. Wnt5a is one of the non-canonical Wnt ligands and is known to be important for BMP-2-mediated osteoblastic differentiation but to be dispensable for Smad1/5/8 phosphorylation [27]. In the present study, FGF-8 suppressed both Smad1/5/8 activation and Wnt5a
expression. In this regard, the inhibition of ALK-2 and -3 using LDN193189 enabled suppression of BMP-2-induced expression of Wnt5a in our preliminary experiments using C2C12 cells, suggesting that the Wnt5a induction caused by BMP-2 is likely to be downstream of Smad1/5/8 phosphorylation and that FGF-8 reduces Wnt5a expression by suppressing Smad1/5/8 signaling.

Relationships between inflammatory bone diseases and FGF have also been shown. Melnyk and colleagues reported that synoviocytes of patients with RA produce basic FGF (bFGF, also called FGF-2) and contain receptors for bFGF and that bFGF derived from synoviocytes per se stimulates cell proliferation in an autocrine manner in cases of RA [35]. Manabe et al. reported that the concentrations of bFGF in synovial fluid were correlated with Larsen’s joint destruction grades, with severe RA patients showing significantly higher levels of bFGF than those in mild RA patients [36]. This finding suggests that increases in endogenous bFGF in the synovial fluid of RA patients may be pathologically associated with joint destruction [36]. However, the interrelationship between FGF-8 levels and RA activity has not been determined. In our preliminary study, we found an increased expression of FGF-8 mRNA by 4.8 folds in the synovia from RA patients compared with that from healthy control, suggesting that FGF-8 is involved in the pathogenesis of RA. Although we still cannot draw a conclusion regarding the role of FGF-8 in RA, a novel role of FGF-8 could be, at least in part, involved in the induction of inflammatory bone and joint disorders. Further study is necessary for determining the pathophysiological relevance of FGF-8 in the progress and development of inflammatory bone damage in RA patients.
TNF-α is known to be involved in many bone and joint diseases including not only RA but also OA [37]. We previously reported that TNF-α inhibited osteoblast differentiation induced by BMP via SAPK/JNK, NFκB and Stat phosphorylation [23, 24, 38]. Although there are some reports indicating significant roles of FGFs in both RA and OA, there has been no study in which the correlation between FGF and TNF-α in these disease models was analyzed. In the present study, we revealed that the inhibitory effects of TNF-α osteoblast differentiation were enhanced under the condition of co-treatment with FGF-8. It was also revealed that FGF-8 inhibits osteoblast differentiation mainly via the ERK pathway, which is biologically different from TNF-α signaling. Although the SAPK/JNK inhibitor also tended to inhibit the effects of FGF-8 on BMP-2-induced expression of osteocalcin, there was no statistically significant difference between the effects of FGF-8 with and without SP600125, and SP600125 had no effect on the expression of type I collagen. Therefore, we concluded that FGF-8 inhibits BMP-2-induced osteoblast differentiation predominantly through ERK. Taken together, our data suggested that FGF-8 and TNF-α collaboratively suppress osteoblast differentiation by distinct mechanisms. However, given that the MEK inhibitor did not completely reverse the effect of FGF-8, further studies are required to clarify the detailed mechanism by which FGF-8 inhibits osteoblast differentiation.

Collectively, the results of the present study showed inhibitory effects of FGF-8 on BMP-2 signaling in the process of osteoblast differentiation, which were, at least in part, activated by the ERK pathway (Fig. 4). The FGF-8-to-
ERK signaling inhibited BMP-2-induced phosphorylation of Smad1/5/8, and the effects were amplified by combined TNF-α activity. These findings suggest that FGF-8 may be involved in the regulatory role of inflammatory bone damages, though the in vivo activity and detailed mechanisms underlying the effects of FGF-8 on osteoblast differentiation are still pending questions.
References


Figure legends

**Fig. 1. FGF-8 inhibited the expression of osteoblast markers induced by BMP-2.**  
A) C2C12 cells (1 × 10^5 viable cells) were treated with BMP-2 in the presence or absence of FGF-8 in a serum-free condition for 24 or 48 h.  
B) C2C12 cells,  
C) MC3T3-E1 cells and  
D) rat primary osteoblasts, the cells were treated with BMP-2 in the presence or absence of FGF-8 and TNF-α in a serum-free condition for 48 h, and then total cellular RNA was extracted. For the quantitation of osteocalcin, ALP, collagen-1, osterix, Runx2 and Id-1 mRNA levels, real-time PCR analysis was performed. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as means ± SEM and analyzed by ANOVA. *P < 0.05 vs. control group and between the indicated groups, and #P < 0.05 vs. BMP-2 group. $P < 0.05$ between the indicated groups by the unpaired t-test.

**Fig. 2. FGF-8 suppressed the BMPR-Smads signaling.**  
A) To analyze the phosphorylation of regulatory Smads by Western blots, C2C12 cells (5 × 10^4 cells) and MC3T3-E1 cells (5 × 10^4 cells) were stimulated with BMP-2, FGF-8 and TNF-α in a serum-free condition for 1 h and then lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using antibodies that detect phosphorylated Smad1/5/8 (pSmad1/5/8) and actin. The relative integrated density of the pSmad1/5/8 protein band was digitized, with the phosphorylated levels being normalized by actin levels, and then the results were expressed as
fold changes. For the quantitation of B) Id-1, C) Wnt5a, D) BMP receptors, E) inhibitory Smads mRNA levels, C2C12 cells (1 × 10^5 cells, B-E) and rat primary osteoblasts (1 × 10^5 cells, E) were treated with BMP-2 and FGF-8 in combination with TNF-α in a serum-free condition for 48 h, total cellular RNA was extracted, and then real-time PCR analysis was performed as shown in the legend of Fig. 1. Results are shown as means ± SEM and analyzed by ANOVA. *P < 0.05 vs. control group and between the indicated groups, and #P < 0.05 vs. BMP-2 group.

Fig. 3. FGF-8 inhibited the expression of BMP-induced osteoblast markers mainly via the ERK pathway. A) C2C12 cells (5 × 10^4 cells) were stimulated with BMP-2, FGF-8 and TNF-α in a serum-free condition for 15 min, and the cells were lysed and subjected to IB analysis using antibodies that detect phospho-FGFR, phospho- and total-ERK1/ERK2, SAPK/JNK, NFκB, P38 and actin. B, C) Cells (1 × 10^5 cells) were treated with FGF-8 and TNF-α in combination with SU5402 in a serum-free condition for 48 h, and then total cellular RNA was extracted and real-time PCR analysis was performed as shown in the legend of Fig. 1. D, E) After preculture with MAPK inhibitors including U0126, SP600125 and NFκB inhibitor and the FGFR inhibitor SU5402 for 1 h, C2C12 cells were treated with FGF-8 in a serum-free condition for 48 h, and then total cellular RNA was extracted and real-time PCR was performed. Results are shown as means ± SEM and analyzed by ANOVA. *P < 0.05 vs.
control group and between the indicated groups. *P < 0.05 vs. BMP-2 group.

n.s.: not significant.

**Fig. 4. Effect of interaction of FGF-8 and TNF-α on BMP-2-induced osteoblast differentiation.** FGF-8 suppressed BMP-2-induced expression of osteoblastic markers. FGFR signaling inhibited BMP-induced Smad1/5/8 phosphorylation and the subsequent expression of Id-1 and Wnt5a, predominantly through ERK activation. TNF-α actions enhanced the inhibitory effects of FGF-8 on BMP-induced osteoblast differentiation by activating SAPK/JNK and NFκB. These pathways were linked to the suppression of BMPR-Smad1/5/8 signaling, leading to the cooperative effects of FGF-8 and TNF-α that counteract BMP-induced osteoblast differentiation.
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Disclosure statement

FO receives speaker honoraria from Novo Nordisk. HM is a consultant for AbbVie, Astellas and Teijin, receives speaker honoraria from Astellas, Boehringer-Ingelheim, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, MSD, Novartis, Pfizer, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, MSD, Novo Nordisk, Takeda, and Tanabe Mitsubishi.
Fig. 1

Fig. 2
Fig. 3

**A**

**B**

**C**

**D**

**E**

Fig. 4