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Interactions between FGF21 and BMP-2 in osteogenesis

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ABSTRACT

Lifestyle-related diseases are increasing and the challenge to create innovative drugs to treat such diseases is a main focus in medical science research. Fibroblast growth factor 21 (FGF21) is a powerful modulator of glucose and lipid metabolism, and is an innovative candidate drug already in clinical trials for type 2 diabetes mellitus and obesity. Bone fragility and impaired fracture healing induced by such lifestyle-related conditions are also a growing problem. Bone morphogenic proteins (BMPs) are well known osteogenic growth factors, and BMP-2 is used to augment bone formation in difficult clinical situations. There are many documented interactions between the FGF and BMP family proteins, although the interaction between FGF21 and BMP-2 remains unknown. The aim of this study was to reveal the effect of FGF21 toward BMP-2-dependent osteogenic activity, using C2C12 cells as a model system. We found that FGF21 enhanced BMP-2-dependent transcription and osteogenesis in the C2C12 cell line, which was confirmed by alkaline phosphatase activity, matrix mineralization, and gene expression. Mechanistically, FGF21 enhanced BMP-2-induced intracellular signaling through Smad proteins, but not through p44/42MAPK proteins. Furthermore, we identified a negative feedback loop in which BMP-2 decreased endogenous FGF21 mRNA expression. In summary, this study demonstrates interactions between BMP-2 and FGF21 pathways exist in vitro, and that FGF21 enhances the osteogenic activity of BMP-2 by up-regulating the BMP-2-dependent Smad signaling pathway.

1. Introduction

The ability of bone to regenerate is well known. However, its ability to restore the original bone's form and function is limited by the size of the bone defect. In larger defects, a bone graft is often used to guide bone regeneration, and surgeons conducting bone grafting procedures have been searching for a bone graft alternatives to avoid the complications of harvesting autogenous bone grafts [1]. Cytokines and growth factors have proven effects on bone tissue regeneration with reduced reliance on bone grafts [2]. The most widely used osteoinductive factors are Bone morphogenic proteins (BMPs), which are members of Transforming Growth Factor-β (TGF-β) family originally identified from bovine bone based on their ability to induce de novo ectopic bone formation [3]. BMP-2 is commercially available as a clinically approved osteoinductive autograft replacement [1], thus BMP-2 is now playing main role in bone tissue engineering.

As the dietary caloric intake is increasing and sedentary lifestyles are becoming more prominent, populations with lifestyle-related diseases including diabetes mellitus (DM) or obesity are increasing year by year. The challenge to create innovative drugs to treat such diseases is a main focus in medical science research. On this front, promising drugs come from the fibroblast growth factor (FGF) family. There are 18 mammalian FGFs (FGF1–FGF10, FGF16–FGF23) that are grouped into 6 subfamilies based on differences in sequence homology and phylogeny [4]. Among these, FGF21 is a member of the FGF19 family that acts in an endocrine manner and is known to be a powerful modulator of glucose and lipid metabolism [4]. FGF21 is an innovative candidate drug for type 2 DM as well as obesity, and it is already in clinical trials.

In many cases, FGF family members have anabolic effects in bone when systemically or locally applied [5,6], and there is a strong interaction between the activities of FGFs and BMPs [7] in most biological processes [8], suggesting cooperative actions of these signaling molecules [9]. These series of evidence strongly suggests that the FGF21 pathway also may interact with BMP-2 signaling, however, the interactions between these two clinically available drugs remain unknown at present. Thus, as the main purpose of this study, we performed a series of experiments to investigate the hypothesis that there is a signaling crosstalk linking FGF21 and BMP-2, leading to the modification of osteogenic activity by BMP-2 in the presence of FGF21 in vitro.

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2. Materials and methods

Animal procedures were approved by the Animal Use and Care Advisory Committee at the University of California, Davis Campus. Samples from human subjects were obtained in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects.

2.1. Luciferase assay for transcriptional activation

To test the effect of FGF21 on BMP-2 dependent transcriptional activation, we assayed the activation of a BMP-2-responsive elements from the Inhibitor of DNA binding 1 (Id1) promoter that had been stably transfected into a C2C12 mouse myoblast (C2C12BRA; kindly provided by Dr. Rifkin at NYU) [10]. Briefly, C2C12BRA cells were seeded at 4×10^3 cells/well in a 96-well plate and incubated with high-glucose Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) with containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL streptomycin for 3 h. Then the medium was replaced with treatment medium, which was DMEM containing 1% FBS mixed with increasing concentrations of recombinant human BMP-2 (INFUSE® Bone Graft, Medtronic, Memphis, TN) and FGF21 (R&D Systems, Minneapolis, MN). After 17 h, luciferase activity was assayed in the cell lysate using the Luciferase Assay System (Promega) with readings normalized to cell seeding density.

2.2. Cell culture

C2C12 cells are clonally derived mouse myoblasts [11] that differentiate into osteoblasts upon treatment with BMP-2 [12]. These cells do not express significant amounts of endogenous BMPs, making them an effective and commonly used model for assessing the osteogenic activity of BMPs [12–14]. Primary human chondrocytes were obtained from the knee joint cartilage of osteoarthritic patients receiving joint replacement surgery, with IRB approval and patient consent. Unless otherwise noted, C2C12 cells and primary chondrocytes were maintained in high-glucose DMEM with 10%FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was changed every 3 days.

2.3. Alkaline-phosphatase (ALP) activity

Alkaline-phosphatase (ALP) activity was measured as follows. Briefly, C2C12 cells were seeded at 2 mL of $4–5 \times 10^4$ cells/mL in a 6-well plate and incubated for 24 h. Then medium was replaced with treatment medium, which was DMEM containing 5%FBS plus 100 ng/mL rhBMP-2 and/or 10, 100, or 400 ng/mL FGF21. After 1 week, the alkaline-phosphatase activity in the cell lysates was measured using an alkaline-phosphatase activity kit (SensoLyte®; San Jose, CA). Protein concentration was measured using the Bradford assay kit (ThermoFisher Scientific, Rockford, IL).

2.4. Matrix mineralization by Alizarin Red

The extent of mineralization of the extracellular matrix was measured semi-quantitatively using Alizarin Red staining, according to previously reported protocols. Briefly, C2C12 cells were treated with the same medium described above for ALP activity, plus 25 μg/mL ascorbic acid and 10 mM β-glycerophosphate. After 14 days of culture, cells were washed, fixed in 95% ethanol, then stained with Alizarin Red S (Sigma) 1% solution in 0.28% ammonia, followed by gentle agitation for 10 min. After imaging, cells were solubilized with 10% (w/v) ethylpyridinium chloride (Sigma) for 15 min and the extracted stain measured by spectrophotometry with an absorbance at 570 nm [15].

2.5. Gene expression analysis

To investigate the transcriptional expression of osteogenesis-related genes and the interactions between BMP-2 and FGF21, RT-PCR was performed using TaqMan primers and probes (Applied Biosystems, Foster City, CA). Briefly, after 3 days treatment, total RNA was isolated using RNeasy Mini Kit (Qiagen,Valencia, CA). 0.5 μg of RNA was reverse transcribed using a High Capacity cDNA Reverese Transcription Kit (Applied Biosystems). The converted cDNA (2.5 μL) samples were amplified in triplicate using real-time PCR (7900HT Fast Real-Time PCR system; Applied Biosystems) in a final volume of 10 μL using TaqMan Fast Universal PCR Master Mix reagent (Applied Biosystems). Pre-designed primers and probes for the following mouse genes were obtained from Applied Biosystems; Mouse osteocalcin (Mm03413826_mH), ALP (Mm00475834_m1), RUNX2 (Mm00501584_m1), osterix (Mm00504574_m1), FGF21 (Mm00840165_g1), and Bmp2 (Mm01340178_m1). Levels of transcripts for Eukaryotic 18S rRNA were used as a reference gene (Applied Biosystems).

2.6. Western blotting

C2C12 cells were recovered in lysis buffer containing 50 mM Tris–HCl (pH7.5), 120 mM NaCl, 2 mM EDTA, 1% NP-40, 5 mM sodium pyrophosphate, 5 mM sodium vanadate, and protease inhibitor mix (Roche Diagnostics, Basel Switzerland) and lysed on ice for 30 min. Centrifugation (12,000*g* for 30 min, 4 °C) removed cell debris, and the protein concentrations in the supernatant measured by Bradford assay. 10 μg protein per lane was subjected to Western blot analysis. Bound antibodies were detected with an HRP-conjugated secondary antibody and visualized with ECL reagents exposed to film. Antibodies used were; anti-phospholyrated-Smad1(Ser463/465) /Smad5(Ser463/465) /Smad8(Ser426/428) (pSmad1/5/8) (#9511), anti-Smad1 (#9743), anti-phospholyrated-p44/42(T202/Y204) (p-p44/42) (#9101S), and anti-total-p44/42 MAPK(t-p44/42) (#9102S) (all from Cell Signaling, Danvers, MA), and anti-GAPDH (AM4300) (Ambion). Band intensities were quantified using Image J version (NIH Image, National Institutes of Health http://rsbweb.nih.gov/ij/), with values normalized to GAPDH.

2.7. Statistical analysis

Statistical analysis was performed using one way analysis of variance, with Fisher's protected least significant difference (PLSD) post hoc test for multiple comparisons of paired samples.

3. Results

3.1. FGF21 enhances BMP2-dependent transcriptional activity

To examine the crosstalk between FGF21 and BMP2 signaling, we first examined whether FGF21 affects the transcriptional response of BMP-2-dependent gene promoters. This was done using a BMP-responsive luciferase reporter stably expressed in C2C12 cells (Fig. 1(A)). As expected, in control conditions overnight treatment with BMP-2 alone up-regulated the luciferase activity. FGF21 alone did not activate BMP-2-dependent transcriptional activation in this assay. Additional FGF21 significantly enhanced BMP-2-dependent transcriptional activity ($^*p < 0.05$). In this simplified experimental system with a luciferase reporter construct and over-

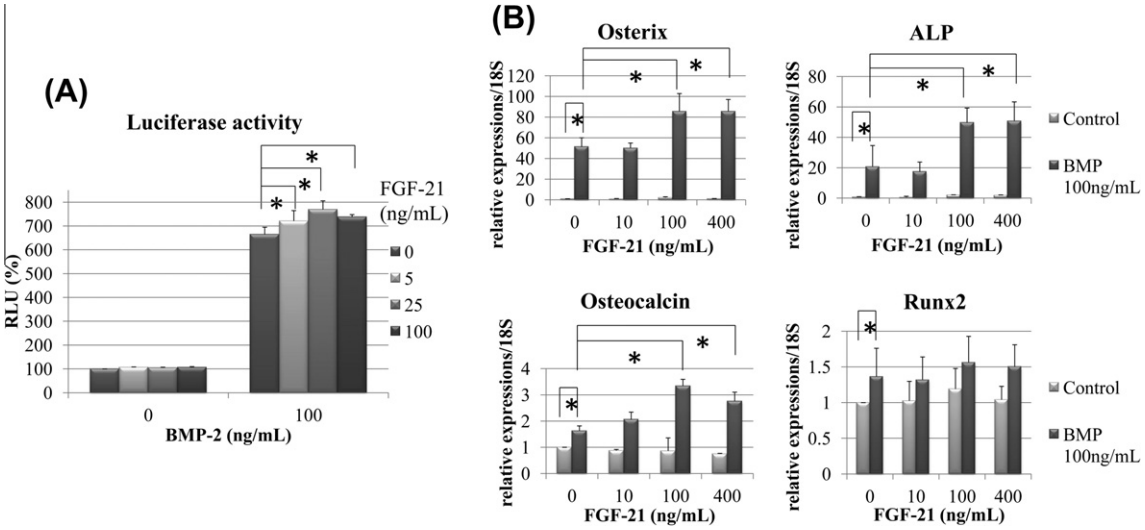

Fig. 1. FGF21 enhances BMP-2-dependent luciferase activation and osteogenic gene expressions in C2C12 cells. (A) The results demonstrated that the additional FGF21 significantly enhanced BMP-2 dependent transcriptional activity compared to BMP-2 alone. (*$p < 0.05$). (B) Osterix, Alp, and Osteocalcin expression were significantly up-regulated by 100 ng/mL of BMP-2 alone, and further enhanced by 100 or 400 ng/mL of FGF-21 at 3 days after BMP-2 and FGF21 treatment. Runx2 expression was slightly but significantly up-regulated by 100 ng/mL of BMP-2, however FGF21 treatment had no effect on Runx2 (*$p < 0.05$).

night exposure to growth factors, these results suggest that FGF21 effectively enhances BMP-2-dependent transcriptional activity.

3.2. Enhancement of BMP-2-dependent osteogenesis by FGF21 in C2C12 cells

Next, we examined whether FGF21 affects the more complex BMP-2-dependent biological process of in vitro osteogenesis. Cells were placed in osteogenic media with BMP-2 alone, FGF21 alone, or BMP-2 and FGF21 in combination. The effect on osteogenic gene expression was measured by RT-PCR after 3 days of differentiation. Alkaline-phosphatase activity was measured after 7 days of differentiation as molecular marker for the early stage of osteoblastic differentiation. Finally, Alizarin Red staining was measured after 14 days of differentiation as a marker of matrix mineralization during the late stages of osteogenesis.

Osteogenic gene expression was quantified by RT-PCR after 3 days of treatment with BMP-2 and/or FGF21. As expected, in control conditions we found that BMP-2 alone up-regulated the expression of osteogenic genes Osterix, ALP, Runx2 and Osteocalcin. FGF21 alone did not activate these genes. The addition of FGF21 significantly enhanced BMP-2-dependent transcription of Osterix, ALP and Osteocalcin compared to conditions with BMP-2 alone (*$p < 0.05$). Although Runx2 expression was slightly but significantly up-regulated by 100 ng/mL of BMP-2 (*$p < 0.05$), addition of FGF21 treatment had no further effect on Runx2 expression (Fig. 1(B)).

Alkaline-phosphatase activity was quantified by hydrolysis of p-Nitrophenyl Phosphate after 7 days of osteogenic differentiation. As expected, in control conditions we found that BMP-2 alone increased the alkaline-phosphatase activity. FGF21 alone did not stimulate alkaline-phosphatase activity. The addition of FGF21 significantly enhanced the BMP-2-dependent alkaline-phosphatase activity above that of BMP-2 alone (*$p < 0.05$) (Fig. 2(A)).

Matrix mineralization was evaluated by Alizarin Red staining after 14 days of differentiation. In control conditions we found that BMP-2 alone significantly increased matrix mineralization. FGF21 alone had no effect on matrix mineralization. The addition of

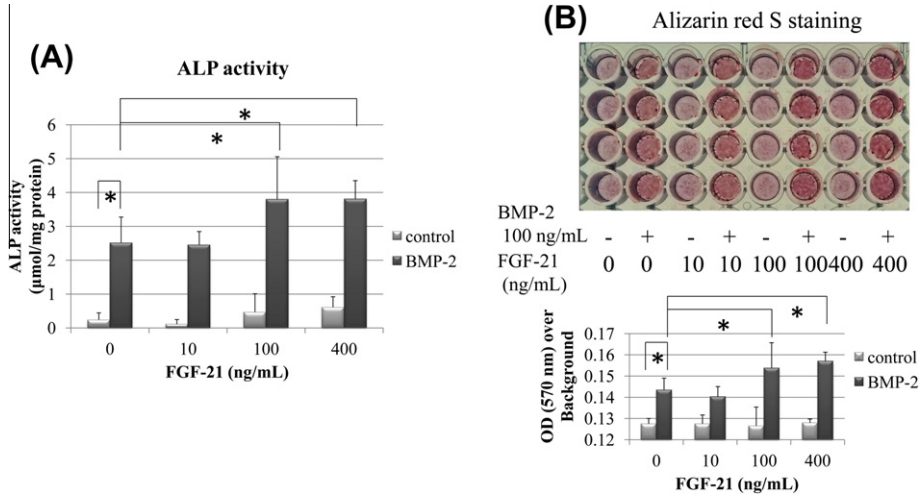

Fig. 2. FGF21 enhances BMP-2-dependent osteogenic differentiation and mineralization in C2C12 cells. (A) At concentrations greater than 100 ng/mL, FGF21 significantly elevates alkaline-phosphatase activity stimulated by 100 ng/mL of BMP-2 in C2C12 cells. (B) 100 or 400 ng/mL of FGF21 enhanced matrix mineralization evaluated by Alizarin Red staining. Top panel shows macroscopic findings of the stained cells, and bottom panel shows quantitative assessment of dye.(*$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

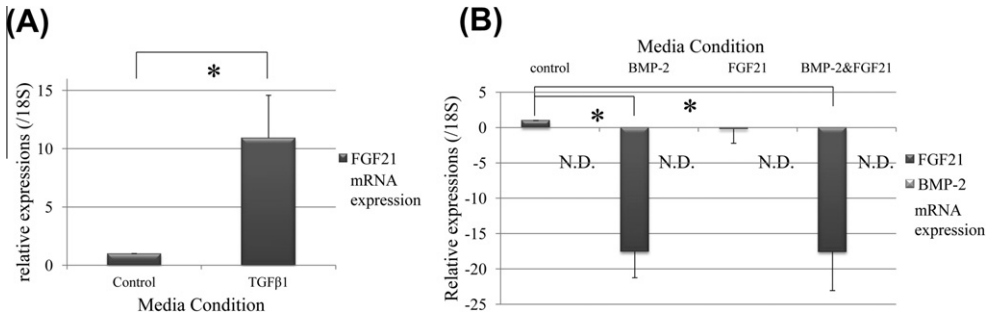

Fig. 3. BMP-2 negatively regulated FGF21 mRNA expression. (A) TGF-β1 (1 ng/mL) significantly up-regulated FGF21 expression. (B) 100 ng/mL of BMP-2 treatment significantly down-regulated the FGF21 expression. Neither 100 ng/mL BMP-2 nor 100 ng/mL FGF21 induced BMP-2 expression in this culture system. (N.D.; not detectable, $^{*}p < 0.05$).

FGF21 at concentrations above 100 ng/mL significantly enhanced BMP-2-dependent matrix mineralization ($^{*}p < 0.05$) (Fig. 2(B)).

These series of results suggests that FGF21 enhances BMP-2-dependeng gene expression, and also enhances the more complex biological process of BMP-mediated in vitro osteogenic differentiation in C2C12 cells.

3.3. BMP-2 negatively regulated FGF21

We next investigated whether BMP-2 affected FGF21 expression, and conversely, whether FGF21 affected BMP-2 expression. As BMP-2 is a TGF-β family member, we first examined whether TGF-β1 affected FGF21 expression in human chondrocytes. We found that 1 ng/mL of TGF-β1 treatment significantly up-regulated FGF21 expressions by 10-fold ($^{*}p < 0.05$, Fig. 3(A)). The result confirmed that FGF21 is a positive response gene for TGF-β1. In contrast, 100 ng/mL of BMP-2 treatment significantly down-regulated FGF21 expression by 17-fold ($^{*}p < 0.05$). FGF21 gene expression was independent of FGF21 treatment (Fig. 3(B)). These results suggested that BMP-2 signaling inhibited FGF21 expression, and establishes FGF21 as a negative response gene for BMP-2. In addition, we found that BMP-2 and FGF21 treatment, alone or in combination, did not induce BMP-2 expression to detectable levels (defined as 40 cycles of RT-PCR from 1ug RNA) in C2C12 cells (Fig. 3(B)).

3.4. FGF21 enhanced BMP-2-dependent Smad1/5/8 phosphorylation, but not p44/42 MAPK

BMPs signal through both Smad-dependent pathways and Smad-independent MAPK pathways, and both of these pathways are active in C2C12 cells [13]. We wanted to determine which BMP signaling pathways are affected by FGF21. We compared BMP-induced phosphorylation of signaling proteins (phospho-Smad-1/5/8 and phospho-p44/42) by western blotting, normalized to the total levels (phosphorylated plus non-phosphorylated) of the respective proteins.

We found that 30 min treatment with 100 ng/mL of BMP-2 significantly increased Smad1/5/8 phosphorylation. FGF21 treatment alone had no effect on Smad phosphorylation. 100 ng/mL of FGF21 significantly enhanced BMP-2-dependent Smad phosphorylation above that of BMP-2 alone ($^{*}p < 0.05$). Total Smad1 levels were constant and independent of the treatment (Fig. 4(A)).

In the MAPK pathway, we found that 30 min of BMP-2 significantly increased p44/42 MAPK phosphorylation ($^{*}p < 0.05$). As reported by others, FGF21 alone stimulated the p44/42 MAPK pathway [16]. Interestingly, FGF21 in the presence of BMP-2 showed no additive effects toward p44/42 phosphorylation. Total p44/42 levels remained constant and independent of the treatment (Fig. 4(B)). Taken together, these results suggested that FGF21 stimulates BMP-2-dependent Smad signaling pathway, but not the non-Smad MAPK pathway.

4. Discussion

Adipose tissues are important sources for hormones and cytokines, known as adipokines, and FGF21 attracts much attention as a relatively new adipokine from broad research fields. Circulating FGF21 levels were elevated in rheumatoid arthritis, coronary heart disease, and Cushing's syndrome [17,18]. One of adipokines, adiponectin increases BMP-2 expressions in osteoblasts [19], however the interactions between FGF21 and BMP-2 remain unknown at present. To our knowledge, this is the first study of the biological interaction between the BMP-2 and FGF21 pathways. Our results establish that FGF21 positively affects BMP-2-dependent osteogenesis via activating Smads but not the p44/42 MAPK pathway in C2C12 cells.

Diabetic patients have increased risks for skeletal fragility [20] and fracture healing complications [21], therefore, the clinical situations in which diabetic patients might be treated by BMP-2 are increasing. Thus, we considered this study to answer the question of whether FGF21 might modify the osteogenic activities of BMP-2. The results provide useful information by demonstrating an interaction between FGF21 and BMP-2, and if these in vitro studies are reproducible in vivo, it provides early evidence that both drugs could be used together without reducing the osteogenic activity of BMP-2.

The effects of the FGF family of proteins in osteogenesis are well investigated, especially for bFGF (FGF-2). Previous reports have confirmed that FGFs including bFGF are essential for bone formation [22–24], although the effect of additive bFGF on osteoblastic differentiation is still controversial [22,23]. However, there is a consensus that supplemental bFGF is important during early in vitro osteoblastic differentiation [25,26] and that sequential administration of bFGF and BMP-2 promotes osteoblastic differentiation compared to BMP-2 alone in rat MSCs [25]. Huang et al. hypothesized that the early application of bFGF might increase the expression of BMP-2 receptors, which have higher binding and activating potential toward BMP ligand [24]. Thus, positive effects for BMP-2 dependent osteogenesis with FGF family members have been reported.

The enhancing effects of FGF21 on BMP2-dependent osteogenesis in our study were opposite to our initial expectations. FGF21 transgenic mice are markedly smaller than their control mice, and have reduced growth hormone-STAT5 signaling [27]. FGF21 can inhibit osteoblastogenesis and stimulates adipogenesis in MSCs [28]. We therefore anticipated that FGF21 has anti-osteogenic activity by BMP-2. Also in this report, administration of

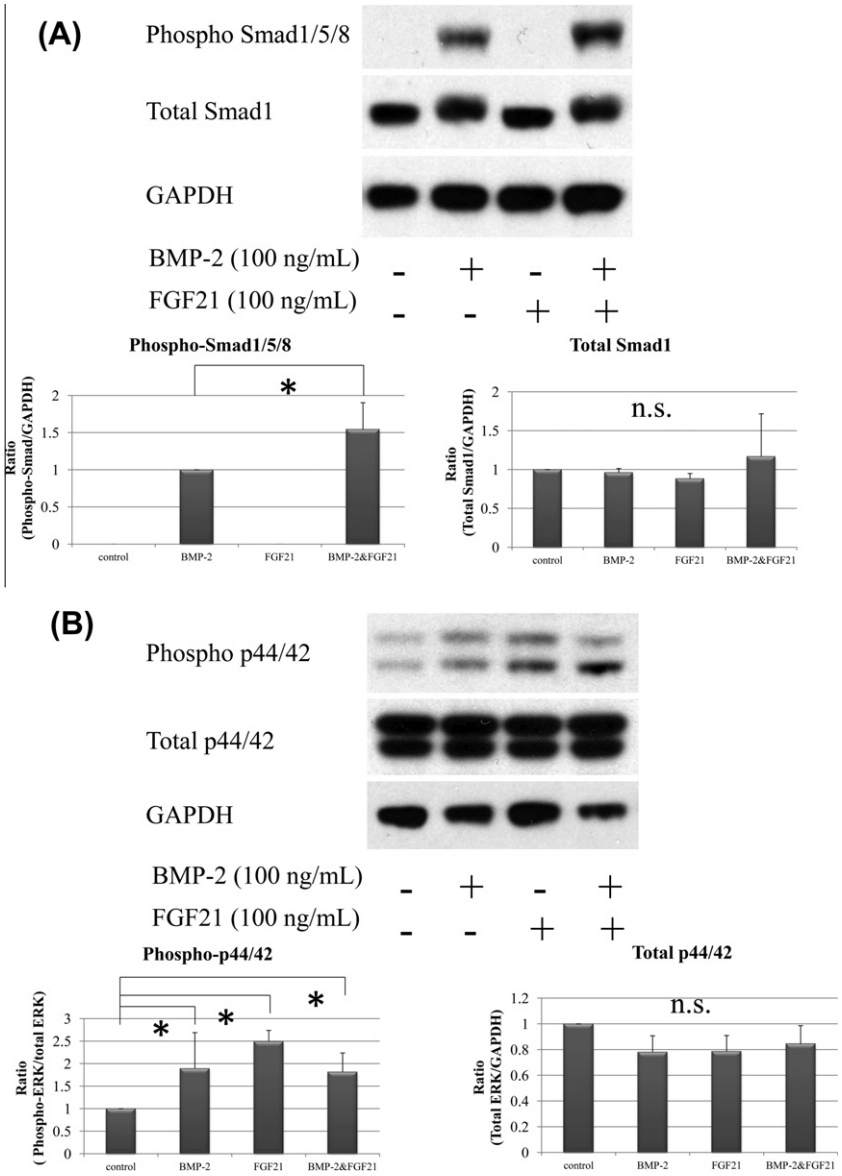

Fig. 4. FGF21 enhances Smad1/5/8 phosphorylation, but not p44/42 MAPK pathway activated by BMP-2. (A) The addition of BMP-2 alone increased pSmad levels. In the presence of FGF21, the same amount of BMP-2 caused greater pSmad1/5/8 levels at the 30-min time point. FGF21 alone had no effect. Total Smad1 levels remained constant. (B) P-p44/42 levels were increased in the presence of BMP-2 and FGF21, however no additive effect was observed. Total p44/42 levels remained constant. Lower panel shows densitometry normalized to GAPDH, ∗ indicates $p < 0.05$ from control at that time. C2C12 cells were treated with BMP-2 (100 ng/mL), and/or FGF21 (100 ng/mL), and protein levels measured by immunoblots of cell lysates.

FGF21 shifts toward canonical BMP-Smad pathway, rather than non-canonical BMP-p44/42 pathway. This result suggests that FGF21 supports BMP-2-Smad-dependent differentiation, rather than the p44/42 MAPK pathway, generally known to be important in cell proliferation [29] in C2C12 cells, leading to the positive effect for BMP-2-dependent osteogenesis.

Although it is recognized that FGF21 works in an endocrine manner, recent papers report that FGF21 may also function in a paracrine/autocrine manner [30,31]. Some studies indicate that FGF21 may preferentially activate the FGF receptor (FGFR)1/βKlotho complex, while other reports showed that it can bind all the four FGFRs [32,33]. In our study, only high concentrations of FGF21 worked in a paracrine manner and augmented BMP-2-dependent osteogenesis. Wu et al. have reported that high concentration dose of 5 and 10 μg/mL FGF21 inhibits collagen type 10 expression in growth plate chondrocytes [31]. They found FGFR1, FGFR3, and βKlotho expression in mouse chondrocytes, and that FGF21 acts in a paracrine manner in chondrocytes [31]. Their paper also indicates that high doses of FGF21 are required to activate chondrocytes signaling, and this high dose may be of uncertain physiological relevance. Our working hypothesis is that the major role for FGF21 is endocrine and that its functions in musculoskeletal tissues warrants further investigation. Our results suggest that higher doses of FGF21 might increase or alter the response to BMP-2.

Interestingly, we found that BMP-2 strongly suppressed FGF21 expression in C2C12 cells. In mesangial cells, TGF-β1 mediated FGF21 mRNA up-regulation was reported [34]. Inhibiting FGF21 expressions also attenuates TGF-β1 pathway [34]. As reported for mesangial cells [34], we found that TGF-β1 increased FGF21 mRNA expression in human chondrocytes, confirming FGF21 is a TGF-β responsive gene. Our study found the opposite is true for BMP-2, which strongly down-regulated FGF21 expression. Others have shown that BMP-2 induces differentiation of PC12 cells via upregulation of FGFR1 expression and augmentation of FGF activity [35]. It is believed that FGF21 has a specific affinity for FGFR1, and BMP-

2 itself might enhance FGF21 signal activation. FGF21-specific signaling response remains unknown, and the regulation of FGF receptors should be examined to confirm this. While the true contribution of BMP-2 to FGF21 remains unknown, the interactions between BMP2 and FGF21 might function to maintain a balance between BMP and FGF signaling.

There are some limitations in this study. We could not control the expression of endogenous FGF21 in our differentiation assay. Upon BMP2 treatment, C2C12 cells differentiate from myoblasts to osteoblasts. FGF21 is strongly in muscle compared to other skeletal components, which was confirmed by our immunohistology (data not shown). The alternation of phenotype by BMP-2 might change the endogenous expressions of FGF21.

In addition, a more detailed mechanism of how FGF21 enhances BMP-Smad pathway, and the effect of FGF21 on BMP ligand/receptors levels should be examined. In the C2C12 cell system, BMP-2 is not expressed, therefore the system is not ideal for detecting whether FGF21 affects BMP-2 expression levels. Our results found that FGF21 treatment did not induce detectable levels of BMP-2 expression. Secondarily, the question whether BMP-2 affects the effect of FGF21 toward DM and obesity needs further investigation. Finally, we need in vivo study on whether FGF21 supports the fracture healing properties of BMP-2. Systemic FGF21 promotes bone loss [28], and administration of BMP-2 with FGF21 should be carefully evaluated.

In summary, this study suggests that FGF21 enhanced BMP-2-dependent osteogenesis in vitro. BMP-2-dependent Smad activation was further increased in the presence of FGF21, which may be the mechanism for enhanced osteogenesis. The effect of FGF21 may be negatively regulated by BMP-2, demonstrating that there is crosstalk between the BMP-2 and FGF21 pathways in both directions.

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