FGF18 inhibits MC3T3-E1 cell osteogenic differentiation via the ERK signaling pathway

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Abstract. Fibroblast growth factor (FGF) 18 is a member of the FGF family and serves a key role in skeletal growth and development. The present study investigated the effect of FGF18 on pre-osteoblast MC3T3-E1 cells and the signaling pathways involved by performing an alkaline phosphatase (ALP) assay and reverse transcription-quantitative polymerase chain reaction. MC3T3-E1 cells incubated in a culture medium supplemented with FGF18 exhibited increased viability when compared with the untreated control cells. In addition, ALP activity was decreased in MC3T3-E1 cells treated with FGF18 plus an osteogenic medium (OM) for 7 and 14 days when compared with untreated and OM-treated controls. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results demonstrated that the expression of osteoblastic-associated genes was significantly repressed in FGF18 plus OM-treated MC3T3-E1 cells, including ALP, collagen type I, osteocalcin, bone sialo protein and osterix. These results suggested that the expression levels of genes associated with osteogenesis were mainly repressed. In addition, combined treatment of MC3T3-E1 cells with OM and FGF18 led to a significant reduction in mineral deposition when compared with the OM-only treated group. Furthermore, FGF18 activated the extracellular signal-regulated kinase pathway in MC3T3-E1 cells, which may have been responsible for the observed decrease in the expression of osteoblastic-associated genes. In conclusion, the results suggest that FGF18 may be involved in MC3T3-E1 cell proliferation and osteoblastic differentiation.

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Introduction

The formation of bone tissue involves two major processes; one of which involves the continuous absorption of old bone tissue by osteoclasts, while the other involves the reconstruction of new bone tissues by osteoblasts (1). Numerous processes are involved in bone formation, including the regulation of osteoblast proliferation, bone matrix maturation, matrix mineralization and apoptosis (2,3). These processes are mediated by bone morphogenetic protein 2, alkaline phosphatase (ALP) and several members of the fibroblast growth factor (FGF) family (4-6).

FGFs are potent mitogens for a wide variety of cells of mesenchymal and neuroectodermal origin (7,8). In addition, FGFs serve a functional role in the differentiation of a variety of cell types, and are involved in a number of developmental processes, including brain patterning, branching morphogenesis and limb development (9). Several FGF family members are active in bone development, including FGF1, FGF2, FGF8, FGF10 and FGF18 (10).

First reported in 1998, FGF18 is a growth factor that exhibits a high homology to FGF8 (11-13). FGF18 is an FGF that has been demonstrated to serve a key role in skeletal growth and development (14). The majority of studies regarding FGF18 have focused on its role in cartilage formation (15,16). A number of studies have demonstrated that FGF18 serves a role in bone formation (17,18), in addition to a positive role in mesenchymal stem cells during bone development (19,20). However, Shimoaka *et al* (21) reported that FGF18 inhibited differentiation and matrix synthesis, and identified the mitogenic action of FGF18 in primary osteoblasts. In order to determine whether these contradicting results may have been

due to use of different cell types, and to determine the effects of FGF18 on osteogenic differentiation, the present study investigated the effect of FGF18 on the viability and differentiation of MC3T3-E1 cells and the potential underlying mechanisms involved. The MC3T3-E1 cell line possesses the ability to self-renew and differentiate into osteoblasts, and is used as a model to study the osteogenic process *in vitro* (22,23).

The aim of the present study was to investigate the role of FGF18 in MC3T3-E1 cells, and examine its effects on the viability and osteoblastic differentiation of these cells *in vitro*.

Materials and methods

Cells culture and reagents. The pre-osteoblastic MC3T3-E1 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA; CRL-2593). L-ascorbic acid, β-glycerophosphate, dexamethasone and the MTT reagent were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The ALP assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China; cat. no. A059-2-96T). Recombinant human FGF18 was obtained from the Engineering Research Center of Bioreactor and Pharmaceutical Development (Ministry of Education, Jilin Agricultural University, Changchun, China). MC3T3-E1 cells were cultured in minimum essential medium Eagle-α (MEM-α; cat. no. 32561037) supplemented with 12% fetal bovine serum (FBS; cat. no. 16000044) and 1% penicillin/streptomycin (cat. no. 10378016; all Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a 5% CO_2 humidified incubator. Osteogenic medium (OM) consisted of 50 μg/ml L-ascorbic acid, 10 mM β-glycerophosphate and 10^{-7} M dexamethasone in MEM-α. Cells were cultured in 24-well cell plates at a density of $2x10^5$ cells/well, and treated with 40 ng/ml FGF18 for 7 and 14 days. The OM or FGF18 plus OM were refreshed every two days for 7 and 14 days. Cells that were cultured with MEM-α supplemented with 12% FBS and 1% penicillin/streptomycin were used as the control.

MTT assay. MC3T3-E1 cells were seeded at $2x10^3$ cells/well in 96-well plates, and cultured in 200 μl complete growth medium supplemented with 0, 5, 10, 20, 40, 80, 160 or 320 ng/ml FGF18 for 72 h. To each well 25 μl MTT solution (5 mg/ml) was then added, and cells were incubated for 4 h. The medium was discarded and 150 μl dimethyl sulfoxide was added to each well. Following incubation for 30 min at room temperature, the absorbance at 490 nm was immediately measured using the SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, LLC, Sunnyvale, CA, USA).

ALP activity. ALP activity was analyzed colorimetrically using an ALP assay kit. Cells were cultured in 24-well cell plates at a density of $1x10^5$ cells/well, and following 7 and 14 days of exposure to control medium, OM or OM plus 40 ng/ml FGF18, the culture medium was removed and cells were washed twice with phosphate-buffered saline (PBS) and solubilized in 0.1% Triton X-100 (buffered in 0.1 M PBS; pH 7.3) at 4˚C for 1 h. Following centrifugation at 200 x g for 5 min at room temperature, ALP activity in the supernatant was determined by measuring the optical density (OD) at 405 and 562 nm using the SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, LLC), and the OD405/OD562 ratio was then calculated. Total protein was quantified using a bicinchoninic acid assay (BCA) kit (cat. no. BCA1-1KT; Sigma-Aldrich; Merck KGaA) to calculate the mean OD/mg protein.

Analysis of osteogenic-associated gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The effects of 40 ng/ml FGF18 on the mRNA expression levels of six osteogenic-associated genes including ALP, collagen type I (Col I), osteocalcin (OCN), bone sialo protein (BSP), osterix (OSX) and runt-related transcription factor 2 (Runx 2) were examined. Total RNA was extracted from $1x10^5$ MC3T3-E1 cells in a 24-well plate using RNAiso Plus reagent (cat. no. D9108A; Takara Biotechnology Co., Ltd., Dalian, China) on days 3, 7 and 14 of culture according to the manufacturer's instructions. The total RNA concentration was determined using a NanoDrop spectrophotometer (cat. no. ND-2000; Thermo Fisher Scientific, Inc., USA). A total of 1 μg RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent kit (cat. no. RR037A; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. qPCR was performed in 20 μl reaction volumes containing SYBR-Green master mix (Roche Applied Science, Penzberg, Germany). The primer sequences (Table I) were designed specifically using Primer Express Software (version 3.0.1; Thermo Fisher Scientific, Inc.), and β-actin served as the endogenous control. Relative mRNA expression was calculated using the comparative $2^{-\Delta\Delta Cq}$ method (24).

Alizarin red S (ARS) staining. At day 14 of treatment with control, OM or OM plus 40 ng/ml FGF18, $1x10^5$ MC3T3-E1 cells were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature, rinsed with PBS and stained with 1 ml/well 0.1% (w/v) ARS (Sigma-Aldrich; Merck KGaA; pH 8.3) for 20 min at room temperature. Cells were then washed with PBS and observed under an inverted light microscope. Stained cells were treated with 10% (w/v) SDS at 37˚C overnight to extract the dye (25), and the level of mineralization was quantified by measuring the absorbance at 405 nm using the SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, LLC).

Western blot analysis. Cells ($3x10^5$) were seeded in 6-well cell plate when confluence reached 75%, and were then treated with OM medium or OM medium plus 40 ng/ml FGF18 for 24 h at 37˚C. The cells were lysed in cold lysis buffer (60 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.25% SDS and 1% Tergitol-type NP40) containing 10 mM NaF, 1 mM Na_3VO_4 and complete protease inhibitor (Roche Diagnostics, Basel, Switzerland) for 30 min on ice and were then centrifuged at 10,000 x g and 4˚C for 15 min. The Enhanced BCA Protein Assay kit (cat no. P0010; Beyotime Institute of Biotechnology, Haimen, China) was used for protein quantification. A total of 40 μg protein was separated by 12% SDS-PAGE and transferred onto a 0.45 μm polyvinylidene difluoride membrane (Whatman plc; GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were blocked with 5% non-fat milk for 20 min at room temperature, and then incubated with the primary antibodies (dilution, 1:1,000) at 4˚C overnight, followed by incubation with the secondary antibody (dilution, 1:3,000) at

Table I. Primer sequences used to detect the expression of *Mus musculus* osteogenic-associated genes.

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	AGAGGGAAATCGTGCGTGAC	CAAGAAGGAAGGCTGGAAAA
ALP	GCTGATCATTCCCACGTTTTC	CTGGGCCTGGTAGTTGTTGT
BSP	CAGAGGAGGCAAGCGTCACT	CTGTCTGGGTGCCAACACTG
OSX	ACCAGGTCCAGGCAACAC	GGGCAGTCGCAGGTAGAA
OCN	CCGGGAGCAGTGTGAGCTTA	ATAGATGCGTTTGTAGGCGGTC
Col I	CCAGCGGTGAAGAAGGAAAGAG	GAACCACGATTGCCAGGAGGAC
Runx2	CCAACTTCCTGTGCTCCGTG	TCTTGCCTCGTCCGCTCC

ALP, alkaline phosphatase; BSP, bone sialo protein; OSX, osterix; OCN, osteocalcin; COL-1, collagen type I; Runx2, runt-related transcription factor 2.

room temperature for 30 min. Enhanced chemiluminescence Plus (cat. no. P0018; Beyotime Institute of Biotechnology) and the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to visualize protein bands, according to the manufacturer's instructions. The rabbit anti-ERK1/2 (cat. no. sc-292838), rabbit anti-phosphorylated (p)-ERK1/2 (cat. no. sc-101760), rabbit anti-β-actin (cat. no. sc-1616) and the horseradish peroxidase-conjugated goat anti-rabbit secondary IgG antibody (cat. no. sc-2004) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). β-actin was used as a loading control.

Statistical analysis. Each treatment was performed in triplicate. The results were analyzed by one-way analysis of variance followed by the Dunnett's post hoc test. Statistical analysis was performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). The results are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FGF18 enhances the viability of MC3T3-E1 cells. The effect of FGF18 on the viability of MC3T3-E1 cells was examined using an MTT assay. As presented in Fig. 1, FGF18 significantly increased the viability of MC3T3-E1 cells in a dose-dependent manner, and the OD value remained stable following 40 ng/ml FGF18. Based on these results, 40 ng/ml FGF18 was considered to be an effective dose, as it enhanced the viability of MC3T3-E1 cells by ~41% at 72 h when compared with untreated controls. This dosage was therefore used in subsequent experiments.

FGF18 decreased ALP activity in MC3T3-E1 cells. The effect of FGF18 on ALP activity in MC3T3-E1 cells was then determined. Following exposure to control media or OM with or without 40 ng/ml FGF18, cells were harvested at 7 and 14 days to assess ALP activity. As shown in Fig. 2, ALP activity in the OM+FGF18 treated group was significantly higher on day 14 when compared with day 7 ($P<0.01$). In addition, cells exposed to OM plus FGF18 demonstrated a significant reduction in ALP activity on day 7 when compared with the OM and control groups ($^{###}P<0.001$ vs. OM; $^{**}P<0.01$ vs. control;

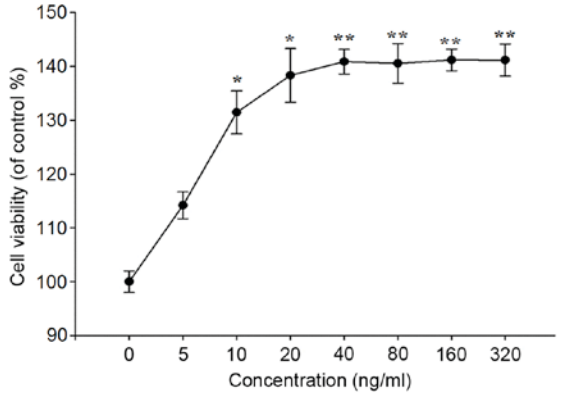

Figure 1. Effect of FGF18 on the viability of MC3T3-E1 cells. MC3T3-E1 cells were treated with 0, 5, 10, 20, 40, 80, 160 and 320 ng/ml FGF18 for 72 h, and cell viability was assessed using an MTT assay. The results are expressed as the mean ± standard deviation (n=3). $^{*}P<0.05$ and $^{**}P<0.01$ vs. control (0 ng/ml FGF18). FGF18, fibroblast growth factor 18.

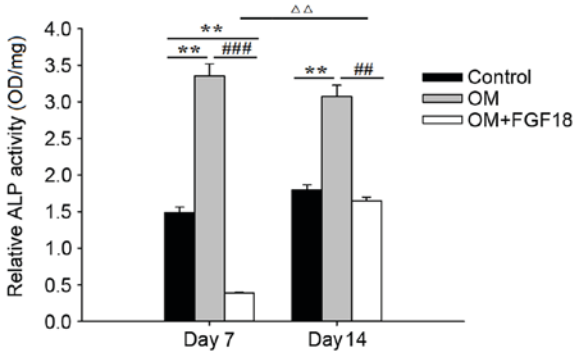

Figure 2. Effect of FGF18 on ALP activity in MC3T3-E1 cells. ALP activity was examined in MC3T3-E1 cells cultured in control, OM or OM plus FGF18 for 7 and 14 days. $^{**}P<0.01$ vs. control; $^{##}P<0.01$ and $^{###}P<0.001$ vs. OM; $^{ΔΔ}P<0.01$ for OM+FGF18, Day 14 vs. Day 7. FGF18, fibroblast growth factor 18; ALP, alkaline phosphatase; OM, osteogenic medium.

Fig. 2). These results suggest that treatment with FGF18 decreases ALP activity in MC3T3-E1 cells.

FGF18 repressed the expression of osteoblastic-associated genes in MC3T3-E1 cells. The effects of FGF18 on the osteogenic differentiation of MC3T3-E1 cells was investigated by

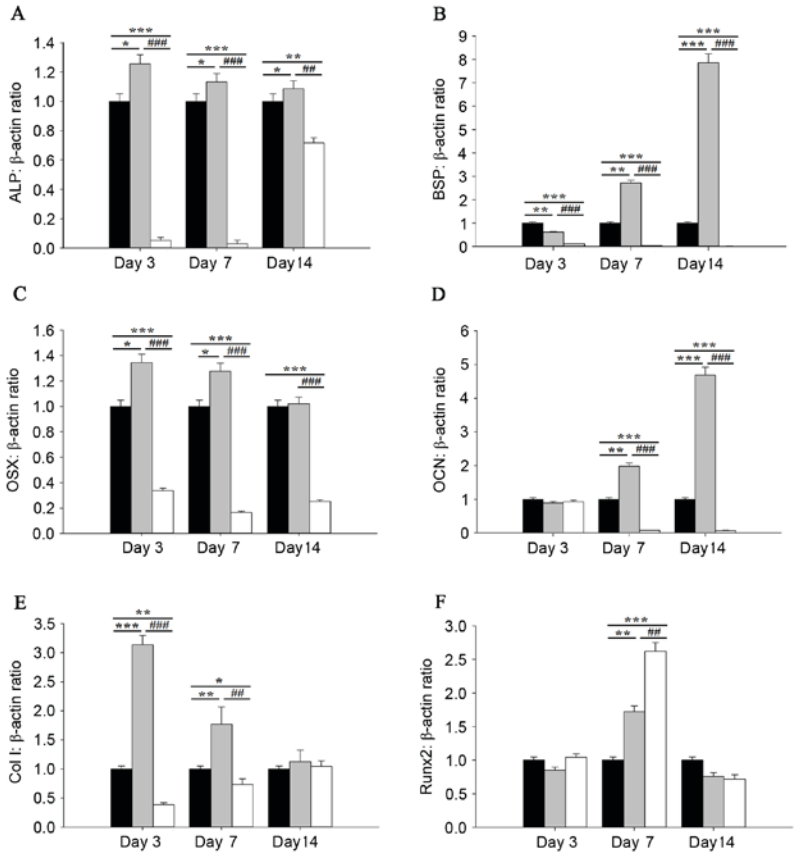

Figure 3. Expression levels of osteogenic-associated genes in MC3T3-E1 cells treated with FGF18. The mRNA expression levels of (A) ALP, (B) BSP, (C) OSX, (D) OCN, (E) COL-1 and (F) Runx2. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. OM. Black bars, control group; gray bars, OM group; white bars, OM+FGF18; FGF18, fibroblast growth factor 18; ALP, alkaline phosphatase; BSP, bone sialo protein; OSX, osterix; OCN, osteocalcin; COL-1, collagen type I; Runx2, runt-related transcription factor 2; OM, osteogenic medium.

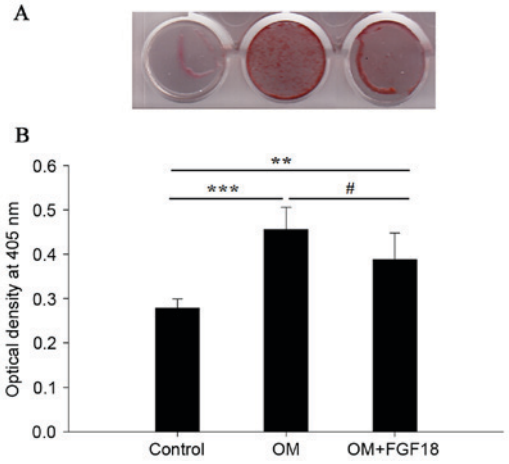

Figure 4. Effect of FGF18 on mineral deposition in MC3T3-E1 cells. (A) Alzarin red S staining and (B) quantification of mineral deposition. **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05 vs. OM. FGF18, fibroblast growth factor 18; OM, osteogenic medium.

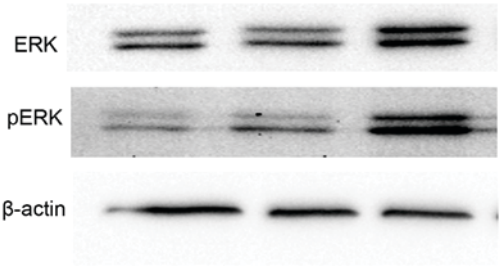

Figure 5. Western blot analysis of FGF18-mediated activation of ERK signaling pathways in MC3T3-E1 cells. β-actin served as a loading control. Lane 1, control group; lane 2, OM group; lane 3, OM+FGF18 group; FGF18, fibroblast growth factor 18; p-ERK, phosphorylated-extracellular signal-regulated kinase.

examining the expression of osteogenic-associated genes, including Col I, Runx2, OSX, OCN, ALP and BSP, at days 3, 7 and 14 of treatment. FGF18 was observed to decrease the expression of ALP, BSP, OSX, OCN and Col I when compared with control and OM-only treated cells (Fig. 3A-E). By contrast, FGF18 treatment significantly enhanced the expression of Runx2 at day 7, whereas its expression was decreased at day 14 when compared with the control and OM groups (Fig. 3F). Runx2 is a key transcription factor involved in osteoblast and chondrogenic differentiation (26). Therefore, it has been hypothesized that Runx2 may be associated with the positive role of FGF18 on chondrogenic differentiation.

FGF18 repressed mineral deposition in MC3T3-E1 cells. To determine the effect of FGF18 on the level of mineralization, ARS staining was employed to visualize mineral deposition in FGF18-treated MC3T3-E1 cells *in vitro*. As shown in Fig. 4,

untreated control MC3T3-E1 pre-osteoblasts exhibited significantly lower levels of mineralization when compared with the OM group. In addition, mineralization was significantly reduced in the OM plus FGF18 group when compared with the OM group (Fig. 4). These results suggest that FGF18 significantly inhibited mineral deposition.

FGF18 activates ERK signaling pathways in MC3T3-E1 cells. The mechanism by which FGF18 functions in osteogenesis was then investigated. As shown in Fig. 5, FGF18 treatment of MC3T3-E1 cells increased the protein expression levels of ERK and phosphorylated-ERK when compared with control and OM groups, which suggests that the ERK signaling pathway was activated. These results suggest that FGF18 may repress MC3T3-E1 differentiation and mineral deposition via the ERK signaling pathway.

Discussion

The results of the present study demonstrated that FGF18 increased the viability of pre-osteoblastic MC3T3-E1 cells and decreased the expression of genes that serve a functional role in osteoblast differentiation and mineral deposition. In addition, FGF18 was observed to increase cell viability in a dose-dependent manner, and increased markedly at a dose of 0-40 ng/ml. At concentrations of FGF18 ≥40 ng/ml, cell viability reached a plateau and no significant difference was observed at an increased dose. Therefore, 40 ng/ml FGF18 was selected as the working concentration for downstream experiments.

In the present study, FGF18 served a negative role in MC3T3-E1 osteogenic differentiation, as it was observed to repress the expression of genes associated with osteogenesis and mineral deposition. FGF18 inhibited ALP activity when compared with the OM-only treated group. In addition, FGF18 treatment decreased the expression of ALP, BSP, OSX and OCN which are early markers of MC3T3 differentiation. OSX expression levels were significantly decreased in the FGF18-treated group when compared with FGF18-untreated groups. OSX is a marker of late osteogenesis, and is abundant in mature osteoblasts (27). Col I and OCN are the two major factors involved in bone matrix mineralization, and Col I is the most abundant protein. By contrast, OCN is the most abundant non-collagenous bone matrix protein, which is expressed in mature osteoblasts. Runx2 is an early transcription factor in osteogenesis, and so its expression is noteworthy when compared with other ostebolastic gene markers (27-29). In the present study, treatment of MC3T3-E1 cells with FGF18 increased Runx2 expression to its highest level at day 7, followed by a marked decrease at day 14, when compared with FGF18-untreated groups. Runx2 is a critical transcriptional regulator of osteoblast and chondrocyte differentiation, as well as a regulator of tumor progression (30). In addition, Runx2 forms a complex with lymphoid enhancer binding factor 1 or transcription factor 4, which subsequently binds to the corresponding binding site in the FGF18 promoter (31). Therefore, the authors hypothesized that the abnormal expression of Runx2 following FGF18 treatment may have been due to the activation of additional cell signaling pathways by FGF18. This requires further investigation in future studies.

The present study demonstrated that FGF18 reduced mineral deposition in MC3T3-E1 cells, as revealed by ARS staining. Following treatment of cells with FGF18, ARS staining was clearly reduced when compared with the OM group. In addition, it was demonstrated that MC3T3-E1 cells might not be a suitable model for assessing osteogenic differentiation. Following 14 days of incubation with OM, MC3T3-E1 cells exhibited positive ARS staining; however, the staining intensity was not as strong as in primary osteoblasts [as previously reported (32)], nor as strong in bone marrow stromal cells (data not shown), thus it may not be a suitable model.

The present study investigated whether FGF18 may function via the ERK signaling pathway to regulate osteogenesis of MC3T3-E1 cells. The results suggested that FGF18 may suppress the osteogenic differentiation of MC3T3-E1 cells via the ERK signaling pathway. This is a novel hypothesis, and it was demonstrated that the protein expression levels of ERK and phosphorylated ERK were markedly enhanced during exposure to OM plus FGF18. A previous report indicated that endogenous Runx2 activity in endothelial cells is phosphatidylinositol 3-kinase/ERK-dependent (33). Therefore, it is possible that the abnormal expression of Runx2 following FGF18 treatment observed in the present study may have been associated with ERK signaling activation. In addition, ERK signaling is known to be involved in mediating cell proliferation and self-renewal (34). Therefore, it is possible that FGF18 repressed the differentiation of MC3T3-E1 cells into mature osteoblasts by maintaining their self-renewing capacity via upregulation of ERK and phosphorylated ERK.

In conclusion, the present study demonstrated that FGF18 increases the viability of MC3T3-E1 cells. In addition, FGF18 serves a negative role in osteogenesis and decreases the expression of osteogenic-associated genes and mineral deposition in MC3T3-E1 cells. The mechanisms by which FGF18 mediates these effects may involve the ERK signaling pathway, as FGF18 was observed to enhance the protein expression levels of ERK and phosphorylated ERK. These results suggest that FGF18 promotes the self-renewal of cells and may suppress MC3T3-E1 osteoblastic maturation via the ERK cell signaling pathway.

Acknowledgements

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