Fat mass and obesity-associated (FTO) gene helps to regulate energy homeostasis in mammals by controlling energy expenditure. In addition, FTO functions in the regulation of obesity and adipogenic differentiation; however, a role in osteogenic differentiation is unknown. This study investigated the effects of FTO on osteogenic differentiation of C3H10T1/2 cells and the underlying mechanism. Expression of osteogenic and endoplasmic reticulum (ER) stress markers were characterized by reverse-transcriptase polymerase chain reaction and western blotting. Alkaline phosphatase (ALP) staining was performed to assess ALP activity. BMP2 treatment increased mRNA expression of osteogenic genes and FTO. Overexpression of FTO increased expression of the osteogenic genes distal-less homeobox5 (Dlx5) and runt-related transcription factor 2 (Runx2). Activation of adenosine monophosphate-activated protein kinase (AMPK) increased FTO expression, and there was a positive feedback loop between FTO and p-AMPK. BMP2 and FTO induced mild ER stress; however, tunicamycin-induced severe ER stress suppressed FTO expression and AMPK activation.

In summary, FTO induces osteogenic differentiation of C3H10T1/2 cells upon BMP2 treatment by inducing mild ER stress via a positive feedback loop with p-AMPK. FTO expression and AMPK activation induce mild ER stress. By contrast, severe ER stress inhibits osteogenic differentiation by suppressing FTO expression and AMPK activation.

Keywords: adenosine monophosphate-activated protein kinase, C3H10T1/2 cells, endoplasmic reticulum stress, fat mass and obesity-associated, osteoblast

INTRODUCTION

Fat mass and obesity-associated (FTO) gene is located on chromosome 16 in humans. FTO helps to regulate energy homeostasis by controlling energy expenditure (Fischer et al., 2009). In 2007, several independent genome-wide association studies and population-based approaches confirmed that associations exist between single nucleotide polymorphisms (SNP) in intron 1 of FTO and obesity (Frayling et al., 2007; Tews et al., 2010). Expression of FTO is high in the early stage of adipogenesis and declines during later stages (Zhao et al., 2014). FTO modulates oxidative stress and the pathogenesis of non-alcoholic fatty liver disease by increasing lipid deposition in liver cells (Guo et al., 2013). However, the
role of FTO in osteogenic differentiation is unknown.

A variety of complex signaling pathways regulate osteogenic differentiation (Komori, 2006; Yamaguchi et al., 2000). Bone morphogenetic proteins (BMPs) are multifunctional proteins that induce osteogenic differentiation and play important roles in various cellular functions, including signal transduction, development, cell growth and repair of bone fractures (Canalis et al., 2003; Wozney, 1998). BMP2 enhances expression of transcription factors such as distal-less homeobox5 (Dlx5) and runt-related transcription factor 2 (Runx2) (Jang et al., 2011b; Javed et al., 2008).

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric enzyme complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits (Oakhill et al., 2009). AMPK regulates energy homeostasis and is ubiquitously expressed (Kanazawa et al., 2008). Activation of AMPK induces osteogenic differentiation via induction of Dlx5-dependent Runx2 expression (Jang et al., 2011b). Consistently, we previously reported that piperine induces osteogenic differentiation via activation of AMPK (Kim et al., 2018; Jang et al., 2011b). An association between AMPK signaling and endoplasmic reticulum (ER) stress has also been reported (Jang et al., 2017; Leclerc et al., 2013).

The ER is vital for synthesis, localization, folding, and secretion of proteins (Gething and Sambrook, 1992). Additionally, ER stress plays an important role in signaling that underlies metabolism, differentiation, and apoptosis of mammalian cells (Ghemrawi et al., 2018; Ozcan et al., 2004). Mild ER stress positively regulates osteogenic differentiation (Jang et al., 2012; Li et al., 2006; Son et al., 2018a); however, tunicamycin (TM) induces severe ER stress by stimulating the unfolded protein response, which results in cellular dysfunction (Li et al., 2006). C/EBP homologous protein (CHOP), a marker of ER stress, induces FTO expression in hepatocytes (Lim et al., 2016). However, the relationship between FTO expression and ER stress in osteoblasts is unknown.

This study demonstrates that BMP2 induces FTO expression by activating AMPK. Thereafter, FTO increases expression of osteogenic genes by inducing mild ER stress.

**MATERIALS AND METHODS**

**Reagents**

Dulbecco’s modified Eagle medium (DMEM), phosphate-buffered saline, penicillin-streptomycin, and 0.25% trypsin-EDTA were purchased from Gibco-BRL (USA). Fetal bovine serum (FBS) was purchased from MP Biomedical (Korea). Compound C was purchased from Abcam (USA). Emerald Amp GRPCR Master Mix was purchased from Takara (Japan), and AmpGene qPCR Green Mix Hi-ROX was purchased from Enzo (USA). Recombinant human BMP2 was purchased from Cowdewi (Korea). Antibodies against BMP2, FTO, CHOP, ATF4 and β-actin were obtained from Santa Cruz Biotechnology (USA). Antibodies against AMPK and phospho-AMPK (p-AMPK) were purchased from Cell Signaling Technology (USA). Antibodies against Dlx5 were purchased from Abcam.

**Cell culture**

The mouse mesenchymal stem cell line C3H10T1/2 (American Type Culture Collection [ATCC], USA) was maintained in DMEM containing 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin in humidified air containing 5% CO2 at 37°C. Differentiation of osteoblasts was induced by the addition of osteogenic medium containing 0.25 g/ml BMP2. The culture medium was replaced every 2 days.

**Reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time PCR analysis**

Total RNA was isolated from cells using TRizol reagent (BioScience Technology, Korea) according to the manufacturer’s instructions. Reverse transcription was performed using 5 μg of total RNA. RT-PCR conditions included initial denaturation at 95°C for 5 min followed by a three-step cycle of denaturation at 95°C for 30 s, annealing at the optimal temperature for each primer pair for 30 s, and extension at 72°C for 30 s. After 25 to 35 cycles, a final extension was performed at 72°C for 5 min. The RT-PCR primer sequences were as follows: β-actin forward, 5′-GACTACCTCATGAAAGTCTC-3′; β-actin reverse, 5′-GATCCACTCTGGTGAAGA-3′; Dlx5 forward, 5′-CAGAAGAGTCGAAAGCTC-3′; Dlx5 reverse, 5′-GGAGCCTGGCCATGAG-3′; Runx2 forward, 5′-TGATCCGGCCCAATCTC-3′; FTO forward, 5′-GGGATGAACTAGGCCATT-3′; FTO reverse, 5′-GGCATGATGGGACCTT-3′; FTO forward, 5′-GGGATGAACTAGGCCATT-3′; FTO reverse, 5′-GGCATGATGGGACCTT-3′; FTO reverse, 5′-GGGATGAACTAGGCCATT-3′

**Transient transfection and luciferase assay**

C3H10T1/2 cells were transiently transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen, USA) as described previously (Son et al., 2018b). Subsequently, the cells were harvested with 0.25 μg/ml BMP2 following 24 h of transfection and assayed using the luciferase reporter assay system (Promega, USA). As an internal control, cytomegalovirus β-galactosidase plasmid was co-transfected into each transfection experiment, and luciferase activity was normalized to the β-galactosidase activity. To confirm the effects of BMP2 on osteogenic medium containing 0.25 g/ml BMP2. The culture medium was replaced every 2 days.

**Silencing of FTO**

The siRNAs for FTO were synthesized chemically (Bioneer, Korea). Silencing of FTO activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Germany).

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Korea), deprotected, annealed, and prepared for transfection according to the manufacturer’s instructions. C3H10T1/2 cells were transfected with the siRNAs using Lipofectamine 2000. The siRNA sequences were as follows: siFTO sense 5′-CUCA-CAGGCCGCUUUAGU-3′; siFTO antisense 5′-ACUAACCGAGGCUGUGAG-3′.

Alkaline phosphatase (ALP) staining
For ALP staining, C3H10T1/2 cells were cultured with BMP2 (0.25 µg/ml) and siFTO (200 nM) for 4 days. Staining was performed using standard protocols. Briefly, cultured cells were fixed with 10% formaldehyde, rinsed twice with de-ionized water, and treated with BCIP/NBT solution (Sigma Aldrich, USA) for 15 min. Following additional washing with phosphate-buffered saline, densitometry analysis was performed on stained cultures using Image J.

Western blot analysis
Cells were harvested using the EzRIPA lysis kit (ATTO Technology, Japan) and centrifuged at 12,000 g for 10 min at 4˚C. Total protein was quantified using the Bradford assay, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. After blocking in 5% skimmed milk prepared in Tris-buffered saline containing Tween 20, the membrane was incubated with specific primary antibodies (1:5,000). Signals were detected using ECL reagent (Advanta-sta, USA). Densitometric analysis of the blotted membrane was performed using a FUSION solo analyzer system (Vilber Lourmat, Germany).

Statistical analysis
All experiments were repeated at least three times. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA). Statistical analysis was performed using the Student’s t-test or ANOVA, followed by Dimcam’s multiple comparison test. All experiments were independently repeated at least three times.

Fig. 1. BMP2 treatment induces FTO expression in C3H10T1/2 cells. (A and B) RT-PCR and real-time PCR analyses were performed using total RNA isolated from C3H10T1/2 cells treated with BMP2 (+, 0.125 µg/ml; ++, 0.25 µg/ml) for 1 day. (C and D) RT-PCR and real-time PCR analyses were performed using total RNA isolated from C3H10T1/2 cells treated with 0.25 µg/ml BMP2 for 12 or 24 h. (E and F) C3H10T1/2 cells were treated with BMP2 for the indicated durations and harvested for western blot analysis using the indicated antibodies. Data represent the mean ± SEM of three individual experiments. All experiments were independently repeated at least three times.

Fig. 2. Overexpression of FTO induces osteogenic differentiation of C3H10T1/2 cells. (A-C) C3H10T1/2 cells were transfected with pcDNA3.1 (2 µg) or pCMV-FTO (+, 1 µg; ++, 2 µg) for 6 h and treated with BMP2 (0.25 µg/ml) for 1 day. (A) RT-PCR analysis was performed using total RNA isolated from cells and primers targeting FTO, Dlx5, Runx2, and β-actin. (B) Real-time PCR was performed using total RNA isolated from cells. (C) Western blot analysis was performed using the indicated antibodies. (D) C3H10T1/2 cells were transfected with pCMV-FTO (+, 0.2 µg; ++, 0.4 µg) or treated with BMP2 (0.25 µg/ml) for 4 days. Data represent the mean ± SEM of three individual experiments. All experiments were independently repeated at least three times.
**RESULTS**

**BMP2 treatment increases FTO expression in C3H10T1/2 cells**

To investigate the expression of FTO during osteogenic differentiation, we treated C3H10T1/2 cells with 0.125 or 0.25 µg/ml BMP2. RT-PCR and real-time PCR analyses demonstrated that BMP2 treatment significantly increased mRNA expression of FTO in a dose-dependent manner (Figs. 1A and 1B). Moreover, BMP2 treatment increased mRNA expression of FTO in a time-dependent manner (Figs. 1C and 1D). Western blot analysis indicated that BMP2 treatment-induced protein expression of FTO in a dose-dependent (Fig. 1E) and time-dependent (Fig. 1F). These results demonstrate that BMP2 induces FTO expression in C3H10T1/2 cells.

**Overexpression of FTO upregulates the osteogenic genes Dlx5 and Runx2 in C3H10T1/2 cells**

To characterize the role of FTO in osteogenic differentiation, we transfected C3H10T1/2 cells with a plasmid harboring FTO. RT-PCR and real-time PCR analyses showed that FTO overexpression upregulated the osteogenic genes Dlx5 and Runx2 in a dose-dependent manner (Figs. 2A and 2B). Western blot analysis confirmed the upregulation of Dlx5 and Runx2 proteins in FTO-overexpressing cells (Fig. 2C). These results indicate that FTO plays a role in osteogenic differentiation.

**Activation of AMPK enhances FTO expression in C3H10T1/2 cells**

To investigate the effect of AMPK activation on FTO expression, we treated C3H10T1/2 cells with BMP2 and AMPK activator CA-AMPK. RT-PCR and real-time PCR analyses demonstrated that AMPK activation significantly increased FTO expression in a dose-dependent manner (Figs. 3A and 3B). Western blot analysis confirmed the upregulation of FTO protein expression in AMPK-activated cells (Fig. 3C). These results suggest that AMPK activation enhances FTO expression in osteogenic cells.

**Knock-down of FTO attenuates BMP2-induced osteogenic differentiation of C3H10T1/2 cells**

To further confirm the role of FTO in osteogenic differentiation, we transfected C3H10T1/2 cells with siFTO. RT-PCR and real-time PCR analyses showed that siFTO treatment significantly reduced FTO expression in a dose-dependent manner (Figs. 4A and 4B). Western blot analysis confirmed the downregulation of FTO protein expression in siFTO-treated cells (Fig. 4C). These results indicate that FTO knock-down attenuates BMP2-induced osteogenic differentiation.

**Conclusion**

In conclusion, our results demonstrate that FTO plays a crucial role in osteogenic differentiation. BMP2 treatment increases FTO expression in C3H10T1/2 cells, and overexpression of FTO upregulates the osteogenic genes Dlx5 and Runx2. Activation of AMPK enhances FTO expression, and knock-down of FTO attenuates BMP2-induced osteogenic differentiation. These findings provide insights into the molecular mechanisms underlying osteogenic differentiation and could have implications for bone regeneration and treatment of osteoporosis.
ing FTO (pCMV-FTO). RT-PCR and real-time PCR analyses showed that overexpression of FTO significantly increased mRNA expression of Dlx5 and Runx2 (Figs. 2A and 2B). Western blot analysis indicated that overexpression of FTO increased protein expression of Runx2 (Fig. 2C). We performed ALP staining to investigate the effect of FTO on ALP activity. ALP activity was significantly increased by FTO overexpression in the BMP2 group compared with that observed in BMP2 group alone (Fig. 2D). These results strongly suggest that FTO positively regulates osteogenic differentiation.

**Knockdown of FTO attenuates BMP2-induced upregulation of the osteogenic gene Runx2 in C3H10T1/2 cells**

To further investigate the role of FTO in osteogenic differentiation, we transfected C3H10T1/2 cells with FTO-targeting siRNA (siFTO). Knockdown of FTO attenuated the BMP2-induced increase in mRNA expression (Figs. 3A and 3B) and protein expression (Fig. 3C) of Runx2. ALP staining was performed to confirm that knockdown of FTO perturbed osteogenic differentiation. This demonstrated that knockdown of FTO attenuated the BMP2-induced increase in ALP activity (Fig. 3D). Taken together, these results strongly suggest that FTO positively regulates osteogenic differentiation by modulating Runx2 expression and ALP activity.

**p-AMPK upregulates FTO in C3H10T1/2 cells**

We next investigated whether AMPK regulates FTO expression in C3H10T1/2 cells. RT-PCR and western blot analyses demonstrated that transfection of pcDNA3.0-cmyc-mAMPKα312, which harbored a constitutively active form of AMPK (CA-AMPK), increased mRNA expression (Fig. 4A) and protein expression (Fig. 4B). Inhibition of AMPK activity using compound C (Com.C) attenuated BMP2-induced upregulation of FTO and p-AMPK (Figs. 4C and 4D). ALP staining was performed to investigate if activation of AMPK affected osteogenic differentiation. This demonstrated that expression of CA-AMPK increased ALP activity (Fig. 4E). Additionally, treatment with Com.C attenuated BMP2-induced ALP activity (Fig. 4F). Furthermore, FTO-luc activity is increased by BMP2 or CA-AMPK but decreases with Com.C (Fig. 4G). Thus, AMPK upregulates FTO.

**FTO induces phosphorylation of AMPK in C3H10T1/2 cells**

Next, we determined if FTO directly activates AMPK in C3H10T1/2 cells. Overexpression of FTO increased phosphorylation of AMPK (Fig. 5A). Consistently, knockdown of FTO abrogated BMP2-induced phosphorylation of AMPK (Fig. 5B).

**FTO and p-AMPK induce mild ER stress**

Mild ER stress induces osteogenic differentiation (Murakami et al., 2009; Son et al., 2018a). BMP2 treatment induced mild ER stress in C3H10T1/2 cells this effect was attenuated by knockdown of FTO or treatment with Com.C (Fig. 6A). Thus, we speculated that FTO and p-AMPK induce mild ER stress in C3H10T1/2 cells. To investigate this, we transfected C3H10T1/2 cells with pCMV-FTO in the presence or absence of Com.C. Expression of ATF4 and CHOP was increased upon transfection of pCMV-FTO and this effect was attenuated in the presence of Com.C (Figs. 6B and 6D). Additionally, expression of ATF4 and CHOP was increased upon transfection of pcDNA3.0-AMPK and this effect was attenuated by knockdown of FTO (Figs. 6C and 6E). These results suggest...
that pAMPK and FTO stimulate osteogenic differentiation by inducing mild ER stress.

**Severe ER stress prevents BMP2-induced upregulation of FTO and p-AMPK**

TM strongly induces ER stress (Jang et al., 2011a; Li et al., 2006). To investigate whether severe ER stress perturbs BMP2-induced upregulation of FTO and p-AMPK, we compared BMP2-induced expression of FTO and p-AMPK in the absence and presence of TM. RT-PCR analysis revealed that TM treatment increased mRNA expression of CHOP but decreased that of FTO (Fig. 7A). Western blot analysis demonstrated that TM treatment abrogated the BMP2 induced increase in p-AMPK (Fig. 7B). ALP staining confirmed that TM treatment attenuated the BMP2-induced increase in ALP activity (Fig. 7C).

**DISCUSSION**

Previously reported that that FTO-knock out mice display postnatal growth retardation, which manifests as reduced body weight and bone mineral density (Guo et al., 2011); however, the molecular mechanism by which FTO stimulates osteogenic differentiation remains unknown. Here, we demonstrate for the first time that FTO stimulates osteogenic differentiation by inducing mild ER stress. The results of the current study illustrate one mechanism underlying the role of FTO in osteogenic differentiation.

A relationship between FTO and AMPK has been previously reported (Pitman et al., 2013; Wu et al., 2017). One study demonstrated that AMPK decreases lipid accumulation in skeletal muscle cells by suppressing FTO expression (Wu et al., 2017). Another study revealed that knockdown of FTO reduces phosphorylation of AMPK in Alzheimer's disease (Pitman et al., 2013). The current study demonstrated that activation of AMPK markedly increased expression of FTO and that a positive feedback loop existed between FTO and p-AMPK in C3H10T1/2 cells (Figs. 4 and 5). However, other tissues have not yet studied positive feedback between FTO and p-AMPK. Therefore, our results in osteoblasts can be highly appreciated.

In osteoblasts, mild ER stress is important for differentiation (Jang et al., 2012; Murakami et al., 2009; Son et al., 2018a) but inhibited by severe ER stress (Jang et al., 2011a, 2014). FTO increases CHOP and other ER stress markers in hepatocyte (Lim et al., 2016). The current study demonstrated that FTO and p-AMPK stimulated osteogenic differentiation by inducing mild ER stress and that induction of severe ER stress upon the application of TM reduced FTO expression and AMPK activation. These results demonstrate that FTO and AMPK are important regulators of osteogenic differentiation induced by mild ER stress and that a positive feedback loop exists between FTO and p-AMPK. In addition, ER stress may be a therapeutic target for the development of drugs to treat bone disorders such as osteoarthritis. Specifically, it may be possible to halt osteogenic differentiation before activation of the positive feedback loop between p-AMPK and FTO by targeting the mechanisms underlying ER stress.

In addition, FTO is a demethylase that was first reported to catalyze the oxidative demethylation of N6-methyladenosine (m6A) in mRNA (Jia et al., 2011). Our results show that the expression of FTO induces mild ER stress. The mechanism between demethylation of FTO and mild ER stress has not been reported yet. However, several studies have reported that ER stress genes altered DNA and histone methylation patterns in or near the promoters of these genes (Lee and Ozcan, 2014; Li et al., 2012; Soeda et al., 2017). Thus, we consider that FTO, an m6A demethylase, regulates demethylation in promoters of ER stress genes.

In summary, FTO induces osteogenic differentiation of C3H10T1/2 cells via a positive feedback loop with p-AMPK. FTO and p-AMPK stimulate osteogenic differentiation by inducing mild ER stress; however, TM-induced severe ER stress suppresses FTO expression and AMPK activation (Fig. 7D).
Disclosure
The authors have no potential conflicts of interest to disclose.

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