Biochemical and Biophysical Research Communications xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ER stress-inducible ATF3 suppresses BMP2-induced ALP expression and activation in MC3T3-E1 cells

Jae-kyung Park [a,1], Hoon Jang [a,b,1], SeongSoo Hwang [c,1], Eun-Jung Kim [a], Dong-Ern Kim [a], Keon-Bong Oh [c], Dae-Jin Kwon [c], Jeong-Tae Koh [d], Kumi Kimura [e], Hiroshi Inoue [e], Won-Gu Jang [f,*], Jeong-Woong Lee [a,b,*]

[a] Research Center of Integrative Cellulomics, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea
[b] Functional Genomics, School of Engineering, University of Science and Technology (UST), Daejeon 305-806, Republic of Korea
[c] Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Suwon, Republic of Korea
[d] Dental Science Research Institute and BK21, School of Dentistry, Chonnam National University, Gwangju 500-757, Republic of Korea
[e] Department of Physiology and Metabolism, Brain/Liver Interface Medicine Research Center, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan
[f] Department of Biotechnology, School of Engineering, Daegu University, Gyeongbuk 712-714, Republic of Korea

ARTICLE INFO

Article history:
Received 18 November 2013
Available online xxxx

Keywords:
Activating transcription factor 3 (ATF3),
Bone morphogenetic protein 2 (BMP2)
Tunicamycin
Osteoblast differentiation

ABSTRACT

Endoplasmic reticulum (ER) stress suppresses osteoblast differentiation. Activating transcription factor (ATF) 3, a member of the ATF/cAMP response element-binding protein family of transcription factors, is induced by various stimuli including cytokines, hormones, DNA damage, and ER stress. However, the role of ATF3 in osteoblast differentiation has not been elucidated. Treatment with tunicamycin (TM), an ER stress inducer, increased ATF3 expression in the preosteoblast cell line, MC3T3-E1. Overexpression of ATF3 inhibited bone morphogenetic protein 2-stimulated expression and activation of alkaline phosphatase (ALP), an osteogenic marker. In addition, suppression of ALP expression by TM treatment was rescued by silencing of ATF3 using shRNA. Taken together, these data indicate that ATF3 is a novel negative regulator of osteoblast differentiation by specifically suppressing ALP gene expression in preosteoblasts.

1. Introduction

The endoplasmic reticulum (ER) has various functions including the synthesis, localization, folding, and secretion of proteins [1]. ER stress, which is included by ultraviolet light, viral infection and several micro-environmental stimuli [2], plays a key role in signal transduction that is important for metabolism, differentiation, and apoptosis of mammalian cells [3]. Tunicamycin (TM) induces ER stress, the unfolded protein response, apoptosis, and cellular dysfunction [4,5]. A recent study demonstrated that TM blocks differentiation of osteoblasts by stimulating ER stress [6].

Osteoblasts are a type of mesenchymal cell and constitute bone along with chondrocytes, osteoclasts, and bone marrow stromal cells [7]. These cells express various markers, such as osteocalcin (OC), alkaline phosphatase (ALP), bone sialoprotein, osterix, collagen type 1, osteopontin and osteonectin [8,9]. Osteoblasts differentiate from bone precursor cells in response to various hormones, cytokines and transcription factors, to form mineralized bone [10,11]. Bone morphogenetic protein-2 (BMP2) stimulates bone formation by activating the Smad1/5/8 pathway and Runt-related transcription factor 2 (Runx2), which controls expression of osteogenic markers such as OC, bone sialoprotein, collagen type 1, and osteopontin [12–14]. BMP2 is a member of the transforming growth factor superfamily and plays important roles in various cellular functions, including signal transduction, development, cell growth, and repair of bone fractures [15–17]. A recent study reported that differentiation of osteoblasts is regulated by a type of ER stress that occurs via BMP2 induction [18].

Activating transcription factor (ATF) 3 is a member of the ATF/cAMP response element-binding protein (CREB) family of transcription factors and is induced by various signals including

Abbreviations: β-GP, β-glycerophosphate; AA, ascorbic acid 2-phosphate; AD-shATF3, anti-ATF3 shRNA; ALP, alkaline phosphatase; ATF, activating transcription factor; BMP2, bone morphogenetic protein-2; CREB, cAMP response element-binding; ER, endoplasmic reticulum; MOI, multiplicity of infection; OC, osteocalcin; TM, tunicamycin.

* Corresponding authors. Fax: +82 53 850 6559 (W.-G. Jang). Address: Research Center of Integrative Cellulomics, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea. Fax: +82 042 860 4608 (J.-W. Lee).

E-mail addresses: myfreeJK@kribb.re.kr (J.-k. Park), Janghoon@kribb.re.kr (H. Jang), hwangss@korea.kr (S. Hwang), eunjungk@paran.com (E.-J. Kim), pawust86@kribb.re.kr (D.-E. Kim), keonoh@korea.kr (K.-B. Oh), daejinkwon@gmail.com (D.-J. Kwon), jtkoh@chonnam.ac.kr (J.-T. Koh), inoue-h@staff.kanazawa-u.ac.kr (H. Inoue), jangwg@daegu.ac.kr (W.-G. Jang), jwlee@kribb.re.kr (J.-W. Lee).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.11.121

growth factors, cytokines, hormones, hypoxia, DNA damage, and a variety of ER stresses [19,20]. Several recent studies suggested that ATF3 functions in general adaptive responses, such as environmental, emotional and nutritional alterations [21–23]. ATF3 is expressed in various cells and its dysfunction is closely related to various inflammatory diseases and cancer [20]. In addition, ATF3 is stimulated by the ER stress inducer, TM [24]. ATF3 plays key roles in atherosclerosis, hyperlipidemia [25,26], adipocyte differentiation [27], and chondrocyte differentiation [28]. However, it is not known whether ATF3 influences the differentiation of osteoblasts. Glucose metabolism is closely correlated with bone metabolism, and TM regulates differentiation of osteoblast.

This study investigated whether ATF3 regulates osteoblast differentiation. BMP2 treatment reduced ATF3 expression at the mRNA and protein levels, and overexpression of ATF3 negatively regulated BMP2-induced ALP gene expression. Furthermore, silencing of ATF3 rescued TM-induced suppression of osteoblast differentiation.

2. Materials and methods

2.1. Reagents

Recombinant human BMP2 protein was obtained from Cowellmedi Co. (Pusan, Korea). TRIzol reagent and Lipofectamine LTX were purchased from Invitrogen (Carlsbad, CA). Anti-ATF3 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (CA). Ascorbic acid 2-phosphate (AA), β-glycerophosphate (β-GP), and TM were purchased from Sigma Aldrich Co. (St. Louis, MO).

2.2. Plasmids and adenovirus

The ALP-Luc (−1783 bp to +135 bp) reporter construct was kindly provided by Dr. Franceshi (University of Michigan). pcDNA3.1-ATF3 and -1850-ATF3-Luc [25] reporter construct were kindly provided by Dr. Kitajima (Gunma University). ATF3 shRNA was designed by iGENE, Ltd (Tsukuba, Japan).

2.3. Cell culture and transient transfection

MC3T3-E1 cells were cultured in α-minimal essential medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) in humidified air containing 5% CO_2 at 37 °C. Cells were transiently transfected with the indicated plasmid DNA using Lipofectamine LTX. The dual-luciferase system (Promega) was used to measure promoter activity. The firefly luciferase signal was normalized to the Renilla luciferase signal. Luminescence was detected using a Wallac 1420 VICTOR2 microplate reader (Perkin Elmer, USA). For viral infection, cells were treated with AD-shATF3 at the designated multiplicity of infection (MOI) in 2% serum. After 4 h, an equivalent volume of medium containing 10% fetal bovine serum was added, and cells were incubated for an additional 24–48 h.

2.4. RT-PCR analysis

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. Reverse transcription was performed using 2 μg total RNA. PCR conditions were as follows: initial denaturation of 95 °C for 5 min; 22–36 cycles of 94 °C for 30 s, the optimised annealing temperature for 30 s, and 72 °C for 30 s; followed by a final extension of 72 °C for 5 min. The primer sequences were as follows: ATF3 forward, 5′-TTCGCCATCCAGAATAAACAC-3′ and reverse, 5′-TTTCTGCAGGCACTCTGTCTT-3′; ALP forward, 5′-CTGCCT ACTTGTGTGGCGTGA-3′ and reverse, 5′-CCACCCATGATCA CGTGC ATA-3′; OC forward, 5′-CTCCTGAGTCTGACAAAGCCTT-3′ and reverse, 5′-GCTGTGACATCCATTACTTGC-3′; GAPDH forward, 5′-ACC ACAGTCCATGCCATCAC-3′ and reverse, 5′-TCCACCACCCTGTTGCT GTA-3′. Quantitative real-time PCR was performed using the TOP-real qPCR SYBR kit and a Rotor-Gene Q thermal cycler (Qiagen, USA). The thermal cycling conditions were as follows: initial denaturation of 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s.

2.5. ALP staining

Cells were fixed in 70% ethanol for 30 min, and then rinsed three times with deionized water. Fixed cells were treated with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP®/NBT) solution (Sigma Aldrich Co.) for 30 min.

2.6. Statistical analysis

All experiments were repeated at least three times, and statistical analysis was performed using a Student's *t*-test or analysis of variance followed by Duncan's multiple comparison tests. A *P*-value < 0.05 was considered significant. Results are expressed as mean ± SD of triplicate independent samples.

3. Results

3.1. ATF3 expression decreases in MC3T3-E1 cells cultured in osteogenic conditions

BMP2 increases the expression levels of ER stress markers such as Bip, C/EBP homologous protein, ATF4, and ATF6 [17,18]. ATF3 is involved in various cellular functions and is induced by ER stress [20]. To identify the role of ATF3 in osteoblast differentiation, we first examined ATF3 expression in MC3T3-E1cells treated with BMP2. RT-PCR analysis revealed that BMP2 reduced the mRNA level of ATF3, but increased the mRNA levels of ALP and OC, which are osteoblast differentiation markers (Fig. 1A). Furthermore, quantitative real-time PCR revealed that these changes in mRNA levels occurred in a time dependent manner (Fig. 1B and C). BMP2 treatment also reduced the protein level of ATF3 (Fig. 1D). As an alternative way of inducing osteoblast differentiation, cells were treated with AA and β-GP following which the mRNA level of ATF3 was reduced and the mRNA levels of ALP and OC were increased (Fig. 1E). Real-time PCR and western blot revealed that these changes occurred (Fig. 1F–H). To confirm that BMP2 and AA/β-GP suppress ATF3 transcription, a transient transfection assay was performed using a luciferase reporter containing the promoter of the ATF3 gene. Consistently, both BMP2 and AA/β-GP decreased TM-induced promoter activity of the ATF3 (Fig. 1I and J). Taken together, ATF3 is significantly reduced by treatment with osteogenesis inducing factors such as BMP2 and AA/β-GP in MC3T3-E1 cells.

3.2. TM-induced ER stress inhibits BMP2-induced osteoblast differentiation and increases ATF3 expression

Our previous study showed that ER-stressor TM suppressed BMP2-induced osteoblast differentiation, and activates ATF6 [6,17]. Therefore, we examined whether ATF3 expression is regulated by ER stress in MC3T3-E1 cells. TM, which potently induces ER stress, significantly inhibited BMP2-induced ALP gene expression in a dose-dependent manner (Fig. 2A). TM treatment also reduced ALP staining, an indicator of ALP activity, in MC3T3-E1 cells in a dose-dependent manner (Fig. 2B). However, TM treatment

Please cite this article in press as: J.-k. Park et al., ER stress-inducible ATF3 suppresses BMP2-induced ALP expression and activation in MC3T3-E1 cells, Biochem. Biophys. Res. Commun. (2013), http://dx.doi.org/10.1016/j.bbrc.2013.11.121