Biochemical and Biophysical Research Communications 463 (2015) 241–247

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

MEK5 suppresses osteoblastic differentiation

Shoichi Kaneshiro [a, b], Dai Otsuki [b], Kiyoshi Yoshida [b], Hideki Yoshikawa [b], Chikahisa Higuchi [b, *]

[a] Department of Orthopaedic Surgery, Japan Community Health Care Organization Osaka Hospital, 4-2-78 Fukushima, Fukushima Ward, Osaka City, Osaka 553-0003, Japan
[b] Department of Orthopaedic Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 30 April 2015
Available online 19 May 2015

Keywords:
MEK5
Osteoblastic differentiation
MEK5 inhibition
MEK5 inhibitor

ABSTRACT

Extracellular signal-regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family and is activated by its upstream kinase, MAPK kinase 5 (MEK5), which is a member of the MEK family. Although the role of MEK5 has been investigated in several fields, little is known about its role in osteoblastic differentiation. In this study, we have demonstrated the role of MEK5 in osteoblastic differentiation in mouse preosteoblastic MC3T3-E1 cells and bone marrow stromal ST2 cells.

We found that treatment with BIX02189, an inhibitor of MEK5, increased alkaline phosphatase (ALP) activity and the gene expression of ALP, osteocalcin (OCN) and osterix, as well as it enhanced the calcification of the extracellular matrix. Moreover, osteoblastic cell proliferation decreased at a concentration of greater than 0.5 μM. In addition, knockdown of MEK5 using siRNA induced an increase in ALP activity and in the gene expression of ALP, OCN, and osterix. In contrast, overexpression of wild-type MEK5 decreased ALP activity and attenuated osteoblastic differentiation markers including ALP, OCN and osterix, but promoted cell proliferation. In summary, our results indicated that MEK5 suppressed the osteoblastic differentiation, but promoted osteoblastic cell proliferation. These results implied that MEK5 may play a pivotal role in cell signaling to modulate the differentiation and proliferation of osteoblasts.

Thus, inhibition of MEK5 signaling in osteoblasts may be of potential use in the treatment of osteoporosis.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Extracellular signal-regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family and is activated by MAPK kinase 5 (MEK5), which is a member of the MEK family [1]. The principal downstream target of MEK5 is ERK5, which is also known as MAPK7 or big MAP kinase 1(BMK1) [2]. Because ERK5 is the only known substrate of MEK5, all effects of MEK5 have been attributed to its ability to activate ERK5 [3].

The MEK5-ERK5 pathway is one of the lesser studied members among the MAPK family. This pathway has been implicated in cell survival, antiapoptotic signaling, angiogenesis, cell motility, differentiation and cell proliferation [4–6]. However, still much remains to be learnt regarding the potential activators of this pathway and the several intermediary molecules involved in signaling both the upstream and downstream activation of MEK5-ERK5.

MEK5-ERK5 is over-expressed or constitutively active in a number of cancers, such as prostate cancer and breast cancer, in comparison with the expression of the pathway in healthy cells [7,8]. On the other hand, the MEK5-ERK5 signaling pathway has been implicated in neurotrophin-mediated protective effects on apoptosis by serum withdrawal in neurons [9,10]. It has also been reported that MEK5-ERK5 is involved in cardiac hypertrophy [11] and cell fusion during skeletal muscle differentiation [12].

Our previous work demonstrated the functions of two other members of the MEK family, MEK1 and MEK2 in osteoblast differentiation. MEK1 could act as a positive modulator for the osteoblast differentiation, whereas MEK2 could act as a negative modulator [13,14].

These findings suggested a potential role of MEK5 in the osteoblastic differentiation. Thus far, this role has not been well elucidated, and no report has explained functions of MEK5 in osteoblasts.

* Corresponding author. Fax: +81 6 6879 3559.
E-mail address: c-higuchi@umin.ac.jp (C. Higuchi).

http://dx.doi.org/10.1016/j.bbrc.2015.05.035
0006-291X/© 2015 Elsevier Inc. All rights reserved.

In this study, we examined the functions of MEK5 on osteoblastic differentiation and proliferation in mesenchymal cell lines.

2. Materials and methods

2.1. Cell culture

Mouse preosteoblastic MC3T3-E1 cells and bone marrow stromal ST2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Road Logan, UT, USA) as the growth medium at 37 °C under a humidified 5% CO_2 atmosphere. ST2 cells were cultured for cell growth in RPMI 1640 (Invitrogen) containing 10% FBS. For each assay, the growth medium was replaced and supplemented with 0.2 mM ascorbic acid (Sigma–Aldrich, St. Louis, MO, USA) and 4 mM β-glycerophosphate (Sigma–Aldrich), which was the differentiation medium. For all assays using ST2 cells, the differentiation medium was added with 50 ng/ml recombinant human BMP-2 (rhBMP-2; osteopharma, Osaka, Japan). The medium was renewed every 3 days.

2.2. Proliferation assay

MC3T3-E1 cells and ST2 cells were cultured in 96-well plates at 2.0×10^4 cells/cm^2 in the differentiation medium. Cell proliferation was assessed using the Premix WST-1 cell proliferation assay system (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. This assay was performed every 24 h for 3 days.

2.3. Alkaline phosphatase (ALP) staining and activity

Cells were seeded in 24-well plates at 2.0×10^4 cells/cm^2. After a 24 h incubation in the growth medium, cells were treated with BIX02189, MEK5 inhibitor (Selleck, Houston, TX, USA), in the differentiation medium for 3 days.

For ALP staining, cells were washed with phosphate-buffered saline (PBS) (Sigma–Aldrich) and fixed for 15 min with 10% formalin at room temperature. After fixation, the cells were incubated with the ProtoBlot II AP System with Stabilized Substrate (Promega, Madison, WI, USA) for 1 h at room temperature.

For measurement of ALP activity, cells were washed twice with PBS and lysed in mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) according to the manufacturer's protocol. ALP activity was assayed using *p*-nitrophenylphosphate as a substrate in an Alkaline Phosphatase Test Wako (Wako Pure Chemicals Industries, Ltd., Osaka, Japan). The protein content was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce).

2.4. Alizarin Red S staining

MC3T3-E1 cells were cultured for 28 days on 24-well plates in the differentiation medium. The cells were subsequently washed twice with PBS, fixed in 10% formalin for 10 min, and stained with Alizarin Red S (Sigma–Aldrich) at pH 6.3 for 1 h. Following staining, the Alizarin Red S solution was discarded and cells washed three times with distilled water. Bound Alizarin Red was dissolved in 200 μl of 100 mM hexadecylpyridium chloride (Sigma–Aldrich). The absorbance of the supernatant was measured at 570 nm.

2.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). First-strand cDNA was synthesized by reverse transcription PCR using SuperScript II RNase H-reverse transcriptase (Life Technologies Japan, Tokyo, Japan). Each cDNA was measured using quantitative real-time PCR of a Light Cycler system (Roche Applied Science, Basel, Switzerland). The SYBR Green assay using a Quantitect SYBR Green PCR Kit (Qiagen), in which each cDNA sample was evaluated in triplicate 20-μl reactions, was used for all target transcripts. Expression values were normalized to GAPDH. The following primers were used;

ALP (forward primer 5′-AATCGGAACAACCTGACTGACC-3′; reverse primer 5′-TCCTTCCAGCAAGAAGAA-3′),
Osteocalcin (forward primer 5′-CTCACTCTGCTGGCCCTG-3′; reverse primer 5′-CCGTAGATGCGTTTGTAGGC-3′),
Osterix (forward primer 5′-AGGCACAAAGAAGCCATAC-3′; reverse primer 5′-AATGAGTGAGGGAAGGGT-3′),
Runx2 (forward primer 5′-GCTTGATGACTCTAAACCTA-3′; reverse primer 5′-AAAAAGGGCCCAGTTCTGAA-3′), and
GAPDH (forward primer 5′-TGAACGGGAAGCTCACTGG-3′; reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′).

2.6. Western blotting

Cells were rapidly lysed on ice using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease and phosphatase inhibitors. The lysates were centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatants were used for the electrophoretic separations, following protein quantitation using BCA protein assay. Western blotting was performed using antibodies against ERK5 (Sigma–Aldrich), phospho-p42/44 MAPK (Thr202/Tyr204) (Cell Signaling Technology), p42/44 MAPK (Cell Signaling Technology), MEK1 (Cell Signaling Technology), MEK2 (Cell Signaling Technology), and MEK5 (Santa Cruz Biotechnology, Inc., Austin, TX, USA). To control protein loading, blots were also treated with β actin antibody (Cell Signaling Technology).

2.7. MEK5 knockdown by RNA interference

MC3T3-E1 cells and ST2 cells were transfected with small interfering RNA (siRNA) using Lipofectamine RNAiMAX (Invitrogen) according to the reverse transfection method in the manufacturer's protocol.

Two different sets of MEK5 siRNA oligos were purchased from Qiagen for MEK5 knockdown.

Cells transfected with siRNA were seeded in 24-well plates at a 1.0×10^4 cells/cm^2. They were incubated for 48 h and expression of MEK1, MEK2, and MEK5 was determined using western blotting. For assays of ALP staining, ALP activity, and quantitative real-time PCR of osteoblastic marker genes, the medium was subsequently replaced with the differentiation medium, and the cells were incubated for 3 days before use in experiments.

2.8. MEK5 overexpression by infection with adenovirus vectors

Adenovirus expressing MEK5 and β-galactosidase were purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Each recombinant adenovirus was plaque purified, expanded, and tittered in 293 cells (Riken Cell Bank). MC3T3-E1 cells were infected and 2 days later, the medium was replaced with differentiation medium for each assay.

2.9. Statistical analysis

All data are expressed as means ± standard deviation (SD). A minimum of 3 independent experiments were performed for each assay. Statistical analysis was performed using a two-sided

unpaired Student's t-test or analysis of variance (ANOVA) for multiple comparisons. Differences between experimental groups were considered significant for p values <0.05.

3. Results

3.1. Inhibition of ERK5 phosphorylation and proliferation of MC3T3-E1 preosteoblastic cells by MEK5 inhibitor BIX02189

Before evaluating the functions of MEK5 in osteoblastic cells, the effect of a MEK5 inhibitor BIX02189 on the phosphorylation of ERK5, a substrate of MEK5, was determined in MC3T3-E1 cells using Western blotting (Fig. 1A). We confirmed two bands, phosphorylated and nonphosphorylated ERK5 (Fig. 1A, upper lane), which was consistent with previous reports. Treatment with BIX02189 decreased the level of ERK5 phosphorylation in a dose-dependent manner (Fig. 1A, upper lane). No effects on phosphorylation of ERK1/2 were concurrently observed (Fig. 1A, middle two lanes).

Fig. 1B shows the results of the proliferation assay, wherein MC3T3-E1 cells treated with varying concentrations of BIX02189 in a dose-dependent manner reduced their proliferation on days 1–3.

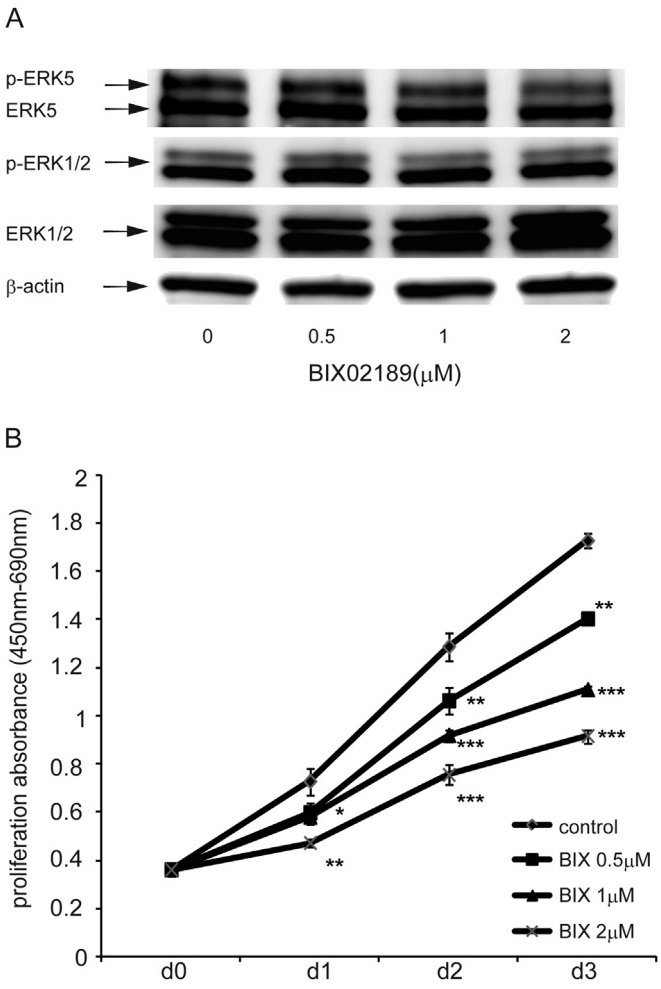

Fig. 1. Effects of MEK5 inhibitor BIX02189 on phosphorylation of ERK5 and proliferation of MC3T3-E1 cells. (A) Phosphorylation levels of ERK5, downstream kinase of MEK5, and other ERK family, ERK1/2 by the treatment with BIX02189 in a dose-dependent fashion. β-Actin was used as the internal control. (B) Proliferation of MC3T3-E1 cells by the treatment with increasing concentrations of BIX02189. Data are means ± SD of 3 independent experiments performed in duplicate (*$P < 0.05$; **$P < 0.001$; ***$P < 0.001$.).

These data indicated that BIX02189 inhibited MEK5-ERK5 signaling pathway without affecting the MEK1/2-ERK1/2 pathway and suggested that inhibition of the MEK5-ERK5 pathway decreased the proliferation of osteoblastic cells.

3.2. Promotive effects of MEK5 inhibitor BIX02189 on osteoblastic differentiation of MC3T3-E1 cells

To confirm the functions of MEK5 on osteoblastic differentiation, the effects of BIX02189 on osteoblastic differentiation in MC3T3-E1 preosteoblastic cells were investigated. Treatment with BIX02189 induced strong ALP staining (Fig. 2A, lower panel) and ALP activity (Fig. 2A, upper graph) in a dose-dependent manner. Furthermore, Alizarin Red S staining indicated that BIX02189 promoted the mineralization of extracellular matrix (ECM) (Fig. 2B, lower panel) and Fig. 2B upper graph showed a dose-dependent increase of mineralization. Quantitative real-time PCR revealed an accelerated dose-dependent mRNA expression of the osteoblastic differentiation markers, including ALP, OCN, and osterix by MEK5 inhibitor (Fig. 2C). On the other hand, the expression of transcription factor Runx2 was not affected by the treatment with inhibitor (Fig. 2C).

These results suggested that MEK5-ERK5 signaling may have a suppressive effect on osteoblastic differentiation in MC3T3-E1 cells.

3.3. Promotive effects of MEK5 knockdown on the osteoblastic differentiation of MC3T3-E1 cells

To further confirm the function of MEK5 in osteoblastic differentiation of MC3T3-E1 cells, we investigated biological changes in cell differentiation following MEK5 knockdown using an RNA interference method. First, low-level MEK5 expression was observed at 48 h after transfection with two different MEK5 siRNAs (Fig. 3A, upper lane). In contrast, expression of the proteins, MEK1 and MEK2 were not affected by MEK5 siRNA (Fig. 3A, middle two lanes). ALP activity and staining were increased by MEK5 knockdown using both siRNAs (Fig. 3B). Similar to the effects observed for MEK5 inhibitor BIX02189, MEK5 knockdown led to the accelerated mRNA expression of ALP, OCN, and osterix but did not significantly affected the mRNA expression of Runx2 in the quantitative real-time PCR assay (Fig. 3C).

These data support the results achieved with BIX02189 experiments in MC3T3-E1 cells.

3.4. Inhibitory effects of adenoviral MEK5 overexpression on the osteoblastic differentiation of MC3T3-E1 cells

To further elucidate the involvement of MEK5 in osteoblastic differentiation, we performed adenoviral overexpression of MEK5 in MC3T3-E1 cells. After confirming overexpression of MEK5 without altering MEK1 and MEK2 protein expression levels (Fig. 4A), the ALP activity and staining were analyzed. Fig. 4B shows weak ALP staining (lower panel) and low ALP activity (upper graph) in cells overexpressing MEK5. Yielding results opposite to those obtained with RNAi experiments, MEK5 overexpression decreased mRNA expression of osteoblastic differentiation markers including ALP, OCN, and osterix (Fig. 4B).

These results indicated that the MEK5 signaling pathway may suppress osteoblastic differentiation.

4. Discussion

The MEK5-ERK5 signaling pathway has been implicated in the regulation of a variety of cellular processes, including

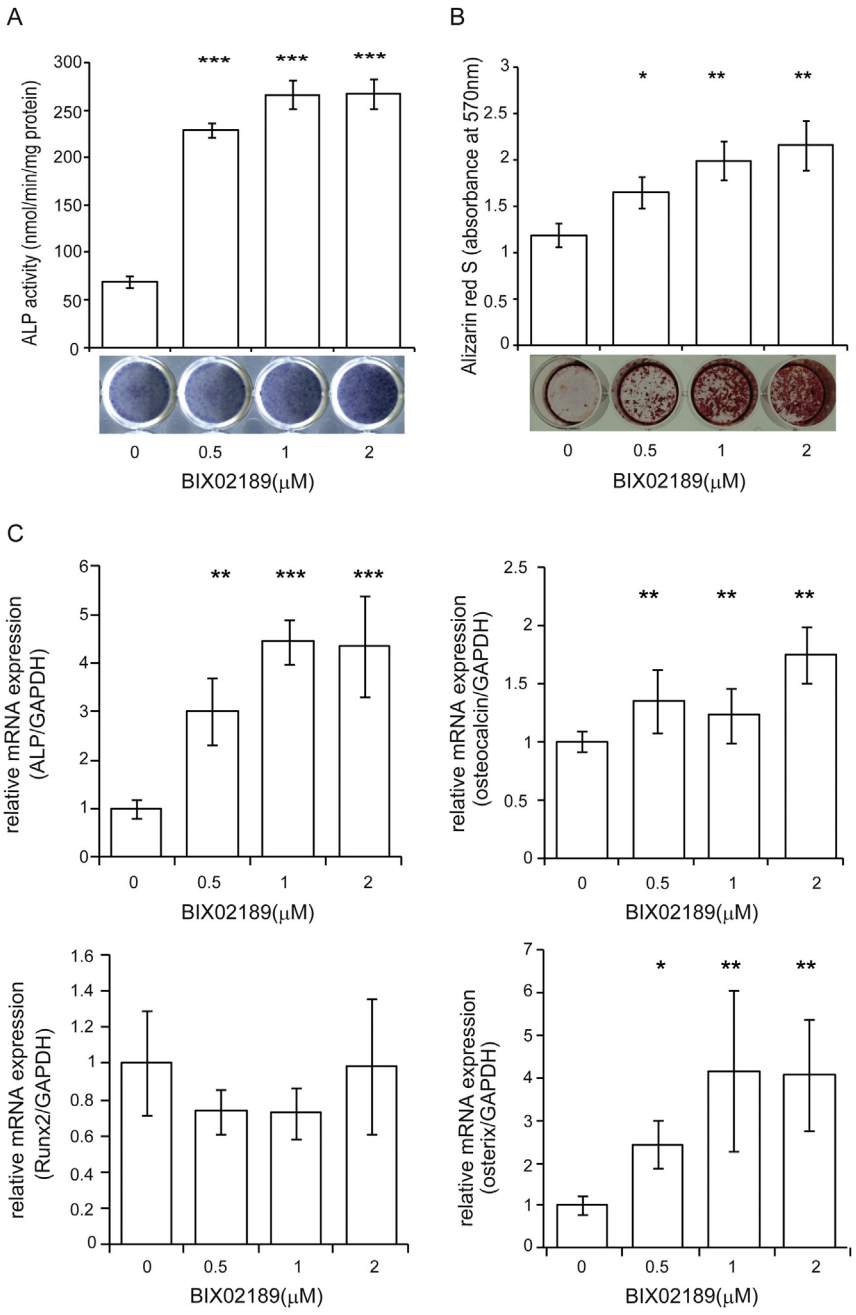

Fig. 2. Promotive effects of BIX02189 on osteoblastic differentiation of preosteoblastic MC3T3-E1 cells. (A) ALP staining and activity of MC3T3-E1 cells treated with BIX02189 for 3 days. (B) Alizarin Red S solution and calcium content quantified in the cellular matrix of MC3T3-E1 cells by the treatment with BIX02189 in the differentiation medium for 28 days. (C) mRNA expression of the osteoblast-related genes: ALP, OCN, Runx2, and osterix by quantitative real-time PCR analysis. The expression of each gene was normalized to GAPDH expression. Data are means ± SD of 3 independent experiments performed in duplicate ($^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.001$.).

proliferation, transformation, survival, and differentiation [2,6,15]. More specifically, mice harboring a null mutation for erk5 gene died around gestational day 10, because of severe cardiovascular defects, prior to the onset of embryonic skeletogenesis [16,17]. Although much experimental work has been performed for elucidating the MEK5-ERK5 pathway and its associated cellular processes, a role of this signaling cascade in osteoblastic differentiation has not yet been reported.

The role of MEK1/2-ERK1/2 signaling pathway in osteoblastic differentiation has been extensively investigated. Several investigators have reported that ERK1/2 activation is required for osteoblastic differentiation [18], whereas others have demonstrated that ERK1/2 activation inhibits osteoblastic differentiation [13,19]. Our previous studies have reported that osteoblastic differentiation is promoted by MEK1/2 inhibition [13]. In addition, our results suggested that the roles of MEK1 and MEK2 in the osteoblastic differentiation may be different [14]. Recent studies involving other cells have revealed different features of the MEK5-ERK5 pathway compared with MEK1/2-ERK1/2 pathway. Moreover, MEK5-ERK5 plays a key role in cardiovascular development and neural differentiation [2,20]. On the basis of prior studies, we focused on a potential role for MEK5-ERK5 in the osteoblastic differentiation.

To the best of our knowledge, the present study has been the first to demonstrate novel functions of MEK5 in osteoblastic

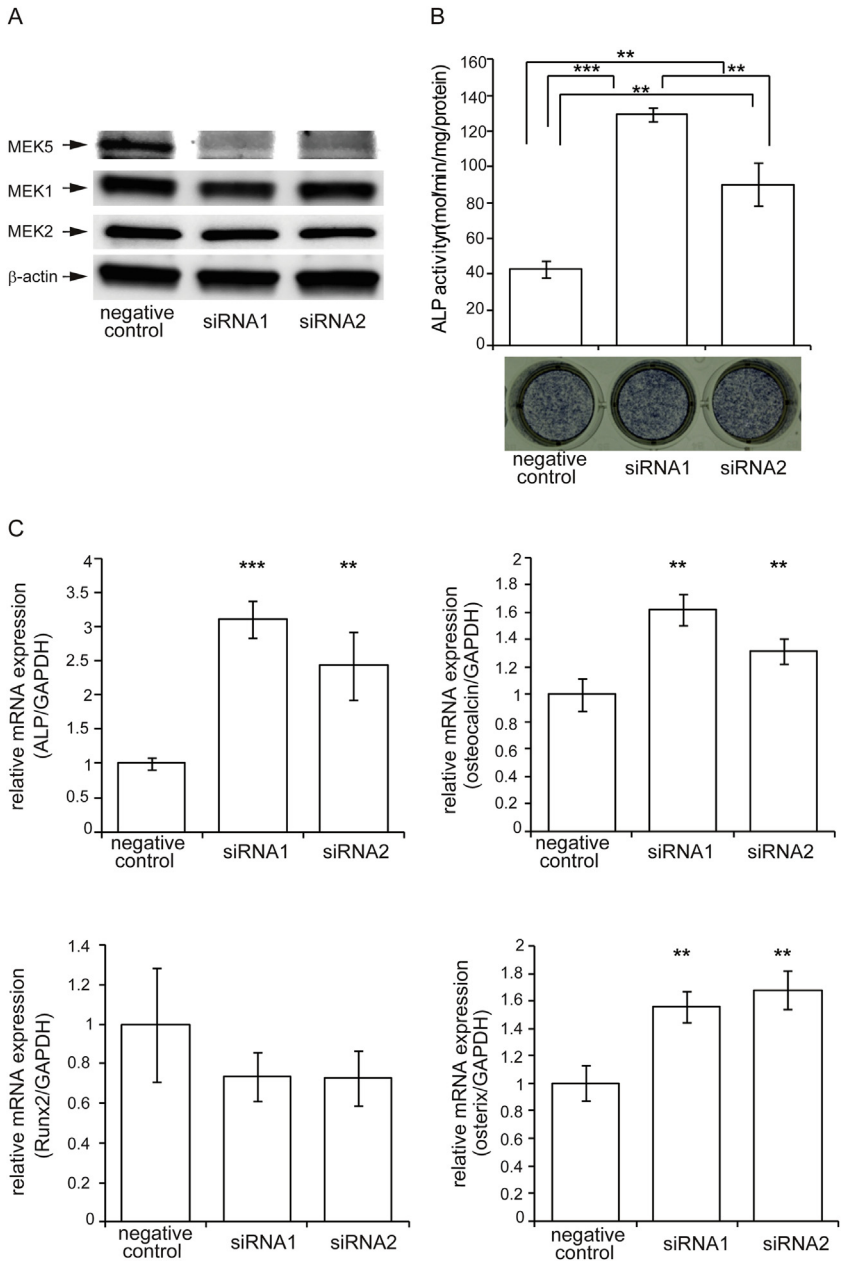

Fig. 3. Acceleration of osteoblastic differentiation of MC3T3-E1 cells by MEK5 Knockdown. (A) Protein expression levels of MEK5, MEK1, and MEK2 by MEK5 knockdown in MC3T3-E1 cells following transfection with control or two MEK5 siRNAs. The cells transfected with siRNA were cultured for 48 h β-Actin was used as the internal control. (B) ALP staining and activity of MC3T3-E1 cells transfected with siRNAs. The cells transfected with siRNA were incubated for 48 h, following which the medium was changed to the differentiation medium. The cells were subsequently incubated for 3 days to evaluate osteoblastic differentiation. (C) mRNA expression of the osteoblast-related genes: ALP, OCN, Runx2, and osterix by quantitative real-time PCR analysis. The expression of each gene was normalized against GAPDH expression. Data are means ± SD of 3 independent experiments performed in duplicate ($^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.001$.).

differentiation and the results of our analysis can be summarized in two principal findings.

First, MEK5 inhibition promoted the osteoblastic differentiation as demonstrated by an increase in ALP activity, ECM calcification, and mRNA expression of ALP, OCN, and osterix in preosteoblastic MC3T3-E1 cells. Another mesenchymal cell line ST2 are more primitive than MC3T3-E1 cells and they are known to differentiate into osteoblast-like cells in a differentiation medium in a manner similar to that of MC3T3-E1 cells [21]. Treatment of ST2 cells with BIX02189 increased ALP activity, with maximum activity observed at a concentration of 0.5 μM rather than that of 1.0 μM, which was the concentration that resulted in maximum activation in MC3T3-E1 cells (Supplemental data). In addition, MEK5 knockdown increased ALP, OCN, and osterix gene expression in MC3T3-E1 cells. Furthermore, proliferation of MC3T3-E1 cells decreased following treatment with high concentrations of the MEK5 inhibitor, suggesting that inhibition of MEK5 suppressed proliferation, while promoting the differentiation of osteoblasts.

In contrast, overexpression of MEK5 decreased ALP activity and reduced the mRNA expression of osteoblastic markers and transcription factors (ALP, OCN and osterix), as well as ECM calcification in MC3T3-E1 cells. Furthermore, overexpression of MEK5 promoted proliferation of this cell lines.

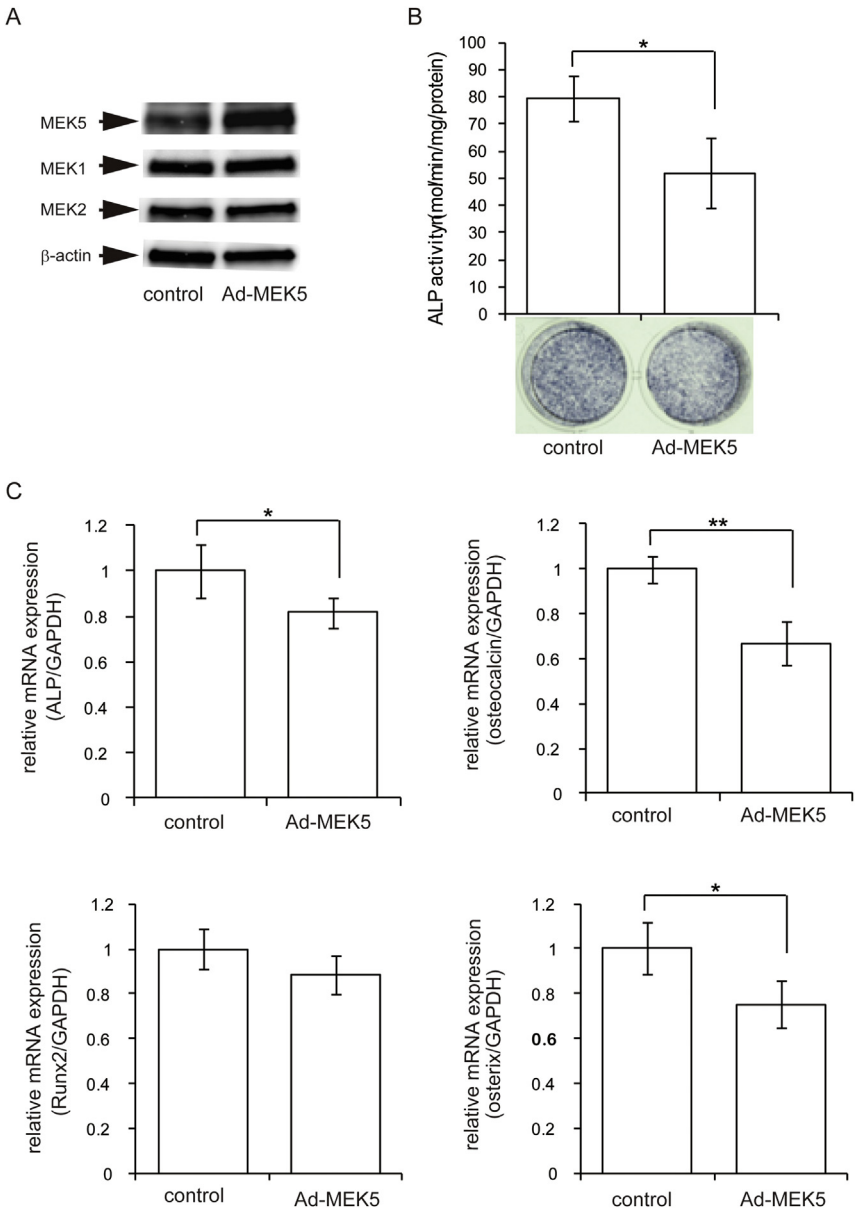

Fig. 4. Inhibitory effects of MEK5 overexpression on osteoblastic differentiation of MC3T3-E1 cells. (A) Protein expression levels of MEK5, MEK1, and MEK2 by MEK5 overexpression in MC3T3-E1 cells. The cells were transfected with adenovirus of MEK5 for 48 h β-Actin was used as the internal control. (B) ALP staining and activity of MC3T3-E1 cells transfected with adenoviruses. The cells transfected with adenoviruses were incubated for 48 h, following which the medium was changed to the differentiation medium. The cells were subsequently incubated for 3 days to evaluate osteoblastic differentiation. (C) mRNA expression of the osteoblast-related genes: ALP, OCN, Runx2, and osterix by quantitative real-time PCR analysis. The expression of each gene was normalized against GAPDH expression. Data are means ± SD of 3 independent experiments performed in duplicate (*$P < 0.05$; **$P < 0.001$.).

In summary, these results suggested that MEK5 had a suppressive effect on the osteoblastic differentiation, which is not dependent on cell-type.

With regard to mechanisms for promotion of osteoblastic differentiation by MEK5 inhibition, inhibition of this pathway appears to stimulate osteoblastic differentiation via osterix-dependent mechanisms. Osterix may function downstream of Runx2 during osteoblastic differentiation [22]. On the other hand, another study found that Runx2 was not involved in induction of osterix [23]. It has also been reported that osterix controls osteoblast differentiation, in part, via a Runx2-independent mechanism [24]. In our present study, expression of Runx2 mRNA was not influenced by inhibition of MEK5. In addition, Runx2 reporter assay may be necessary to reveal the dependency of Runx2-osterix axis.

In a recent study, it has been reported that in MC3T3-E1 cells, fluid shear stress stimulated ERK5 phosphorylation and osteoblastic differentiation, and this differentiation was down-regulated by the treatment with BIX02189, the MEK5-ERK5 inhibitor. In that study, it was concluded that the MEK5-ERK5 pathway mediates osteoblastic differentiation promoted by fluid shear stress [25]. However, this is not consistent with our results, although we used the same cell line and reagents. We found that the inhibitor suppressed not only the phosphorylation of ERK5 but also suppressed the phosphorylation of ERK1/2 on using a high dose of the inhibitor (more than 2 μM) (data not shown). Because a high dose (10 μM) of the inhibitor was used in that study, we speculated that the ERK1/2 pathway also could mediate in their experiments. In the present study, we used the inhibitor within the concentration, which selectively suppresses MEK5-ERK5 pathway. In addition,

we obtained consistent results from the experiments of selective knockdown and overexpression of MEK5.

Understanding of the MEK5 pathway relative to other MAP kinases may also help in tailoring appropriate drug treatments for many cancers. Current studies suggest that there may be some redundancies and cross-talk among members of the MAP kinase family [5,26–29]. Future studies or re-examination of existing data using newer pathway-specific inhibitors may help to elucidate the specific signaling role of MEK5-ERK5 in these tissues [2,28]. This understanding may help with the development of specific treatments and possibly minimize the side effects of future treatments. Several features of the MEK5-ERK5 pathway may provide a novel target for future therapeutics. Structurally, the C-terminus of ERK5 is much larger than that of other MAP kinases and contains both auto-inhibitory and nuclear-shuttling functions [15,30–33]. This structure lends itself to the development of specific drug targets, which may not interfere with the functions of other MAP kinases'. This specificity may allow for targeted therapeutics to inhibit this pathway, while minimizing the inhibition of other MAP kinases that may be critical to the survival of healthy cells. Therefore, MEK5 inhibitors could be developed for the treatment of osteoporosis in patients with and without RA.

In conclusion, the present study indicates that MEK5 suppresses osteoblastic differentiation, while it promotes osteoblastic proliferation. Our study provides new insights into the potential treatment options for osteoporosis, focusing on osteoblastic differentiation in vitro. Thus, inhibition of MEK5 signaling in osteoblasts may be of potential use in the treatment of osteoporosis.

Conflict of interest

Shoichi Kaneshiro, Dai Otsuki, Kiyoshi Yoshida, Hideki Yoshikawa, and Chikahisa Higuchi have no conflict of interest.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 25462369.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.035.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.035.

References

[1] J.M. English, C.A. Vanderbilt, S. Xu, et al., Isolation of MEK5 and differential expression of alternatively spliced forms, J. Biol. Chem. 270 (1995) 28897–28902.
[2] M. Hayashi, J.D. Lee, Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice, J. Mol. Med. Berl. 82 (2004) 800–808.
[3] Y. Kato, V.V. Kravchenko, R.I. Tapping, et al., BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C, EMBO J. 16 (1997) 7054–7066.
[4] M. Hayashi, C. Fearns, B. Eliceiri, et al., Big mitogen-activated protein kinase 1/extracellular signal-regulated kinase 5 signaling pathway is essential for tumor-associated angiogenesis, Cancer Res. 65 (2005) 7699–7706.
[5] O.L. Roberts, K. Holmes, J. Muller, et al., ERK5 and the regulation of endothelial cell function, Biochem. Soc. Trans. 37 (2009) 1254–1259.
[6] X. Wang, C. Tournier, Regulation of cellular functions by the ERK5 signalling pathway, Cell. Signal. 18 (2006) 753–760.
[7] P.B. Mehta, B.L. Jenkins, L. McCarthy, et al., MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion, Oncogene 22 (2003) 1381–1389.
[8] J.C. Montero, A. Ocana, M. Abad, et al., Expression of Erk5 in early stage breast cancer and association with disease free survival identifies this kinase as a potential therapeutic target, PLoS One 4 (2009) e5565.
[9] F.L. Watson, H.M. Heerssen, A. Bhattacharyya, et al., Neurotrophins use the Erk5 pathway to mediate a retrograde survival response, Nat. Neurosci. 4 (2001) 981–988.
[10] J.E. Cavanaugh, J. Ham, M. Hetman, et al., Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity, and cAMP in neurons, J. Neurosci. 21 (2001) 434–443.
[11] G.S. Francis, Pathophysiology of chronic heart failure, Am. J. Med. 110 (Suppl. 7A) (2001) 37S–46S.
[12] K. Sunadome, T. Yamamoto, M. Ebisuya, et al., ERK5 regulates muscle cell fusion through Klf transcription factors, Dev. Cell 20 (2011) 192–205.
[13] C. Higuchi, A.A. Myoui, N. Hashimoto, et al., Continuous inhibition of M PK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix, J. Bone Min. Res. 17 (2002) 1785–1794.
[14] S. Kaneshiro, K. Ebina, K. Shi, et al., IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways in vitro, J. Bone Min. Metab. 32 (2014) 378–392.
[15] S. Nishimoto, E. Nishida, MAPK signalling: ERK5 versus ERK1/2, EMBO Rep. 7 (2006) 782–786.
[16] C.P. Regan, W. Li, D.M. Boucher, et al., Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 9248–9253.
[17] L. Yan, J. Carr, P.R. Ashby, et al., Knockout of ERK5 causes multiple defects in placental and embryonic development, BMC Dev. Biol. 3 (2003) 11.
[18] S. Gallea, F. Lallemand, A. Atfi, et al., Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells, Bone 28 (2001) 491–498.
[19] A. Suzuki, J. Guicheux, G. Palmer, et al., Evidence for a role of p38 MAP kinase in expression of alkaline phosphatase during osteoblastic cell differentiation, Bone 30 (2002) 91–98.
[20] S. Nishimoto, M. Kusakabe, E. Nishida, Requirement of the MEK5-ERK5 pathway for neural differentiation in Xenopus embryonic development, EMBO Rep. 6 (2005) 1064–1069.
[21] E. Otsuka, A. Yamaguchi, S. Hirose, et al., Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid, Am. J. Physiol. 277 (1999) C132–C138.
[22] K. Nakashima, X. Zhou, G. Kunkel, et al., The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation, Cell 108 (2002) 17–29.
[23] M.H. Lee, T.G. Kwon, H.S. Park, et al., BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2, Biochem. Biophys. Res. Commun. 309 (2003) 689–694.
[24] T. Matsubara, K. Kida, A. Yamaguchi, et al., BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation, J. Biol. Chem. 283 (2008) 29119–29125.
[25] L.G. Zhao, S.L. Chen, Y.J. Teng, et al., The MEK5/ERK5 pathway mediates fluid shear stress promoted osteoblast differentiation, Connect. Tissue Res. 55 (2014) 96–102.
[26] J.H. Ostrander, A.R. Daniel, K. Lofgren, et al., Breast tumor kinase (protein tyrosine kinase 6) regulates heregulin-induced activation of ERK5 and p38 MAP kinases in breast cancer cells, Cancer Res. 67 (2007) 4199–4209.
[27] H. Song, X. Jin, J. Lin, Stat3 upregulates MEK5 expression in human breast cancer cells, Oncogene 23 (2004) 8301–8309.
[28] N. Mody, J. Leitch, C. Armstrong, et al., Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway, FEBS Lett. 502 (2001) 21–24.
[29] M.E. Ramos-Nino, S.R. Blumen, T. Sabo-Attwood, et al., HGF mediates cell proliferation of human mesothelioma cells through a PI3K/MEK5/Fra-1 pathway, Am. J. Respir. Cell Mol. Biol. 38 (2008) 209–217.
[30] Y. Kato, R.I. Tapping, S. Huang, et al., Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor, Nature 395 (1998) 713–716.
[31] M. Buschbeck, A. Ullrich, The unique C-terminal tail of the mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling, J. Biol. Chem. 280 (2005) 2659–2667.
[32] J.D. Lee, R.J. Ulevitch, J. Han, Primary structure of BMK1: a new mammalian map kinase, Biochem. Biophys. Res. Commun. 213 (1995) 715–724.
[33] G. Zhou, Z.Q. Bao, J.E. Dixon, Components of a new human protein kinase signal transduction pathway, J. Biol. Chem. 270 (1995) 12665–12669.