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Increased extracellular and intracellular Ca^{2+} lead to adipocyte accumulation in bone marrow stromal cells by different mechanisms

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ABSTRACT

Mesenchymal stem cells found in bone marrow stromal cells (BMSCs) are the common progenitors for both adipocyte and osteoblast. An increase in marrow adipogenesis is associated with age-related osteopenia and anemia. Both extracellular and intracellular Ca^{2+} ($[Ca^{2+}]_o$ and $[Ca^{2+}]_i$) are versatile signaling molecules that are involved in the regulation of cell functions, including proliferation and differentiation. We have recently reported that upon treatment of BMSCs with insulin and dexamethasone, both high $[Ca^{2+}]_o$ and high $[Ca^{2+}]_i$ enhanced adipocyte accumulation, which suggested that increases in $[Ca^{2+}]_o$ caused by bone resorption may accelerate adipocyte accumulation in aging and diabetic patients. In this study, we used primary mouse BMSCs to investigate the mechanisms by which high $[Ca^{2+}]_o$ and high $[Ca^{2+}]_i$ may enhance adipocyte accumulation. In the process of adipocyte accumulation, two important keys are adipocyte differentiation and the proliferation of BMSCs, which have the potential to differentiate into adipocytes. Use of MTT assay and real-time RT-PCR revealed that high $[Ca^{2+}]_i$ (ionomycin)-dependent adipocyte accumulation is caused by enhanced proliferation of BMSCs but not enhanced differentiation into adipocytes. Using fura-2 fluorescence-based approaches, we showed that high $[Ca^{2+}]_o$ (addition of $CaCl_2$) leads to increases in $[Ca^{2+}]_i$. Flow cytometric methods revealed that high $[Ca^{2+}]_o$ suppressed the phosphorylation of ERK independently of intracellular Ca^{2+}. The inhibition of ERK by U0126 and PD0325901 enhanced the differentiation of BMSCs into adipocytes. These data suggest that increased extracellular Ca^{2+} provides the differentiation of BMSCs into adipocytes by the suppression of ERK activity independently of increased intracellular Ca^{2+}, which results in BMSC proliferation.

1. Introduction

Bone marrow-derived mesenchymal stem cells that are found among bone marrow stromal cells (BMSCs) are the common progenitors for both adipocytes and osteoblasts [1–4]. Clinically, increases in marrow adipogenesis are known to be associated with osteoporosis, diabetes mellitus, and age-related osteopenia [5]. We recently reported that upon treatment of BMSCs with insulin and dexamethasone, high extracellular Ca^{2+} ($[Ca^{2+}]_o$) enhanced adipocyte but not osteoblast accumulation in BMSCs. These observations suggested that the increases in $[Ca^{2+}]_o$ that are caused by bone resorption may accelerate adipocyte accumulation rather than osteoblastic bone formation in aging and/or diabetic patients [6].

Both extracellular and intracellular Ca^{2+} are versatile signaling molecules that are involved in the regulation of a number of cell functions, including proliferation, differentiation, and cell death. In the previous study, we showed that both high $[Ca^{2+}]_o$ and intracellular Ca^{2+} ($[Ca^{2+}]_i$) enhance the accumulation of adipocytes. However, the molecular mechanisms by which high $[Ca^{2+}]_o$ and

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high $[Ca^{2+}]_i$ may mediate an increase in the adipogenic induction of lipid accumulation remain unclear. In the process of accumulation of bone marrow adipocytes, two important keys are adipocyte differentiation and the proliferation of BMSCs, which have the potential to differentiate into adipocytes. In this study, we show that enhanced adipocyte accumulation by high $[Ca^{2+}]_i$ is caused by enhanced proliferation of BMSCs but not enhanced differentiation. We also show that high $[Ca^{2+}]_o$ enhances both the proliferation of BMSCs through increment of $[Ca^{2+}]_i$ and the adipocyte differentiation independently of increased $[Ca^{2+}]_i$, which result in adipocyte accumulation. Although the involvement of mitogen-activated protein kinases (MAPKs) in the regulation of osteogenic differentiation has been demonstrated in osteoblasts and mesenchymal stem cells [7–9], the role of MAPK pathways in the regulation of bone marrow adipogenic differentiation by Ca^{2+} remains unclear. Here, we show that increased extracellular Ca^{2+} but not intracellular Ca^{2+} enhances BMSC adipocyte differentiation through the suppression of extracellular-signal-regulated kinase (ERK) activity. These findings regarding enhanced adipogenic differentiation and subsequent inhibition of osteogenic differentiation by high extracellular Ca^{2+} via suppression of the ERK pathway may have the potential to be the target for treatment of osteopenia and anemia.

2. Materials and methods

2.1. Cell culture

The cell culture methods were described previously [10,11]. Briefly, male C57Bl/6 mice (Charles River Japan, Kanagawa, Japan) were euthanized by cervical dislocation, and bone marrow cells were collected from the tibia and femur and were cultured at 37 °C in 5% CO_2/95% air. We selectively maintained adherent cells (BMSCs) by removing the floating cells when changing the medium. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experimental protocol was approved by the Animal Care and Use Committee of Juntendo University.

2.2. Measurement of adipocyte accumulation

To induce adipocyte differentiation, cells were seeded onto 24-well plates at 3×10^4 cells per well and treated with 10 μg/ml insulin and 0.25 μM dexamethasone for 14 days. For Oil Red O staining and extraction, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and then rinsed twice with PBS. The cells were treated with 60% isopropanol for 1 min and then with Oil Red O (Sigma–Aldrich, MO, USA) that was dissolved in 60% isopropanol for 20 min. This process was followed by three rinses with 60% isopropanol. Pictures of the cells were taken, and the Oil Red O dye in lipid droplets was eluted by 500 μL of isopropanol. The absorbance at 520 nm was finally measured with a microtiter plate reader.

2.3. Measurement of cell numbers

Cells were seeded onto 96-well plates at 5×10^3 cells or 1×10^4 cells per well. After 14 days, the cell counts were estimated using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay [12], which is a modification of the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) assay. In brief, 100 μL of medium and 10 μL of WST-8 reagent (Dojindo, Kumamoto, Japan) were added to the wells. After 4 h incubation at 37 °C, the absorbance at 450 nm was recorded with a microtiter plate reader.

2.4. Measurement of intracellular free calcium concentrations

Cells were plated onto glass-bottom dishes and loaded with 5 μM fura-2 acetoxymethylester (AM) (Dojindo) suspended in balanced salt solution (BSS) containing 115 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM glucose (the pH was adjusted to 7.4 with NaOH) in the dark for 30 min. The loaded cells were rinsed three times in BSS, and data acquisition and analysis was carried out using AquaCosmos 2.0 (Hamamatsu Photonics, Hamamatsu, Japan). Solutions were superfused at a rate of 2 ml/min.

2.5. Flow cytometry analysis

Cells were treated with or without 10 nM phorbol-myristate-acetate (PMA), 9 mM $CaCl_2$, and 100 nM ionomycin for 10 min. Following the stimulation, the cells were washed and resuspended at 4×10^6 cells per mL in PBS with 1% fetal bovine serum (FBS). The cells were fixed by adding an equal volume of Cytofix Buffer (Becton Dickinson, NJ, USA) at 37 °C for 10 min. After washing with 1% FBS in PBS, the cells were stained with phycoerythrin (PE)-conjugated mouse anti-ERK1/2 (pT202/pY204) (Becton Dickinson) antibody at room temperature for 30 min. Cells were washed and analyzed with a FACScan flow cytometer (Becton Dickinson).

2.6. Quantitative real-time RT-PCR analysis

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The cDNA was then amplified using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (both from Applied Biosystems). The TaqMan probes and primers (all from Applied Biosystems) for pre-adipocyte factor-1 (Pref-1, assay identification number Mm00494477_m1), CCAAT-enhancer binding protein α (C/EBPα, assay identification number Mm00514283_s1), peroxisome proliferator-activated receptor γ (PPARγ, assay identification number Mm01184322_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assay identification number Mm99999915_g1) were used. PCR mixtures were pre-incubated at 50 °C for 2 min, which was followed by an incubation at 95 °C for 20 s and then 40 cycles of 95 °C for 3 s and 60 °C for 30 s using the Applied Biosystems 7500 Fast real-time PCR system. The real-time data were analyzed using the 7500 software (Applied Biosystems).

2.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Homogeneity of variances and mean values were confirmed with a Bartlett test and a one-way ANOVA, respectively. Significance was evaluated with a Tukey's post hoc test, and differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Increases in both extracellular and intracellular Ca^{2+} enhance adipocyte accumulation in bone marrow stromal cells

We examined whether both extracellular and intracellular Ca^{2+} enhance adipocyte accumulation. We cultured BMSCs that were treated with insulin and dexamethasone for 14 days and then evaluated lipid accumulation by measuring by Oil Red O staining and extraction. Extracellular Ca^{2+} at a concentration of 9 mM $CaCl_2$ (high $[Ca^{2+}]_o$) enhanced adipocyte accumulation in BMSCs (Fig. 1A,

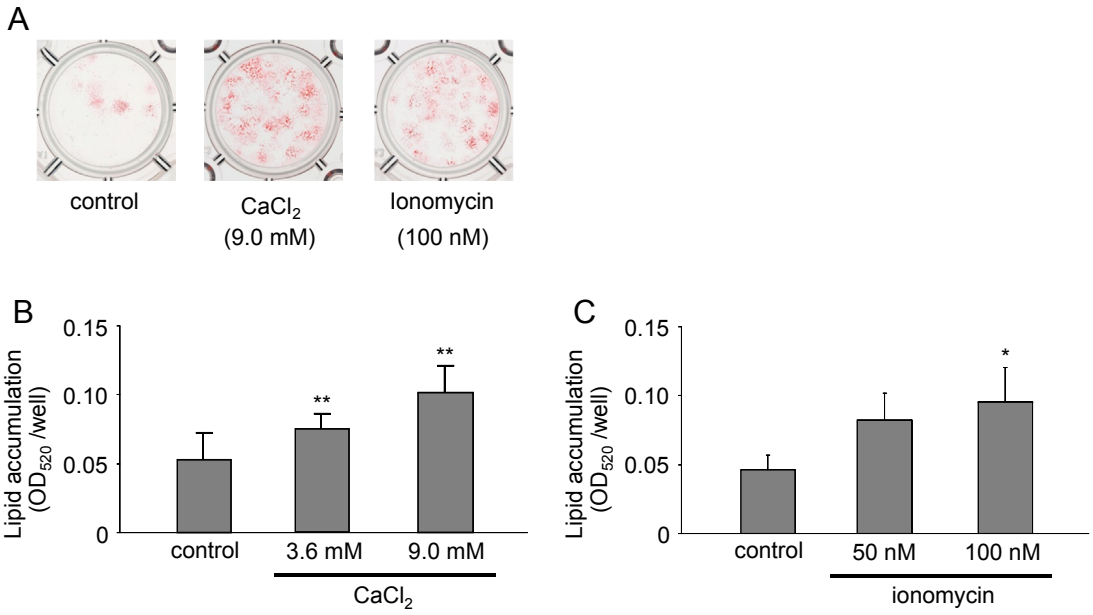

Fig. 1. Both high $[Ca^{2+}]_o$ and high $[Ca^{2+}]_i$ enhance the accumulation of adipocytes in bone marrow stromal cells. Bone marrow stromal cells (BMSCs) were cultured in the presence of insulin and dexamethasone for 14 days. (A) Typical photomicrographs of Oil Red O staining of cells are shown. The levels of total adipocyte accumulation following treatment with the indicated $CaCl_2$ (B) or ionomycin (C) were measured by Oil Red O extraction. Values are means ± SEM, n = 9 (*P < 0.05, **P < 0.01 vs. control).

B). In addition, 100 nM ionomycin (high $[Ca^{2+}]_i$) enhanced adipocyte accumulation in BMSCs (Fig. 1A, C).

3.2. Increased extracellular Ca^{2+} enhances both BMSC proliferation and adipocyte differentiation, and increased intracellular Ca^{2+} enhances BMSC proliferation but not adipocyte differentiation

Both adipocyte differentiation and the proliferation of BMSCs, which have a potential to differentiate into adipocytes, are important processes that occur upon bone marrow adipocyte accumulation. To determine whether both extracellular and intracellular Ca^{2+} affect the proliferation of BMSCs, 5×10^3 cells were seeded, and the cell count was evaluated after 14 days of culture, using a modification of MTT assay. Both 9 mM $CaCl_2$ and 100 nM ionomycin significantly increased the cell numbers (Fig. 2A).

To determine whether both extracellular and intracellular Ca^{2+} alter the expression levels of adipocyte markers of BMSCs, we used real-time quantitative RT-PCR. One day following treatment with insulin and dexamethasone, a significant increases in the mRNA levels of Pref-1 (Fig. 2B-upper right), C/EBPα (Fig. 2B-lower left), and PPARγ (Fig. 2B-lower right) were observed. Extracellular Ca^{2+} at a concentration of 9 mM showed a significant additional increase in the mRNA levels of C/EBPα and PPARγ but not Pref-1 (Fig. 2B). However, 100 nM ionomycin did not affect the expression levels of adipogenic markers (Fig. 2B). These data suggest that the mechanisms that lead to an accumulation of adipocytes are different between high $[Ca^{2+}]_o$ and high $[Ca^{2+}]_i$. Enhanced adipocyte accumulation depends on high $[Ca^{2+}]_o$ is caused by both enhanced proliferation of BMSCs and enhanced adipocyte differentiation. On the other hand, adipocyte accumulation depends on high $[Ca^{2+}]_i$ is caused by enhanced proliferation but not adipocyte differentiation.

3.3. High extracellular Ca^{2+} both increases in intracellular Ca^{2+} and suppresses the phosphorylation of ERK in bone marrow stromal cells

Using fura-2 fluorescence-based approaches, we showed that both 8 mM $CaCl_2$ and 100 nM ionomycin leads to increases in $[Ca^{2+}]_i$ (Fig. 3A). ERK is known to regulate proliferation and differentiation. We next determined whether Ca^{2+} affects the activity of ERK in bone marrow cells using flow cytometry. PMA was used as a positive control for the phosphorylation of ERK. PMA treatment for 10 min increased fluorescence intensity (Fig. 3B). Treatment with 9 mM $CaCl_2$ partially suppressed the phosphorylation of ERK that was activated by PMA (Fig. 3B). However, 100 nM ionomycin did not affect the phosphorylation of ERK that was activated by PMA (Fig. 3B). These results suggest that high extracellular Ca^{2+} increases in intracellular Ca^{2+}. In addition, high extracellular Ca^{2+} suppresses the phosphorylation of ERK independently of increased $[Ca^{2+}]_i$.

3.4. Inhibition of the phosphorylation of ERK enhances the differentiation of bone marrow stromal cells into adipocytes

We next assessed the effects of ERK inhibition on the proliferation of BMSCs using mitogen-activated protein kinase/ERK kinase (MEK) inhibitors. Treatment with the inhibitor U0126 (5 μM) suppressed the proliferation of BMSCs (Fig. 4A). Similarly, a significant suppression in cell number was observed upon treatment with the inhibitor PD0325901 (20 nM, Fig. 4A). We next determined whether the MEK inhibitors alter the expression of adipocyte markers. Following a 1 day treatment with insulin and dexamethasone, BMSCs exhibited a significant increase in the mRNA levels of Pref-1 (Fig. 4B-upper right), C/EBPα (Fig. 4B-lower left), and PPARγ (Fig. 4B-lower right). Treatment with 5 μM U0126 resulted in a significant additional increase in the mRNA levels of C/EBPα and PPARγ but not Pref-1 (Fig. 4B). Similar effects were observed in cells that were treated with 20 nM PD0325901 (Fig. 4B). These results suggest that increased extracellular Ca^{2+} levels but not intracellular Ca^{2+} levels enhance the differentiation of BMSCs into adipocyte through the suppression of ERK activity.

4. Discussion

In the present study, primary mouse BMSCs were used to investigate the mechanisms by which high $[Ca^{2+}]_o$ and high $[Ca^{2+}]_i$ enhance adipocyte accumulation during treatment with both

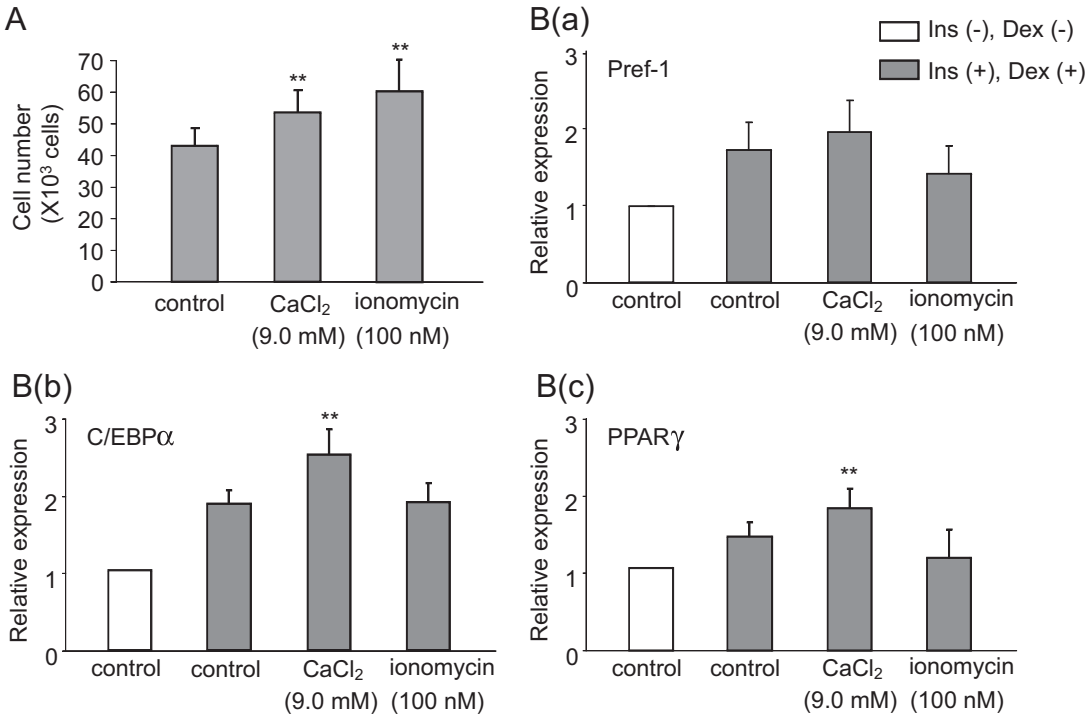

Fig. 2. Increased extracellular Ca^{2+} enhances both BMSC proliferation and adipocyte differentiation, and increased intracellular Ca^{2+} enhances BMSC proliferation but not adipocyte differentiation. (A) BMSCs were cultured with the indicated $CaCl_2$ or ionomycin at 5×10^3 cells per well in the presence of insulin and dexamethasone. After 14 days, the cell number was evaluated using a modification of the MTT assay. Values are means ± SEM, n = 16 (**P < 0.01 vs. control). (B) BMSCs were cultured without insulin or dexamethasone. After 7 days, the cells were cultured for 1 day treated with the indicated $CaCl_2$ or ionomycin with or without insulin and dexamethasone. Total RNA was isolated, and the quantitative mRNA levels of pre-adipocyte factor-1 (Pref-1), CCAAT-enhancer binding protein α (C/EBPα), and peroxisome proliferator-activated receptor γ (PPARγ) were determined using real-time quantitative RT-PCR. Values are means ± SEM, n = 8 (**P < 0.01 vs. control group cultured with insulin and dexamethasone).

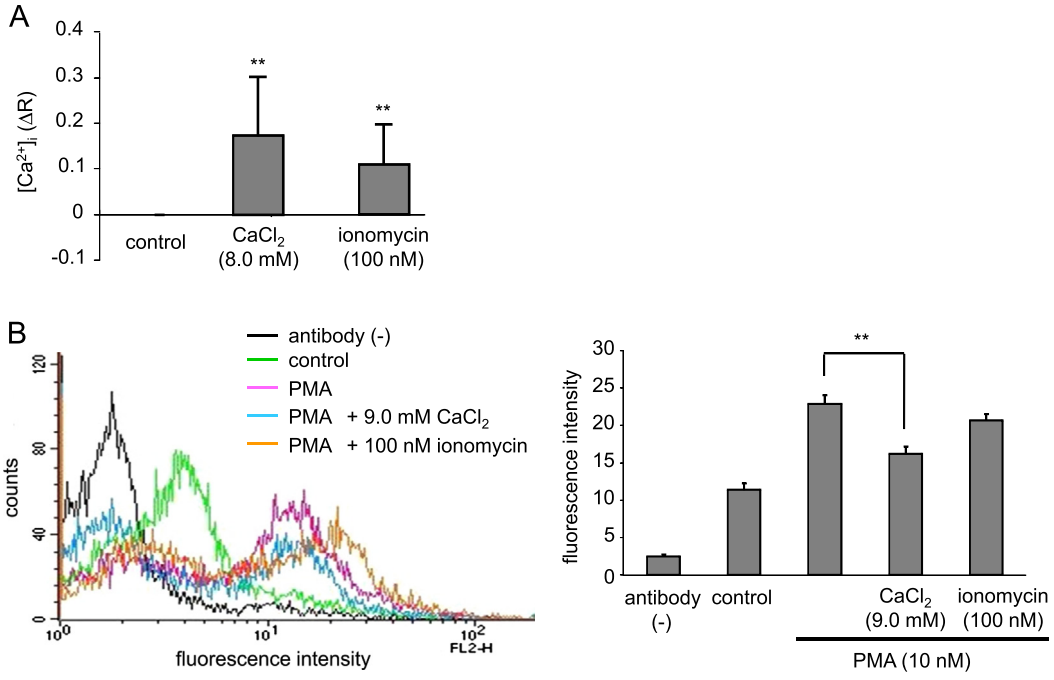

Fig. 3. High extracellular Ca^{2+} both increases in intracellular Ca^{2+} and suppresses the phosphorylation of ERK in BMSCs. (A) $[Ca^{2+}]_i$ of BMSCs was measured using the fura-2 method. Changes in $[Ca^{2+}]_i$ caused by addition of $CaCl_2$, or ionomycin are shown. Values are the mean ± SEM, n = 26–58 (**P < 0.01 vs. control). (B) Isolated bone marrow cells were treated with or without 10 nM phorbol-myristate-acetate (PMA) and the indicated $CaCl_2$ or ionomycin. After 10 min, cells were stained with phycoerythrin (PE)-conjugated anti-phospho-extracellular-signal-regulated kinase (ERK) antibody and analyzed by flow cytometry. Typical histograms of cells treated with vehicle (green), PMA (pink), PMA and high $[Ca^{2+}]_o$ (blue), PMA and ionomycin (yellow), and without antibody (black) are shown in left panel. Fluorescence intensity is shown in right panel. Values are means ± SEM, n = 9 (**P < 0.01 vs. PMA treated group).

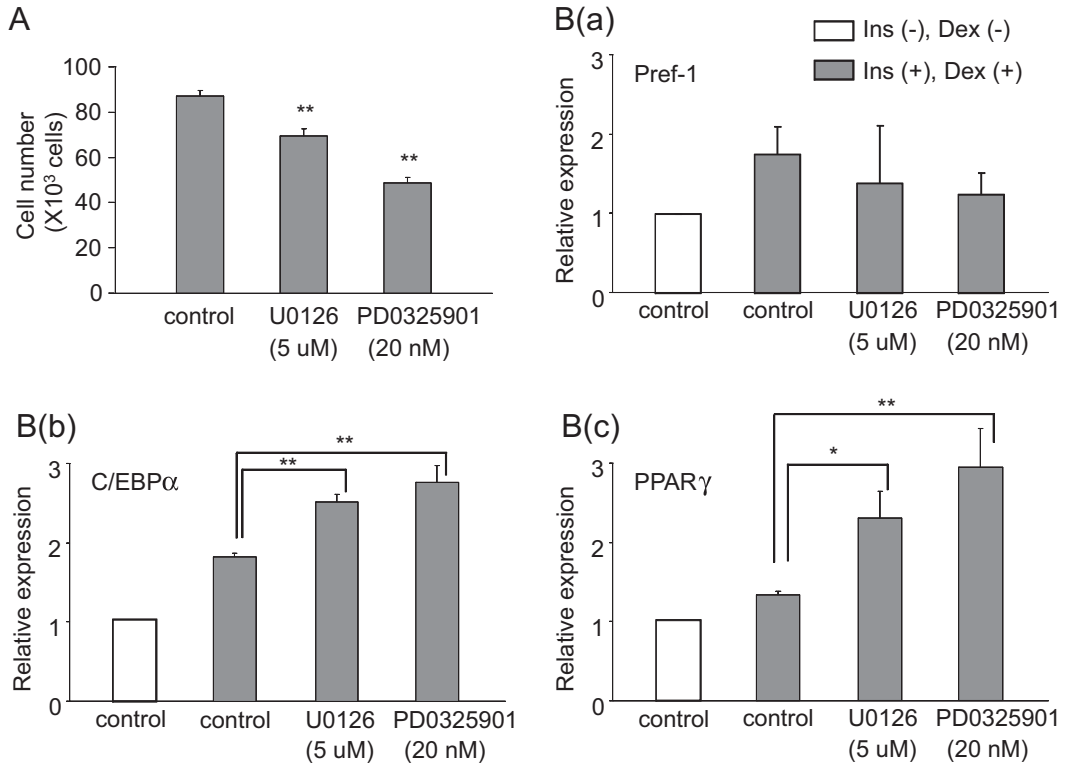

Fig. 4. ERK inhibition by MEK inhibitors both suppresses the proliferation of BMSCs and enhances the differentiation of BMSCs into adipocytes. (A) BMSCs were cultured with the indicated mitogen-activated protein kinase/ERK kinase (MEK) inhibitors (U0126 and PD0325901) at 1×10^4 cells per well in the presence of insulin and dexamethasone. After 14 days, the cell counts were evaluated using a modification of the MTT assay. Values are means ± SEM, n = 8 (**P < 0.01 vs. control). (B) BMSCs were cultured without insulin or dexamethasone. After 7 days, the cells were cultured for 1 day treated with the indicated MEK inhibitors with or without insulin and dexamethasone. Total RNA was isolated, and the quantitative mRNA levels of Pref-1, C/EBPα, and PPARγ were determined using real-time quantitative RT-PCR. Values are means ± SEM, n = 4 (*P < 0.05, **P < 0.01 vs. control group cultured with insulin and dexamethasone).

insulin and dexamethasone. High $[Ca^{2+}]_o$ increased $[Ca^{2+}]_i$, but suppressed the phosphorylation of ERK independently of intracellular Ca^{2+} in BMSCs. Increases in $[Ca^{2+}]_i$ (both high $[Ca^{2+}]_o$ and the Ca^{2+} ionophore ionomycin) enhanced the proliferation of the BMSCs. Inhibition of ERK with chemical inhibitors (U0126 and PD0325901) of mitogen-activated protein kinase/ERK kinase (MEK) enhanced the differentiation of BMSCs into adipocytes. These data suggest that increased extracellular Ca^{2+} levels enhance both the proliferation of BMSCs through increases in intracellular Ca^{2+} levels and adipocyte differentiation through the suppression of ERK activity independently increased intracellular Ca^{2+}.

Both extracellular and intracellular Ca^{2+} ions are versatile signaling molecules that are involved in the regulation of many cell functions, including proliferation, differentiation, and cell death. In the previous study, we showed that both high $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ enhance the accumulation of adipocytes. In the present study, we first showed that the mechanism of adipocyte accumulation is different between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$. In the process of accumulation of bone marrow adipocytes, both the proliferation of BMSCs and adipocyte differentiation of BMSCs are important. High $[Ca^{2+}]_i$ enhanced proliferation but not adipocyte differentiation. On the other hand, high $[Ca^{2+}]_o$ enhanced both proliferation and adipocyte differentiation. Since the increase of $[Ca^{2+}]_i$ by ionomycin enhanced proliferation only, high $[Ca^{2+}]_o$ may provide the differentiation of BMSCs to adipocytes by the suppression of ERK activity independently of subsequent increase of $[Ca^{2+}]_i$ by high $[Ca^{2+}]_o$, which results in BMSC proliferation.

Stem cells and regenerative medicine are studied by many researchers, especially since the discovery of iPS cells by the Yamanaka group [13]. BMSCs are also promising stem cells for regenerative medicine because of differentiation potency. BMSCs are multipotent and differentiate into osteoblasts, adipocytes, chondrocytes, and smooth muscle cells [1–4]. However, BMSCs cannot be isolated in large quantities and need to be expanded in culture. It is known that Ca^{2+} plays a central role in proliferation [14–18]. Consistent with these reports, our results showed that increased $[Ca^{2+}]_I$ enhanced the proliferation of BMSCs. It is important that the enhanced proliferation was observed without having effects on differentiation into adipocytes (Fig. 2) which have the demerit to be associated with osteoporosis, diabetes mellitus, and age-related osteopenia [5]. Thus, the reagents that can be used to increase $[Ca^{2+}]_i$ may be one method to increase BMSC number with little effects on adipocyte differentiation.

MAPKs play important roles in cellular responses to growth factors, cytokines, and environmental stress. There are at least three distinct groups of MAPKs: ERK1/2, Jun amino-terminal kinase (JNK1/2/3), and p38 MAPKs (p38). The inhibition of ERK pathway has been reported to suppress the osteogenic differentiation of bone marrow mesenchymal stem cells [8,9]. Because both osteoblasts and adipocytes that have a common precursor, the pluripotent mesenchymal stem cell found in BMSCs, it appears that inhibition of ERK might enhance the adipocyte differentiation of BMSCs. In the present study, we showed that inhibition of ERK using two MEK inhibitors (U0126 and PD0325901) enhanced the differentiation of BMSCs into adipocytes (Fig. 4). However, there are conflicting reports on the role of the mitogen-activated protein kinase (MAPK) pathway in adipocyte differentiation of BMSCs including mesenchymal stem cells. Several reports [19,20] showed that activation of ERK enhance adipocyte differentiation, while others [8,21] reported the inhibition of adipocyte differentiation by ERK activation in BMSCs or bone marrow mesenchymal stem cells. This discrepancy

in ERK activity may reflect the degree of differentiation or maturity of the cells that is acted on by ERK. Pref-1 is a pre-adipocyte marker, and both C/EBPα and PPARγ are important adipogenic transcription factors in pre-adipocytes. Activated C/EBPα and PPARγ form a positive transcriptional feedback loop, which induce the expression of one another and coordinate downstream adipocyte developmental biology. We showed that both high Ca^{2+} and ERK inhibition by MEK inhibitors increased the expression of C/EBPα and PPARγ and did not affect the expression of Pref-1 (Figs. 2 and 4). These data indicate that both high Ca^{2+} and ERK inhibition affect the differentiation of pre-adipocytes into adipocytes but do not affect the differentiation of stem cells into pre-adipocytes. Kim et al. reported that Pref-1 activates the MEK/ERK pathway to prevent adipocyte differentiation by inhibiting PPARγ induction [22]. In addition, ERK has been reported to suppress the transcriptional activity of PPARγ by direct phosphorylation, thereby inhibiting adipocyte differentiation [23,24]. These studies support our observation that increased extracellular Ca^{2+} levels enhance BMSC adipocyte differentiation through the suppression of ERK activity.

In summary, we have shown that increased extracellular Ca^{2+} levels enhance BMSC adipocyte differentiation through the suppression of ERK activity. Increased $[Ca^{2+}]_o$-mediated suppression of ERK signaling may be a new target for therapy for anemia and fractures that are caused by accelerated marrow adipocyte accumulation in patients during aging or with diabetes or osteoporosis. We have also shown that increased $[Ca^{2+}]_i$ enhanced the proliferation of BMSCs without affecting the differentiation into adipocytes. Reagents that are used to increase in $[Ca^{2+}]_i$ may therefore be a method to increase the number of BMSCs, which are promising stem cells for use in regenerative medicine under conditions of undifferentiation.

Conflict of interest

Dr. Daida receives grants and personal fees from Teijin Pharma, grants and personal fees from AstraZeneca K.K., grants and personal fees from Shionogi & Co., Ltd., grants and personal fees from KOWA PHARMACEUTICAL COMPANY LTD., grants and personal fees from Pfizer Co., Ltd., grants and personal fees from Dainippon Sumitomo Pharma Co., Ltd., grants and personal fees from Otsuka Pharmaceutical Co., Ltd., grants and personal fees from sanofi-aventis K.K., grants and personal fees from MSD K.K., grants and personal fees from Novartis Pharma K.K., grants and personal fees from Astellas Pharma Inc., grants and personal fees from Nippon Boehringer Ingelheim Co., Ltd., grants and personal fees from Takeda Pharmaceutical Co., Ltd., grants and personal fees from Bristol-Myers Squibb, grants and personal fees from Mochida Pharmaceutical Co., Ltd., grants and personal fees from Bayer Yakuhin, Ltd, grants and personal fees from Daiichi-Sankyo Company, Limited , grants and personal fees from Mitsubishi Tanabe Pharma Corp., grants and personal fees from Kissei Pharmaceutical Co., Ltd., outside the submitted work.

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