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# Eluted zinc ions stimulate osteoblast differentiation and mineralization in human dental pulp stem cells for bone tissue engineering

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A B S T R A C T

*Objective:* Zinc is an essential element for proliferation, differentiation and survival in various cell types. In a previous study, we found that zinc ions released from zinc-modified titanium surfaces (eluted zinc ions; EZ) stimulate cell viability, osteoblast marker gene expression and calcium deposition in human bone marrow-derived mesenchymal cells (hBMCs). The aim of the present study was to investigate the effects of EZ on osteoblast differentiation among dental pulp stem cells (DPSCs) in vitro.
*Method:* In this study, we evaluated the effects of EZ on osteogenesis in DPSCs. Osteoblast and osteoclast marker gene expression was evaluated by real-time PCR. We also evaluated alkaline phosphatase (ALP) staining and calcium deposition.
*Results:* We found that EZ stimulated osteoblast marker gene (type I collagen, alkaline phosphatase (ALP), osteocalcin (OCN) and Runx2) expression, vascular endothelial growth factor A (VEGF-A), and TGF-beta signaling pathway-related gene expression after 7 days of incubation. Osteoclastogenesis occurs in a receptor for activated nuclear-factor kappa B ligand (RANKL)/osteoprotegerin (OPG)-independent manner. Real-time PCR analysis revealed that EZ did not affect RANKL or OPG mRNA expression. It was also revealed that EZ induced alkaline phosphatase (ALP) staining and calcium deposition in DPSCs. Collectively, these results demonstrate the potential for clinical application to prospective treatment of bone diseases.

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## 1. Introduction

Zinc is an essential trace element that has been confirmed to have effects on osteoblast differentiation and skeletal development. Zinc also acts as a signaling molecule and affects intracellular signaling pathways (Hirano et al., 2008), and has been demonstrated to have a stimulatory effect on osteogenesis due to alkaline phosphatase synthesis and collagen synthesis (Kawakubo, Matsunaga, Ishizaki, Yamada, & Hayashi, 2011). These processes induce osteoblast differentiation and bone formation (Seo, Cho, Kim, Shin, & Kwun, 2010; Yamaguchi & Weitzmann, 2011; Yamaguchi, Goto, Uchiyama, & Nakagawa, 2008; Kawamura et al., 2000;Ito et al., 2005), and zinc-deficient conditions induce downregulation of osteoblast marker gene expression, alkaline phosphatase activity, calcium deposition and bone growth in vitro and in vivo (Hambidge & Krebs, 2007; Kwun et al., 2010). Based on the effects of zinc ions on osteoblast differentiation and bone formation, we have developed a zinc-modified titanium implant that releases zinc ions from its surface, and the shear strength between bone and zinc-modified titanium implant was 5-fold higher than that of non-zinc-modified titanium implant (Alvarez, Fukuda, & Yamamoto, 2010). In a previous study, we found that zinc ions released from the zinc-modified titanium surface (eluted zinc ions; EZ) stimulated cell viability, osteoblast marker gene expression and calcium deposition in human bone marrow-derived mesenchymal cells (hBMCs) (Yusa et al., 2011). We also observed advantageous effects of EZ on osteoblast differentiation in hBMCs, as compared to zinc chlorides. These results confirmed

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the potential of EZ as a nutritional factor for osteoblast differentiation and bone regeneration.

Bone regeneration using cell-based therapies require stem cell sources for differentiation into osteoblasts and osteocytes. Embryonic stem (ES) cells have the ability to differentiate into all cell types within the body (Smith et al., 1988;Evans & Kaufman, 1981;Martin, 1981). Induced pluripotent stem (iPS) cells can be reprogrammed into an ES-like state by transduction with a combination of transcriptional factors. iPS cells are thus capable of differentiating into cell types characteristic of the three layers in vitro and in vivo, and are a very useful cell source for the cell-based therapy (Egusa et al., 2010; Takahashi & Yamanaka, 2006; Takahashi et al., 2007). ES cells and iPS cells are also cell sources for bone regenerative therapy (Tong, Brown, & Krebsbach, 2007; Buttery et al., 2001;Bilousova et al., 2011). Bone marrow-derived mesenchymal stem cells are widely used for in vitro investigation of osteoblast differentiation (Ohgushi et al., 1996;Kihara, Oshima, Hirose, & Ohgushi, 2004), and mesenchymal stem cells derived from umbilical cord and adipose tissue also have the capacity for differentiation into osteoblasts (Zhang et al., 2009;Ongaro et al., 2014;Wen et al., 2014).

Dental pulp stem cells (DPSCs) are somatic stem cells from dental pulp tissues, which also contain fibroblasts, collagen fiber, nerves, blood vessels with histiocytes, macrophages, granulocytes, mast cells, plasma cells and odontoblasts. They are located in the coronal and root portions of the tooth, and are able to differentiate into osteoblasts, chondrocytes and adipocytes under specific cell culture conditions. These characteristics of DPSCs are similar to those of bone marrow-derived mesenchymal stem cells (BMCs). Furthermore, DPSCs also display a high proliferation rate (Miura et al., 2003) and this characteristic ability makes them a suitable stem cell source for tissue engineering.

The aim of the present study was to investigate the effects of EZ on osteoblast differentiation of DPSCs in vitro. Development of osteogenesis was determined by analyzing osteoblast marker gene expression, alkaline phosphatase activity and matrix mineralization.

## 2. Materials and methods

### 2.1. Isolation of EZ from the surface of zinc-modified titanium

EZ were isolated as described previously (Yusa et al., 2011). Briefly, to isolate EZ from the surface of the zinc-modified titanium, zinc-modified titanium was fabricated by chemically treating a commercially pure titanium sponge with a solution containing a $[Zn(OH)_4]^{2-}$ complex. Surface properties of the zinc-modified titanium were analyzed as in our previous report (Alvarez et al., 2010). After the zinc-modified titanium sponge was prepared, 100 g was soaked in 200 ml of ultra-pure water and placed in a mechanical shaker bath at 37 °C for 3 days to release zinc ions. Zinc ion concentration was measured without dilution using inductively coupled plasma atomic emission spectrometry (ICP-AES) (SPS 7700; Seiko Instruments Inc., Japan).

### 2.2. Cell cultures

A normal human deciduous tooth was extracted, and DPSCs were isolated as reported elsewhere (Suchánek et al., 2007). Dental pulp tissue from human deciduous teeth was kindly provided by professor Yukio Kato (Hiroshima University) and following protocols approved by the Hiroshima University Ethics Authorities (No. D88-2). DPSCs from the third to sixth passages were used in this study. DPSCs were cultured in standard medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (D6046; Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (CELLect GOLD; ICN Biomedicals Inc., USA) and 1% penicillin/streptomycin (Gibco, Invitrogen Co., USA) at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. To assess cell morphology, DPSCs were labeled with PKH26 (Sigma) red fluorescent cell linker before seeding. Cell morphology was observed under a phase-contrast microscope (OLYMPUS IX 70; Olympus, Japan).

### 2.3. In vitro differentiation experiments

After the cultures reached subconfluence, experimental treatments were initiated in order to induce osteoblast differentiation. DPSCs were provided with osteogenic medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS) (CELLect GOLD; ICN Biomedicals Inc.) and 1% penicillin/streptomycin (Gibco, Invitrogen Co.), 10 mM β-glycerophosphate (Sigma), 0.28 mM ascorbic acid 2-phosphate (Wako Pure Chemical, Japan) and 100 nM dexamethasone (Sigma). Then, 3.2 nM EZ or 3.2 nM $ZnCl_2$ was applied to cell cultures, as 3.2 nM EZ stimulated osteoblast differentiation in hBMCs most effectively in a previous report (Yusa et al., 2011). Medium with 3.2 nM EZ or 3.2 nM $ZnCl_2$ was changed every 3 days.

### 2.4. Cell growth assay

Cell growth assays were performed by seeding DPSCs at a concentration of $4 \times 10^4$ cells/well in 6-well dishes, followed by incubation in a humidified atmosphere of 5% $CO_2$, 95% air at 37 °C. After cells reached subconfluence, standard medium was changed to osteogenic medium. Cell numbers were counted after the indicated days using trypsin ethylenedimamineterraacetic acid (EDTA) with a TC10™ Automated Cell Counter (Bio-Rad, USA).

### 2.5. RNA preparation and gene expression analysis

DPSCs were seeded on 6-well plates at a concentration $3 \times 10^5$/well. After reaching subconfluence, standard medium was changed to osteogenic medium with EZ or $ZnCl_2$ for 7 days. Total RNA was prepared from cultured cells using RNAiso (Takara Bio, Japan) according to the manufacturer's protocol. RNA concentrations were calculated by absorption ratio ($OD_{260}$/$OD_{280}$) using a NanoDrop Spectrometer (LMS Co., Ltd., Japan). First-strand cDNA was synthesized using a Superscript III fast-strand synthesis system (Invitrogen Co.) according to the manufacturer's protocol. Real-time PCR was perf\ormed with a Thermal Cycler Dice Real Time System Single TP850 (Takara Bio) using SYBR Premix Ex Taq II (Takara Bio). Primers used were: 5′-CCCCTGGAAAGAATGGAGATG-3′, 5′-TCCAAACCACTGAAACCTCTG-3′ [collagen, type I, alpha 1 (type I collagen)]; 5′-CACACTCCTCGCCCTATTG-3′, 5′-GTCTCTTCACTACCTCGCTG-3′ [osteocalcin (OCN)]; 5′-CCTGGACCTCGTTGACACCT-3′, 5′-GTCCCCTGGCTCGAAGAGA-3′ [ALP liver/bone/kidney variant 1 (ALP)]; 5′-GGAGGGACTATGGCATCAAAC-3′, 5′-CTCACGTCGCT-CATTTTG C-3′ [runt-related transcription factor 2 (Runx2)]; 5′-TGGATGCCTTGAATAATAAGCAGGA-3′, 5′-AATTTGCGGCACTTGTG-GAA-3′ [receptor for activated nuclear-factor kappa B ligand (RANKL)]; 5′-AATCAACTCAAAAATGTGGAATAGATGT-3′, 5′-CGTAAACTTTGTAGGAACAGCAA-3′ [osteoprotegerin (OPG)]; 5′-TGACAGGGAAGAGGAGGAGA-3′, 5′-CGTCTGACCTGGGGTAGAGA-3′ [vascular endothelial growth factor A (VEGF-A)]; and 5′-CGCTCTCTGCTCCTCCTGTT-3′, 5′-CCATGGTGTCTGAGCGATGT-3′ [GAPDH].

The following cycling parameters were used: 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 5 s, 60 °C for 30 s; and a dissociation stage. Relative expression ratio of the markers was calculated based on the (ddct) comparative threshold cycle (CT)

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