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Ecklonia cava extract promotes osteogenic differentiation of human periodontal ligament stem cells

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Abstract

Ecklonia cava extract promotes osteogenic differentiation of human periodontal ligament stem cells

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Introduction

In the field of tissue engineering, dental stem cells isolated from human teeth have been differentiated into various cells in order to promote regeneration of tooth structures and periodontal tissue. Furthermore, many researches have been conducted to discover

materials, especially natural products with fewer adverse side-effects or toxicity, that promote stem cell differentiation and periodontal tissue regeneration. Recently, implementation of naturally occurring bioactive compounds from seaweeds has been carried out for stimulating proliferation and differentiation of stem cells. In this study, *Ecklonia cava*, an edible brown alga species found along the southern coast of Korea, was investigated as a possible osteogenic product. Various biological activities of *Ecklonia cava* have been reported including antioxidation, anti-inflammatory, anticancer, antihypertensive, and antidiabetic effects. However, no previous study has investigated the effects of *Ecklonia cava* on the differentiation of human periodontal ligament stem cells (hPDLSCs). Thus, the aim of this study was to investigate the effects of *Ecklonia cava* on the osteogenic differentiation of hPDLSCs.

Materials and Methods

hPDLSCs were isolated and cultured from human third molars. To characterize the cultured hPDLSC, flow cytometric analysis was performed using mesenchymal stem cell (MSC) markers. Then, the multilineage differentiation capacity of hPDLSC was confirmed with Alizarin Red S stain, Oil Red O dye and Alcian Blue stain as indicators of osteogenic, adipogenic, and chondrogenic differentiation,

respectively. To examine the effects of *Ecklonia cava* extract (ECE) on hPDLSC viability, hPDLSCs were incubated with various concentrations of ECE for 1, 3, and 7 days. Then, to investigate the effects of ECE on the osteogenic differentiation of hPDLSCs, gene expression levels of the osteoblastic marker genes, *Col1*, *ALP*, *OPN*, *OCN*, and *Runx2*, were examined by real-time PCR, and protein expression levels of OCN, OPN, Col1, and Runx2 were detected by Western blot. In addition, possible mechanism underlying the osteogenic differentiation process after ECE treatment was explored by measuring the expression levels of Smad4, p-Smad2/3, p-p38, p38, p-ERK1/2 and ERK1/2 using Western blot.

To evaluate the capacity of hPDLSCs for bone formation in response to ECE, hPDLSCs were mixed with HA/TCP without or with ECE and then subcutaneously transplanted into the dorsal surface of immunocompromised mice. For histological analysis, samples were acquired 10 weeks after transplantation, and hematoxylin and eosin (H&E) staining and immunohistochemical analysis were used to evaluate the differentiated bone-like tissue.

Results

Characterization of hPDLSCs as mesenchymal stem cells (MSCs) and multilineage differentiation potential of hPDLSCs were confirmed.

For the effects of ECE on hPDLSC proliferation, ECE increased proliferation of hPDLSCs without any cytotoxicity after 1 and 3 days of culture. After 7 days of culture, cell proliferation declined below the control value starting at 100 μg/ml of ECE. Real-time PCR showed that expressions of *ALP*, *Col1*, *OCN, OPN,* and *Runx2* increased in ECE-treated group compared to untreated group, and protein expression levels of OCN, OPN, Col1, and Runx2 increased as well. In addition, Western blot analysis demonstrated that ECE may stimulate osteogenesis by activation of p38 and ERK1/2 signaling pathways.

For *in vivo* study, histological analysis with H&E staining showed increased area of bone-like tissue layer in the ECE-treated group, and immunohistochemical analysis confirmed the generation of bone-like tissue with increased OSX, OPN, and OCN expressions.

Conclusion

In this study, the effect of ECE on osteogenic differentiation of hPDLSCs was investigated *in vitro* and *in vivo*. The results of the experiments confirmed that ECE-treated group had greater ability to promote osteogenic differentiation of hPDLSCs compared to the control group. Therefore, ECE is suggested to be a potential natural compound with therapeutic properties against bone-related

complications and diseases that urge further studies to elucidate its detailed mechanism of action and bioavailability.

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Contents

I. Introduction

Significant bone loss due to either traumatic or non-traumatic causes may not heal spontaneously and generally requires further intervention to achieve proper tissue regeneration[1]. Autografts have been used as standard clinical material for bone regeneration, but limited availability and donor site complications have raised concerns[2,3]. On the other hand, allografts have been used as substitute with successful bone healing[2]. However, the results were inferior and failed to meet clinical demands compared to autografts, and possible transmission of disease and other infectious agents were reported[4].

As an alternative to autografts and allografts, tissue engineering has emerged as an innovative and promising technique in the treatment of various bone defects. Bone tissue engineering approach involves mesenchymal stem cells with high proliferative and osteogenic potentials and appropriate scaffold and is now generally regarded as a possible alternative to autogenous bone grafting[5]. Due to easy accessibility, different types of dental tissues with multilineage differentiation capabilities, including peridontal ligament (PDL), pulp, apical papilla, and alveolar bone, have been used to isolate human dental mesenchymal stem cells[6-8]. PDL is a group of specialized connective tissue that attaches a tooth to the alveolar bone

and consists of fibroblasts, epithelial cells, undifferentiated mesenchymal cells, bone cells, and cementum cells. Human periodontal ligament stem cells (hPDLSCs) were considered to be one of the most favorable cell source in cell-based regenerative therapy since hPDLSCs have the ability to differentiate toward various specialized cells and can be differentiated into osteoblasts upon induction of growth factors. Therefore, hPDLSCs have become the most common stem cells applied in bone tissue engineering and clinical practice[7,9].

In addition, growth factors combined with bone substitute materials may improve cell proliferation leading to enhanced bone regeneration capacity. Various growth factors, such as bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), and platelet-rich plasma (PRP), have been widely used to enhance the healing process and promote bone regeneration[10]. Nevertheless, the application of these growth factors alone show certain shortcomings[11]. For example, some of the concerns associated with the sole use of BMPs are high cost, high dosage requirement, rapid degradation, and adverse clinical side effects[12,13]. Therefore, discovering new osteogenic products or drugs with higher efficacy, fewer adverse side-effects, and lower cost is critical for promising bone regeneration therapy.

Taking into account these limitations, implementation of naturally occurring bioactive compounds from seaweeds, herbs, fruits and

vegetables for stimulating proliferation and differentiation of stem cells *in vitro,* may provide a new perspective in regenerative medicine. *Ecklonia cava*, a member of the family Laminariaceae, is an edible brown alga species which is found along the southern coast of Korea and is recognized as a rich source of bioactive derivatives such as fucoidan and phlorotannins[14]. Various biological activities of *Ecklonia cava* have been reported including antioxidation, anticancer, antidiabetic, antimicrobial, anticoagulative, anti-human immunodeficiency virus, antihypertensive, inhibition of matrix metalloproteinase enzyme, protective effect against UVB-induced oxidative stress, anti-inflammatory and antiallergic activities[15]. Recently, anti-osteoclastogenic and osteoblastogenic effects of *Ecklonia cava* were reported which suggested its therapeutic potential in bone-associated diseases, such as osteoporosis, periodontitis, rheumatoid arthritis, and Paget's disease[16,17]. The balance between bone-resorbing osteoclasts and bone-synthesizing osteoblasts contributes to bone homeostasis and should be highly regulated to prevent dysregulation of bone remodeling which may be associated with the onset of the bone-related diseases[18]. However, these studies have only investigated the *in vitro* effects of *Ecklonia cava*, necessitating evaluation of this natural product on the regulation of bone metabolism *in vivo.*

Until now, no previous study has investigated the effects of *Ecklonia cava* on the differentiation of hPDLSCs and osteoblasts. Based on the previous *in vitro* study regarding the possible osteoblastogenic effect of *Ecklonia cava*[17], it was hypothesized that *Ecklonia cava* could enhance the bone regeneration capacity of hPDLSCs. Thus, the aim of this study was to investigate the effects of *Ecklonia cava* on the osteogenic differentiation of hPDLSCs and to explore the possible mechanism underlying the process, as a part of continuous research to explicate natural products from marine sources with significant bioactivities.

II. Materials and Methods

Primary cell culture

Human third molars were collected from three healthy young males (18-25 years old) under the protocol approved by the institutional review board of Seoul National University Dental Hospital, Seoul, South Korea (IRB Number 05004). Periodontal ligament tissues were gently separated from the root surfaces of the extracted human third molars and digested in a solution of 3 mg/ml collagenase type I (Worthington Biochem, Freehold, NJ) and 4 mg/ml dispase (Boehringer, Mannheim, Germany) for 1 hour at 37℃. Single-cell suspensions were obtained by passing the cells through a 40-μm strainer (Falcon BD Labware, Franklin Lakes, NJ) supplemented with 10% FBS (Gibco BRL), 100 unit/ml penicillin, and 100 mg/ml streptomycin (Biofluids, Rockville, MD), and incubated at 37℃ in 5% CO_2. The medium was changed after the first 24 hours, and then every 3 days. The isolated cells formed single cell-derived colonies. All primary cells used in this study were from passage 3 and 4.

Flow cytometric analysis

To confirm stem cell characteristics of the cultured cells from the human periodontal ligament, expression of mesenchymal stem cell surface markers was assessed by flow cytometry according to the previously described method[19].

Briefly, cells in their third passage (1.0×10^6 cells) were fixed with 3.7% paraformaldehyde for 10 min and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (ICN Biomedicals, Aurora, OH) for 30 minutes for blocking. Cells were then incubated with specific antibodies for CD34, CD13, CD90, or CD146 at 4℃ for 1 hour, followed by incubation with fluorescence secondary antibodies at room temperature for 1 hour. All antibodies were purchased from BD Biosciences (San Jose, CA). The percentages of CD13-, CD90-, and CD146-positive and CD34-negative cells were measured with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the results were analyzed using CellQuest Pro software (Becton Dickinson Immunocytometry Systems).

Osteogenic, chondrogenic, and adipogenic differentiation

To confirm the multilineage differentiation ability of hPDLSCs, induction for osteogenic, chondrogenic and adipogenic differentiation was done. The cells were cultured in osteogenic differentiation medium with 50 μg/ml ascorbic acid, 10 mM ß-glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich), StemPro Chondrogenic and StemPro Adipogenic differentiation media (Gibco BRL) for 21 days. At the third week of osteogenic, adipogenic, and chondrogenic induction, the cells were stained with 2% Alizarin Red S stain (Sigma-Aldrich) at pH 4.2 for detection of mineralization, 0.3% Oil Red O dye (Sigma-Aldrich) and 1% Alcian Blue stain (Sigma-Aldrich) to detect proteoglycans, fat vacuoles, and Nissl bodies as indicators of osteogenic, adipogenic, and chondrogenic differentiation, respectively. The stained cells were visualized under an inverted light microscope (Olympus U-SPT; Olympus, Tokyo, Japan).

Preparation of Ecklonia cava extract

Ecklonia cava extract (ECE, powder) was provided by Seojin Biotech Co. Ltd. (Suwon, Korea). ECE was prepared using 70% ethanol extraction, and the total phlorotannins and dieckol contents

were 67% and 10.6%, respectively[20]. ECE powder was dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich) to obtain 100 mg/ml stock solutions, which was filtered through a 0.2-μm pore size Corning® syringe filter (Corning Inc., Corning, NY).

Cell viability assay

Cell proliferation was quantified using WST-8 assay kit (Qunati-MAX™ WST-8 Cell Viability Assay Kit; Biomax Co., Ltd., Korea) using tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that reacts with dehydrogenase in living cells to form an orange-colored, water-soluble formazan product.

Briefly, hPDLSCs (3.0×10^3 cells/well) were seeded in 96-well plates and cultured for 24 hours. Various concentrations of ECE were added in 100μl of culture medium per well for final concentrations of 0, 25, 50, 100, 200, 1000 μg/ml of ECE and cultured for 1, 3, and 7 days. Pre-mixed optimized dye solution (10μl) was added at the end of the treatment. Cells were incubated at 37℃ in 5% CO_2 for 1 hour. Then, 100μl of the reaction products were measured using an ELISA plate reader at 450 nm (650 nm as reference). Each condition was prepared

in triplicate, and the values were expressed as O.D. (mean O.D. ± standard deviation).

RNA preparation and real-time polymerase chain reaction analysis

To evaluate the gene expression levels in differentiated hPDLSCs, 5.0×10^5 cells were seeded in a 60-mm culture dish and cultured for 7 days under osteogenic differentiation induction conditions without or with 25 μg/ml and 50 μg/ml of ECE. Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and cDNA was synthesized from 2 μg of total RNA using reverse transcriptase (Superscript II Preamplification System; Invitrogen, Gaithersburg, MD). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green PCR Master Mix (ABI Prism 7500 sequence detection system; Applied Biosystems). The reaction conditions were 40 cycles of 15 seconds of denaturation at 95℃ and one minute of amplification at 60℃. All reactions were repeated three times and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). To compare the gene expression levels of the control and the ECE treated groups, the cycle threshold values were calculated and compared. The relative mRNA expression levels in hPDLSCs and

their ECE treated counterparts were then compared in a histogram. The expression levels of *ALP*, *Col1*, *OCN*, *OPN*, *Runx2*, and *GAPDH* were evaluated. The specific primer sets used in this analysis are listed in Table 1.

Western blot analysis

hPDLSCs (5.0×10^5 cells/dish) were seeded in a 60-mm culture dish and cultured for 7 days in osteogenic differentiation medium without or with 25 μg/ml and 50 μg/ml of ECE. Cell lysate protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein (30 mg/lane) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). Primary antibodies against β-actin (Sigma), Runx2 (Cell Signaling Technology), OPN (Abcam), Col1 and OCN (Santa Cruz Biotechnology) were used in this study. In addition, primary antibodies against phospho-p38 (p-p38), p38, phospho-ERK1/2 (p-ERK1/2), ERK1/2 (Cell Signaling Technology), p-Smad2/3 and Smad4 (Santa Cruz Biotechnology) were used to investigate the mechanism underlying ECE-induced osteogenic differentiation. Previous reports have demonstrated that Smad, p38 and ERK MAPK signaling pathways play significant roles in the osteogenic differentiation[21,22]. The blots were developed using horseradish

peroxidase-conjugated secondary antibodies (Cell Signaling Technology), and the proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare).

Transplantation and histological analysis

Animal study was reviewed and approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-190426-13). hPDLSCs (1.0×10^7 cells) were mixed with 100 mg of hydroxyapatite/β-tricalcium phosphate ceramic particles (HA/TCP, Dentium, Seoul, South Korea) with or without 25mg/ml ECE. Before the transplantation, one drop of fibrin (TISSEEL®, Baxter, IL, USA) was added to the transplant and manipulated into bolus shape for easier handling. The mixture of cells and scaffold was then transplanted subcutaneously into the immunocompromised mice (n = 6) (NIH-bg-nu/nu-xid; Harlan Sprague Dawley, Indianapolis, IN).

For histological analysis, samples were acquired 10 weeks after transplantation and fixed in 3.7% paraformaldehyde solution for 48 hours at 4°C before decalcification with 12% EDTA (pH 7.4) at 4°C. Semiserial 5-mm sections were prepared for hematoxylin and eosin (H&E) staining and examined under a light microscope (Olympus U-SPT; Olympus, Tokyo, Japan). Same sized digital images of the

histological sections in each group were obtained and analyzed. Quantitative evaluation of bone formation was done with histomorphometry using Trainable Weka Segmentation plugin of ImageJ software as previously described[23,24].

For immunohistochemical analysis, slides with tissue sections were stained with Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA), and Ventana ChromoMap Kit (Ventana Medical Systems, AZ, USA) was used for detection. Sections were deparaffinized with EZ Prep solution (Roche, Basel, Switzerland). Slides were incubated with rabbit polyclonal antibodies against OSX (1:500; Abcam, Cambridge, UK), OPN (1:500; Abcam, Cambridge, UK), and OCN (1:100; Santa Cruz Biotechnology, TX, USA) for 32 minutes at 37℃, and a secondary antibody for 20 minutes at 37℃. Slides were incubated in DAB + H_2O_2 substrate for 8 minutes at 37℃. The stained sections were counterstained with hematoxylin. Same sized digital images of OSX, OPN, and OCN staining in each group were obtained and analyzed. Quantitative evaluation of OSX, OPN, and OCN stained area was done with histomorphometry using Trainable Weka Segmentation plugin of ImageJ software.

Statistical analysis

All statistical analysis was performed using SPSS for Windows (version 22.0, SPSS Inc., Chicago, USA). For *in vitro* experiments, normal data with equal variance was analyzed using one-away analysis of variance (ANOVA). For histomorphometry of *in vivo* experiment, unpaired Student's t-test was used. Significance was defined as $p < 0.05$.

III. Results

Characterization of hPDLSCs

hPDLSCs were isolated and cultured successfully from human third molars. To characterize the cultured hPDLSC, flow cytometric analysis was performed using mesenchymal stem cell (MSC) markers, including CD13, CD90, and CD146. Flow cytometric analysis showed that 99.70% of hPDLSCs expressed CD13, 99.79% expressed CD90, 81.73% expressed CD146, and 1.72% expressed CD34 (Fig. 1).

Next, the multilineage differentiation capacity of hPDLSC with osteogenic, chondrogenic and adipogenic media was investigated *in vitro*. After 3 weeks of osteogenic induction, hPDLSC formed extensive Alizarin Red S-positive mineral deposits throughout the adherent layers. Furthermore, the cells were capable of undergoing adipogenic and chondrogenic differentiation after incubation with adipogenic-inductive and chondrogenic-inductive supplements for 3 weeks. Oil Red O and Alcian Blue staining showed lipid droplet formation and Alcian Blue-positive nodules, respectively (Fig. 2).

Effect of ECE on cell proliferation and cytotoxicity of hPDLSCs

To examine the effect of ECE on proliferation and cytotoxicity of hDPLSCs *in vitro*, 0, 25, 50, 100, 200, and 1000 μg/ml of ECE were added to hPDLSCs. After 1 and 3 days of culture, ECE increased proliferation of hPDLSCs without any cytotoxicity. After 7 days of culture, the O.D. value declined below the control value and showed decreasing trend in cell proliferation starting at 100 μg/ml of ECE (Fig. 3).

ECE induces osteogenic differentiation of hPDLSCs in vitro

The effect of ECE on the osteogenic differentiation of hPDLSCs was investigated *in vitro*. The expression levels of the related osteoblastic marker genes, *Col1*, *ALP*, *OPN*, *OCN*, and *Runx2*, were examined by real-time PCR. The expression levels of the osteogenic differentiation associated markers, *Col1*, *ALP*, *OPN*, *OCN*, and *Runx2*, increased during the differentiation process ($p < 0.05$) (Fig. 4). In addition, the protein expression levels of OCN, OPN, Col1, and Runx2 were significantly elevated in ECE-treated hPDLSCs compared to untreated control ($p < 0.05$) (Fig. 5).

ECE promoted osteogenic differentiation of hPDLSCs via p38 and ERK1/2 activation

The roles of Smad, p38 and ERK in ECE-induced osteogenic differentiation of hPDLSCs were investigated. After ECE treatment, the expression levels of Smad4, p-Smad2/3, p38 and ERK1/2 were detected by Western blot. Treatment of hPDLSCs with ECE led to significant increase in p-p38 and p-ERK1/2 MAPK compared to control ($p < 0.05$) (Fig. 6). On the other hand, the expression levels of Smad4, p-Smad 2/3, ERK1/2 and p38 were similar following ECE treatment. Therefore, there was no difference in the protein levels of Smad4, p-Smad 2/3, p38 and ERK1/2 MAPK in hPDLSCs between the control and ECE treatment groups. Western blot analysis indicated that exposure of the cells to ECE significantly increased the levels of p-p38 and p-ERK1/2 without altering the total amounts of these proteins. This result showed that ECE may induce osteogenic differentiation via activation of p38 and ERK1/2.

Effect of ECE on osteogenic differentiation of hPDLSCs in vivo

An *in vivo* transplantation model was used to assess the capacity of hPDLSCs for bone formation in response to ECE. At 10 weeks

after transplantation, the ECE-treated group showed significant generation of bone-like tissue, whereas the control group generated a limited amount of bone-like tissue (Fig. 7A,B). Histomorphometric analysis showed that the area of bone-like tissue in the ECE-treated group was 3.82-fold larger than that in the control group (p = 0.010627) (Fig. 7C).

To additionally explore the effect of ECE on the osteogenic differentiation of hPDLSC, immunohistochemical analysis was used to evaluate the differentiated bone-like tissue. The expressions of osteoblast differentiation markers, including OSX, OPN, and OCN, were significantly increased in the ECE-treated group compared with the control group (Figs. 8-10). Histomorphometric analysis showed that the bone-like tissue area in the ECE-treated group was 1.5-fold larger for OSX (p = 0.001604), 2.69-fold larger for OPN (p = 0.001288), and 1.53-fold larger for OCN compared to the control group (p = 0.000198) (Figs. 8E-10E). These *in vivo* results confirm that ECE promotes osteogenic differentiation of hPDLSCs.

IV. Discussion

It is well-documented that unlike embryophytes, or land plants, marine organisms have unique metabolism pathways and are capable of producing structurally novel and biologically active metabolites[25]. Seaweeds, in particular, provide various biological functional activities, and there has been a considerable interest in expanding the potential application of seaweeds, including functional foods and biomedical fields such as pharmaceutical science and tissue engineering[26-28].

Ecklonia cava, a type of brown seaweed, is abundantly produced in the costal areas of Korea and Japan and is not available in other parts of the world. Therefore, this valuable natural product is commonly used in Korea in the field of food ingredients, animal feed, fertilizers and medicine[29]. It has been recognized that the total polyphenolic compounds in *Ecklonia cava* are distinctly more plentiful than in other brown seaweeds[30-32]. The polyphenolic compounds of brown seaweeds are known as phlorotannins, and various phenolic secondary metabolites can be isolated from *Ecklonia cava* such as eckol (a closed-chain trimer of phloroglucinol), 6,6′-bieckol (a hexamer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer) and triphlorethol-A, which have been associated with the biological activities[33,34]. Phlorotannins are known for diverse health benefits

including but not limited to antioxidation, anticancer, antidiabetic, antimicrobial, anticoagulative, anti-human immunodeficiency virus, antihypertensive, inhibition of matrix metalloproteinase enzyme, protective effect against UVB-induced oxidative stress, anti-inflammatory and antiallergic activities[15]. However, the effects of ECE on osteogenic differentiation in hPDLSCs have never been investigated. In this context, it was hypothesized that ECE may be a more potent inducer of osteogenic differentiation compared with other seaweeds, and the present study was carried out to demonstrate that ECE promoted osteogenic differentiation of hPDLSCs *in vitro* and *in vivo.*

The *in vitro* investigation has shown that ECE promotes the proliferation of hPDLSCs. ECE enhanced the viable cell count of hPDLSCs in a dose-dependent manner up to 3 days of cell culture. However, after 7 days of culture, 100 μg/ml of ECE treatment decreased the cell viability, suggesting cytotoxicity for hPDLSCs starting at 100 μg/ml of ECE. Based on the cell viability assay result, 2 concentrations of ECE (25 μg/ml and 50 μg/ml) were chosen to conduct rest of the study.

Osteogenic differentiation is a complex process involving the development of mineralized extracellular matrix. There are many regulators influencing the osteogenic differentiation such as ALP,

Col1, OPN, and OCN, and ALP is an essential enzyme that contributes to high concentration of phosphate at an earlier stage of mineral deposition[35]. Col1 is a necessary factor involved in the formation of bone extracellular matrix by connecting the cell surface integrins to other extracellular matrix protein[36]. Then OPN attaches to the matrix surface and stimulates the interaction between cells and the extracellular matrix and hydroxyapatite formation, assisting the regulation of osteoblast-like cells, calcium deposition and bone formation[37]. Finally, OCN is a protein predominantly found in bone that mainly plays role in the late stage of osteogenesis[38]. In this study, ECE was found to promote the osteogenic differentiation of hPDLSCs in a dose-dependant manner. The gene expression levels significantly increased at the higher dose of 50 μg/ml, with the exception of OPN. Also, qRT-PCR data showed that ECE significantly increased the mRNA expression level of *ALP* and upregulated the mRNA levels of *Col1*, *OPN*, *OCN*, and *Runx2* in hPDLSCs, by which mineralization is enhanced. Consistent with these results, the protein expressions of Col1, OPN, OCN, and Runx2 were also elevated with ECE treatment in a dose-dependent manner. Therefore, ECE promoted cell differentiation and maturation of osteoblasts, indicating that ECE is beneficial in preventing

bone-associated diseases such as osteoporosis and periodontitis, by promoting osteoblast function.

To further examine the effect of ECE on the osteogenic differentiation of hPDLSCs, the signaling pathways responsible for osteogenic differentiation were investigated. Transforming growth factor-beta (TGF-β) signaling pathway is known to have integral function in skeletal development and bone homeostasis and converts signals to both the canonical Smad-dependent signaling pathway and the non-canonical-Smad-independent signaling pathway (such as p38 and ERK) to regulate mesenchymal stem cell differentiation during skeletal development, bone formation and bone homeostasis[39]. Both the Smad and p38 MAPK signaling pathways come together at transcription factors, such as Runx2, to promote osteoblast differentiation[39]. The Smad-dependent signaling, phosphorylated Smad2/3 complexes with Smad4 and co-translocates into the nuclei, where they gather co-factors to control target gene expression. According to Wu *et al.*, the TGF-β‐Smad signaling promotes proliferation, chemotaxis, and early differentiation of osteoprogenitor cells and inhibits osteoblast maturation and mineralization[39]. However, the results of this study show conflicting data since p-Smad2/3 and Smad4 were not influenced by ECE treatment, suggesting that ECE does not follow the Smad-dependent signaling pathway.

In the non-Smad-dependent pathway, phosphorylated TGF-β -activated kinase 1 recruit TAK1-binding protein 1 to trigger the MKK-p38 MAPK or MKK‑ERK1/2 signaling cascade[39]. It is already well-known that the MAPK superfamily, including ERKs and p38 kinases, coordinates signals from a various range of extracellular stimuli, and plays important roles in cellular functions such as proliferation, differentiation, and cell death in diverse cell types[40]. In the present study, it was demonstrated that expression levels of osteogenic markers were increased by ECE treatment when p38 and ERK1/2 signaling pathways were promoted. Although the levels of total p38 and ERK1/2 did not significantly change following ECE treatment, the levels of p-p38 and p-ERK1/2 were markedly increased compared to untreated cells. This might result from the increased phosphorylation of ERK1/2 and p38 proteins after activation of the signaling pathways. The results of this study implies that ECE takes the Smad-independent signaling pathway to induce osteogenic differentiation. Therefore, once this pathway is activated, the transcription factor, Runx2, is stimulated to initiate the osteogenesis process by upregulating the expression levels of Col1, OPN, OCN, which coincides with the data of this study. Overall, these results suggested that ECE triggers the Smad-independent signaling pathway

of p-p38 and p-ERK1/2 to promote hPDLSC osteogenic differentiation.

To confirm the results of *in vitro* study, the effects of ECE on osteogenic differentiation of hPDLSCs and hard tissue formation were investigated *in vivo*. It is well-known that MSC transplantation is a promising therapeutic application for periodontal regeneration since it can provide abundant amount of necessary cells to differentiate into the desired tissues, such as cementum, alveolar bone, dentin, and collagen fibers[41,42]. Previous studies demonstrated that HA/TCP had the ability to induce hard tissue formation of MSCs[43,44]. Therefore, many studies have been carried out to regenerate hard tissues by mixing HA/TCP with MSCs. Based on these past attempts, histological analysis of transplanted HA/TCP combined with hPDLSCs with or without ECE in immunocompromised mice was conducted. The results of the animal study demonstrated that ECE induced hPDLSCs to regenerate bone-like tissue at the interface between the scaffold and cellular portion through H&E staining. Furthermore, OSX, OPN, and OCN expressions were significantly increased in the bone-like tissue area of the ECE-treated group, suggesting that OSX, OPN, and OCN may play important role in ECE induced osteogenesis.

In conclusion, the present study demonstrated that ECE could enhance the osteogenic differentiation of hPDLSCs. The underlying molecular mechanism showed that non-Smad-dependent pathway involving p38 MAPK or ERK1/2 signaling cascade may be responsible for the inductive effect of ECE. Also, the effect of ECE on osteogenic differentiation was confirmed via area of bone-like tissue formation shown by *in vivo* transplantation model. As a result, ECE is considered as a natural alternative for bone regeneration treatment in the clinical setting.

V. Conclusion

In this study, the effect of ECE on osteogenic differentiation of hPDLSCs was investigated *in vitro* and *in vivo*. The results of the experiments confirmed that

1) ECE promoted proliferation and osteogenic differentiation of hPDLSCs, as shown in WST-8 assay, qRT-PCR, and Western blot analysis.

2) Also, Western blot analysis demonstrated that ECE may stimulate osteogenesis by p38 and ERK1/2 signaling pathways.

3) ECE has a greater ability to promote generation of bone-like tissue compared to the control group, as confirmed by the *in vivo* transplantation model using immunocompromised mice.

4) As a result, ECE is suggested to be a potential natural compound with therapeutic properties against bone-related complications and diseases that urge further studies to elucidate its detailed action mechanism and bioavailability.

References

1. Liao S, Murugan R, Chan CK, Ramakrishna S. Processing nanoengineered scaffolds through electrospinning and mineralization suitable for biomimetic bone tissue engineering. *J Mech Behav Biomed Mater*. 2008;1(3):252-260.

2. Kim J, Jeong SY, Ju YM, et al. In vitro osteogenic differentiation of human amniotic fuild-derived stem cells on a poly(lactide-co-glycolide) (PLGA)-bladder submucosa matrix (BSM) composite scaffold for bone tissue engineering. *Biomed Mater*. 2013;8(1):014107.

3. Wang W, Yeung KWK. Bone grafts and biomaterials substitutes for bone defect repair: A review. *Bioact Mater*. 2017;2(4):224-247.

4. Stevenson S, Horowitz M. The response to bone allografts. *J Bone Joint Surg Am*. 1992;74(6):939-950.

5. Hofmann A, Ritz U, Verrier S, et al. The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds. *Biomaterials*. 2008;29(31):4217-4226.

6. Jo YY, Lee HJ, Kook SY, et al. Isolation and characterization

of postnatal stem cells from human dental tissues. *Tissue Eng*. 2007;13(4):767-773.

7. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet*. 2004;364(9429):149-155.

8. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2000;97(25):13625-13630.

9. Gay IC, Chen S, MacDougall M. Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthod Craniofac Res*. 2007;10(3):149-160.

10. Schliephake H. Clinical efficacy of growth factors to enhance tissue repair in oral and maxillofacial reconstruction: a systematic review. *Clin Implant Dent Relat Res*. 2015;17(2):247-273.

11. Zhang N, Wu YP, Qian SJ, Teng C, Chen S, Li H. Research progress in the mechanism of effect of PRP in bone deficiency healing. *Scientific World Journal*. 2013;2013:134582.

12. Oryan A, Alidadi S, Moshiri A, Bigham-Sadegh A. Bone morphogenetic proteins: a powerful osteoinductive compound with non-negligible side effects and limitations. *Biofactors*. 2014;40(5):459-481.

13. Zara JN, Siu RK, Zhang X, et al. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. *Tissue Eng Part A*. 2011;17(9-10):1389-1399.

14. Wijesekara I, Yoon NY, Kim SK. Phlorotannins from Ecklonia cava (Phaeophyceae): biological activities and potential health benefits. *Biofactors*. 2010;36(6):408-414.

15. Wijesinghe WA, Jeon YJ. Exploiting biological activities of brown seaweed Ecklonia cava for potential industrial applications: a review. *Int J Food Sci Nutr*. 2012;63(2):225-235.

16. Kim S, Kang SS, Choi SI, Kim GH, Imm JY. Ecklonia cava Extract Containing Dieckol Suppresses RANKL-Induced Osteoclastogenesis via MAP Kinase/NF-κB Pathway Inhibition and Heme Oxygenase-1 Induction. *J Microbiol Biotechnol*. 2019;29(1):11-20.

17. Oh JH, Ahn BN, Karadeniz F, Kim JA, Lee JI, Seo Y, Kong CS. Phlorofucofuroeckol A from Edible Brown Alga *Ecklonia Cava* Enhances Osteoblastogenesis in Bone Marrow-Derived Human Mesenchymal Stem Cells. *Mar Drugs*. 2019;17(10):543.

18. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. *Bone*. 2007;40(2):251-264.

19. Jin H, Park JY, Choi H, Choung PH. HDAC inhibitor trichostatin A promotes proliferation and odontoblast differentiation of human dental pulp stem cells. *Tissue Eng Part A*. 2013;19(5-6):613-624.

20. Kim S, Kang SS, Choi SI, Kim GH, Imm JY. Ecklonia cava Extract Containing Dieckol Suppresses RANKL-Induced Osteoclastogenesis via MAP Kinase/NF-κB Pathway Inhibition and Heme Oxygenase-1 Induction. *J Microbiol Biotechnol*. 2019;29(1):11-20.36.

21. Ye G, Li C, Xiang X, et al. Bone morphogenetic protein-9 induces PDLSCs osteogenic differentiation through the ERK and p38 signal pathways. *Int J Med Sci*. 2014;11(10):1065-1072.

22. Xu DJ, Zhao YZ, Wang J, He JW, Weng YG, Luo JY. Smads, p38 and ERK1/2 are involved in BMP9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. *BMB Rep*. 2012;45(4):247-252.

23. Arganda-Carreras I, Kaynig V, Rueden C, et al. Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. *Bioinformatics*. 2017;33(15):2424-2426.

24. Malhan D, Muelke M, Rosch S, et al. An Optimized Approach to Perform Bone Histomorphometry. *Front Endocrinol*

(Lausanne). 2018;9:666.

25. Heo SJ, Ko SC, Cha SH, et al. Effect of phlorotannins isolated from Ecklonia cava on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation. *Toxicol In Vitro*. 2009;23(6):1123-1130.

26. Wijesinghe W, Jeon YJ. Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweed: A review. *Carbohydr Polym*. 2012;88(1):13-20.

27. Li B, Lu F, Wei X, Zhao R. Fucoidan: structure and bioactivity. *Molecules*. 2008;13(8):1671-1695.

28. Cho YS, Jung WK, Kim J, Choi IW, Kim SK. Beneficial effects of fucoidan on osteoblastic MG-63 cell differentiation. *Food Chem*. 2009;116(4):990-994.

29. Ahn GN, Kim KN, Cha SH, Son CB, Lee J, Heo MS, Yeo IK, Lee NH, Jee YH, Kim JS, Heu MS, Jeon YJ. Antioxidant activities of phlorotannins purified from Ecklonia cava on free radical scavenging using ESR and H_2O_2-mediated DNA damage. *Eur Food Res Technol*. 2007;226(1-2):71-79.

30. Heo SJ, Park EJ, Lee KW, Jeon YJ. Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresour Technol*. 2005;96(14):1613-1623.

31. Heo SJ, Jeon YJ, Lee JH, Kim HT, Lee KW. Antioxidant effect of enzymatic hydrolyzate from a kelp, Ecklonia cava. *Algae*. 2003;18(4):341-347.

32. Heo SJ, Lee KW, Song CB, Jeon YJ. Antioxidant activity of enzymatic extracts from brown seaweeds. *Algae*. 2003;18(1):71-81.

33. Kang KA, Lee KH, Chae S, et al. Eckol isolated from Ecklonia cava attenuates oxidative stress induced cell damage in lung fibroblast cells. *FEBS Lett*. 2005;579(28):6295-6304.

34. Kang KA, Lee KH, Chae S, et al. Triphlorethol-A from Ecklonia cava protects V79-4 lung fibroblast against hydrogen peroxide induced cell damage. *Free Radic Res*. 2005;39(8):883-892.

35. Guo Z, Kang S, Chen D, et al. MAPK signaling pathway alters expression of midgut ALP and ABCC genes and causes resistance to Bacillus thuringiensis Cry1Ac toxin in diamondback moth. *PLoS Genet*. 2015;11(4):e1005124.

36. Lian JB, Stein GS. Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Crit Rev Oral Biol Med*. 1992;3(3):269-305.

37. Zhang R, Zhang Z, Pan X, Huang X, Huang Z, Zhang G.

ATX-LPA axis induces expression of OPN in hepatic cancer cell SMMC7721. *Anat Rec (Hoboken)*. 2011;294(3):406-411.

38. Rocha ÉD, de Brito NJ, Dantas MM, Silva Ade A, Almeida Md, Brandão-Neto J. Effect of Zinc Supplementation on GH, IGF1, IGFBP3, OCN, and ALP in Non-Zinc-Deficient Children. *J Am Coll Nutr*. 2015;34(4):290-299.

39. Wu M, Chen G, Li YP. TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Res*. 2016;4:16009.

40. Zhao Y, Song T, Wang W, et al. P38 and ERK1/2 MAPKs act in opposition to regulate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. *Plos One*. 2012;7(8):e43383.

41. Hynes K, Menicanin D, Gronthos S, Bartold PM. Clinical utility of stem cells for periodontal regeneration. *Periodontol 2000*. 2012;59(1):203-227.

42. Kawaguchi H, Hirachi A, Hasegawa N, et al. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J Periodontol*. 2004;75(9):1281-1287.

43. Ohgushi H, Okumura M, Tamai S, Shors EC, Caplan AI. Marrow cell induced osteogenesis in porous hydroxyapatite

and tricalcium phosphate: a comparative histomorphometric study of ectopic bone formation. *J Biomed Mater Res*. 1990;24(12):1563-1570.

44. Arinzeh TL, Tran T, Mcalary J, Daculsi G. A comparative study of biphasic calcium phosphate ceramics for human mesenchymal stem-cell-induced bone formation. *Biomaterials*. 2005;26(17):3631-3638.

Table

Table 1. Primer Sequences for Real-Time Polymerase Chain Reaction

Gene	GenBank No.	Sequences
ALP	BC090861	5′-GGACATGCAGTACGAGCTGA-3′ 5′-GCAGTGAAGGGCTTCTTGTC-3′
Col1	XM_012651	5′-CTGACCTTCCTGCGCCTGATGTCC-3′ 5′-GTCTGGGGCACCAACGTCCAAGGG-3′
OCN	X53698	5′-GTGCAGAGTCCAGCAAAGGT-3′ 5′-TCAGCCAACTCGTCACAGTC-3′
OPN	J04765	5′-CCCACAGACCCTTCCAAGTA-3′ 5′-ACACTATCACCTCGGCCATC-3′
Runx2	NM_001015051	5′-CGCATTCCTCATCCCAGTAT-3′ 5′-GACTGGCGGGGTGTAAGTAA-3′
GAPDH	NM_002046	5′-ACCACAGTCCATGCCATCA-3′ 5′-TCCACCACCCTGTTGCTGT-3′

ALP, alkaline phosphatase; Col1, type 1 collagen; OCN, osteocalcin; OPN, osteopontin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Figures
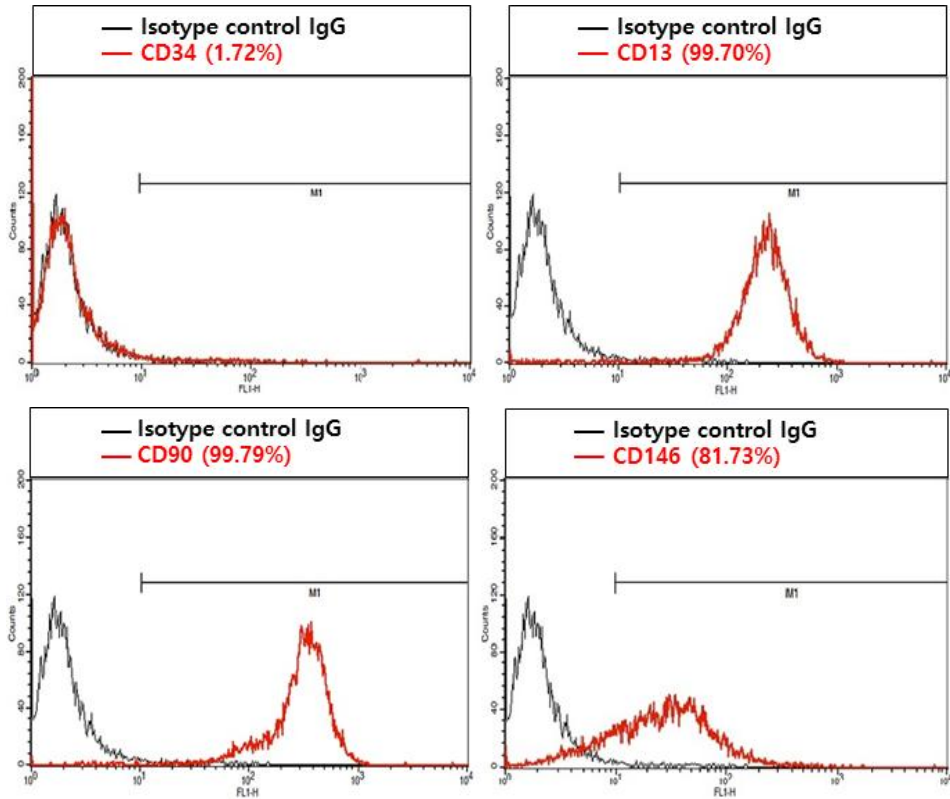
Figure 1. Characterization of human periodontal ligament stem cells (hPDLSCs) as mesenchymal stem cells (MSCs).

MSCs markers, including CD34, CD13, CD90, and CD146, were used for fluorescence-activated cell sorting analysis of hPDLSCs. To analyze the populations of CD34-negative, CD13-positive, CD90-positive, and CD146-positive cells, the percentages of the cells to the right of the M1 gate were measured (n = 3).
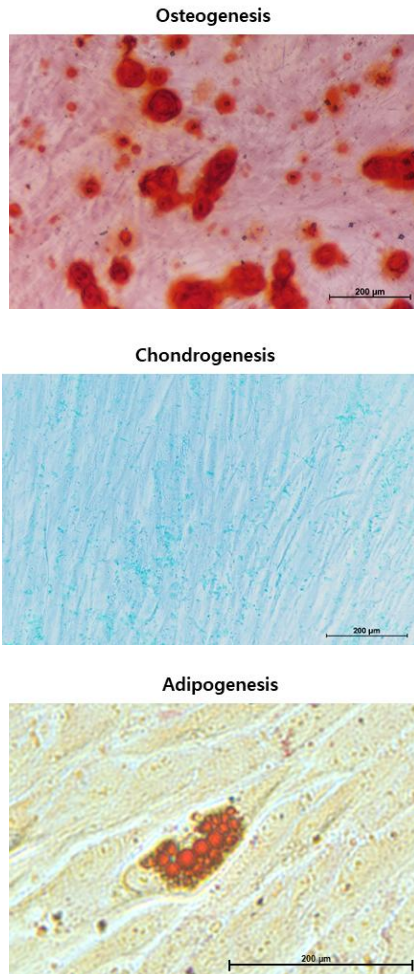
Osteogenesis

Chondrogenesis

Adipogenesis

Figure 2. Multilineage differentiation of human periodontal ligament stem cells (hPDLSCs).

To investigate the multilineage differentiation potential of hPDLSCs, the cells were cultured with osteogenic, chondrogenic, and adipogenic differentiation media for 21 days *in vitro*. Alizarin Red S staining, Alcian Blue staining, and Oil Red O staining respectively showed the progress of each group differentiation.
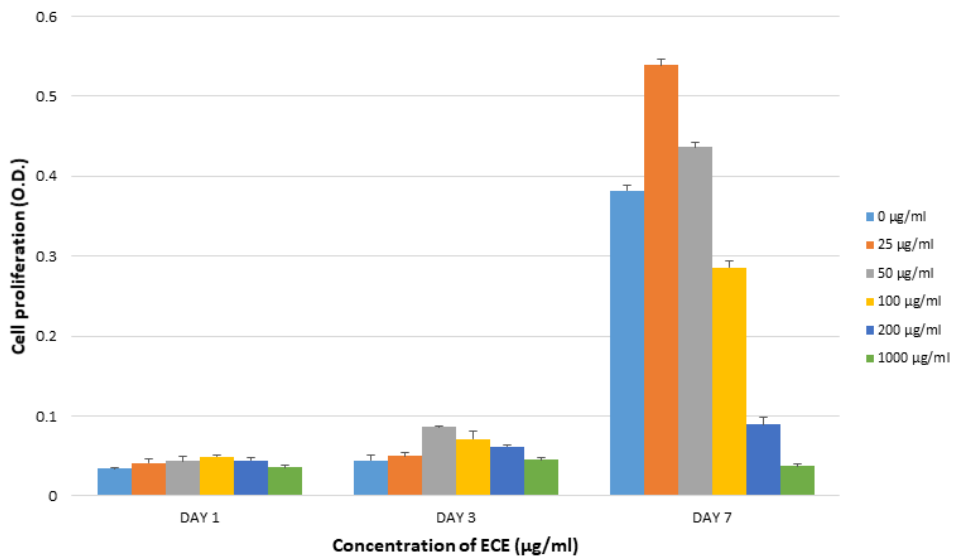
Figure 3. Effect of *Ecklonia cava* extract (ECE) on cell proliferation and cytotoxicity of human periodontal ligament stem cells (hPDLSCs) *in vitro*.

To examine the effect of ECE on hPDLSC proliferation and cytotoxicity *in vitro*, hPDLSCs were incubated with 0, 25, 50, 100, 200, or 1000 μg/ml of ECE for 1, 3, and 7 days. After culture, cell viability was evaluated using WST-8 assay. After 1 and 3 days of culture, ECE increased proliferation of hPDLSCs without any cytotoxicity. After 7 days of culture, cell proliferation declined below the control value starting at 100 μg/ml of ECE.
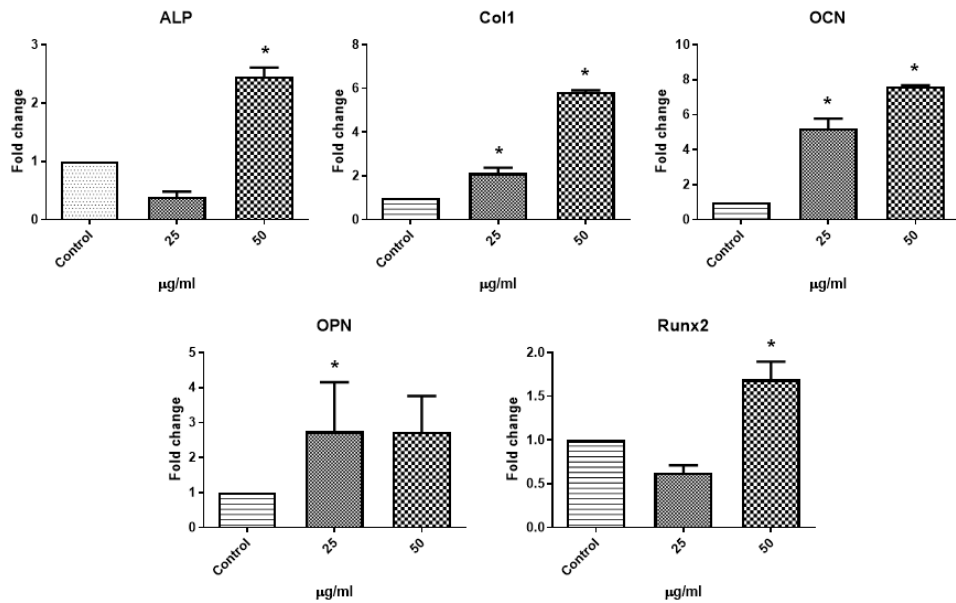
Figure 4. Effect of *Ecklonia cava* extract (ECE) on osteogenic differentiation markers via quantitative real-time polymerase chain reaction (qRT-PCR).

Human periodontal ligament stem cells were incubated in the presence of the indicated concentrations of ECE with osteogenic differentiation medium for 7 days. qRT-PCR of 5 osteogenic differentiation markers was done. Expressions of *ALP*, *Col1*, *OCN, OPN,* and *Runx2* increased at concentration of 50 μg/ml. Data are representative of three independent experiments and are expressed as the mean ± SD; *$p < 0.05$ vs. control.
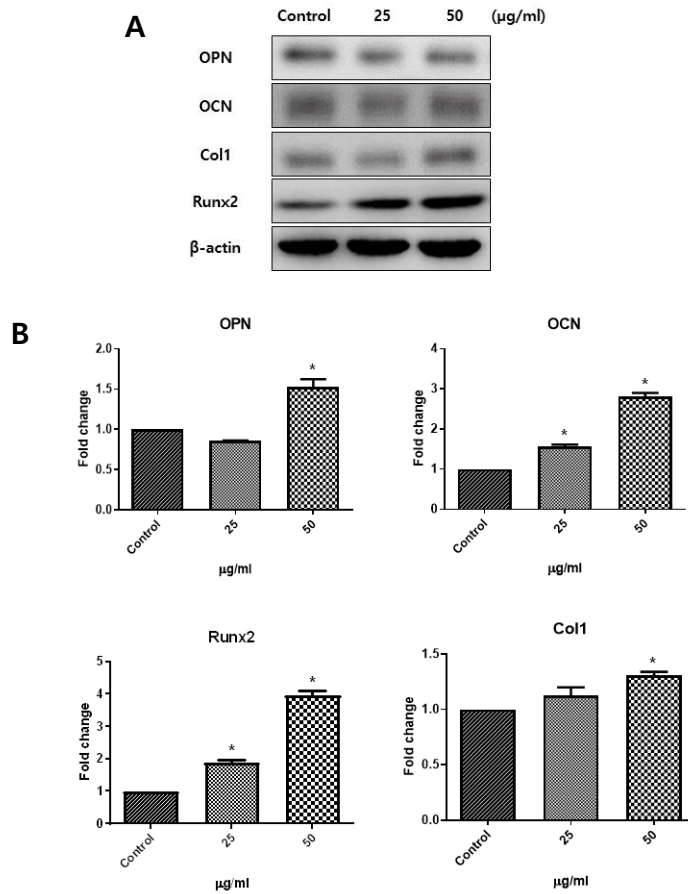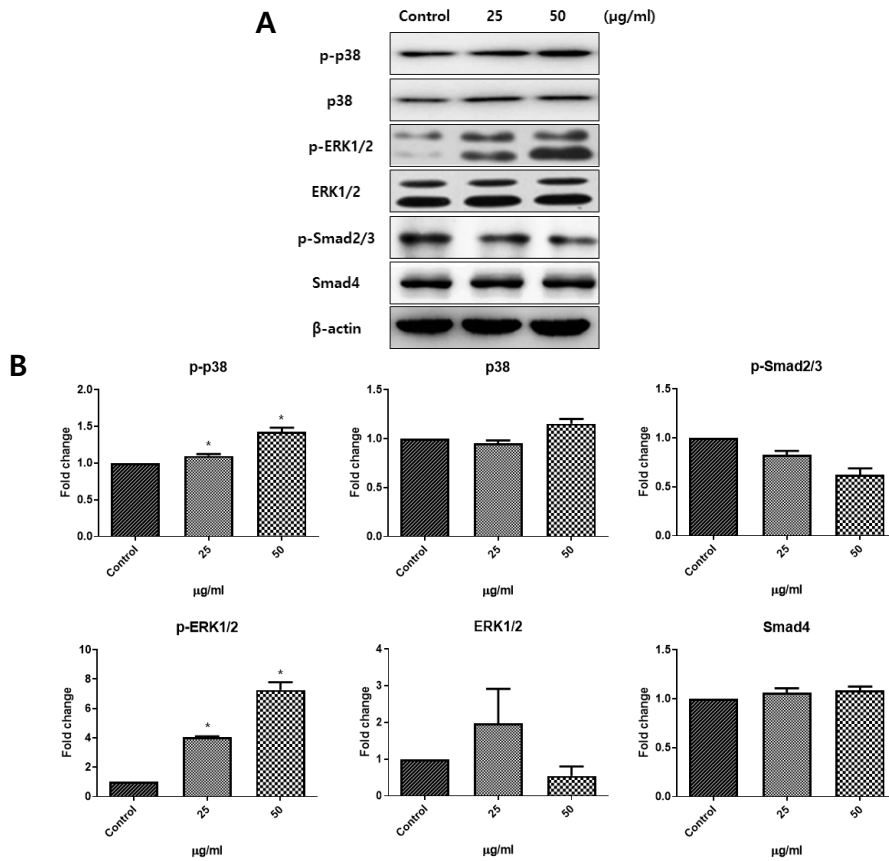
Figure 5. Effect of _Ecklonia cava_ extract (ECE) on protein expressions of OCN, OPN, Col1, and Runx2.

Human periodontal ligament stem cells were cultured in osteogenic differentiation medium without or with ECE (25 μg/ml and 50 μg/ml) for 7 days. The expression levels of OCN, OPN, Col1, and Runx2 were detected by Western blot. ß-actin served as loading control. Data are representative of three independent experiments and are expressed as the mean ± SD; *$p < 0.05$ vs. control.

Figure 6. *Ecklonia cava* extract (ECE) induced osteogenic differentiation in human periodontal ligament stem cells (hPDLSCs) via p38 and ERK1/2 signaling pathways. hPDLSCs were cultured in osteogenic differentiation medium without or with ECE (25 µg/ml and 50 µg/ml) for 7 days. Whole-cell lysates were subjected to Western blot analysis with indicated antibodies; ß-actin served as an internal control. Data are representative of three independent experiments and are expressed as the mean ± SD; *$p < 0.05$ vs. control.
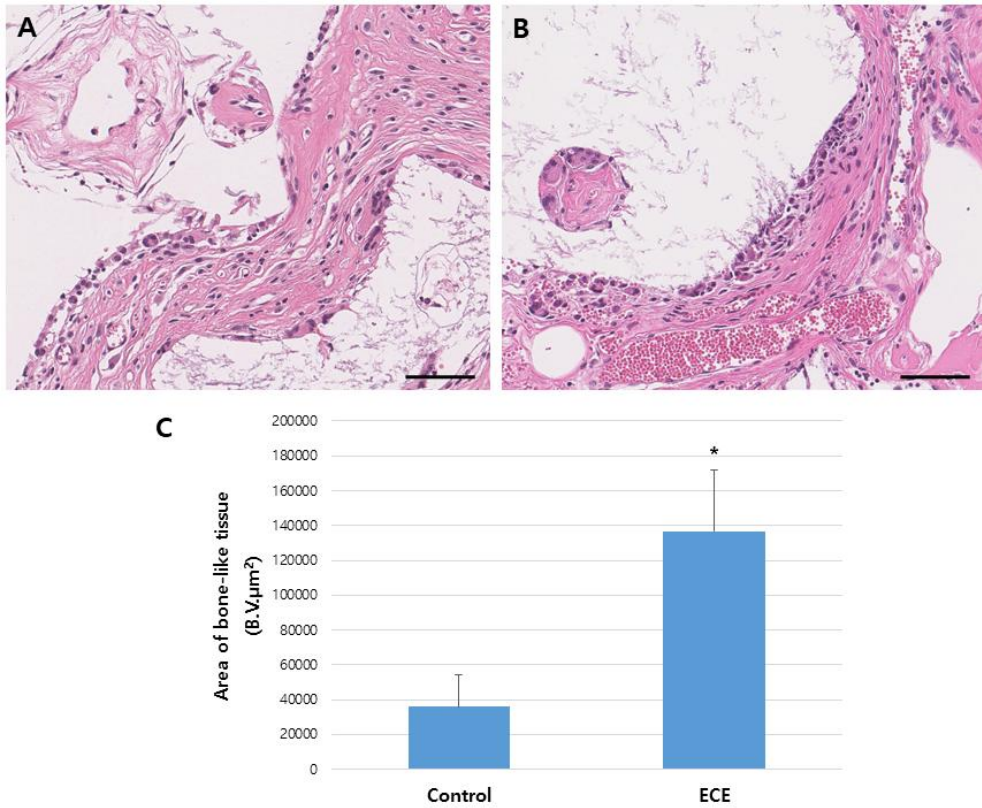
Figure 7. Histological analysis with Hematoxylin & Eosin (H&E) staining.

(A) H&E staining of bone-like tissue in control group. (B) H&E staining of increased bone-like tissue in *Ecklonia cava* extract (ECE)-treated group. (C) Histomorphometric analysis showed that bone-like tissue area in the same gross area in ECE-treated group was 3.82-fold larger than that in control group. The difference in data showed statistical significance ($*p < 0.05$). Scale bar = 90μm.
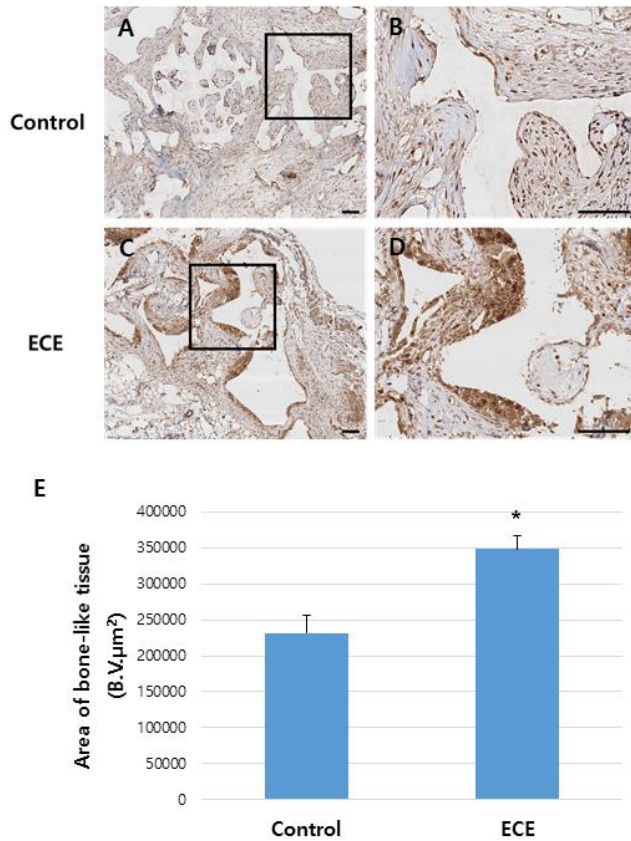
Figure 8. Immunohistochemical analysis of OSX expression.
(A) OSX expression in control group at 80x magnification. (B) OSX expression in control group at 200x magnification. (C) OSX expression in *Ecklonia cava* extract (ECE)-treated group at 80x magnification. (D) OSX expression in ECE-treated group at 200x magnification. (E) Histomorphometric analysis showed that OSX expression in the bone-like tissue area of ECE-treated group was 1.5-fold larger than that in control group (∗$p < 0.05$). Scale bar = 100μm.
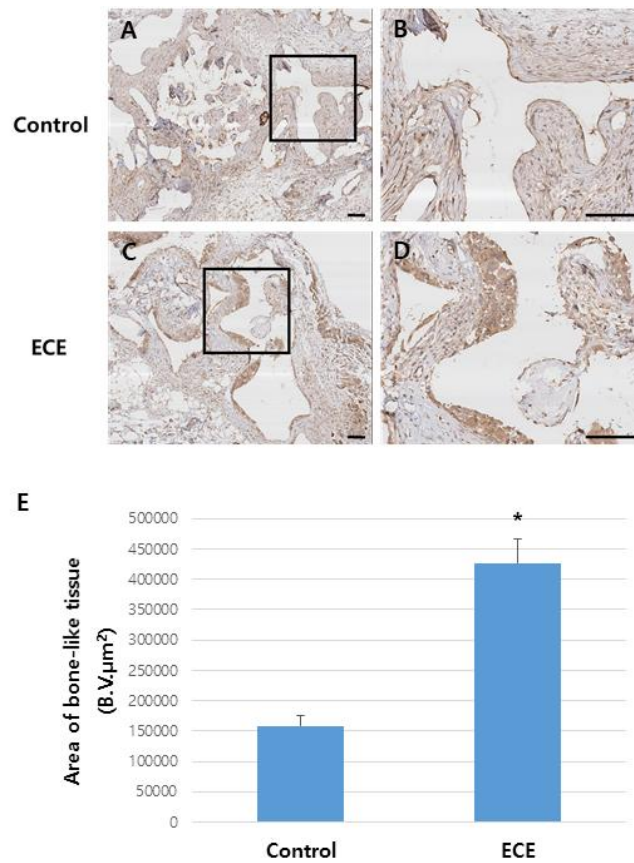
Figure 9. Immunohistochemical analysis of OPN expression.
(A) OPN expression in control group at 80x magnification. (B) OPN expression in control group at 200x magnification. (C) OPN expression in *Ecklonia cava* extract (ECE)-treated group at 80x magnification. (D) OPN expression in ECE-treated group at 200x magnification. (E) Histomorphometric analysis showed that OPN expression in the bone-like tissue area of ECE-treated group was 2.69-fold larger than that in control group (*$p < 0.05$). Scale bar = 100μm.
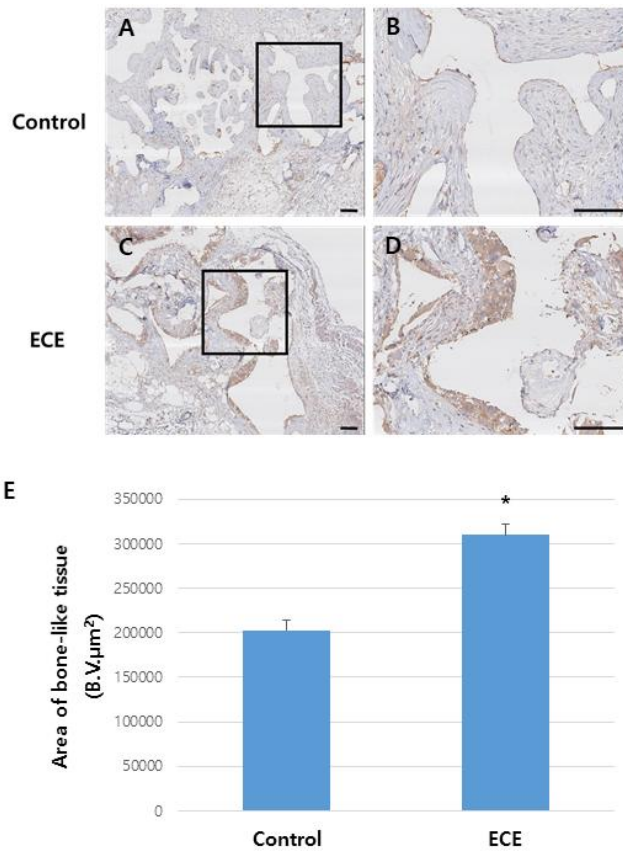
Figure 10. Immunohistochemical analysis of OCN expression.
(A) OCN expression in control group at 80x magnification. (B) OCN expression in control group at 200x magnification. (C) OCN expression in *Ecklonia cava* extract (ECE)-treated group at 80x magnification. (D) OCN expression in ECE-treated group at 200x magnification. (E) Histomorphometric analysis showed that OCN expression in the bone-like tissue area of ECE-treated group was 1.53-fold larger than that in control group (*$p < 0.05$). Scale bar = 100μm.

국문초록

치아의 여러 부위에서 줄기세포를 추출하여 다양한 세포로 분화시키고 이를 이용한 치아 및 치주조직의 재생에 대한 연구가 많이 진행되어 왔다. 더 나아가 줄기세포의 분화 및 치주조직의 재생을 촉진하는 인자들, 특히 부작용이나 독성이 적은 천연물에 대한 연구도 활발하게 이루어지고 있다. 본 연구에서 사용한 감태는 다시마목 미역과의 갈조류로 우리나라 동해안과 제주도 근해에 널리 자생하고 있다. 현재까지 보고된 감태에 대한 연구로는 항산화, 항염증, 항암, 항고혈압 및 항당뇨 효능 등이 보고되어왔지만 사람치아줄기세포에 미치는 영향에 대한 연구는 이루어진 바가 없어 본 연구에서는 감태 추출물이 치주인대 줄기세포에 어떠한 영향을 주는지 알아보고자 한다.

성인 미성숙 제3대구치로부터 추출한 치주인대 줄기세포를 배양한 뒤 중간엽 줄기세포의 표지자 확인을 위해 유세포분석법을 시행하였다. 추출된 세포를 골아세포, 지방세포, 연골세포로 분화시킨 후 각각의 염색을 시행하여 치주인대 줄기세포의 다분화능력을 확인하였다.

세포 증식 및 독성에 대한 영향을 조사하기 위하여 감태 추출물을 농도별로 처리하여 1일, 3일 및 7일 동안 배양한 후 세포 생존능 분석을 시행하였다. 적정 감태 추출물의 농도를 확인한 후 골분화가 유도된 세포를 대상으로 quantitative RT-PCR을 통해 조골세포 분화 유전자인 *Col1*, *ALP*, *OPN*, *OCN* 및 *Runx2*의 발현 정도 및 Western blot으로 OCN, OPN, Col1 및 Runx2 단백질 발현 정도를 확인하였다. 또한

Western blot으로 Smad4, p-Smad2/3, p-38, p38, p-ERK1/2 및 ERK1/2의 발현 정도를 측정함으로써 감태 추출물 처리 후 골형성 분화 과정의 기본이 되는 메커니즘을 조사하였다.

체내에서의 분화를 확인하기 위해 치주인대 줄기세포를 HA/TCP와 혼합하여 면역 억제된 쥐의 등에 10주 동안 이식하였다. 감태 추출물에 의한 치주인대 줄기세포의 골분화를 조사하기 위해 조직학적 분석과 면역조직화학법을 이용한 분석을 시행하였다.

유세포분석법을 통해 성인 미성숙 제3대구치로부터 추출한 치주인대 줄기세포가 중간엽 유래 줄기세포의 특징을 갖고 있고 또한 Alizarin Red S 염색, Alcian Blue 염색, Oil Red O 염색을 통해 골조직, 연골조직, 지방조직으로 분화가 가능한 것을 확인하였다.

감태 추출물을 농도별로 처리하였을 때 배양 1일 및 3일 후에는 세포 독성이 나타나지 않았으며 배양 7일에서는 100 μg/ml 농도부터 세포 독성이 나타나는 것으로 관찰되었다. Quantitative RT-PCR을 통해 감태 추출물 처리군에서 *ALP, Col1, OCN, OPN* 및 *Runx2*의 발현이 처리되지 않은 군에 비해 증가하고, OCN, OPN, Col1 및 Runx2의 단백질 발현도 증가함을 보여 주었다. 또한, Western blot 분석은 감태 추출물이 p38 및 ERK1/2 신호 경로의 활성화에 의해 골형성을 유도 할 수 있음을 입증 하였다.

면역 억제된 쥐를 이용한 생체내 실험에서 H&E 염색을 사용한 조직학적 분석은 감태 추출물 처리 군에서 뼈-유사 조직층의 면적이 증가한 것으로 나타났고, 면역조직화학 분석은 OSX, OPN 및 OCN 발현의 증가

로 뼈-유사 조직의 생성을 확인하였다.

본 연구에서는 생체외 및 생체내 실험을 통하여 감태 추출물이 사람 치주인대 줄기세포의 골분화를 촉진하는 것을 확인하였다. 결론적으로 이 실험의 임상적 의미는 감태 추출물은 치조골 재생에 효과적인 영향을 미칠 것으로 사료되며 향후 감태 추출물의 정확한 작용기전 및 생체 이용률을 밝히기 위한 추가 연구가 필요할 것으로 생각된다.

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주요어 : 감태, 치주인대 줄기세포, 조직재생

학 번 : 2016-33606