Enhanced osteogenic differentiation of human periodontal ligament stem cells by suberoylanilide hydroxamic acid

YUNZE XUAN1,#; BIN JIN1,#; SAYAN DEB DUTTA3,5; MENGMENG LIU2; ZAIXIAN SHEN1; XIWEN LIU3; YANG KANGJUAN4,*; LIM KI-TAEK5,*

1 Department of Stomatology, Affiliated Hospital of Yanbian University, Yanji, 133000, China
2 Department of Immunology and Molecular Microbiology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, 08826, South Korea
3 Affiliated Hospital of Yanbian University, Yanji, 133000, China
4 Department of Cell Biology and Medical Genetics, Yanbian University Medical College, Yanji, 133000, China
5 Department of Biosystems Engineering, Kangwon National University, Chuncheon, 24341, South Korea

Key words: Suberoylanilide hydroxamic acid, Histone deacetylase, Osteoblast, hPDLSCs, Alkaline phosphatase, Periodontal bone regeneration

Abstract: Periodontitis is a type of chronic inflammation in the gingival tissue caused by infectious bacteria colonizing the surface of the teeth, leading to the destruction of tooth-supporting tissues and loss of alveolar bone. Suberoylanilide hydroxamic acid (SAHA), a class of histone deacetylase (HDAC) inhibitor, has the potential to stimulate osteoblast differentiation by acetylating histone proteins, and thus suppressing the expression of adipogenic and chondrogenic genes. However, the effect of SAHA on the differentiation of human periodontal ligament stem cells (hPDLSCs) is yet to be elucidated. Herein, we investigated the effects of SAHA on in vitro proliferation and differentiation of hPDLSCs by MTT assay, Alizarin Red-S, and alkaline phosphatase staining, and real-time PCR. Notably, 300 ng/mL SAHA treatment enhanced the proliferation and mineralization of hPDLSCs, indicating their osteogenic potential. Moreover, a significant enhancement of osteogenesis gene markers and proteins was observed. We also demonstrated that ERK is a positive regulator of Runx2 transcription factors during osteoblast differentiation. These results indicate that SAHA may be a useful osteogenic induction agent for periodontal bone regeneration.

Introduction

Periodontitis is a destructive inflammation in the surrounding and supporting structures of the teeth that results in tissue injury, including loss of connective tissue and alveolar bone (Straka et al., 2015; Larsson et al., 2015). Consequently, periodontitis often leads to loose teeth and is the primary cause of tooth loss. At present, more and more scientists are focusing on tissue reconstruction, and strive to preserve physiologically functional teeth in the mouth for an extended period of time. In recent years, the potential application of mesenchymal stem cells (MSCs) for tissue regeneration has attracted much attention in various medical studies. It is reported that there are abundant mesenchymal stem cell pools in the oral cavity (Huang et al., 2009). Human dental pulp mesenchymal stem cells (hDMSCs) can be isolated in the form of dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), apical papillary stem cells (SCAP), and alveolar bone marrow stem cells (ABMSCs); they are derived from various dental tissues, including immature wisdom teeth, exfoliated deciduous teeth, apical papilla, periodontal ligament, and dental follicle tissue (Huang et al., 2009; Potdar and Jethmalani, 2015). The periodontal ligament (PDL) is a soft connective tissue located between the cementum and the alveolar bone, providing tooth nutrition to maintain homeostasis and regeneration of periodontal tissue. PDL contains different cell types as well as a group of stem cells (Alvarez et al., 2015). The PDLSCs express the mesenchymal stem cell markers CD13, CD90, and CD146, as well as bone-associated markers such as alkaline phosphatase (ALP) and bone sialoprotein (BSP), and have the potential to differentiate into osteoblasts, osteoblast-like cells, and periodontal ligament fibroblasts. PDLSCs eventually form typical alveolar bone/periodontium-like structures under defined culture conditions and are

*Address correspondence to: Kangjuan Yang, yangkj@ybu.edu.cn; Ki-Taek Lim, ktlim@kangwon.ac.kr
#These authors contributed equally to this work

Received: 18 November 2019; Accepted: 28 April 2020

Doi: 10.32604/biocell.2020.09170 www.techscience.com/journal/biocell

This work is licensed under a Creative Commons Attribution 4.0 International License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
considered to be potential sources of MSCs (Park et al., 2011, 2013). Although several methods have been studied to transport PDLSCs into periodontal defects, including cell mass, micro-tissue, and cell sheet engineering in vivo (Park et al., 2011; Jin and Choug, 2016), few studies described the clinical application of PDLSCs as they are difficult to handle during surgery, and there are challenges in retaining the transferred PDLSCs at the surgical site.

Histone deacetylase (HDAC) inhibitors were reported to inhibit proliferation and promote the differentiation of cancer cells in tumors and hematological malignancies. The mechanism of HDAC inhibitors involves the regulation of gene expression by increasing the acetylation of histones and non-histones (Marks, 2007; Kouraklis and Theocharis, 2006; Lee et al., 2015). HDAC inhibitors interact with lysine residues of histone proteins (H2A, H2B, H3, and H4), resulting in the removal of acetyl moieties. Acetylation results in the formation of positive charges on lysine residues, which inactivate the function of the native protein. Thus, histone deacetylation is generally associated with the suppression of gene expression (Richon, 2006; Duncan et al., 2015). Moreover, it was reported that HDAC inhibitors could induce Runx2-dependent osteoblast differentiation by suppressing adipocyte differentiation in vitro (Fischer et al., 2010; Dudakov et al., 2013).

Suberoylanilide hydroxamic acid (SAHA), a new class of broad-spectrum histone deacetylase inhibitor, was reported to enter phases I and II clinical trials for application in cancer treatment (Marks, 2007). SAHA resembles HDAC in structure and function; it inhibits cell cycle progression and induces apoptosis to disrupt gene function (Richon, 2006). Moreover, SAHA induces cardiomyogenic differentiation of human dental follicle-derived stem cells by regulating the cardiomyogenic transcription factors TNNT2, α-SMA, and Desmin (Sung et al., 2016). Previously, SAHA was reported to enhance bone resorption and reduce mineral density (Sato et al., 2001; Kwon et al., 2012). However, the role of SAHA in odontoblastic differentiation and corresponding signaling pathways have not been elucidated extensively. Thus, our present study investigated the effects of SAHA on osteoblast differentiation as well as the mineralization potential of hPDLSCs in vitro. Further, we demonstrated that SAHA induces osteoblast differentiation via ERK/MAPK signaling pathways.

Materials and Methods

Human PDLSC cultures

Human periodontal ligaments were extracted and obtained from the third molar tooth of adult humans. All the experiments regarding dental implantations, stem cell isolation, and maintenance were approved by the Department of Stomatology, Institutional Review Board, affiliated with the Hospital of Yanbian University (Yanbian, China; IRB number 2019201). Written consent letters were obtained from all the donors. Human periodontal ligaments were gently scraped with forceps from the third molar and digested for 1 h with 3 mg/mL collagenase type-1 (Worthington Biochem. Co., NJ, USA) and 4 mg/mL dispase (Boehringer, UK) at 37°C. After digestion, the remaining cells were cultured in an alpha modification of Eagle’s medium (α-MEM; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, USA), 2 mM glutamine, 100 mM ascorbic acid-2-phosphate, and 1% antibiotics (Gibco-BRL; 100 U/mL penicillin and 100 mg/mL streptomycin) and incubated at 37 ± 1°C with 5% CO2. The medium was replaced every 48 h. For our study, we selected hPDLSCs from three different donors. The second or third passage of cells was used for all the experiments.

Cytotoxicity evaluation

The cytotoxicity of hPDLSCs in the presence of SAHA was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit per the manufacturer’s instructions (Promega, Wisconsin, USA). Briefly, hPDLSCs (3 × 10³ cells/well) were cultured in 96-well plates for 48 h. Varying amounts of SAHA were added to 100 µL of the culture medium per well to achieve final concentrations of 0, 100, 300, 500, 800, 1000, and 2000 ng/mL. After incubation, a 15 µL premixed dye solution was added and incubated at 37°C for 4 h followed by addition of 100 µL stop solution. Finally, the end product was analyzed by measuring the optical density (OD) at 595 nm (Infinite M Nano 200 Pro; TECAN, Switzerland). All samples were run in triplicate (n = 3), and data are presented as mean ODs ± standard deviations. Statistical significance was considered at *p < 0.05.

Flow cytometry

For flow cytometry, 1 × 10⁶ were fixed with 3.7% paraformaldehyde (Sigma-Aldrich, USA) for 10 min and resuspended in 1X phosphate-buffered saline (PBS; Gibco-BRL) containing 1% bovine serum albumin (BSA; ICN Biomedical Corp., CA, USA) for 30 min to block nonspecific antibody-binding sites. Next, cells were incubated with specific primary antibodies (CD34, CD13, CD90, or CD146) for 1 h at 4°C, followed by incubation with fluorescent secondary antibodies for 1 h at room temperature. All the antibodies were purchased from BD Biosciences (San Jose, CA, USA). Finally, the percentages of CD13, CD90, CD146 positive, and CD34 negative cells were measured using a FACS Caliber flow cytometer (BD Immunocytometry Systems, SJ, USA). Results were analyzed using the Cell Quest Pro software (BD Systems, SJ, USA).

Osteogenic, chondrogenic, and adipogenic differentiation

PDLSCs (1 × 10⁴/well) were cultured in 24-well plates containing DMEM medium supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 1% antibiotics (Gibco-BRL). Next, the cells were transferred to osteogenic (50 mg/mL ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone), chondrogenic (1% chondrogenic basal medium, 1% chondrogenic supplement, and 5 µg/mL gentamicin reagent), or adipogenic (1% adipocyte basal medium, 1% adipocyte supplement, and 5 µg/mL gentamicin reagent) differentiation medium (StemPro, Gibco-BRL) and cultured for three weeks. Subsequently, the cells were stained with 2% Alizarin Red S (ARS; pH 4.2; Sigma-Aldrich, USA), 1% Alcian Blue (Sigma-Aldrich), and 0.3% Oil Red O dye (Sigma-Aldrich) for the detection of
mineralized nodules (calcium deposition), proteoglycans, and fat vacuoles as indicators of osteogenic, chondrogenic, and adipogenic differentiation, respectively. Stained cells were visualized under an inverted optical microscope (Olympus U-SPT; Olympus, Japan).

### Alkaline phosphatase staining

For ALP activity, hPDLSCs were cultured (4 × 10^4 cells/well) in 24-well plates with αMEM supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich) for seven days with or without SAHA (300 ng/mL) treatment. Before staining, cells were fixed in 10% formalin (DUKSAN Chemicals Co., Gyeonggi-do, Republic of Korea), rinsed with 1X PBS twice, incubated with 0.1% TritonX-100 for 5 min, and finally stained using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich) according to the manufacturer’s protocol. After seven days of incubation, cells were gently washed twice with ice-cold PBS and lysed with 0.01% sodium dodecyl sulfate for 30 min. The supernatant was collected, and an assay buffer containing 2 mM magnesium chloride (MgCl₂), 0.5 M adenosine monophosphate (AMP; pH 10.0), and 9 mM p-nitrophenyl phosphate (pNPP) was used to measure ALP activity. Absorbance was measured at 405 nm. All samples were run in triplicate (n = 3). Data are presented as mean ODs ± standard deviations, and statistical significance was considered at *p < 0.05.

### Alizarin Red-S staining

To evaluate the biomineralization potential of hPDLSCs, ARS staining was performed as described previously (Dutta et al., 2019). Briefly, hPDLSCs (4 × 10^4 cells/well) were seeded in 24-well plates in α-MEM containing 10% FBS and 1% antibiotics at 37 ± 1°C until reaching 50–60% confluence. The hPDLSCs were cultured in differentiation media with antibiotics at 37 ± 1°C until reaching 50%

### RNA isolation and real-time polymerase chain reaction (RT-qPCR) analysis

To study the gene expression in the presence of SAHA, hPDLSCs (1 × 10^6 cells/well) were cultured for two weeks in osteogenic induction medium. The RNeasy Mini Kit (Qiagen, Germany) was used to extract total RNA from the samples. RNA and cDNA sample purities and concentrations were determined spectrophotometrically (Infinite® M Nano 200 Pro; TECAN). Approximately, 2 μg of RNA was used to synthesize cDNA. RNA expression was quantified using RT-qPCR with an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The reaction conditions included 40 cycles of denaturation for 15 s at 95°C followed by amplification at 60°C. All reactions were run in triplicate (n = 3) and normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative mRNA expression levels of the control and SAHA-treated groups were calculated and compared using the ΔΔCT method. For real-time PCR, the specific primers for OPN, OCN, BSP, Coll, Runx2, ALP, and GAPDH, were synthesized as listed in Tab. 1.

### Statistical analysis

Statistical analysis was carried out by one-way ANOVA using Statistical Package for Social Science 22.0 software (SPSS 22.0, Inc., USA). All data shown are mean ± SD of triplicate (n = 3) experiments, and statistical significance was considered as *p < 0.05.

### Results

#### Characterization of hPDLSCs cells

Human PDLSCs were isolated from the third molar tooth of humans and cultured in α-MEM medium. After seven days of primary culture, fibroblast-like colonies were observed (Fig. 1A). Passage three (P₃) cells were used for the characterization study. Next, to investigate the immunophenotypic markers associated with hPDLSCs, we performed flow cytometry. FACS analysis showed that approximately 99.93% of hPDLSCs expressed CD13, 99.85% expressed CD90, 84.13% expressed CD146, and 10.71% expressed CD34 markers (Fig. 1B). The relative intensity of antibody binding to cells was quantified to determine the percentage of positive cells. CD34, preliminary recognized as a negative marker, represented primitive hematopoietic progenitors and endothelial cells. In contrast, CD13, CD90, and CD146 were putative positive markers of MSCs. These results indicate that hPDLSCs displayed morphological as well as phenotypic characteristics of MSCs.
To investigate the effects of SAHA on osteoblast differentiation in vitro, we performed Alizarin Red-S (ARS) and alkaline phosphatase (ALP) staining. The hPDLSCs were cultured in osteogenic differentiation medium and treated with or without SAHA (0 or 300 ng/mL) for two weeks. After two weeks, the plates were washed and stained with 40 mM ARS and ALP staining kit to evaluate mineralization efficiency. Interestingly, the SAHA-treated hPDLSCs showed more intense color after ALP (Fig. 4A) and ARS (Fig. 4B) staining compared to control samples. The ALP activity was significantly increased in SAHA-treated (300 ng/mL) cells compared to control. Furthermore, ARS staining showed a significant increase in calcium content in the presence of 300 ng/mL SAHA, as shown in Fig. 4C.

### Osteoblast-specific genes and proteins expression

To evaluate the gene and protein expression related to osteoblast differentiation, we conducted real-time PCR and western blotting. The expression levels of relative markers, including osteopontin (OPN), osteocalcin (OCN), collagen type I (Col1), alkaline phosphatase (ALP), bone sialoprotein (BSP), and runt-related transcription factor 2 (Runx2), were significantly increased after treatment with 300 ng/mL SAHA as shown in Fig. 5A. To explore the protein expression of SAHA-treated hPDLSCs, we evaluated expression levels by western blotting. We found that the expression of transcription factors, such as glioma-associated protein (GLI1), runt-related transcription factor-2 (RUNX2), and osterix (OSX), were significantly elevated by SAHA treatment as compared to the control group (Fig. 5B). GLI1 was known previously to induce early osteoblast differentiation in a RUNX2-dependent and independent manner in early osteoblast differentiation (Hojo et al., 2012).

### SAHA induces osteoblast differentiation via ERK/MAPK signaling pathway

To investigate the signaling mechanism related to osteoblast differentiation, we also evaluated the expression of ERK/MAPK signaling pathway-associated proteins, such as extracellular receptor kinase (ERK), phosphorylated ERK (p-ERK), mitogen-activated protein kinases (p-38 and p-p38), extracellular receptor kinase (ERK), and p38 mitogen-activated protein kinase (p38 MAPK). Western blotting results showed that the expression of p-ERK and p38 MAPK were significantly increased in SAHA-treated hPDLSCs as compared to control samples (Fig. 6A). The expression of p38 MAPK was significantly increased after treatment with 300 ng/mL SAHA (Fig. 6C).

### Table 1: Prime sequences used for real-time polymerase chain reaction (RT-qPCR)

| No. | Genes | Genbank No. | Sequences (5’-3’) | References |
|-----|--------|-------------|-------------------|------------|
| 1   | OPN    | J04765      | CCCACAGACCTCTCAAGTA ACACATATTACCTCGGAGC | Young et al., 1990 |
| 2   | OCN    | X53698      | GTGCCAGAATCTCAAGGAGGT TGCCCAGCTTCGCTCAAGTC | Kiefer et al., 1990 |
| 3   | Col1   | XM_012651   | CTTGACCTTCTCCGCTGATGTCC GTCTGGGGCACAACCTGTCC | NCBI Annotation Project, 2002 |
| 4   | ALP    | BC090861    | GGACATGCACTAGAGCTGA GCAGTGAAGGGCAGTCTGTC | Strausberg et al., 2002 |
| 5   | BSP    | NM_004967   | CAAAGCACAGAGGGGAGAGG CGTACTCCCCTCTGTATTTCA | Oldberg et al., 1988 |
| 6   | Runx2  | NM_00101505 | CGCATTTCTCTATCCCAATAT GACTGGCGGGGTTGTAAGTAA | Xu et al., 2019 |
| 7   | GAPDH  | NM_00525678 | ACCACAGTCCATGGCATCAC TCCACCCCGTGGTGGA | Mondragón et al., 2019 |

**Abbreviations:** OPN, osteopontin; OCN, osteocalcin; Col1, type 1 collagen; ALP, alkaline phosphatase; BSP, bone sialoprotein; Runx2, runt-related transcription factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### Tri-lineage differentiation potential of hPDLSCs

To observe the multilineage differentiation potential of hPDLSCs at P3, the cells were allowed to differentiate in osteogenic, chondrogenic, or adipogenic medium in vitro. After three weeks of osteogenic induction, hPDLSCs successfully differentiated into osteocytes. ARS staining showed an intense color due to the mineral deposition throughout the adhesion layer (Fig. 2A). Furthermore, the cells were also able to undergo adipogenic and chondrogenic differentiation after treatment with specific adipogenic and chondrogenic supplements. Alcian Blue and oil Red-O staining showed the synthesis of proteoglycans (Fig. 2B) and lipid droplets (Fig. 2C), respectively.

**SAHA treatment promotes hPDLSCs proliferation**

It was reported earlier that SAHA induced the proliferation of dental pulp stem cells (DPSCs) without altering their morphology (Duncan et al., 2012). The effect of SAHA on in vitro proliferation of hPDLSCs was evaluated by MTT assay, as indicated in Fig. 3. Human PDLSCs were seeded in 96-well plates and treated with different concentrations of SAHA (0, 100, 300, 500, 800, 1000, and 2000 ng/mL). After 24 h incubation, cell proliferation was not inhibited with increasing concentrations of SAHA (Fig. 3A). Notably, 2000 ng/mL SAHA still had no cytotoxicity to hPDLSCs as compared to the control samples after 48 h of incubation, as indicated in Fig. 3B. In particular, there was a significant increase in cell viability at 300 ng/mL concentration (*p < 0.05*) as compared to the control samples (*p < 0.05*) after 24 and 48 h. Thus, our results suggested that 300 ng/mL is the optimum SAHA concentration for hPDLSCs proliferation.
and c-Jun-N-terminal kinases (JNK and p-JNK). The relative expression of ERK was upregulated after treatment with 300 ng/mL SAHA. While a significant increase (*p < 0.05) of ERK was observed in the SAHA-treated group (Fig. 6), there was no significant increase in the expression of MAPK signaling pathway-associated protein, p-p38, as compared to the control samples. Our findings further demonstrate that SAHA enhances the osteogenic differentiation by the MAPK/ERK signaling pathway and that RUNX2, which is downstream of MAPK signaling, plays a crucial role in this process.

**Discussion**

HDAC inhibitors are promising drugs for the treatment of cancer as they have been shown to have antitumor activities, such as the ability to inhibit cell proliferation (Martinez-Iglesias et al., 2008). SAHA, a new class of HDAC inhibitors, has been identified recently as an effective anticancer drug. SAHA enhances gene expression by increasing the acetylation of histones and non-histone proteins. It was also reported that SAHA could modulate differentiation (Jeon et al., 2006). Kwon et al. (2012) reported that SAHA (5 µM) induced the differentiation potential of mouse dental papilla cells (MDPC23) in vitro. Moreover, they showed that SAHA increased the expression of DSPP during odontoblast differentiation. SAHA-induced growth, viability, apoptosis, and mineralization of dental pulp cells (DPCs) has also been reported (Duncan et al., 2015; Wang et al., 2014), which led us to hypothesize that SAHA induces the differentiation of
FIGURE 2. The tri-lineage differentiation potential of hPDLSCs. The cells were cultured in osteogenic, chondrogenic, and adipogenic medium for three weeks, respectively. Alizarin Red-S (A), Alcian blue (B), and Oil Red-O (C) staining showed the progress of differentiation (Magnification, 10×).

FIGURE 3. Cytotoxicity evaluation of hPDLSCs in the presence of SAHA at indicated time intervals. To investigate the effect of SAHA on hPDLSCs cell proliferation in vitro. The cells were treated with SAHA for 24 h (A) and 48 h (B). The proliferation value was represented as mean optical density (mean O.D.) of 595 nm. All data shown are mean ± SD of triplicate experiments (n = 3), statistical significance was considered as *p < 0.05.

FIGURE 4. The mineralization efficiency of hPDLSCs in the presence of SAHA; the hPDLSCs were cultured in osteogenic differentiation medium without or with SAHA (0 and 300 ng/mL). (A) ALP staining After 5 days of incubation (Magnification, 10×). (B) Alizarin Red-S staining and mineralization was performed after 1 and 2 weeks of incubation (Magnification x10). All data shown are mean ± SD of triplicate experiments (n = 3). Statistical significance was considered as *p < 0.05.
human periodontal ligament stem cells (hPDLSCs) in vitro. Consistent with our hypothesis, we demonstrated that SAHA stimulates the osteogenic differentiation of hPDLSCs by inducing their proliferation.

SAHA can promote the differentiation of hPDLSCs into osteoblasts and result in the formation of alveolar bone. Recently, “The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy,” proposed a criterion to define MSCs. MSCs must express markers such as CD10, CD13, CD29, CD44, CD59, CD90, and CD146, and lack expression of negative markers, such as CD14, CD34, CD45, HLA-DR. (Lei et al., 2014). Human PDLSCs isolated from the periodontal ligament of the third molars also exhibit fibroblast-like appearance upon confluence. Dental stem cells have also been shown to express some stemness markers such as Nanog, Oct4, and Sox2 by RT-qPCR. Our results suggest that hPDLSCs isolated from molar teeth are characteristic of hMSCs as they highly express positive markers such as CD10, CD13, CD90, and CD146 and do not express negative markers, such as CD34.

CD146 was initially recognized as an adhesion molecule in melanoma cells but is currently used as a marker for the identification of endothelial cell lineages (Sung et al., 2016). Notably, the presence of CD146 positive hPDLSCs showed significant proliferative and osteogenic potential (Zhu et al., 2013). Thus, MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro. Previously, it was reported that human PDLSCs contain a population of postnatal multipotent stem cells that can be isolated using explant cultures or enzymatic digestion and expanded in vitro (Choi et al., 2015; Kim et al., 2015; Osathanon et al., 2013; Saghiri et al., 2018; Seo et al., 2004; Lee et al., 2014; Zhao and Liu, 2014). Our results show that hPDLSCs differentiate successfully into osteocytes, chondrocytes, and adipocytes after in vitro culture in supplemented medium, as reported previously (Sun et al., 2016). These results indicate that hPDLSCs are the richest sources of stem cells for osteoinduction, and SAHA could enhance the differentiation potential of hPDLSCs. Further, our investigation showed that SAHA can increase ALP activity and calcium deposition in hPDLSCs, as noted in previous reports (Kwon et al., 2012). These results further elucidated the effect of SAHA on the differentiation of hPDLSCs.

In this study, SAHA showed significant effects on enhancing matrix mineralization and osteoblast-specific gene marker expression in hPDLSCs. Initially, SAHA was chosen for in vitro differentiation of hPDLSCs because it is clinically relevant and exhibits a stronger osteoinduction potential than other HDAC inhibitors. Osteogenic and odontogenic differentiation is related to a variety of bone-related markers, such as Runx2, Osterix, ALP, OCN, OPN, and Col1 (Fu et al., 2014; Ge et al., 2018; d’Alessandro et al., 2013; Jin et al., 2015). Previously, it was reported that HDAC inhibitors like SAHA stimulate osteoblast differentiation by increasing the acetylation of Runx2, a transcription factor critical for osteoblast differentiation and migration (Schroeder et al., 2004). Acetylation of Runx2 increases protein stability and up-regulates the expression of another transcription factor, Dpp. However, in mature odontoblasts, Runx2 was found to downregulate the expression of Dpp (Kwon et al., 2012). Notably, mutations in the Runx2 gene were found to inhibit the terminal differentiation of odontoblasts, indicating the role of Runx2 in osteoblast differentiation (Miyazaki et al., 2008). Kwon et al. (2012) reported that a 5 µM concentration of SAHA was sufficient to modulate the
expression of Runx2. They also showed that SAHA promotes the acetylation of histone H3. Another study reported that HDAC-8 regulates osteogenesis of hMSCs by regulating the level of H3K9Ac and Runx2 acetylation (Fu et al., 2014). In consideration of these previous studies, we investigated the effects of SAHA (300 ng/mL) on relative mRNA expression of OPN, OCN, Col1, BSP, ALP, and Runx2 in hPDLSCs. Notably, an increase in Runx2, OCN, BSP, OPN, and Col1 mRNA expression was noticed, further indicating that 300 ng/mL of SAHA is effective for osteoblast-specific gene marker expression. Similarly, it was reported that Valproic acid (VPA) and Trichostatin A (TSA) enhanced the expression of OCN and BSP in human DPSCs (Paino et al., 2013; Duncan et al., 2013). Furthermore, our results indicate that SAHA also upregulated the level GLI1, OSX, and Runx2 proteins. These data suggest that SAHA might regulate Runx2 and OSX expression during the differentiation of hPDLSCs. Runx2, core-binding factor 1 (CBFA1)/osteogenic specific factor 2 (OSF2), is a member of the Runt domain transcription factor family and is critical for embryo development. Runx2 binds to specific promoters and regulates the transcription of several genes (e.g., Oct4, Nanog) that play a crucial role in osteoclast formation, chondrocyte development, bone matrix formation, and subsequent bone mineralization (Lee et al., 2011b; Hu et al., 2015; Xia et al., 2011). Osterix is a zinc-finger-containing transcription factor encoded by the Sp7 gene that regulates gene expression in committed osteoblast precursor cells. Osterix is also a transcription factor essential for osteoblast differentiation, acting downstream of Runx2 (Shi et al., 2016). In addition to OSX, GLI1 is thought to play a crucial role in mediating transcriptional responses in early osteoblast differentiation (Hojo et al., 2012). During osteogenic differentiation, both Runx2 and Osterix, along with GLI1, regulate the expression of

![FIGURE 6. (A) Screening for the ERK/MAPK signaling associated proteins (JNK, p-JNK, p38, p-P38, ERK, and p-ERK) by western blot analysis. (B) Blots were quantified by ImageJ software and data was normalized to control (α-tubulin). All data shown are mean ± SD of triplicate experiments (n = 3). Statistical significance was considered as *p < 0.05.](image-url)
significant bone marker genes, including OPN, OCN, and type I collagen expressed by chondrocytes and osteoblasts.

In our present study, SAHA showed a significant increase in ERK/MAPK signaling-related protein expression that is critical for osteoblast-osteosteat development. Previous reports have shown that the mitogen-activated protein kinase (MAPK) signaling pathway plays an essential role in the regulation of osteogenic differentiation of BMSCs (Lee et al., 2011a; Ren et al., 2015; Xia et al., 2011; Iwasaki et al., 2013; He et al., 2016; Li et al., 2013; Yu et al., 2012; Jun et al., 2010). The MAPK family proteins, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (p38), have been reported to induce osteogenic differentiation (He et al., 2016; Yu et al., 2012). In particular, ERK1/2 is activated continuously during cell transformation, proliferation, and survival. Also, p38 is involved in a variety of cellular responses including cell cycle control, inflammatory responses, apoptosis, and the differentiation of several cell types, including osteoblasts (Ren et al., 2015; Li et al., 2013).

Interestingly, previous findings suggested that Runx2 is a downstream regulator of the ERK1/2 pathway. Phosphorylation of ERK1/2 induces the expression of Runx2 during osteoblast differentiation and skeletal development (Yin et al., 2015; Jeon et al., 2006; Schroeder et al., 2004; Feng et al., 2009). In conjunction with that research, our study revealed that the phosphorylation levels of ERK and the activation of Runx2 were upregulated in the SAHA treated groups. However, the expression of p-JNK and p-p38 did not increase in our study. Collectively, our data strongly suggests that SAHA promotes the phosphorylation of ERK1/2, which in turn upregulated the transcriptional activity of Runx2, leading to the osteogenesis in hPDLSCs.

Our study was limited in that we used the periodontal ligament stem cells instead of other dental cells, such as dental pulp stem cells or apical papillary cells. Although we have investigated the role of SAHA on hPDLSC differentiation, it is also critical to investigate the effects of SAHA on chondrogenesis, neurogenesis, and adipogenesis. Data from this study demonstrates that SAHA enhances hPDLSC differentiation by regulating the expression of Runx2, OSX, and other transcription factors via the ERK/MAPK signaling pathway. However, to investigate the therapeutic potential of SAHA, further studies on other types of dental cells are required.

Conclusions

This study investigated the role of SAHA on the in vitro differentiation of human periodontal ligament stem cells (hPDLSCs). We investigated the cytotoxicity, in vitro differentiation (Alizarin Red-S and alkaline phosphatase), RT-qPCR, and western blotting as direct indicators of osteogenesis. Here we present an outline of SAHA-induced hPDLSC differentiation based on preliminary findings. The cell viability of SAHA-treated cells (300 nM) was increased by 20% (p < 0.05) as compared to the control groups (p < 0.05) after 48 h incubation. As a result, both cell viability and proliferation were increased significantly at 24 and 48 h. Gene expression is directly related to functional protein synthesis.

Osteoblast-specific gene expression studies revealed that the expression of Runx2, ALP, OCN, and COL1 was significantly increased (p < 0.05) in the SAHA-treated cells after two weeks in culture. These results were also validated by Western blot analysis. The MAPK/ERK1/2 family protein expression was also studied to understand the role of MAPK signaling during osteoblast differentiation. Interestingly, ERK and p-JNK expression were significantly increased by 12.8% (p < 0.05) and 8.7% (p < 0.05), followed by a 24% increase in Runx2 expression (p < 0.05), respectively. These results suggest that ERK might be a positive regulator of Runx2 transcription factors. In conclusion, the present findings suggest that SAHA treatment (300 ng/mL) can stimulate osteoblast maturation by the MAPK/ERK1/2 signaling pathway.

Author Contributions: X. Y. Z., S. D. D., J. B., and L. M. M. contributed to the data acquisition, analysis, and writing of the manuscript; J. B., X. Y. Z., and J. Z. H. designed the study, and S. D. D. revised the manuscript. All the authors gave final approval and agreed to be accountable for all aspects of the work.

Funding Statement: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) and funded by the Ministry of Education (No. 2018R1A6A1A03025582) and the National Research Foundation of Korea (NRF- 2019R1D1A3A0310828). The authors are also thankful to the Yanbian University Youth Project of China (No. 201804).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

Alvarez R, Lee HL, Wang CY, Hong C (2015). Characterization of the osteogenic potential of mesenchymal stem cells from human periodontal ligament based on cell surface markers. International Journal of Oral Science 7: 213–219. DOI 10.1038/ijos.2015.42.

Choi JK, Hwang HI, Jang YJ (2015). The efficiency of the in vitro osteo/dentinogenic differentiation of human dental pulp cells, periodontal ligament cells and gingival fibroblasts. International Journal of Molecular Medicine 35: 161–168. DOI 10.3892/immm.2014.1986.

d’Alessandro L, Petriti M, Ferrante M, Di Marco S, Trubiani O, Spoto G (2013). Cyclic nucleotide phosphodiesterase activity in stem cells of human periodontal ligament (PDL-MSCs) before and after osteogenic induction. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology 116: e317–e323. DOI 10.1016/j.oooo.2012.02.023.

Dudakov A, Evans JM, Li Y, Middha S, McGee-Lawrence ME, Wijnen AJV, Westendorf JJ (2013). Histone deacetylase inhibition promotes osteoblast maturation by altering the histone H4 epigenome and reduces Akt phosphorylation. Journal of Biological Chemistry 288: 28783–28791. DOI 10.1074/jbc.M113.489732.

Duncan HF, Smith AJ, Fleming GJ, Cooper PR (2012). Epigenetic modulation of dental pulp stem cells: implications for...
regenerative endodontics. International Endodontic Journal 49: 431–446.

Duncan HF, Smith AJ, Fleming GP, Partridge NC, Shimizu E, Moran GP, Cooper PR (2015). The histone-deacetylase-inhibitor suberoylanilide hydroxamic acid promotes dental pulp repair mechanisms through modulation of matrix metalloproteinase-13 activity. Journal of Cellular Physiology 2015: 798–816.

Duncan HF, Smith AJ, Fleming GP, Cooper PR (2013). Histone deacetylase inhibitors epigenetically promote reparative events in primary dental pulp cells. Experimental Cell Research 10: 1534–1543. DOI 10.1016/j.yexcr.2013.02.022.

Dutta SD, Patel DK, Seo YR, Park CW, Lee SH, Kim JW, Kim J, Hu N, Feng C, Jiang Y, Miao Q, Liu H (2015). Regulative effect of histone deacetylase inhibitors-1 accelerates odontoblastic differentiation of human stem cells from apical papilla. Tissue Engineering Part A 22: 721–732. DOI 10.1089/ten.tea.2015.0273.

Jin H, Chong HW, Lim KT, Jin B, Jin C, Chung JH, Chong PH (2015). Recombinant human plasmogen activator-inhibitor-1 promotes cementogenic differentiation of human periodontal ligament stem cells. Tissue Engineering Part A 21: 2817–2828. DOI 10.1089/ten.tea.2014.0399.

Jun JH, Yoon WJ, Seo SR, Woo KM, Kim GS, Ryoo HM, Baek JH (2010). BMP2-activated Erk/MAP kinase stabilizes Runx2 by increasing p300 levels and histone acetyltransferase activity. Journal of Biological Chemistry 285: 36410–36419. DOI 10.1074/jbc.M110.142307.

Kim SY, An SY, Lee JS, Heo JS (2015). Zanthoxylium schinifolium enhances the osteogenic potential of periodontal ligament stem cells. In Vitro Cellular & Developmental Biology-Animal 51: 165–173.

Kiefer MC, Saphire AC, Bauer DM, Barr PJ (1990). The cDNA and derived amino acid sequences of human and bovine bone Gla protein. Nucleic Acids Research 18: 1909.

Kouraklis G, Theoharisi S (2006). Histone deacetylase inhibitors: a novel target of anticancer therapy. Oncology Reports 15: 489–494.

Kwon A, Park HJ, Baek K, Lee HL, Park JC, Woo KM, Ryoo HM, Baek JH (2012). Suberoylanilide hydroxamic acid enhances odontoblast differentiation. Journal of Dental Research 91: 506–512. DOI 10.1177/002203451243367.

Larsson L, Castilho RM, Giannobile WV (2015). Epigenetics and its role in periodontal diseases: a state-of-the-art review. Journal of Periodontology 86: 556–568. DOI 10.1002/jop.2014.04559.

Lee CH, Huang YL, Liao JF, Chiou W (2011a). Ugonin K promotes osteoblastic differentiation and mineralization by activation of p38 MAPK- and ERK-mediated expression of Runx2 and osterix. European Journal of Pharmacology 688: 383–389. DOI 10.1016/j.ejphar.2011.06.059.

Lee JH, Lee DS, Chong HW, Shon WJ, Seo BM, Lee EH, Cho JY, Park JC (2011b). Odontogenic differentiation of human dental pulp stem cells induced by preeameloblast-derived factors. Biomaterials 32: 9706–9706. DOI 10.1016/j.biomaterials.2011.09.007.

Lee JS, Yi JK, An SY, Heo JS (2014). Increased osteogenic differentiation of periodontal ligament stem cells on polydopamine film occurs via activation of integrin and PI3K signaling pathways. Cellular Physiology and Biochemistry 34: 1824–1834. DOI 10.1159/000366381.

Lee ZH, Kim HJ, Ryoo HM (2015). A novel osteogenic activity of suberoylanilide hydroxamic acid is synergized by BMP-2. Journal of Bone Metabolism 22: 51–56. DOI 10.11005/jbm.2015.22.2.51.

Lei M, Li K, Li B, Gao LN, Chen FM, Jin Y (2014). Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation. Biomaterials 35: 6332–6343. DOI 10.1016/j.biomaterials.2014.04.071.

Li L, Han M, Li S, Wang L, Xu Y (2013). Cyclic tensile stress during physiological occlusal force enhances osteogenic differentiation of human periodontal ligament cells via ERK1/2-Elk1 MAPK pathway. DNA and Cell Biology 32: 488–497. DOI 10.1089/dna.2013.2070.
SAHA-INDUCED OSTEOGENESIS IN DENTAL STEM CELLS

Sato Y, Ishida S, Mootooka H, Takayama K, Tomita Y, Maeda H, Satoh (2001). Decreased bone mass and increased bone turnover with valproate therapy in adults with epilepsy. Neurology 57: 1–3. DOI 10.1212/WNL.57.1.1.

Schroeder TM, Kahler RA, Li X, Westerdorf JJ (2004). Histone deacetylase 3 interacts with Runx2 to repress the Osteocalcin promoter and regulate osteoblast differentiation. Journal of Biological Chemistry 279: 41998–42007. DOI 10.1074/jbc.M403702200.

Saghir MA, Asatourian A, Sorenson CM, Shibli N (2018). Mice dental pulp and periodontal ligament endothelial cells exhibit different proangiogenic properties. Tissue and Cell 50: 31–36. DOI 10.1016/j.tice.2017.11.004.
Yin L, Cheng W, Qin Z, Yu H, Yu Z, Zhong M, Sun K, Zhang W (2015). Effects of naringin on proliferation and osteogenic differentiation of human periodontal ligament stem cells in vitro and in vivo. Stem Cells International 2015:1–9. DOI 10.1155/2015/758706.

Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW (1990). cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics 7:491–502.

Yu Y, Mu J, Fan Z, Lei G, Yan M, Wang S, Tang C, Wang Z, Yu J, Zhang G (2012). Insulin-like growth factor 1 enhances the proliferation and osteogenic differentiation of human periodontal ligament stem cells via ERK and JNK MAPK pathways. Histochemistry and Cell Biology 137: 513–525. DOI 10.1007/s00418-011-0908-x.

Zhao BJ, Liu YH (2014). Simvastatin induces the osteogenic differentiation of human periodontal ligament stem cells. Fundamental & Clinical Pharmacology 28: 583–592. DOI 10.1111/fcp.12050.

Zhu W, Tan Y, Qiu Q, Li X, Huang Z, Fu Y, Liang M (2013). Comparison of the properties of human CD146+ and CD146−periodontal ligament cells in response to stimulation with tumour necrosis factor α. Archives of Oral Biology 58: 1791–1803. DOI 10.1016/j.archoralbio.2013.09.012.