HISTONE DEACETYLASE INHIBITOR INDUCES THE ODONTOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS: COMPARISON OF TRICHOSTATIN A (TSA) AND SUBEROYLANILIDE HYDROXAMIC ACID (SAHA)

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Oral Biology
Common Course
FACULTY OF DENTISTRY
Chulalongkorn University
Academic Year 2020

สารยับยั้งเอนไซม์ฮีสโตนดีอะซิทิลเลสกระตุ้นการพัฒนาเนื้อเยื่อฟันจากเซลล์โพรงประสาทฟัน: เปรียบเทียบระหว่างไตรโคสแตตินเอ (ทีเอสเอ) และซับอิรอยลานิไลด์ ไฮดรอซามิคแอซิด (เอสเอเอชเอ)

นางอินดรานี สุลิสโยวาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีววิทยาช่องปาก ไม่สังกัดภาควิชา/เทียบเท่า
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563

Thesis Title	HISTONE DEACETYLASE INHIBITOR INDUCES THE ODONTOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS: COMPARISON OF TRICHOSTATIN A (TSA) AND SUBEROYLANILIDE HYDROXAMIC ACID (SAHA)
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Field of Study	Oral Biology
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อินดรานี สุลิสโยวาติ : สารยับยั้งเอนไซม์ฮีสโตนดีอะเซทิลเลสกระตุ้นการพัฒนาเนื้อเยื่อฟันจากเซลล์โพรงประสาทฟัน: เปรียบเทียบระหว่างไตรโคสแตตินเอ (ทีเอสเอ) และซับอิรอยลานิไลด์ ไฮดรอซามิคแอซิด (เอสเอเอชเอ). (HISTONE DEACETYLASE INHIBITOR INDUCES THE ODONTOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS: COMPARISON OF TRICHOSTATIN A (TSA) AND SUBEROYLANILIDE HYDROXAMIC ACID (SAHA)) อ.ที่ปรึกษาหลัก : รศ.ทญ. ดร.รัชนี อัมพรอร่ามเวทย์, อ.ที่ปรึกษาร่วม : รศ. ทญ. ดร.ชลิดา ลิ้มจีระจรัส

เซลล์เนื้อเยื่อโพรงประสาทฟันมนุษย์ ได้รับการพิสูจน์แล้วว่ามีความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์ชนิดอื่นๆ ในกลุ่มของเซลล์กระดูกได้ เมื่อได้รับการกระตุ้นโดยสารกลุ่มที่มีฤทธิ์ยับยั้งเอนไซม์ฮีสโทนดีแอเซทิลเลส ได้แก่ ไตรโคสแตตินเอ (TSA) และซับอีรอดย์ลานิไลน์ ไฮดรอกซามิกแอซิด (SAHA) อย่างไรก็ตาม ยังไม่มีการศึกษาเปรียบเทียบคุณสมบัติการกระตุ้นให้เซลล์เกิดกระบวนการแปรสภาพไปเป็นเซลล์สร้างเนื้อฟันของสารทั้งสองชนิด การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อพิสูจน์และเปรียบเทียบคุณสมบัติการกระตุ้นให้เซลล์เกิดกระบวนการแปรสภาพไปเป็นเซลล์สร้างเนื้อฟันและการสะสมแร่ธาตุของไตรโคสแตตินเอ และซับอีรอดย์ลานิไลน์ ไฮดรอกซามิกแอซิดในความเข้มข้นที่แตกต่างกัน ที่มีต่อเซลล์เนื้อเยื่อโพรงประสาทฟันมนุษย์ โดยกระบวนการทดสอบประกอบไปด้วยการทดสอบความเป็นพิษต่อเซลล์ด้วยเทคนิค MTT การตรวจวัดระดับการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทส การย้อมสีแร่ธาตุด้วยสีย้อม alizarin red การวัดระดับการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างเนื้อฟันด้วยวิธี RT-qPCR และวัดการเคลื่อนที่ของเซลล์ด้วยวิธี Scratch wound healing assay ผลการศึกษาพบว่าสารทั้งสองชนิดไม่มีความเป็นพิษต่อเซลล์เนื้อเยื่อโพรงประสาทฟันมนุษย์ และมีคุณสมบัติในการกระตุ้นให้เซลล์เกิดกระบวนการแปรสภาพไปเป็นเซลล์สร้างเนื้อฟัน โดยพบว่ามีการเพิ่มขึ้นอย่างมีนัยสำคัญของระดับการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทสและการตกตะกอนแร่ธาตุเมื่อทำการเพาะเลี้ยงเซลล์ร่วมกับไตรโคสแตตินเอความเข้มข้น 400 นาโนโมลาร์ หรือซับอีรอดย์ลานิไลน์ ไฮดรอกซามิกแอซิดความเข้มข้น 1 ไมโครโมลาร์ รวมถึงมีการเพิ่มขึ้นอย่างมีนัยสำคัญของยีน NFI-C, KLF4, DMP1, DSPP, COL1, ALP, BSP, OC, VEGF และ p21 ที่ระยะเวลา 1, 3, 5 และ 7 วัน และยังสามารถกระตุ้นการเคลื่อนที่ของเซลล์เนื้อเยื่อโพรงประสาทฟันมนุษย์เมื่อทำการเพาะเลี้ยงเป็นระยะเวลา 3 วันได้อีกด้วย จากผลการศึกษาสรุปได้ว่า สารไตรโคสแตตินเอ และซับอีรอดย์ลานิไลน์ ไฮดรอกซามิกแอซิดมีคุณสมบัติที่คล้ายคลึงกัน และสามารถกระตุ้นเซลล์เนื้อเยื่อโพรงประสาทฟันมนุษย์ ให้เกิดกระบวนการแปรสภาพไปเป็นเซลล์สร้างเนื้อฟันและการสะสมแร่ธาตุได้ สารทั้งสองชนิดมีความเหมาะสมสำหรับประยุกต์ใช้ในการกระตุ้นการสร้างใหม่ของเนื้อฟัน.

สาขาวิชา	ชีววิทยาช่องปาก	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6075846732 : MAJOR ORAL BIOLOGY
KEYWORD: TSA, SAHA, odontoblast differentiation, human dental pulp cells

Indrani Sulistyowati : HISTONE DEACETYLASE INHIBITOR INDUCES THE ODONTOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS: COMPARISON OF TRICHOSTATIN A (TSA) AND SUBEROYLANILIDE HYDROXAMIC ACID (SAHA). Advisor: Assoc. Prof. RUCHANEE AMPORNARAMVETH, D.D.S., Ph.D Co-advisor: Assoc. Prof. Chalida Limjeerajarus, D.D.S., Ph.D

Human dental pulp cells (hDPCs) have shown their plasticity to differentiate into odontoblast-like cell lineages under the treatment of two-members of hydroxamates HDAC inhibitors (HDACis), Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA). However, a comparison of the potency for stimulating odontoblast-like differentiation and mineralization process among these two HDACis has not been reported. Therefore, we aimed to confirm and compare the stimulatory effect of TSA and SAHA in inducing odontoblast-like differentiation and promoting mineralized-nodule formation. The primary cultured hDPCs was used for MTT assay, ALP activity assay, and alizarin red staining in the presence and absence of TSA and SAHA, with various concentration. Odontoblast-related gene expression was observed by RT-qPCR. Scratch wound healing assay was performed to observe the TSA and SAHA effects on cell migration. Either TSA or SAHA treatment have no effect on hDPCs viability as observed by MTT assay. The presence of TSA and SAHA induced odontoblast-like differentiation, confirmed by; the significant increase of ALP activity and mineral deposition during TSA 400 nM or SAHA 1μM treatment, the significant acceleration pattern of NFI-C, KLF4, DMP1, DSPP, COL1, ALP, BSP, OC, VEGF, and p21 gene expressions analyzed by RT-qPCR, at 24h, 72h, 7d and 5d. Furthermore, Scratch wound healing assay displayed the enhanced cell migration at 72h after TSA or SAHA treatment. Our findings showed that TSA and SAHA might have similar stimulatory effect in inducing odontogenic differentiation and mineralization process of hDPCs and proposed another potential application of TSA and SAHA to promote dentin regeneration.

Field of Study:	Oral Biology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious and The Most Merciful. Alhamdulillah. The author is grateful to Allah (subhana wa ta'ala) for bestowing her with wellness, blessing, and understanding to accomplish this thesis.

The author also wishes to express her sincere gratitude and warm appreciation to the following persons who had much contributed in supporting and helping her in the completion of this valuable piece of work and achievement of master's degree.

Prof. Prasit Pavasant, DDS., Ph.D., head of department, for his word of encouragement and for giving the author a long period of time to accomplish this study.

Assoc. Prof. Ruchanee Ampornaramveth, DDS., Ph.D., thesis adviser, for always giving support, guidance, valuable suggestions and improvements that are helpful to her in the completion and success of this study.

Assoc. Prof. Chalida Limjeerajarus, DDS., Ph.D., thesis co-adviser, for providing valuable support, advices, and suggestions to improve this study.

Asst. Prof.Weerachai Singhatanadgit, D.D.S., Ph.D., chairman of the committee, and Assoc. Prof. Neeracha Sanchavanakit, DDS., Ph.D., committee member, who had provided valuable information and knowledge in their own field and shared their ideas in the way of improvement this study.

Dr. Teerawat Sukpaita, doctoral student, who had shared his skill and provided a guidance in the way of performing the research as part of this thesis.

To her teachers in Oral Biology Program, for their worthy support, help, cooperation, and time in the term of providing the authors all needed information.

In addition, the author thank the worthy support, help, and cooperation provided by laboratory members, the technical and support staff in the Department of Oral and Maxillofacial Surgery, Department of Microbiology, and Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University.

To all who are not mentioned but provided support and help in the completion of this study, thank you very much.

Indrani Sulistyowati

TABLE OF CONTENTS

Page

List of tables

Page

List of figures

Page

INTRODUCTION

The breakdown of dentin layer integrity triggered by a deep dental cavity may lead to dental pulp inflammation (pulpitis) (1) and the pulp-capping material application may be required to stimulate reparative dentin formation as a dental repairing system and protect pulp tissue from further inflammation. (2) Importantly, the quality of reparative dentin structure is highly influenced by the signaling molecules contained in the material applied. The discontinuities and absence of tubular structure in reparative dentin mediated by the conventional pulp-capping material (3-12) can reduce dentin permeability and is incapable of providing long-term protection of the pulp tissue in later life. (2, 13)

Dental pulp cells exhibited their ability to differentiate into odontoblast-like cells and generate reparative dentin formation in response to the damaged dentin. This potency indicates that pulp tissue contains odontogenic progenitor or stem cells which can differentiate into multiple cell lineages including odontoblast, osteoblast, and adipocyte. (14, 15) Nevertheless, every cell in an individual carries the same genetic information encoded in the chromosome, so the presence of a specific signaling molecule is essential to control the cell fate decision, proliferation, and differentiation involved in reparative dentin formation. (14-18)

Epigenetic events such as histone modification (19) is one of the ways to turn ‘on’ or ‘off’ the gene expression through modifying the chromosome structure without changing the DNA sequences. (20) This event takes control of directing ‘the destination’ lineage of cells (15, 18) and involves two enzymes; histone acetyltransferases (HATs) and histone deacetylases (HDACs) generating histone acetylation and deacetylation, respectively. (19, 20)

The acetylation by HATs is associated with gene activation through nucleosome relaxation. While, the deacetylation by HDACs is associated with gene repression through nucleosome compaction. (19-21) Eighteen members of HDACs have been found in humans and divided into four classes depending on their structural similarities (21-23) in which class I HDAC (HDAC1, 2, and 3) and class II HDAC (HDAC4, 5, and 9) have been expressed in dental pulp tissue (24).

Nowadays, Trichostatin A (TSA) and Suberoylanilide Hydroxamic Acid (SAHA or Vorinostat), hydroxamate acid HDAC inhibitors (HDACis) members (20, 23, 25), are the most studied HDACis in the field of dentinogenesis-induction but, only SAHA has an approval from the United States Food and Drug Administration (FDA) for clinical use in the treatment of cutaneous t-cell lymphoma (CTCL) (20, 25). Both are involved in changing the balance between HATs and HDACs by blocking the active site of HDACs. This blockade promotes the relaxation of nucleosome structure and facilitates the binding of transcriptional machinery proteins to target genes resulting in gene activation. (23, 26)

Huang et al. (27) has reported the direct involvement of TSA in the TGF-β pathway playing an important role in inducing dentinogenesis at the early stage of odontoblast-like cell differentiation through up-regulating the expression of odontoblast differentiation-related gene markers including the nuclear factor I-C (NFI-C), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), and osteocalcin (OC). TSA also enhances ALP activity and mineralization in a dose-dependent manner. (28) While, SAHA treatment in MDPC23 odontoblast-like cell line demonstrated the enhancement of odontoblast differentiation through enhanced DSPP gene expression mediated by the

increased NFI-C expression in mRNA and protein level which subsequently, induced mineralization in a dose-dependent manner. (26) These findings indicated that TSA and SAHA might induce odontoblast differentiation and promote mineralization through enhanced dentin extracellular matrix production in pulp-derived stem cells (28) and transformed cell lines (26, 27, 29). However, a comparison of the stimulatory effects on dentinogenesis-induction between TSA and SAHA has not been reported in primary cells of dental pulp.

This present study aims to investigate and compare the stimulatory effect of TSA and SAHA in inducing odontoblast-like differentiation of human dental pulp cells (hDPCs) and mineralized nodule formation, through the involvement of odontoblast differentiation-related genes. Therefore, this study may provide valuable information for the initial development of a biocompatible material to induce dentin regeneration.

LITERATURE REVIEW

The discovery in 1940 – 1953 revealed that DNA (deoxyribonucleic acid) molecules is a carrier of hereditary information which encodes all the instruction needed for regulating cellular activity. Each eucaryotic cell contains double-stranded DNA in its nucleus which has only 5 – 8 μm in diameter, yet double-stranded DNA is about 2 m in length. (30) To tuck this very long DNA in the nucleus, the specialized protein called the histone octamer and histone-like protein (H1) is responsible for DNA packaging, which organizes the DNA into several coil and loop formations and avoids it from becoming unruly creases. (19, 30)

Because double-stranded DNA and histone proteins are differently charged, DNA packaging occurs. The negatively charged phosphate in DNA's backbone allows DNA to bind to and interact with the positively charged histone octamer. When DNA is folded, the H1 protein tightly holds them together and forms the nucleosome structure. (30) The histone octamer is composed of two molecules of each H2A, H2B, H3, and H4 protein, and each histone protein, except H1, contains histone tails (called N-terminal tails) that protrude from its core and provide approximately 30 amino acid residues that are essential for nucleosome interaction. (17, 19, 30)

Double-stranded DNA in the compact structure of the nucleosome prevents DNA from interacting with other enzymes and proteins which generate the cellular genetic activity including DNA replication, DNA repair, and transcriptional activity. Therefore, N-terminal tails play a role as signaling mediator to manipulate the accessibility of DNA strands which are in nucleosome form. Through the modification of N-terminal tails such as acetylation, methylation, phosphorylation, ubiquitination, glycosylation, and ADP-ribosylation. (19) These

lead to the opening and closing of the nucleosome. The relaxation of nucleosome structure by histone modification facilitates the accessibility for the transcriptional machinery to bind with DNA strand and leads to the upregulation of gene expression. In contrast, nucleosome compaction inhibits the binding of transcriptional machinery by DNA strands, resulting in a decrease in gene expression. Therefore, this event may regulate the change of gene expression. (19, 30, 31)

Epigenetic is interpreted as the event of the alternation of gene expression through modifying the nucleosome structure and DNA strand accessibility, without changing the DNA sequences, and histone modification is one of the ways to this event. Another mechanisms involved in regulating the accessibility and alternation of nucleosome structure is DNA methylation, the presence of non-coding microRNAs and chromatin remodeling which act as the switch in gene expression. (20, 30) As a result, epigenetic influences cell fate commitment and differentiation by activating and repressing gene expression involved in cell lineage decision-making as described in Waddington's Landscape. (17, 30, 32, 33) The Prevoius study (34) demonstrated the involvement of epigenetic change in inducing mesenchymal stem cells (MSCs) differentiation into chondrocyte lineage through the increase of chondrogenesis-related gene markers and the decrease of MSC gene markers.

Environmental factors such as stress, lifestyle, smoking, microbial infection, and disease stage can also trigger epigenetic events. (17, 32) In normal situations, this event occurs throughout life in balance to organize the gene transcription needed for cell development, differentiation, and maintenance. (17, 20) However, it can turn to devastating effects in response to pathological conditions and lead to inflammation and oncogenesis.

(17, 30) According to a review by Lavu et al. (35), epigenetic may contribute to the pathogenesis of periodontal disease through DNA methylation in cytokine genes and may be modulated by the presence of bacteria in gingival epithelia. This issue may indicate that epigenetic changes can also be influenced by therapeutic agents.

Recent advances in therapeutic strategy, several inhibitor enzymes are available with United States Food and Drug Administration (FDA) approval such as suberoylanilide hydroxamic acid (SAHA) and romidepsin inhibiting histone deacetylation and 5-azacytidine targeting DNA methylation and these have been used as anticancer drugs in clinical application. (20, 23)

3.1 Epigenetic and Histone Acetylation

Epigenetic is the event referring to the alternation of nucleosome structure which results in activating and repressing gene expression without changing the DNA sequences. As mentioned above, the alternation of nucleosome structure can be modulated by various post-translational histone modification to N-terminal tails including acetylation, methylation, ubiquitination, phosphorylation, and poly (ADP)-ribosylation. Histone modification by acetylation is one of the most clearly understood mechanisms in transcriptional regulation which involves two enzymes such as histone acetyl transferases (HATs) and histone deacetylases (HDACs) controlling histone acetylation and deacetylation, respectively. (19, 20, 23)

3.1.1 Histone Acetyltransferases (HATs)

Histone acetyl transferases (HATs) are able to transfer the acetyl group from acetyl coenzyme A (17) to lysine residues of N-terminal tails of H3 and H4. (15, 31) This action switches the positively charged of histone core into the negatively charged, which leads to the relaxation of DNA strands and mediates the activation of transcriptional activity. Hence, HATs act as transcriptional co-activator (36) and relate to the increase of gene expression by allowing the binding of transcriptional machinery proteins (such as RNA polymerase II, proteins which are associated and giving positive and negative effects on transcriptional activity) to the promoter area of target genes. (15, 23, 25, 37) Several HATs have been discovered, such as PCAF, p300/CREB-binding protein, GCN5, TAFII250, ACTR, and SRC-1. (19, 23) Among them, p300/CREB-binding protein and PCAF/GCN5 are the most studied HATs during tissue development.

The involvement of p300 and PCAF has been observed in mediating the activated TGF-β pathway. TGF-β plays an important role in growth arrest of normal and cancer cells. (27) In vivo experiment, TGF-β1 ligand promotes the maturation stage of enamel development and induces odontoblast differentiation and dentin mineralization. (38) To initiate the TGF-β pathway, two receptors, TGF-β type I (TβRI) and type II (TβRII) receptors embedded in the cell surface were involved in the binding of TGF-β ligand. In brief, ligand binding to receptors activates the TGF- pathway, which then recruits and collaborates with the Smad pathway to promote cell growth arrest and cell differentiation (14) by translocating these complexes into the nucleus. Recently, the interaction of HDAC1 and p300 in the promoter area of TβRII regulates its expression.

Deacetylation of H3 and H4 in the TβRII promoter down-regulates its transcription, resulting in TGF-βRII repression. Whereas, acetylation in TGF-βRII promoter through the p300 and PCAF cooperation could induce the increase of its transcription and modulate the TGF-β pathway. (27)

p300 plays an important role in regulating gene expression, which controls several cellular activities, including cell fate decision and differentiation in human dental pulp cells (HDPCs). In vitro experiment, p300 maintains the pluripotency of HDPCs by up-regulating the transcriptional activity of NANOG and SOX2, the pluripotency gene marker when HDPCs were seeded in the non-inductive medium. The expression of odontoblastic marker genes such as DMP-1, DSPP, OPN, and OCN was reduced by p300 over-expression. In contrast, when HDPCs were seeded in inductive medium, the expression of odontoblast marker genes was significantly increased in mRNA and protein level. p300 was recruited to enhance the acetylation of lysine 9 residue of H3 (H3K9Ac) in the promoter area of DSPP and OC genes, resulting in the transcription of those genes. The high level of ALP activity has been seen on p300-overexpressed HDPCs cultured in inductive medium. Corresponding to this result, the over-expressed p300 elevates the formation of mineralized nodules, indicating that p300 can enhance odontoblast differentiation and mediate the secretion of dentin extracellular matrix, inducing dentin formation. (39)

3.1.2 Histone Deacetylases (HDACs)

While HATs induce more ‘open’ nucleosome structure associated with gene activation, histone deacetylases (HDACs) are associated with gene repressor. (19) It removes the acetyl group from lysine residues of N-terminal tails and generates the tightly condensation of nucleosome structure. Deacetylation inhibits the binding of transcriptional machinery proteins to DNA strands and leads to the repression of gene expression. (22, 31) In humans, there are eighteen members of HDACs divided into four classes depending on their structural similarities and two families, depending on their dependency, $Zn^{2+,}$ and NAD+-dependent. (31, 40) Class I (such as HDAC1, 2, 3, and 8), class IIA (such as HDAC4, 5, 7, and 9), class IIB (such as HDAC6 and 10) and class IV HDACs (such as HDAC11) are included on the the Zn^{2+}-dependent subfamily which can be inhibited by HDAC inhibitors (HDACis) composed of zinc binding structure such as Trichostatin A (TSA) and vorinostat [suberoylanilide hydroxamic acid (SAHA)]. Class III HDACs, on the other hand, such as sirtuins, are NAD+-dependent HDACs that require the coenzyme nicotinamide adenine dinucleotide (NAD+) for function and are not affected by HDACis. (20, 41)

Different expression levels of several members of class I HDAC (such as HDAC1, 2, and 3) and class II HDAC (such as HDAC4, 5, and 9) have been seen in dental pulp tissue. In the extracted third molar teeth, HDAC2 and 9 were strongly expressed within mature odontoblast nuclei. HDAC1 and 3 were weakly expressed in the cell bodies of mature odontoblasts. Whereas HDAC4 was moderately expressed in some odontoblast and dental pulp. (24)

Furthermore, the involvement of histone modification via HDAC3 and p300 has been demonstrated during odontoblast differentiation in mice dental pulp cells (mDPCs). Both were recruited by KFL4 transcription factors, resulting in different effects on cell fate decision of odontoblasts. The recruitment of HDAC3 to KFL4 is associated with the repression of the target gene's expression. In contrast, the interaction between HDAC3 and KFL4 was significantly decreased in mDPCs during odontoblastic induction. While the recruitment of p300 to KFL4 in the DMP1 promoter area occurred, this mechanism promoted the odontoblast differentiation. (42) The balance of HATs and HDACs expression regulates the alternation of nucleosome structure and the transcription process and arranges several cellular activities including cell cycle, DNA repair, hormone signaling, and cell fate decision.(15, 39, 40) Whereas the imbalance between HATs and HDACs expression is associated with several diseases, metabolic disorders, and cancer. (15, 23)

3.2 HDAC Inhibitors (HDACis)

HDAC inhibitors (HDACis), the epigenetics-modifying agents (41), change the balance between HATs and HDACs to more acetylase lysine residue by blocking the active site of HDACs and resulting in the gene activation through facilitated the binding of transcriptional machinery proteins to target gene. (26, 31) The increased gene expression affects several cellular activities such as inhibiting cell proliferation, inducing cell death (43), enhancing cell differentiation, and controlling cell fate decision. (26, 40) HDACis are now used in cancer

treatment due to their ability to reduce inflammation (40) and angiogenesis, reactivate the silenced tumor suppressor gene, up-regulate the DNA repair gene, and modulate immune response. (23, 25, 40)

HDACis have been categorized into five groups depending on their chemical structure, such as hydroxamic acids (hydroxamates), short chain fatty (aliphatic) acids, benzamides, cyclic tetrapeptides, and sirtuin inhibitors. (23) Trichostatin A (TSA) has been discovered as the first natural hydroxamate for inhibiting HDAC activity. Suberoylanilide hydroxamic acid (SAHA or vorinostat), another member of hydroxamates, has a similar structure to TSA and is the first HDACis which has been approved by the United States Food and Drug Administration (FDA) to treat Cutaneous T-Cell Lymphoma (CTCL). (20, 23, 25) Up to date, Depsipeptide from the cyclic tetrapeptides group and Belinostat from the hydroxamates group, have been approved by FDA for the treatment of CTCL and PTCL (Peripheral T-Cell Lymphoma), respectively. (40, 44)

Several HDACis exhibited inhibitory effects on specific HDACs. For instance, hydroxamate groups including TSA and SAHA are able to inhibit Class I and II HDACs. TSA can perform an inhibitory effect at low concentration (nmol/L). While, SAHA inhibits HDACs in the concentration of μmol/L. Depsipeptide is a member of a natural cyclic peptide product which selectively inhibits HDAC1 and 2 in the low concentration (nmol/L). The inhibitory effect on Class I and IIA HDACs was also performed by aliphatic acids at mmol/L concentration. Whereas, MS-275 (Entinostat) and MGCD0103 (Mocetinostat) are members of the Benzamides group which is a selective inhibitor for HDAC 1, 2, 3 and Class I and IV HDACs at the concentration of μmol/L, respectively. (23, 35, 40)

A zinc-dependent HDACs have a catalytic pocket containing a zinc-binding site, a long hydrophobic channel, and an internal cavity adjacent to the pocket. (32) In contrast, the structure of HDAC is made up of three parts: a functional zinc-binding group, a cap or enzyme binding group with an aromatic structure, and a linker or spacer group containing a hydrophobic compound that connects the cap and the zinc-binding group. (32, 41, 44)

To inhibit deacetylation, HDACi binds to the catalytic pocket of HDACs by inserting their linker. Once in the position, the functional zinc-binding group interacts with zinc ion at the active site of HDACs and prevents deacetylation. The cap allows the HDACis to attach to the HDACs. Subsequently, it blocks and interacts with the amino acid residue at the entrance of the N-acetylated lysine binding channel of HDACs. HDACis accumulate the acetylation on histone protein via removing the zinc ion with its hydroxy group at the active site of HDACs, which leads to dysfunctional the charge-relay system. (25, 31, 32, 41, 42) Among all HDACis members, TSA and SAHA are the most studied HDACi in the field of dentinogenesis-induction (shown at Table I).

Table 1. The stimulatory effect of HDAC inhibitors in inducing dentinogenesis.

HDAC Inhibitors	Cell Type	Up-regulation	Down-regulation	Refference
TSA	Human pancreatic cancer cell lines BxPC-3, PANC-1, CFPAC-1, and MIA PaCa-2	- p300 - TβRII gene expression	- HDAC1	Huang et al. 2004
	MDPC-23	- Cell proliferation and cell viability	- p53	Duncan et al. 2012

		- p21, DMP-1 expression - Mineralized nodules formation		
	DPCs of extracted rodent incissors	- Cell proliferation and cell viability - p21 expression - Mineralized nodules formation - DSPP, BMP-2, and BMP-4 expression		Duncan et al. 2012
	hDPCs	- Cell proliferation - ALPase activity - Mineralized nodules formation - The expression of odontogenic gene marker: ● NFI-C ● DSPP ● DMP-1 - Others gene: ● phospho-Smad2/3 ● Smad4 ● BSP ● Osteocalcin (OC)	- HDAC3	Jin et al. 2013
	hDPSCs	- H3 and H4 acetylation - Cell number - Cell migration - p21 expression - Chemokines molecules: ● CXCR4 ● FGF2		Luo et al. 2017

		• MCP-1 • SDF-1) - Adhesion molecules: • FN • ICAM-1 • VCAM-1 • Integrin β1		
	mDPCs	- Transcription ability of KLF4 - Trancription of *Dmp1* and *Sp7* gene	- HDAC3	Huangheng et al. 2019
SAHA	MDPCs, OD-11	- Matrix mineralization - Odontoblast differentiation gene marker (in mRNA level): • NFI-C (in mRNA and protein level) • DSPP • DMP-1 • ALP • Nestin		Kwon et al. 2012
	DPCs of extracted rodent incissors	- Matrix mineralization - MMP-9 - MMP-13 - Mineralization gene: • Nestin • BSP • BMP-4 • Adrenomedullin - Cell migration		Duncan et al. 2016
LMK-235	hDPCs	- ALPase activity		Liu et al. 2017

		- Odontoblast differentiation gene marker (in mRNA level): ● ALP ● RUNX2 ● DSPP - Mineralized nodules formation		
VPA	MDPC-23	- Cell proliferation and cell viability - DMP-1 expression - BMP-4 expression - TGF-β1 - Mineralized nodules formation	- p53	Duncan et al. 2012
	DPCs of extracted rodent incissors	- Cell proliferation and cell viability - p21 expression - Mineralized nodules formation - The expression of odontoblast differentiation gene marker: ● NFI-C ● DSPP ● DMP-1 ● BMP-2 ● BMP-4 ● Nestin		Duncan et al. 2012
	hDPSCs	- H3 and H4 acetylation - Cell number		Luo et al. 2017

		- Cell migration - p21 expression - Chemokines molecules: • CXCR4 • FGF2 • MCP-1 • SDF-1) - Adhesion molecules: • FN • ICAM-1 • VCAM-1 • Integrin β1		

3.3 Tooth Development

The tooth begins at the embryonic stage and continues at the postnatal stage, even after the tooth erupts in the oral cavity. (45, 46) The rigid dentin builds up the tooth, together with other tissues such as enamel and cementum, and acts as a protective barrier to maintain dental pulp tissue vitality which resides in the pulp chamber. (47) Briefly, the matured odontoblast cells initiate dentin formation by producing and secreting dentin extracellular matrix such as type I collagen (the main scaffold for dentin tissue during mineral deposition) and several non-collagenous proteins including dentin sialoprotein (DSP), dentin phosphoprotein (DPP), dentin matrix protein 1 (DMP1), osteocalcin (OC), bone sialoprotein (BSP), and osteopontin (OPN), as well as, by generating mineralization. (14, 16, 45, 48) Uniquely, tooth mineralization begins in embryonic life then continues throughout life. (48)

During embryonic life, a series of signaling interactions occurs between epithelial cells and neural crest-derived mesenchymal cells to initiate tooth development. Epithelial cell proliferation begins at the site of the future tooth on embryonic day 10-11 and promotes subsequent epithelial thickening. The thickened epithelial penetrates beneath the mesenchymal, forming an epithelial bud surrounded by condensing mesenchyme. At this stage, signals move back and forth from epithelial to mesenchymal cells, passing through the basal membrane. This process regulates cytodifferentiation of amelogenesis and odontogenesis. (46) Sonic hedgehog (Shh) and Wnt signaling and growth factors such as FGF and BMPs induce the expression of specific transcription factors to promote the expression of other growth factors and specific molecules, and generate further tooth development. (45)

Cross-talk signaling also takes control to regulate tooth morphology formation in the late stage of bell stage. The terminal differentiation occurs in the inner enamel epithelium (IEE) and mesenchymal cells at the periphery layer of the dental papilla. After IEE differentiation into ameloblast cells, mesenchymal cells differentiate into odontoblast cells. A signal, sent by the cuboidal pre-ameloblast to the cuboidal pre-odontoblast cells, induces mesenchyme mitosis. Then, the daughter cells migrate toward the basal membrane and differentiate into odontoblast cells described by the elongation of the cell body, cytological polarization, and formation of a single cellular process. (48) Meanwhile, the parent cells maintain their stemness and contribute to the tooth repair system when the tooth is injured. (45, 48) The matured odontoblast cells are able to produce and secrete dentin extracellular matrix to undergo mineralization in the primary dentin formation. (2, 48) After root formation

is complete and the tooth has erupted and functioned, dentin formation continues physiologically to form the secondary dentin. Furthermore, tertiary dentin formation occurs as a result of external stimuli such as dental caries, tooth ware, and dental restoratives used. (2, 45, 46, 48)

3.3.1 Reparative Dentin Formation

In response to mild dental cavity, the survived odontoblast cells can stimulate dentin extracellular matrix production to generate new dentin tissue formation, called as the reactionary dentin. Reactionary dentin displays tubular structure with anatomical and biological similarities to primary dentin. The more severe dental cavity may cause odontoblast death and necessitates the application of dental pulp-capping material to the damaged dentin. In this part, signaling molecules contained in the material stimulate dental pulp cells to differentiate into odontoblast-like cells and promote new dentin tissue formation, called the reparative dentin. However, the quality of reparative dentin formation is influenced by the molecule itself. The discontinuities and absence of tubular structure in reparative dentin can reduce dentin permeability and does not provide long-term protection of dental pulp tissue vitality from further infection. (2, 16)

3.3.2 The Involvement of pulp-capping material during reparative dentin formation

Depending on signaling molecule contained in the pulp-capping material, reparative dentin may be displayed in various structures such as fibrodentin, osteodentin, and tubular mineralized matrix. (16) Fibrodentin is characterized by an

atubular structure formed by cuboidal or spindle-shape pulp cells during reparative dentin formation. Occasionally, the cells are entrapped within reparative dentin and resemble a bone-like structure, called an osteodentin structure. Tubular mineralized matrix formation is induced by odontoblast-like cells through elongated and polarized odontoblast at the peripheral of pulp exposure-site and provides adequate protection and hermetic seal for pulp tissue from bacterial invasion or external stimuli in the long-term.(2, 3, 16, 49)

Previously, $Ca(OH)_2$ (4-8), mineral trioxide aggregate (MTA) (3, 9-12), hydrogel (6), composite resin (CR) (7), and resin-modified glass ionomer (RMGI) (7) used as pulp-capping material has induced fibrodentin and osteodentin structure, and promoted tunnel defect in reparative dentin. (Table I) Tunnel defect refers to a discontinuities in dentin bridge structure and allows bacteria to invade pulp tissue which associate with treatment failure. (3-8, 49)

Table 2. The effect of direct pulp-capping material in reparative dentin formation.

The Author	Model Organism	Direct Pulp Capping Material	Result
Cox et al. 1996	235 teeth of adult monkeys	$Ca(OH)_2$	- Tunnel defect in dentin bridges - Recurring pulp inflammation - Associated with the presence of inflammatory cells and stained bacterial profile
Faraco and Holland. 2006	30 teeth of dogs	$Ca(OH)_2$ and MTA	$Ca(OH)_2$ group: - A complete dentin bridges with tubular type and no pulp inflammation in only 3 teeth

			- 12 of 15 teeth exhibited a chronic inflammation and a microabscess in the coronary pulp in 4 specimens - Particels of capping material were dispersed in the tissue or inside the macrophage cytoplasm MTA group: - A complete dentin bridges with tubular type was seen in all teeth - 8 of 15 teeth exhibited a small necrotic pulp tissue - The odontoblastic layers line the underlying dental pulp - No micro-organism and inflammatory infiltrate appearance
Murray et al. 2006	135 teeth of adult monkeys	$Ca(OH)_2$, Composite Resin (CR), Resin Modified Glass Ionomer (RMGI)	- Tunnel defect (82% with $Ca(OH)_2$, 42% with RMGI, 0% with CR) - Operative debris (77% with $Ca(OH)_2$, 57% with RMGI, 29% with CR) - Pulp inflammation (68% with $Ca(OH)_2$, 22% with RMGI, 26% with CR) - Bacterial leakage (50% with $Ca(OH)_2$, 19% with RMGI, 20% with CR)
Hwang et al. 2008	Fisrt maxillary molar of male Sprague-Dawley rats	MTA	- Showed irregular and atubular form within reparative dentin, compared with untreated group - The entrapped odontoblast cells within reparative dentin were strongly expressed BSP in protein and mRNA level - TβRI and Smad2/3 protein were detected in both untreated and treated group

			- In treated group, TGF-β1 was significantly increased BSP mRNA but has little effect on DSPP mRNA
Kyung-San et al. 2008	20 extracted human third molars	MTA and Dycal	- Induced reparative dentin formation in all teeth samples of MTA group but only in 6 of 10 samples of Dycal group - MTA group showed thicker reparative dentin than Dycal group - Induced the expression of DSP and HO-1 in the newly formed odontoblast-like cells and pulp fibroblast
Khalil et al. 2013	36 first maxillary first molars of Wistar rats	MM-MTA and ProRoot MTA	Induced a complete reparative dentin except in one tooth sample of MM-MTA and two samples of ProRoot MTA
Njeh et al. 2016	48 first maxillary molars of male Sprague-Dawley rats	Dycal and Hydrogel	Dycal group: - Increased the number of aggregated pulp cells - Caused a moderate inflammatory reaction to pulp tissue and resulted a necrotic pulp adject to reparative dentin - Induced reparative dentin formation except in mesial and distal area of the pulp horn - Exhibited tunnel defect persisting across reparative osteodentin - The thickness of reparative dentin varied between 100 and 200 μm Hydrogel group: - Induced the formation of complete reparative dentin except mesial area of the pulp horn

			Hydrogel group: - Caused a mild inflammatory reaction to pulp tissue and resulted calcospheritic structure - The thickness of reparative dentin varied between 50 and 150 μm Both group: - Showed the presence of chondrocyte-like cells
Nowicka et al. 2016	28 third molars from 17 humans	$Ca(OH)_2$ (CH) and Single Bond Universal (SBU)	- 2 patient in CH group complained a spontaneous minor pain and 1 patient complained a moderate pain - Both group showed porosities and tunnel defect - Fibrodentin and osteodentin were visible in SBU group with the absence of new odontoblast layer - Odontoblast and dontoblast-like cells were visible in CH group - Both group exhibited a chronic infalmmation
Cai et al. 2017	hDPCs	ProRoot MTA, Portland Cement (PC), Dycal under the presence and absence of bacterial exposure	- Enhanced the cell number except Dycal group in both condition - Stimulated tubular structure in the mineralized matrix - The expression of odontoblast differentiation genes varied depending on the material and bacterial exposure ▪ The absence of bacterial exposure ○ DSPP and OCN was expressed in PC and MTA group at day 1 but only OCN was disappeared at day 14

			○ ALP expression was inhibited in all treated group at day 1 and 14 ▪ The presence of bacterial exposure ○ DSPP was expressed in PC and Dycal group ○ OCN was inhibited in all treated group ○ ALP expression was expressed only at day 1 in PC and MTA group
Rodrigues et al. 2017	hDPCs	White MTA Angelus and MTA Plus	- Both MTA had o cytotoxic effect to cell viability - hDPCs strongly expressed osteogenic gene markers including BMP-2 and OC under treatment of both MTA but not ALP expression - Both MTA showed lower ALPase activity than control - Only MTA Angelus showed higher percentage of mineralized area

In response to damaged dentin, the dental pulp cells can differentiate into odontoblast-like cells and form reparative dentin to replace the damaged area at the site of pulp exposure. This potency indicates that dental pulp contains odontogenic progenitor or stem cells which are involved in reparative dentin formation. Previously, dental pulp cells obtained from the third molar have demonstrated odontoblast-like differentiation which promotes reparative dentin formation when it is transplanted into immunocompromised mice. (43) Under appropriate conditions, dental pulp stem cells, indeed, can differentiate into multiple cell lineages including ectodermal lineages (such as cementoblast,

odontoblast, melanocyte, neural cell), endodermal lineages (such as hepatocytes), and mesodermal lineage (such as adipocyte, myoblast, chondrocyte, osteoblast, endothelial cell). (2, 15)

The tooth repair system also requires dental pulp cell migration and differentiation into odontoblast lineages. However, this process involves different processes with tooth development in the embryonic stage controlled by the crosstalk signaling between oral epithelium and mesenchyme and mediated by the basal membrane. Whereas, during tooth repair, odontoblast differentiation occurs in the absence of oral epithelium. (14, 16, 45, 50)

3.4 Hierarchy of odontogenic-marker genes associated with *Nfic-Klf4-Dmp1-Dspp* cascades

NFI-C, the member of the nuclear factor I (NFI) family (51), is found in odontoblast and pre-odontoblast during tooth development and considered as transcription factors. In the prenatal stage during the tooth bud development, the NFI-C gene has been strongly expressed in the mesenchymal cells of the dental papilla. Subsequently, at the beginning of root tooth formation, NFI-C gene expression has been found in either oral epithelial or mesenchymal cells of the tooth, including ameloblast and odontoblast. During root tooth formation, at the postnatal stage, the NFI-C gene has been strongly expressed within odontoblast and pre-odontoblast of the molar of mice and periodontal ligament and developing bone. (52)

Previous studies (51, 53) have reported that NFI-C involvement occurred during odontoblast differentiation and dentin formation. NFI-C deficiency in mice resulted in

decreased bone density and tooth deformities such as molar root agenesis and thin and brittle dentin formation of mandibular and maxillary incisors. (52, 53) The NFI-C overexpression is linked with the up-regulation of other odontoblast gene markers such as KFL4, DMP-1, and DSPP mediating the secretion and mineralization of dentin extracellular matrix to form reparative dentin. (51)

It has been suggested that the odontoblast differentiation and dentin formation might be controlled by *Nfic-Klf4-Dmp1-Dspp* cascades. NFI-C protein is recruited to activate the transcription and KLF4 gene expression via direct binding to the KLF4 promoter area. Subsequently, KLF4 gene expression induces odontoblast differentiation through the up-regulation of DMP1 gene expression by binding to the DMP1 promoter area. DSPP gene expression subsequently is induced to promote the dentin extracelular matrix secretion. Therefore, NFI-C is considered to take control of the initiation of odontoblast differentiation and dentin extracellular matrix production. (42, 51, 54)

The expression of KLF4, DMP1, and DSP protein was significantly decreased in NFI-C-deficient odontoblast compared with wild type odontoblast. In contrast, the NFI-C and KLF4 gene overexpression induced mineralization in MDPC-23 odontoblast cells by up-regulating DSPP gene expression. In the absence of the NFI-C gene, DSPP gene expression is activated by KLF4. In contrast, DSPP gene expression was significantly decreased in KLF4-deficient cells. This finding corresponds to another result that NFI-C could not induce DSPP gene transcription in the absence of KLF4 gene, indicating that NFI-C cooperates with KLF4 to regulate the expression of DMP1 and DSPP gene in odontoblast. (51)

Kwon et al. (26), on the other hand, proposed that NFI-C protein was directly recruited to the DSPP promoter area, resulting in increased DSPP gene expression in MDPC23 cells. The NFI-C gene knockdown by siRNA reduced NFI-C mRNA level and caused the decrease of odontogenic marker gene expression such as DSPP, DMP1, and Nestin.

Odontoblast maturation is required to promote the dentin extracellular matrix production and secretion such as the collagenous and non-collagenous protein and induce mineralization. Among all non-collagenous proteins, dentin matrix protein (DMP) 1, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are commonly expressed during dentin formation. DSP was involved in initiating dentin mineralization while DPP was involved in the maturation of dentin formation. DMP-1 induces mineral deposition along with the collagen fibrin. (14, 55)

DSP and DPP are encoded by a single gene called dentin sialophosphoprotein (DSPP). Mutation in the DSPP gene exhibited dentinogenesis imperfecta and dentin displasia phenotype. The severe tooth defect caused by enlarged predentin, as well as, thin dentin layer and dentin hypomineralization was found in DSPP-defieceint mice. DSPP- and DMP1-deficient mice also exhibited a similar phenotype including the enlargement of pulp chamber, the failure maturation in predentin and dentin, and the dentin hypomineralization.(55-57)

3.5 HDACis (TSA and SAHA) involvement in odontoblast differentiation

3.5.1 Trichostatin A (TSA)

Jin et al. (28) has reported that TSA induced cell proliferation in hDPCs culture and enhanced ALP activity and mineralization in a dose-dependent manner, indicating that TSA promoted hDPCs differentiation into odontoblast cells. It was confirmed by the increased expression of odontogenic marker genes such as NFI-C, DSPP, and DMP-1. The increased phospho-Smad2/3, Smad 4, Bone sialoproptein (BSP) and Osteocalcin (OC) expression were also observed in TSA treatment. In addition, TSA is also able to inhibit HDAC3 activity and trigger cell differentiation. Corresponding to these results, other studies (58, 59) have reported that TSA enhanced the expression of transcriptional factors, p21, which leads to cell growth arrest and induces cell differentiation via generating the transcription of the target gene. The mineralization was significantly increased by TSA in dose and time-dependent manners. In addition, the fluctuating expression of osteopontin, DMP-1, DSPP, BMP-2, BMP-4 and Nestin has been seen during TSA treatment.

Furthermore, TSA has shown the increase of dental pulp tissue repair in response to the damaged dentin. Low concentration of TSA (2 and 20 nM/L) elevated the expression of chemokines and adhesion molecules, leading to the enhancement of cell proliferation, cell migration, and cell adhesion activity. This stimulatory effect is essential to generating reparative dentin. The damaged dentin stimulates dental pulp cell proliferation, as well as cell migration and attachment to replace the injured odontoblast. (22)

Huang et al. (27) has reported that the expression and activity of HDAC1 and p300/PCAF take control in regulating the TGF-β pathway through the transcriptional activation of TβRII. TSA treatment in dose dependent-manner led to the increase of TβRII transcriptional activity through decreasing HDAC1 binding and increasing the recruitment of transcriptional co-activator such as p300 and PCAF in the TβRII promoter area. TSA also enhanced KFL4 activity in the DMP1 gene promoter area via inhibiting HDAC3 activity and subsequently recruiting p300 transcriptional co-activator to cooperate with KFL4, generating the increase in transcription activity of the DMP1 gene. (42) Those results showed that TSA could inhibit HDACs activity and enhance acetylation in the promoter area of the target gene by recruiting HATs as a transcriptional co-activator to promote the transcription process.

3.5.2 Suberoylanilide hydroxamic acid (SAHA)

SAHA stimulates the mineralization of dentin extracellular matrix in MDPC23 odontoblast-like cell line via enhancing odontoblast differentiation. SAHA enhances NFI-C gene transcription resulting in the over-expression of NFI-C protein. The NFI-C protein directly binds to the DSPP gene in its promoter area to initiate the up-regulation of other odontogenic marker genes including DMP-1 and Nestin, which are essential for stimulating the production of dentin extracellular matrix. NFI-C gene knockdown by siRNA inhibits SAHA to stimulate the over-expression of DSPP gene, indicating that SAHA cooperates with NFI-C transcriptional co-activator to promote DSPP gene expression. (26)

Duncan et al. (41) has suggested that SAHA induced the increased MMP-13 expression, a member of the matrix metalloproteinases (MMPs) family, through HDAC4 inhibition. MMP-13, dose dependently, was involved during reparative dentin formation through enhanced cell migration and matrix mineralization.

MATERIALS AND METHODS

4.1 Human Dental Pulp Tissue Preparation

Right after extraction, four healthy third molars collected from four patients (18-25 years of age) at Department of Oral Surgery, Faculty of Dentistry, Chulalongkorn University under procedures and informed consent approved by The Human Research Ethics Committee from Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2020-013) were rinsed with sterile saline solution then, soaked in the growth (non-induction) medium (Dulbecco's Modified Eagle Medium (DMEM; #11960, Gibco® Life Technologies) supplemented with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 5 mg/mL amphotericin B). Teeth were transferred into the laboratory at 4°C then, cleaned with sterile phosphate buffered saline (PBS) under the sterile condition. Each tooth was held by dental forceps and a scalpel blade was used for cleaning off periodontal ligament on the root surface.

Dental pulp tissue was gently separated from tooth fragments after opening the crown with a hammer. It was rinsed with sterile PBS for debridement and prevention of contamination, and cut off into small pieces (approximately 1-2 mm fragment). Human dental pulp cells (hDPCs) were further isolated with an outgrowth method.

4.2 Human Dental Pulp Cells Isolation

Each piece of dental pulp tissue was washed with sterile PBS, twice, then placed into a 25 mm culture dish containing 1 mL of growth medium to support dental pulp tissue

and cell attachment. Those culture dishes were incubated at 37°C with 5% CO_2 with periodically medium changes every 2-3 days. hDPCs at 3rd-6th passage and 80% confluent were used for this present study.

4.3 HDACis Preparation

Master stocks 5mM Trichostatin A (TSA; #58880, Sigma – Aldrich) and Suberoylanilide Hydroxamic Acid (SAHA; #149647, Sigma – Aldrich) in dimethyl sulfoxide (DMSO; #D2650, Sigma® Life Science) were diluted in PBS before further dilution to experimental concentration in culture medium (50 nM, 200 nM, 400 nM, and 800 nM TSA and 0.5 μM, 1 μM, 3 μM, 5 μM, and 10 μM SAHA).

4.4 Cell Viability Assay

Cell viability of hDPCS was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; #M6494, Invitrogen, USA) assay. hDPCs were seeded at the density of 2 x 10^4 cells/cm^2 in 96-well plates containing growth medium for 24 hours, at 37°C. Cells were incubated in serum-free medium for a further 4 hours at 37°C before being exposed with various HDACis concentrations (50 nM, 200 nM, 400 nM, and 800 nM TSA; 0.5 μM, 1 μM, 3 μM, 5 μM, and 10 μM SAHA) supplemented in osteogenic (mineralized induction) medium (DMEM 10% supplemented with 50 μg/mL ascorbic acid, 5 mM β-Glycerophosphate, and 250 μM dexamethasone). Whereas control groups were treated with growth medium and osteogenic medium without HDACis.

MTT powder was diluted in serum-free DMEM without phenol red until it reached the concentration of 0.5 mg/mL. At experimental day-3 (72 hours), all medium was removed, and cells were rinsed with PBS, once. 100 µL MTT solution was added to each well, then incubated at 37°C for 30 minutes. After completely removing the MTT solution, the existing formazan crystal was dissolved by adding 100 µL of the glycine buffer/DMSO solution in a ratio of 1:9. The orbital shaker was used to dissolve the formazan crystal, completely, for 5 minutes. The absorbance was read at 570 nm within an hour using a Spectrophotometer and the values were expressed as optical density (OD).

4.5 Mineralization Assay

hDPCs were seeded at the density of 5 x 10^4 cells/cm^2 in 24-well plates containing growth medium at 37°C. After 24 hours (experimental day-0), cells were washed with sterile PBS and exposed with HDACis-supplemented osteogenic medium in various HDACis concentrations (50 nM, 200 nM, and 400 nM TSA; 0.5 µM and 1 µM SAHA) for a further 21 days. The control groups were treated with growth and osteogenic medium without HDACis. The medium was changed every 2-3 days.

On experimental day-14 and -21, the mineralized nodule formation was examined by staining with Alizarin Red. Cells were washed with ice-cold PBS, twice, then fixed with ice-cold methanol for 10 minutes at room temperature (RT). After completely removing methanol, the fixed cells were rinsed with deionized water, twice, and subsequently stained by adding 300 µL of 1% alizarin red solution to cover the cellular monolayer. Five minutes after staining at RT, the mineralized nodule formation was observed under an inverted

microscope (Nikon Eclipse TS100, Nikon Instruments Inc., Melville, NY) and quantified with ImageJ Software version 1.51.

4.6 Scratch Wound Healing Assay

Cell migration of hDPCs was evaluated by scratch wound healing assay. hDPCs at the density of 5 × 10^4 cells/cm^2 were seeded into 24-well plates and incubated in the general medium at 37°C for 24 hours. After 4 hours of serum starvation (counted as experimental day 0), a scratch wound was made through the confluent layer of cells using a 200 μL pipette tip in the middle of each well plate. Then, cells were washed with serum-free medium, twice, to remove cell debris. hDPCs in the experimental group were incubated with serum-free medium supplemented with 400 nM TSA and 1 μM SAHA for a further 72 hours. Whereas the control group was treated with serum-free medium without HDACis.

Using an inverted microscope, photomicrographs of the scratch were taken at 0, 24, 48 and 72 hours after the scratch wounding. The scratch-wound closure rate was determined using ImageJ Software version 1.51. The distance between cells that have migrated into the wounded area and their respective starting points, were determined. The distance of wound closure at 24, 48, and 72 hours post-scratch wounding were quantified and compared with the control group.

4.7 RNA Extraction

hDPCs at the density of 5 x 10^4 cells/cm^2 were seeded in 6-well plates containing the growth medium for 24 hours to allow cell attachement. 80% cell confluence was used

for the experiment. After 4 hours of serum starvation at 37°C, cells were treated for 10 days with osteogenic medium supplemented with 400 nM TSA and 1 μM SAHA, with medium changes every 2-3 days. The control groups were treated in osteogenic medium without HDACis.

The total RNA was harvested at 24 hours, 72 hours, 7 days and 10 days post-induction with Trizol reagent (#K00054-0200, Biotechrabbit) to lysate cells. After adding 1 mL of Trizol to each well, cells were scraped and homogenized using a pipette tip. The lysate was incubated at RT for 5 minutes, then transferred into a 1.5 mL tube. 200 μL of chloroform was added and vortex vigorously for approximately 15 seconds to homogenize. The mixture was incubated for 2-3 minutes at RT before centrifuged at 4°C at 12.000 x g for 15 minutes. After centrifugation, the mixture was separated into three layers. The aqueous phase which refers to total RNA was replaced into a new 1.5 mL tube. 500 μL Ice-cold Isopropanol was added into each tube then incubated at -20°C for 2-4 hours. Furthermore, the samples were centrifuged for 10 minutes at 4°C at 12.000 x g.

RNA precipitation was formed into a white gel-like pellet at the bottom of a 1.5 mL tube. The supernatant was removed gently and the pellet was rinsed with 1 mL of ice-cold 70% ethanol, twice and 100% ethanol, once as the last washing step. The pellet was centrifuged at 7.500 x g at 4°C for 5 minutes between the first and last washing steps. After removing the supernatant, the RNA pellet was dried at RT until completely dry for approximately 5-10 minutes. Lastly, the RNA pellet was dissolved in 10-20 μL Nuclease free-water (#P1193, Promega, USA) by passing the solution through a pipette tip a few times. RNA

concentration was quantified by the NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE).

4.8 Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To observe the stimulatory effect of HDACis in inducing the expression of odontoblast-like differentiation-related gene markers in hDPCs, Real Time RT-PCR was performed. First strand cDNA was constructed by using reverse transcriptase reaction by ImProm-II Reverse Transcription System (#A3800, Promega Corporation, Madison, WI).

Real Time RT-PCR was performed using CAPITAL^{TM} qPCR Green Mix HRox (#BR0501901, BiotecRabbit) with a reaction volume of 20 µL containing 375 nM of primers and 1 µL of RT product. Real time thermal cycle (CFX96 TouchTM Real Time PCR System, Bio – Rad) was used through the PCR protocol as follows: initial activation at 95^{o}C for 2 – 3 minutes, denaturation at 95^{o}C for 15 seconds, and extension at 60-65^{o}C for 30 seconds for 40 cycles. Bio-Rad CFX Maestro 1.1 manager software was used to analyze relative gene expression.

PCR primers for nuclear factor I-C (NFIC), krüppel-like factor 4 (KLF4), dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP1) were used to observe the expression of odontoblast-like differentiation-related gene markers. Furthermore, PCR primers for type I collagen (COL1), alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OC) were used to observe the expression of general mineralization-related gene markers and vascular endothelial growth factor (VEGF) was used to detect the capability of blood vessel formation in hDPCs under HDACis treatment. Furthermore, a PCR primer for the

p21 gene was used to determine whether HDACis has a stimulatory effect in inducing cell cycle arrest and leading to cell differentiation in odontoblast differentiation by generating the expression of downstream genes. All gene expression was analyzed and normalized with the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table I).

The threshold cycle (CT) from each sample was counted using the real-time cycler software then, changed from CT to fold change of genes by using the Bio-Rad CFX Maestro 1.1 manager software.

Table 3. PCR primer sequences of target genes.

Primers	**Sequence (5' → 3')**
VEGF	Forward : CCTTGCTGCTCTACCTCCAC Reverse : ATCTGCATGGTGATGTTGGA
p21	Forward : TCAGGGTCGAAAACGGCG Reverse : CCTCTTGGAGAAGATCAGCCG
NFI-C	Forward : GACCTGTACCTGGCCTACTTTG Reverse : CACACCTGACGTGACAAAGCTC
COL-1	Forward : GTGCTAAAGGTGCCAATGGT Reverse : ACCAGGTTCACCGCTGTTAC
ALP	Forward : CGAGATACAAGCACTCCCACTTC Reverse : CTGTTCAGCTCGTACTGCATGTC
DSPP	Forward : TCACAAGGGAGAAGGGAATG Reverse : TGCCATTTGCTGTGATGTTT
DMP-1	Forward : ATGCCTATCACAACAAACC

	Reverse : CTCCTTTATGTGACAACTGC
BSP	Forward : ATGGCCTGTGCTTTCTCAATG Reverse : AGGATAAAAGTAGGCATGCTTG
OC	Forward : CTTTGTGTCCAAGCAGGAGG Reverse : CTGAAAGCCGATGTGGTCAG
KFL4	Forward : CTGAACAGCAGGGACTGT Reverse : GTGTGGGTGGCTGTTCTTTT
GAPDH	Forward : TGAAGGTCGGAGTCAACGGAT Reverse : TCACACCCATGACGAACATGG

4.9 Alkaline Phosphatase Acivity Assay

ALP activity of hDPCs during odontoblast-like differentiation was measured at day-3, -5, -7, and -10 post-induction. hDPCs at the density of 5×10^4 cells/cm^2 were seeded in 24-well-plate containing growth medium. After 24 hours (at experimental day-0), cells were exposed to osteogenic medium without or with 400 nM TSA and 1 μM SAHA for 10 days, with medium changes every 2-3 days.

Briefly, cell lysate from each sample was collected by adding ALP lysis buffer (10 mM Tris – HCl pH 10, 2 mM $MgCl_2$, 0.1% Triton X-100) before freezing at -80°C for 10 minutes. After thawing, 100 μL of cell lysate was incubated in the substrate mixture of 10 μL P-Nitrophenyl Phosphate (PNPP; N7653, Sigma Life Science) and 100 μL 0.1 M aminopropanol in 2 mM of $MgCl_2$, at 37°C for 30 minutes until the color was turned to yellow. The reaction

was stopped by adding 900 µL 0.1 M NaOH to each well. The absorbance of the mixture was measured at 410 nm wavelength using a microplate reader.

For the protein assay, those samples were treated in the same manner as in the ALP assay until the samples were frozen. The total protein was analyzed using the BCA Protein Assay Kit (#3380463, EMD Millipore Corp., USA). After thawing, 200 µL bicinchoninic acid solution was added to the samples and incubated at 37°C for 30 minutes. The absorbance of samples was measured at 562 nm wavelength using a Spectrophotometer and the amount of total protein was quantified against a standard curve. Furthermore, ALP activity of samples was obtained by normalizing the ALP activity product with total protein content then compared with the control group.

4.10 Statistical analysis

All experiments were repeated three to four times. We calculated the mean and standard deviation (SD) for each set of data. For statistical analysis, One-Way ANOVA and Tukey's HSD post hoc test were applied to compare either experimental groups with control or among one experimental group to another. Significant differences were considered as $p < 0.05$.

RESULT

5.1 Effect of TSA and SAHA treatment in hDPCs viability

To evaluate the effect of HDACis (TSA and SAHA) treatment on hDPC viability, MTT assay was performed in osteogenic medium containing various HDACis concentrations (50 nM, 200 nM, 400 nM, and 800nM TSA and 0.5 μM, 1 μM, 3 μM, 5 μM, and 10 μM SAHA) for 72 hours. Meanwhile, growth and osteogenic medium without TSA/SAHA were used to treat the control group.

After 72 hours of treatment, hDPCs viability was not influenced by 50 nM, 200 nM and 400 nM TSA; 0.5 μM and 1 μM SAHA treatments as shown by comparing with the control group, treated in osteogenic medium without HDACis. In contrast, the higher concentration of HDACis significantly influenced hDPCs viability, compared with the control. Cell viability was significantly decreased in the higher concentration of TSA (800 nM) and SAHA (3 μM, 5 μM, and 10 μM) (Figure 1).

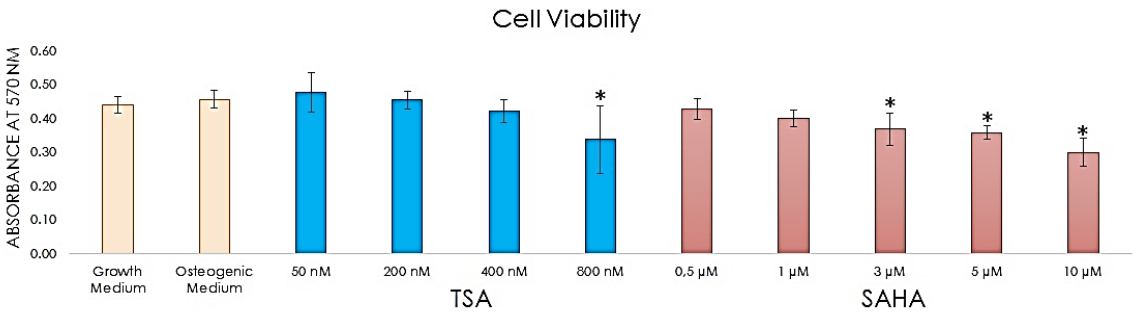


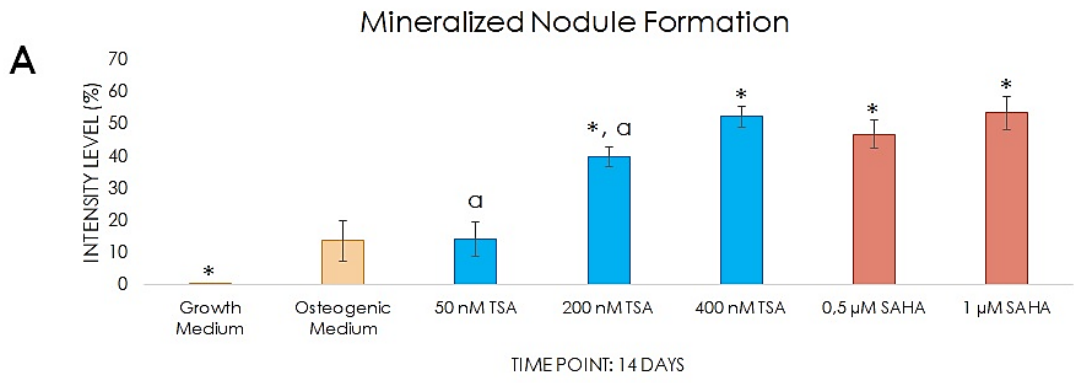
Figure 1. Cell viability of hDPCs cultured in the presence and absence of TSA and SAHA after 72 hours was assessed with MTT assay. (One-way ANOVA, Tukey's HSD post hoc test, *Asterisks*: significant different compared with osteogenic medium, as a control, at $p < 0.05$, n = 4).

Due to 50 nM, 200 nM, and 400 nM TSA; 0.5 μM and 1 μM SAHA showed no impact on hDPCs viability as seen in the comparison with control, we decided to use these candidate doses for further experiment.

5.2 The enhancement of mineralized nodule formation under HDACis treatment in hDPCs

To observe the stimulatory effect and determine the best concentration of HDACis (50 nM, 200 nM, and 400 nM TSA; 0.5 μM and 1 μM SAHA) in inducing odontoblast-like differentiation and inducing mineralization *in vitro*, we stained hDPC culture with Alizarin Red at experimental day-14 and -21. No mineralized nodule was formed in the growth medium-treated group even up to 21 days of treatment, compared with osteogenic medium without HDACis, as a control.

During 14 days of mineralization induction, there was no significant difference in the 50 nM TSA-treated group, indicating that it had a similar effect to the osteogenic medium. Meanwhile, the mineralized nodule formation was significantly elevated on 200 nM and 400 nM TSA; 0.5 μM and 1 μM SAHA, compared with osteogenic medium (Figure 2A-B).



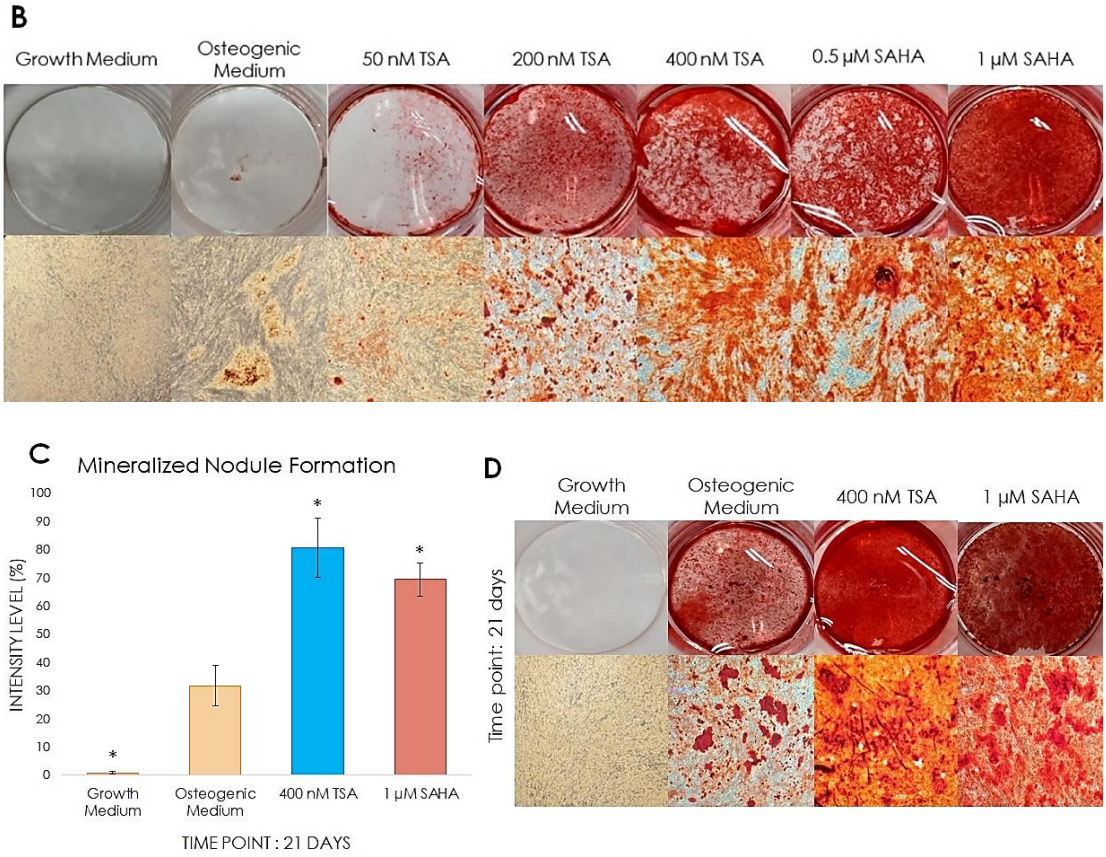


Figure 2. The effect of TSA and SAHA on mineral deposition in hDPCs in growth and osteogenic medium with/without TSA or SAHA. (**A, C**) Quantitative analysis was measured by ImageJ Software representing the percentage of the alizarin red staining intensity. (**B, D**) Alizarin Red Staining at day-14 and -21 post-induction. (One-way ANOVA, Tukey's HSD post hoc test, *Asterisks*: significant difference compared with osteogenic medium at $p < 0.05$, *a*: significant difference among TSA-treated groups, compared with 400 nM TSA at $p < 0.05$, *Hashtag*: significant difference between TSA- and SAHA-treated group at $p < 0.05$, n = 4).

Statistical analysis data showed that 400 nM TSA exhibited the highest formation of mineralized nodules than other TSA, which indicates that it might be a potent concentration to boost mineralization. However, 0.5 μM- and 1 μM SAHA-treated group might provide a similar effect in inducing mineralized nodule formation. In contrast, the optical density (OD) value measured by Spectrophotometer revealed that 1 μM SAHA treatment increased the mineralization, significantly, compared with SAHA 0.5 μM (data not shown). No significant

difference between TSA and SAHA was found at 14 days post-induction. As a result, we observed the mineralization for the next 7 days (experimental day-21) using 400 nM TSA and 1 μM SAHA which is assumed as the optimal concentration inducing mineralization.

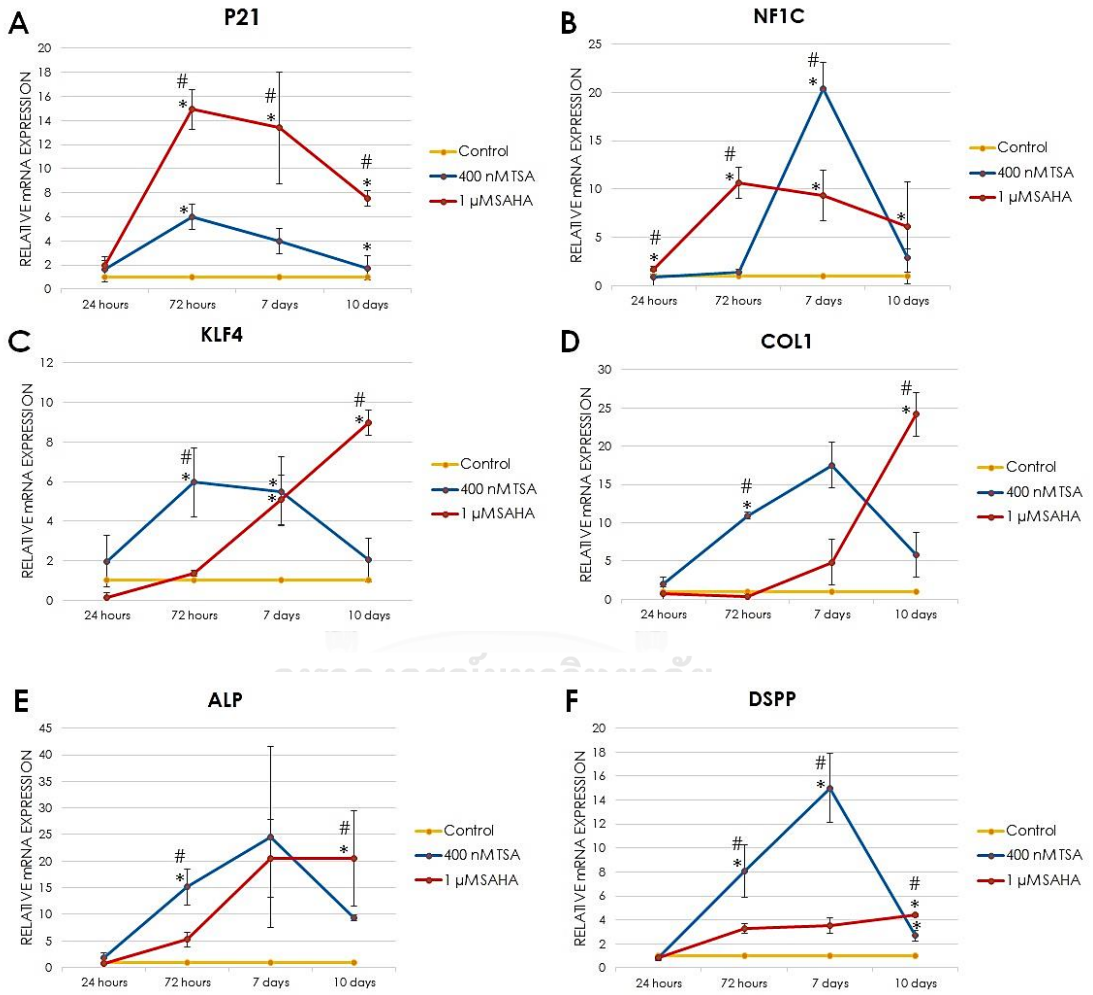
At experimental day-21, the mineralized nodule formation in 400 nM TSA- and 1 μM SAHA-treated group was significantly higher than the induction medium-treated group but, no significant difference was found in the comparison between both HDACis-treated groups (Figure 2C-D). This indicated that TSA and SAHA, at observed dose, might provide a similar effect in inducing hDPCs differentiation into odontoblast-like cells and generating the mineralization process, as seen in osteogenic medium-treated group.

5.3 Pattern of odontoblast-related gene expression of hDPCs under HDACis treatment

To confirm the odontogenesis effect of TSA and SAHA, the expression of odontoblast differentiation-related gene markers, under treatment of osteogenic medium containing HDACis (400 nM TSA and 1 μM SAHA), was evaluated by RT-qPCR and compared with control treated with osteogenic medium without HDACis.

During the onset of differentiation and up to the next 10 days, the HDACis-treated group showed the expression pattern of general mineralization-related gene markers such as COL1, ALP, BSP, and OC and odontoblast differentiation-related gene markers including NFI-C, KLF4, DMP1, and DSPP (Figure 3A-J). TSA and SAHA displayed a similar alteration pattern of P21 gene expression which induces cell cycle arrest and triggers cell differentiation through inducing the transcription of the downstream gene (58). The significant elevation in

P21 gene expression was found up to 6- and 14.9-fold in the TSA- and SAHA-treated group after 72 hours of treatment, respectively, compared with control. Then, it gradually declined at the later stage (day-7 and -10) to 4- and 1.7-fold in TSA and 13.4- and 7.5-fold in SAHA treatment. In the comparison between TSA and SAHA, we found that SAHA significantly up-regulated P21 gene expression during treatment, compared with TSA.



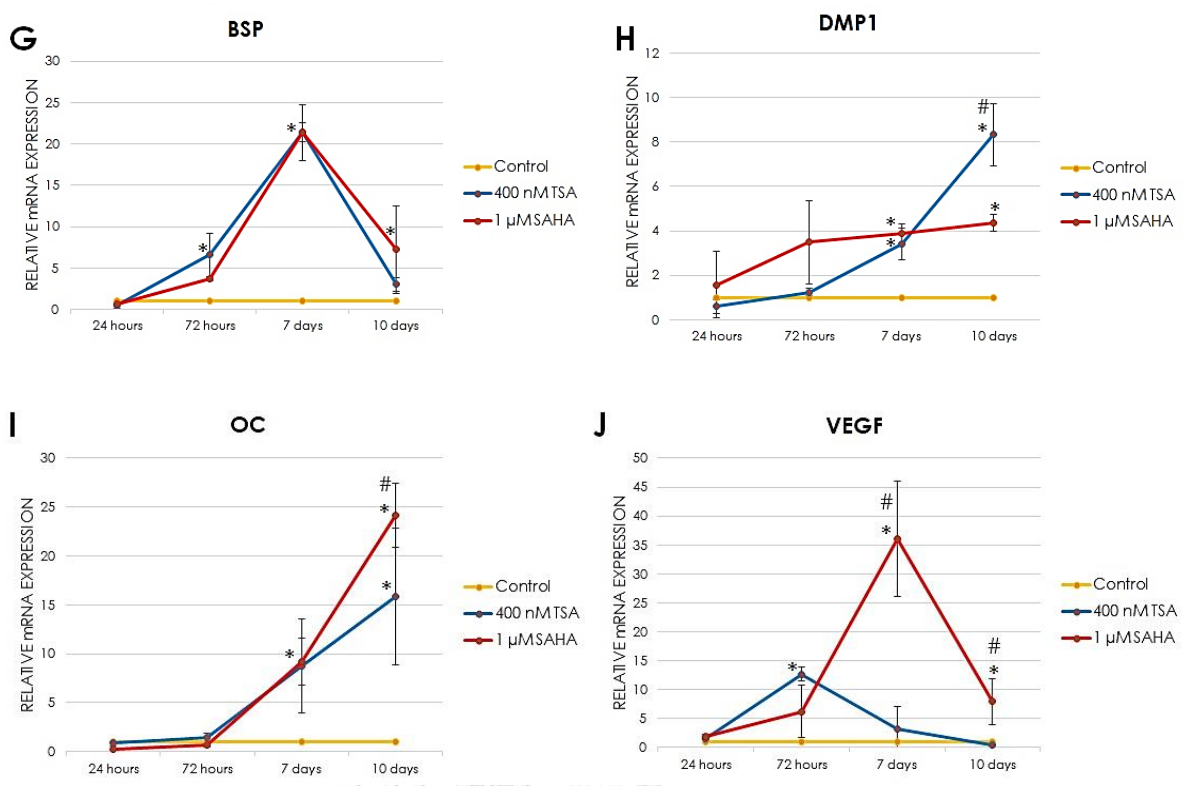


Figure 3. *The relative expression of odontoblast-related gene markers during odontogenesis was confirmed by RT-qPCR at 24h, 72h, 7d and 10d post-induction.* (**A**) P21: protein 21, (**B**) NFI-C: Nuclear Factor I C, (**C**) KLF4: Krüppel-like factor 4, (**D**) COL1: Type I Collagen, (**E**) ALP: Alkaline Phosphatase, (**F**) DSPP: Dentin Sialophosphoprotein, (**G**) DMP1: Dentin Matrix Protein 1, (**H**) BSP: Bone Sialoprotein, (**I**) OC: Osteocalcin, and (**J**) VEGF: Vascular Endothelial Growth Factors (One-way ANOVA, Tukey's HSD post hoc test, *Asterisks*: significant difference compared with control at $p < 0.05$, *Hashtag*: significant difference between TSA- and SAHA-treated group at $p < 0.05$, n = 3).

The NFI-C gene was expressed to initiate the expression of downstream gene related to odontoblast differentiation (42, 51, 54) and KLF4 gene (42) is one of them. HDACis treatment showed a similar acceleration pattern of NFI-C and KLF4 gene expressions at the early stage of differentiation. SAHA 1 μM and TSA 400 nM treatments significantly enhanced NFI-C gene expression from 1.7- up to 10.6-fold at 24 – 72 hours and 1.5- up to 20.4-fold at 72 hours – 7 days of treatment, respectively, then followed by the gradually decreased NFI-

C gene expression at the later stage. The increased expression of KLF4 gene was also seen up to 6-fold at 72 hours and 36-fold at 7 days of treatment under TSA and SAHA treatment.

HDACis treatment in hDPCs enhanced the expression of COL1, ALP, DSPP, and BSP genes in the similar pattern to the expression of NFI-C and KLF4 genes. The 1 μM SAHA-treated group showed the significant up-regulated expression of COL1, ALP, DSPP, and BSP genes up to 24-, 20.5-, 4.4-, and 7-fold at experimental day-10, respectively. Whereas, after 10 days of 400 nM TSA treatment, the expression of COL1, ALP, DSPP, and BSP genes were significantly increased by up to 5.8-, 9-, 2.7-, and 3-fold, respectively. It should be noted that SAHA demonstrate better effect on the expression of COL1, ALP and DSPP genes significantly on the later time point (day-10) when comparing with TSA. A significant difference was not found in BSP gene expression in the comparison of SAHA to TSA.

DMP1 gene expression was also gradually elevated under HDACis treatment during the onset of differentiation. However, the 400 nM TSA-treated group showed a significant increase level in DMP1 gene expression up to 8.3-fold at day-10 post-treatment when compared to 1 μM SAHA which was only expressed up to 4.7-fold, 1.7 times lower.

The linear acceleration pattern of OC gene expression was found up to 9-fold increased after 7 days of treatment with 400 nM TSA and 1 μM SAHA, and even increased until the later stage (10 days). At this time point, OC gene expression in SAHA has increased up to 24-fold, 1.5 times higher than TSA. Furthermore, during odontoblast differentiation, HDACis treatment resulted in a different pattern of VEGF gene expression, a gene marker for new blood vessel formation (60). The 400 nM TSA-treated group showed significantly increased expression of VEGF gene after 72 hours (up to 12.7-fold) compared to the control,

which then dropped at day-7 to 3-fold and disappeared at day-10. The 1 μM SAHA group, on the other hand, maintained the accelerated VEGF gene expression level from 72 hours up to 7 days of treatment. There was a significant increase in VEGF gene expression up to 36-fold (at day-7), 12 times higher, compared with the TSA-treated group. But, its expression was down-regulated to 7.5-fold after 10 days of SAHA treatment.

In comparison, between TSA and SAHA, SAHA appeared to show more effect during the treatment. Notably, NFI-C gene expression in SAHA was significantly 10-fold higher than TSA at 72 hours post-treatment. Furthermore, COL1 gene expression in SAHA was upregulated up to 24-fold, 4 times higher than TSA at day-10. A similar effect was also found in ALP gene expression in which SAHA treatment maintained ALP gene expression at 20-fold at the later stage. Meanwhile, in TSA, ALP gene expression was significantly down-regulated to 10-fold, 2 times lower than SAHA. SAHA also showed the significant elevation of DSPP gene expression up to 4.4-fold at the later stage, 1.6 times higher than TSA.

Real Time RT-PCR ensured the significant up- and down-regulation of P21, NFI-C, KLF4, DMP1, DSPP, COL1, ALP, BSP, OC, and VEGF during the respective time points. These findings demonstrated that HDACis treatment might result in the acceleration pattern of odontoblast differentiation-related gene expression and lead to the enhancement in hDPCs differentiation into odontobalst-like cells.

5.4 Change of ALP Activity during odontoblast-like differentiation in HDACis-treated hDPCs

To verify TSA and SAHA-induced ALP Activity during mineralized induction, ALP assay was measured at experimental day-3, -5, -7 and -10. During TSA (400 nM) and SAHA (1 μM) treatment in osteogenic medium, both of the HDACis group showed different patterns of ALP activity level. ALP activity of 400 nM TSA-treated groups was significantly increased starting from the early stage until the intermediate stage (experimental day-3 and day-5, respectively) of odontoblast differentiation, compared with the control group. Its activity, however, decreased at later time-points (at experimental day-7 and -10). In contrast, the significant acceleration pattern of ALP activity was shown in the 1 μM SAHA-treated group starting from the early stage and continued to increase up to day-10 post-induction, compared with the control and TSA-treated groups (Figure 4).

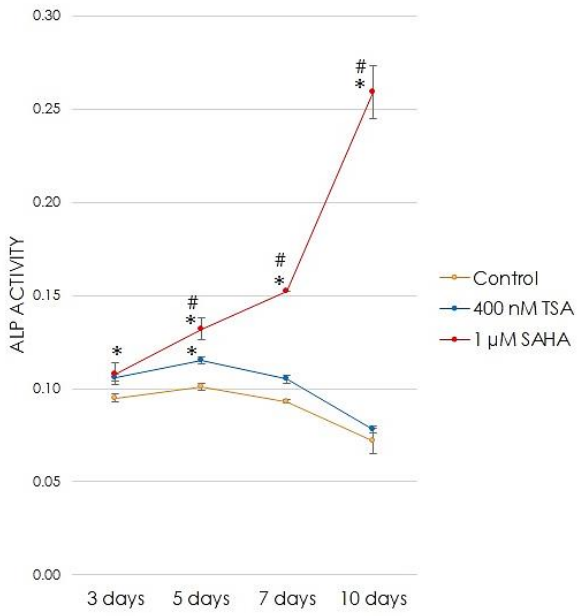


Figure 4. *The effect of TSA or SAHA on the acceleration pattern of ALP Activity during odontogenesis.* (One-way ANOVA, Tukey's HSD post hoc test, *Asterisks*: significant difference

compared with control at $p < 0.05$, *Hashtag*: significant difference between TSA- and SAHA-treated group at $p < 0.05$, n = 4).

This finding supported Alizarin Red Staining result, which showed that TSA and SAHA significantly enhanced the mineralized nodule formation up to day-21 post-induction, compared with control. We also assumed that SAHA might have more effect in enhancing ALP activity compared with TSA.

5.5 The stimulatory effect of HDACis in inducing hDPCs migration

Scratch wound healing assay was performed for 72 hours in serum starvation condition for 72 hours, to test the effect of HDACis (400 nM TSA and 1 μM SAHA) on hDPC migration. A percentage of wound closure described cell migration which was calculated as the difference of the distance between the separated monolayer cells at the wound-made time versus every next 24 hours. The significant acceleration in wound closure was found only 72 hours post-treatment, compared with control. However, no significant difference was shown in inducing cell migration under TSA-SAHA treatment at that time-point (Figure 5A-B).

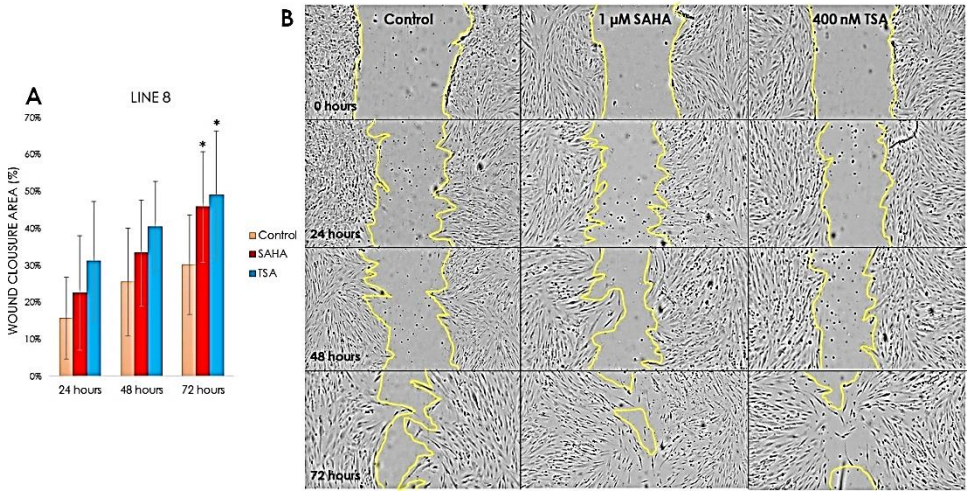


Figure 5. Scratch wound healing assay displayed cell migration during 72 hours in serum-free medium with/without TSA or SAHA. (**A**) Quantitative analysis of the wound closure area expressed

as the percentage, (**B**) hDPCs migration at 0, 24, 48 and 72 hours of treatment; scale bar is 200 mm. (One-way ANOVA, Tukey's HSD post hoc test, *Asterisks*: significant difference compared with control at $p < 0.05$, n = 3).

DISCUSSION

In the present study, we demonstrated the involvement of HDAC inhibitor TSA and SAHA in inducing hDPCs differentiation into odontoblast lineages without interfering with cell viability and mineralized nodule formation through enhanced the expression level of odontoblast differentiation-related gene markers as well as by increasing ALP activity. TSA and SAHA, the epigenetic-modifying agents belonging to hydroxamic acid inhibitors group (25), remove zinc ion on the HDAC active site to generate the inhibition of class I and II HDACs activity. (23, 31, 40) This process prevents deacetylation on lysine residue of histone core N-terminal tails (32), causing the balance between HATs and HDACs to shift to more acetylate lysine residue (31). Histone acetylation converts the positively charged of histone core into the negatively charged lysine residue of histone tails, resulting in nucleosome structure relaxation (23, 42) and providing a binding site for transcriptional machinery protein to target gene (26) to induce cellular activities such as cell proliferation, cell death, cell differentiation, and cell fate decision. (31, 41)

Reviews of HDACis (23, 40) have mentioned that TSA shows the inhibitory effect at the low concentration of nmol/L while SAHA requires μmol/L concentration to generate histone acetylation. In the present study, we found the high concentration of TSA (800 nM) and SAHA (3, 5, and 10 μM) significantly reduced cell viability, indicating that it might have a cytotoxic effect on hDPCs. According to these findings, we assumed that TSA and SAHA in the concentration of 50, 200, and 400 nM; and 0.5 μM and 1 μM, respectively, might be proper candidate doses for treatment *in vitro* without affecting cell viability.

Our finding correspond to previous studies reporting that 1 μM SAHA (26) and 50, 100, 200, 400 nM TSA (21, 28) treatment didn't show cytotoxic effect and cause cell death in either cell line (MDPC-23) or primary cell (hDPSCs and hPDLCs). However, the high concentration of TSA (800 nM) has an impact on cell viability. (21) Contrastingly, SAHA treatment on murine dental pulp-derived cell line (MDPC-23) (26) at the concentration of 3 and 5 μM hasn't affected cell viability after 48 hours. The low concentration of TSA (25 nM and 400 nM) was toxic for living MDPC-23 showed by the reduction of cell proliferation. (29) This might be caused by the difference in cell type, primary cell vs transformed cell line.

After obtaining the safe doses for hDPCs viability, we evaluated TSA and SAHA effect on the mineralization under mineralized induction medium. At experimental day-14, the higher formation of mineralized nodule was found on 400 nM TSA- and 1 μM SAHA-treated groups, significantly, compared with control and other HDACis groups. However, up to 21 days of observation, the enhanced mineralization on TSA (400 nM) vs SAHA (1 μM) groups did not show a significant difference. These indicated that both HDACis concentration seemed to have similar odontogenesis effects in inducing mineralization as shown by alizarin red staining quantification and suggested that HDACis dose selection should be considered to enhance odontoblast-like differentiation. Notably, the large mineralized nodules and strong alizarin red staining occurred at the range of TSA and SAHA concentrations below those concentrations which reduce cell viability, significantly. Our results also correspond with previous studies demonstrating that 400 nM TSA and 1 μM SAHA treatment triggered the increased mineralization. (21, 26)

Further evidence found in this present study was the significantly elevated relative expression of p21 gene at the early stage of differentiation, the significantly elevated expression level of odontoblast-related marker NFI-C, KLF4, DMP1, and DSPP and general mineralized-related marker COL1, ALP, BSP, and OC at the given-time point, indicating that TSA and SAHA triggers mineralization through the inducement of cell cycle arrest and differentiation. Previously, TSA (28, 29) and SAHA (26) treatment have been demonstrated to dose-dependently reduce cell growth mediated by the increased level of p21 gene expression and the acceleration of odontoblast differentiation and mineralization.

TSA and SAHA exhibited a distinct significant difference in each odontogenesis-related gene expression at the given-time point except BSP gene expression which does not have a significant difference over time. Overall, both HDACis demonstrate stimulatory effect on almost all odontogenesis-related gene expression. All these together, we assumed that TSA and SAHA might have similar odontogenesis effects by inhibiting the HDAC active side as well as leading to histone acetylation and nucleosome relaxation. The HDACis-mediated transcriptional activity induces the expression of target genes related to odontoblast differentiation and generates mineralized nodule formation.

Nfic-Klf4-Dmp1-Dspp cascade may take control to regulate the odontoblast differentiation as previously reported. NFI-C gene expression initiates the expression of downstream genes. The binding of NFI-C gene-associated protein to the promoter area of KLF4 gene induces its expression and hence, DMP1 gene is expressed via the recruitment of KLF4 gene-associated protein to DMP1 gene promoter area. DMP1 thereupon induces the expression of the DSPP gene. (42, 51, 54) DMP1 and DSPP gene expression encourage

odontoblast maturation which is responsible for dentin extracellular matrix production and secretion including the collagenous and non-collagenous proteins as well as the mineralization. (14, 55) In the present study, HDACis treatment showed the increased expression of NFI-C gene was subsequently followed by the up-regulation of KLF4, ALP, DSPP, DMP1 and BSP genes and led to the enhancement of mineralization in hDPCs. Previous study (51) has reported that NFI-C was recruited to the KLF4 promoter area to induce KLF4 gene transcription which engages in mediating DSPP and DMP1 gene expression, because when NFI-C gene was knocked down in MDPC23 odontoblast cells, the protein level of KLF4, DMP1, and DSP were reduced. In contrast, the over-expression of NFI-C and KLF4 genes induced mineralization by up-regulating DSPP and DMP1 gene expression. A different suggestion was put forward by Kwon et al. (26), demonstrating that NFI-C protein was directly recruited to the DSPP promoter area and leading to the increase of DSPP gene expression in MDPC23 cells. The NFI-C gene knockdown by siRNA reduced NFI-C mRNA level and caused the decrease of odontogenic marker gene expression such as DSPP, DMP1, and Nestin.

Alkaline phosphate has been identified as a mineralization initiator by providing phosphate to initiate the mineralization process, and thus high ALP activity has been linked to cells forming mineralized tissue. (61) TSA enhanced ALP activity from the early onset of differentiation (72 hours) and gradually reduced at the later stage, which corresponds with the observed relative expression of ALP gene by Real Time RT-PCR. The acceleration pattern of ALP gene expression was found up to 7 days of TSA treatment and it was dropped subsequently at day-10. Whereas, SAHA enhanced ALP gene expression time-dependently from the early to intermediate stage of differentiation and maintained its expression up to

10 days. The acceleration pattern of ALP activity was also found in SAHA during odontoblast differentiation. Whereas, the increase in ALP activity in TSA came quickly at the beginning but, it decreased at the later stage. Lastly, the observed increase in VEGF gene and wound closure rate confirmed that the inhibitory effect of HDACis to promote histone acetylation, might mediate new blood vessel growth (60) and cell migration, respectively.

Evidently, HDACis can induce cell differentiation in pluripotent and adult stem cell populations (28), primary dental pulp cells/osteobalst (21, 59), and odontoblast/osteoblast cell lines (26, 29, 62). Notably, under appropriate condition and concentration, HDACis triggers the expression of a series of closely odontogenesis-related marker genes and mineralization process *in* vitro (26, 28, 29, 59) and *in* vivo (28). These findings may provide valuable information for the initial development of a potentially biocompatible material to induce reparative dentin formation that can be applied in direct application of vital pulp treatment. However, the authors recognized that more research is required regarding the HDACis effects on other cell types because HDACis is also known to regulate odontogenesis (26, 28, 29, 59), osteogenesis (21, 62), and chondrogenesis (63). The suitable carrier material for HDACis which can efficiently deliver HDACis agent to the damaged site of dental pulp tissue and release HDACis in appropriate concentration, should also be considered before clinical application, as well as the cross-talk effect between HDACis and the existing restorative material.

CONCLUSION

TSA and SAHA, pan HDAC inhibitors, promoted hDPCs differentiation into odontoblast-like cells and mineralized nodule formation in a dose-dependent manner without interfering with cell viability. The observed increase in gene expression related to odontoblast differentiation and maturation indicated that these two HDACis can epigenetically mediate odontoblast-like differentiation. The large mineralized nodules, strong positive alizarin red staining, and the elevated ALP activity also provided further evidence for the odontogenesis effects of HDACis. Because HDACis has been approved by FDA and used in several clinical treatments, they provide interesting candidates for regenerative biomaterials in the field of dental treatment.

REFERENCES

1. Kearney M, Cooper PR, Smith AJ, Duncan HF. Epigenetic Approaches to the Treatment of Dental Pulp Inflammation and Repair: Opportunities and Obstacles. Front Genet. 2018;9:311.

2. Tziafas D, Smith AJ, Lesot H. Designing new treatment strategies in vital pulp therapy. J Dent. 2000;28(2):77-92.

3. Hwang YC, Hwang IN, Oh WM, Park JC, Lee DS, Son HH. Influence of TGF-beta1 on the expression of BSP, DSP, TGF-beta1 receptor I and Smad proteins during reparative dentinogenesis. J Mol Histol. 2008;39(2):153-60.

4. Cox CF, Sübay RK, Ostro E, Suzuki S, SH. S. Tunnel defects in dentin bridges: their formation following direct pulp capping. . Operative Dentisry. 1996;21:4-11.

5. Faraco IM, Jr., Holland R. Response of the pulp of dogs to capping with mineral trioxide aggregate or a calcium hydroxide cement. Dent Traumatol. 2001;17(4):163-6.

6. Njeh A, Uzunoğlu E, Ardila-Osorio H, Simon S, Berdal A, Kellermann O, et al. Reactionary and reparative dentin formation after pulp capping: Hydrogel vs. Dycal. Evidence-Based Endodontics. 2016;1(1).

7. Murray PE, Garcia-Godoy F. The incidence of pulp healing defects with direct capping materials. Am J Dent. 2006;19(3):171-7.

8. Nowicka A, Lagocka R, Lipski M, Parafiniuk M, Grocholewicz K, Sobolewska E, et al. Clinical and Histological Evaluation of Direct Pulp Capping on Human Pulp Tissue Using a Dentin Adhesive System. Biomed Res Int. 2016;2016:2591273.

9. Cai S, Zhang W, Tribble G, Chen W. Reactions of human dental pulp cells to capping agents in the presence or absence of bacterial exposure. J Oral Sci. 2017;59(4):621-7.

10. Min KS, Park HJ, Lee SK, Park SH, Hong CU, Kim HW, et al. Effect of mineral trioxide aggregate on dentin bridge formation and expression of dentin sialoprotein and heme oxygenase-1 in human dental pulp. J Endod. 2008;34(6):666-70.

11. Khalil IT, Sarkis T, Naaman A. MM-MTA for direct pulp capping: a histologic comparison with ProRoot MTA in rat molars. J Contemp Dent Pract. 2013;14(6):1019-23.

12. Rodrigues EM, Cornelio ALG, Mestieri LB, Fuentes ASC, Salles LP, Rossa-Junior C, et al. Human dental pulp cells response to mineral trioxide aggregate (MTA) and MTA Plus: cytotoxicity and gene expression analysis. Int Endod J. 2017;50(8):780-9.

13. Nie X, Tian W, Zhang Y, Chen X, Dong R, Jiang M, et al. Induction of transforming growth factor-beta 1 on dentine pulp cells in different culture patterns. Cell Biol Int. 2006;30(4):295-300.

14. Teti G, Salvatore V, Ruggeri A, Manzoli L, Gesi M, Orsini G, et al. In vitro reparative dentin: a biochemical and morphological study. Eur J Histochem. 2013;57(3):e23.

15. Rodas-Junco BA, Canul-Chan M, Rojas-Herrera RA, De-la-Pena C, Nic-Can GI. Stem Cells from Dental Pulp: What Epigenetics Can Do with Your Tooth. Front Physiol. 2017;8:999.

16. Tziafas D. Characterization of Odontoblast-like Cell Phenotype and Reparative Dentin Formation In Vivo: A Comprehensive Literature Review. J Endod. 2019;45(3):241-9.

17. Thomas Pollard WE, Jennifer Lippincott-Schwartz, and Graham Johnson. Cell Biology. Third Edition ed. Philadelphia: Elsevier; 2016.

18. Lin Y, Zheng L, Fan L, Kuang W, Guo R, Lin J, et al. The Epigenetic Regulation in Tooth Development and Regeneration. Curr Stem Cell Res Ther. 2018;13(1):4-15.

19. Guidi CJ, Imbalzano AN. Chromatin Remodeling and Cancer. In: Pardee GSSaAB, editor. Cell Cycle and Growth Control. Second Edition ed: John Wiley & Sons, Inc; 2004. p. 265-95.

20. Duncan HF, Smith AJ, Fleming GJ, Cooper PR. Epigenetic modulation of dental pulp stem cells: implications for regenerative endodontics. Int Endod J. 2016;49(5):431-46.

21. Huynh NC, Everts V, Pavasant P, Ampornaramveth RS. Inhibition of Histone Deacetylases Enhances the Osteogenic Differentiation of Human Periodontal Ligament Cells. J Cell Biochem. 2016;117(6):1384-95.

22. Luo Z, Wang Z, He X, Liu N, Liu B, Sun L, et al. Effects of histone deacetylase inhibitors on regenerative cell responses in human dental pulp cells. Int Endod J. 2018;51(7):767-78.

23. Hyun-Jung Kim, Bae S-C. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res. 2011;3:166-79.

24. Klinz FJ, Korkmaz Y, Bloch W, Raab WH, Addicks K. Histone deacetylases 2 and 9 are coexpressed and nuclear localized in human molar odontoblasts in vivo. Histochem Cell Biol. 2012;137(5):697-702.

25. Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone Deacetylase Inhibitors as Anticancer Drugs. Int J Mol Sci. 2017;18(7):1414.

26. Kwon A, Park HJ, Baek K, Lee HL, Park JC, Woo KM, et al. Suberoylanilide hydroxamic acid enhances odontoblast differentiation. J Dent Res. 2012;91(5):506-12.

27. Huang W, Zhao S, Ammanamanchi S, Brattain M, Venkatasubbarao K, Freeman JW. Trichostatin A induces transforming growth factor beta type II receptor promoter

activity and acetylation of Sp1 by recruitment of PCAF/p300 to a Sp1.NF-Y complex. J Biol Chem. 2005;280(11):10047-54.

28. 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):613-24.

29. Duncan HF, Smith AJ, Fleming GJ, Cooper PR. Histone deacetylase inhibitors induced differentiation and accelerated mineralization of pulp-derived cells. J Endod. 2012;38(3):339-45.

30. Bruce Alberts DB, Karen Hopkin , Alexander Johnson , Julian Lewis , Martin Raff , Keith Roberts , and Peter Walter. Essential Cell Biology. Third Edition ed. New York: Garland Science, Taylor & Francis Group; 2010.

31. Huynh NC, Everts V, Ampornaramveth RS. Histone deacetylases and their roles in mineralized tissue regeneration. Bone Rep. 2017;7:33-40.

32. Duncan HF, Smith AJ, Fleming GJ, Cooper PR. HDACi: cellular effects, opportunities for restorative dentistry. J Dent Res. 2011;90(12):1377-88.

33. Tronick E, Hunter RG. Waddington, Dynamic Systems, and Epigenetics. Front Behav Neurosci. 2016;10:107.

34. Bhattacharya S, Zhang Q, Andersen ME. A deterministic map of Waddington's epigenetic landscape for cell fate specification. BMC systems biology. 2011;5:85-.

35. Vamsi Lavu, Vettriselvi Venkatesan, Rao SR. The epigenetic paradigm in periodontitis pathogenesis. Journal of Indian Society of Periodontology. 2015;19:142-9.

36. Herlofsen SR, Bryne JC, Hoiby T, Wang L, Issner R, Zhang X, et al. Genome-wide map of quantified epigenetic changes during in vitro chondrogenic differentiation of primary human mesenchymal stem cells. BMC Genomics. 2013;14:105.

37. Bin Mohsin AH, Barshaik S. Epigenetics in Dentistry: A Literature Review. Journal of Clinical Epigenetics. 2017;03(01).

38. El-Zainy MA, Nour El-Haddad K, Obeid RF. Efficacy of transforming growth factor- β on development of ameloblasts and odontoblasts in tooth germ of young albino rat. Future Dental Journal. 2017;3(2):39-46.

39. Wang T, Liu H, Ning Y, Xu Q. The histone acetyltransferase p300 regulates the expression of pluripotency factors and odontogenic differentiation of human dental pulp cells. PLoS One. 2014;9(7):e102117.

40. Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res. 2007;5(10):981-9.

41. Duncan HF, Smith AJ, Fleming GJ, Partridge NC, Shimizu E, Moran GP, et al. The Histone-Deacetylase-Inhibitor Suberoylanilide Hydroxamic Acid Promotes Dental Pulp Repair Mechanisms Through Modulation of Matrix Metalloproteinase-13 Activity. J Cell Physiol. 2016;231(4):798-816.

42. Tao H, Lin H, Sun Z, Pei F, Zhang J, Chen S, et al. Klf4 Promotes Dentinogenesis and Odontoblastic Differentiation via Modulation of TGF-beta Signaling Pathway and Interaction With Histone Acetylation. J Bone Miner Res. 2019;34(8):1502-16.

43. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in

<em>vivo</em&gt. Proceedings of the National Academy of Sciences. 2000;97(25):13625.

44. Sara Saffar Soflaei, Amir Abbas Momtazi-Borojeni, Muhammed Majeed, Giuseppe Derosa, Pamela Maffioli, Sahebkar A. Curcumin: A Natural Pan-HDAC Inhibitor in Cancer. Current Pharmaceutical Design. 2018;24:123-9.

45. Tompkins K. Molecular mechanisms of cytodifferentiation in mammalian tooth development. Connect Tissue Res. 2006;47(3):111-8.

46. Miletich I, Sharpe PT. Neural crest contribution to mammalian tooth formation. Birth Defects Res C Embryo Today. 2004;72(2):200-12.

47. Asgary S, Nazarian H, Khojasteh A, Shokouhinejad N. Gene expression and cytokine release during odontogenic differentiation of human dental pulp stem cells induced by 2 endodontic biomaterials. J Endod. 2014;40(3):387-92.

48. Smith AJ, Lesot H. Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair? Crit Rev Oral Biol Med. 2001;12(5):425-37.

49. Tziafas D. Basic mechanisms of cytodifferentiation and dentinogenesis during dental pulp repair. Int J Dev Biol. 1995;39(1):281-90.

50. Li Y, Lü X, Sun X, Bai S, Li S, Shi J. Odontoblast-like cell differentiation and dentin formation induced with TGF0²1. Archives of oral biology. 2011;56 11:1221-9.

51. Lee HK, Lee DS, Park SJ, Cho KH, Bae HS, Park JC. Nuclear factor I-C (NFIC) regulates dentin sialophosphoprotein (DSPP) and E-cadherin via control of Kruppel-like factor 4 (KLF4) during dentinogenesis. J Biol Chem. 2014;289(41):28225-36.

52. Steele-Perkins G, Butz KG, Lyons GE, Zeichner-David M, Kim HJ, Cho MI, et al. Essential role for NFI-C/CTF transcription-replication factor in tooth root development. Mol Cell Biol. 2003;23(3):1075-84.

53. Lee DS, Park JT, Kim HM, Ko JS, Son HH, Gronostajski RM, et al. Nuclear factor I-C is essential for odontogenic cell proliferation and odontoblast differentiation during tooth root development. J Biol Chem. 2009;284(25):17293-303.

54. Roh SY, Park JC. The role of nuclear factor I-C in tooth and bone development. J Korean Assoc Oral Maxillofac Surg. 2017;43(2):63-9.

55. Yamakoshi Y. Dentinogenesis and Dentin Sialophosphoprotein (DSPP). J Oral Biosci. 2009;51(3):134.

56. Suzuki S, Haruyama N, Nishimura F, Kulkarni AB. Dentin sialophosphoprotein and dentin matrix protein-1: Two highly phosphorylated proteins in mineralized tissues. Arch Oral Biol. 2012;57(9):1165-75.

57. Chen S, Unterbrink A, Kadapakkam S, Dong J, Gu TT, Dickson J, et al. Regulation of the Cell Type-specific dentin sialophosphoprotein gene expression in mouse odontoblasts by a novel transcription repressor and an activator CCAAT-binding factor. J Biol Chem. 2004;279(40):42182-91.

58. Ouellet S, Vigneault F, Lessard M, Leclerc S, Drouin R, Guerin SL. Transcriptional regulation of the cyclin-dependent kinase inhibitor 1A (p21) gene by NFI in proliferating human cells. Nucleic Acids Res. 2006;34(22):6472-87.

59. Duncan HF, Smith AJ, Fleming GJ, Cooper PR. Histone deacetylase inhibitors epigenetically promote reparative events in primary dental pulp cells. Exp Cell Res. 2013;319(10):1534-43.

60. Saghiri MA, Asatourian A, Sorenson CM, Sheibani N. Role of angiogenesis in endodontics: contributions of stem cells and proangiogenic and antiangiogenic factors to dental pulp regeneration. J Endod. 2015;41(6):797-803.

61. Woltgens JH, Lyaruu DM, Bronckers AL, Bervoets TJ, Van Duin M. Biomineralization during early stages of the developing tooth in vitro with special reference to secretory stage of amelogenesis. Int J Dev Biol. 1995;39(1):203-12.

62. Dudakovic A, Evans JM, Li Y, Middha S, McGee-Lawrence ME, van Wijnen AJ, et al. Histone deacetylase inhibition promotes osteoblast maturation by altering the histone H4 epigenome and reduces Akt phosphorylation. The Journal of biological chemistry. 2013;288(40):28783-91.

63. Lee J, Im GI. Effects of Trichostatin A on the Chondrogenesis from Human Mesenchymal Stem Cells. Tissue Eng Regen Med. 2017;14(4):403-10.

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