Journal of Dental Sciences 19 (2024) 2323–2331

Available online at www.sciencedirect.com

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Original Article

Sphingosine-1-phosphate receptor 1-mediated odontogenic differentiation of mouse apical papilla-derived stem cells

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Received 24 January 2024; Final revision received 5 February 2024
Available online 13 February 2024

KEYWORDS
Odontogenic differentiation;
Regenerative endodontics;
Sphingosine-1-phosphate (S1P);
S1P receptor 1;
Stem cells on the apical papilla

Abstract *Background/purpose:* Sphingosine-1-phosphate (S1P) exhibits receptor-mediated physiological effects by facilitating the differentiation of mesenchymal stem cells toward the osteoblast lineage. This study aimed to determine the effect of S1P on odontogenic differentiation of mouse immortalized stem cells of dental apical papilla (iSCAP) and assess the distribution of the S1P receptor 1 (S1PR1) in the apical papilla and the root canal wall of immature rat molars.
Materials and methods: Immunostaining for S1PR1 was conducted at the apex of the rat mandibular first molar and within the root canal wall. The iSCAP was treated with S1P and bone morphogenetic protein (BMP)-9 (for comparison), and the expression levels of the odontogenic differentiation marker were evaluated via real-time reverse-transcriptase quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. Mineralization and lipid droplet formation were evaluated via Alizarin red and Oil red O staining.
Results: S1PR1-positive cells were expressed in areas of both apical papilla and dentin-pulp interface of root canal wall. During the odontogenic differentiation of iSCAP, S1P and BMP-9 increased the expression of the differentiation marker mRNA and secreted proteins including dentin sialophosphoprotein, dentin matrix phosphoprotein 1, and matrix extracellular phosphoglycoprotein. The S1PR1 signaling pathway is involved in the action of S1P, but not that of BMP-9. S1PR1 signaling also facilitated mineralization in iSCAP and suppressed the

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https://doi.org/10.1016/j.jds.2024.02.004

differentiation of these cells into adipocytes.
Conclusion: S1P induced odontogenic differentiation of iSCAP through S1PR1. Furthermore, S1PR1-positive cells were expressed in the apical papilla of immature rat molars and in the dentin-pulp interface where odontoblast-like cells exist.

Introduction

Currently, investigators are actively exploring the field of regenerative endodontics, and pulp revascularization is a technique involving the induction of odontogenic differentiation[1] in stem cells derived from the apical papilla (SCAP) located at the root apex. For immature permanent teeth with pulp necrosis, inducing bleeding at the root apex and guiding SCAP into the root canal result in changes in the root canal wall thickness, elongation of the root, and closure of the apex. This process is expected to elicit vital responses in the dental pulp.[2] Nevertheless, according to prior studies, the hard tissue that develops at the root is not dentin but rather bone or cementum.[3] Consequently, the mechanism underlying the differentiation of SCAPs into odontoblasts must be better understood. SCAPs exhibit notable tissue regeneration capabilities and can differentiate into odontogenic, neurogenic, and adipogenic lineages, similar to dental pulp stem cells (DPSCs).[1] Recent findings indicate that transforming growth factor (TGF)-β2/Smad signals play a pivotal role in promoting the differentiation of SCAPs into odontoblasts, concurrently contributing to elevated expression of dentin sialophosphoprotein (DSPP) and dentin matrix phosphoprotein 1 (DMP1).[4] However, the specific details have not been elucidated.

Dentin constitutes the principal calcified tissue in teeth, with odontoblasts actively synthesizing and secreting both collagenous proteins and non-collagenous proteins (NCPs) to establish the extracellular matrix (ECM). NCPs play a crucial role in regulating the mineralization process. Notably, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are products of the DSPP gene.[5] DSPP is the most expressed NCPs and is used as an early odontogenic differentiation marker. DMP1 is a differentiation marker that has a similar structure to DSPP. DMP1 is an acidic ECM protein expressed in dentin and bone.[6] According to prior studies, DMP1 binds to αvβ3 integrin on the cell surface in the ECM of human mesenchymal stem cells and osteoblast-like cells, thereby participating in the formation of adhesion points.[7] Matrix extracellular phosphoglycoprotein (MEPE), which belongs to the SIBLING family of proteins, is expressed in both dentin and bone. MEPE is implicated in dentin formation and mineralization, serving as a marker for late-stage odontogenic differentiation. Furthermore, MEPE is considered to play a significant role in the homeostasis of the dentin-pulp complex.[8]

Sphingosine-1-phosphate (S1P), a lipid mediator present in living organisms, is generated by phosphorylation of sphingosine to sphingosine kinase (Sphk) 1 and Sphk2.[9] By activating the S1P signaling pathway via S1P receptors (S1PR1-5), S1P functions as a signaling molecule, affecting physiological processes, such as cell differentiation, proliferation, and migration.[9] S1P, its receptors, and enzymes responsible for its synthesis and degradation have been demonstrated to stimulate bone formation and suppress bone resorption.[10] The activation of S1PR1/Akt/β-catenin and S1PR2/RhoA/ROCK signaling pathways facilitates osteoblast differentiation, leading to an augmentation in bone mass within the cancellous bone of the mouse tibia.[11–13] In addition, S1P can activate the differentiation of mesenchymal stem cells into osteoblasts. Regarding the differentiation of multipotent stem cells, we revealed that S1P promotes osteogenic differentiation by increasing the expression of low-density lipoprotein receptor-related protein (LRP) 5[14] and suppresses adipogenic differentiation by inhibiting cAMP accumulation mediated by S1PR1 signals.[15] S1P has also been demonstrated to promote osteogenic differentiation of bone marrow stem cells (BMSCs) via mouse S1PR2.[16] Conversely, in terms of the development and maintenance of teeth, which share similarities with bones as hard tissues, S1P has been reported to augment migration in DPSCs[17] and contribute to stem cell homeostasis through S1P/Akt/ERK signals in dental pulp cells.[18] However, the impact of S1P signaling on odontogenic differentiation has not been elucidated. Based on the literature, S1PR1 is expressed in rat apical periodontitis lesions and plays a role in bone resorption.[19,20] Nonetheless, the expression of S1P receptors at the site of SCAPs or odontoblasts in the root apex remains undocumented. Therefore, we investigated the distribution of the S1PR1 in periapical region of immature rat molars and evaluated the effect of S1P on odontogenic differentiation of SCAPs. Recently, an immortalized mouse SCAP line was successfully established, and the effect of bone morphogenetic protein-9 (BMP-9) on the odontogenic differentiation of SCAP was documented.[21] Therefore, we compared the effects of S1P to those of BMP-9.

Materials and methods

Reagents

S1P ready made solution (Sigma, St. Louis, MO, USA) and BMP-9 (Recombinant Mouse BMP-9; R&D Systems, Inc., Minneapolis, MN, USA) were employed in this experiment. W146 (Cayman Chemical, Ann Arbor, MI, USA) was used as the S1PR1 inhibitor.[12,22] Immunohistochemical staining was carried out with the following antibodies: anti-S1PR1 (1:100, Cayman Chemical) as primary antibodies and

horseradish peroxidase-conjugated polymer anti-rabbit antibody (Agilent Technologies, Santa Clara, CA, USA) as the secondary antibody.

Periapical staining of immature rat molars

Five-week-old rats with immature molars were used in the experiment (n = 3). Sequential tissue specimens comprising paraffin sections with a thickness of 4 μm around the mesial roots of mandibular first molars were prepared using conventional methods. Hematoxylin and eosin staining was performed according to the manufacturer's instructions. The sections were deparaffinized, subjected to antigen retrieval in citrate buffer (pH 6.0) at 95 °C for 40 min, and blocked. Thereafter, the cells were incubated with primary antibodies against S1PR1 overnight at 4 °C and then with the secondary antibodies. Finally, the sections were stained with diaminobenzidine and the nuclei were counterstained with hematoxylin. The sections were observed using an optical microscope (BX51TRF: Olympus Corporation., Tokyo, Japan). The experiment was approved by the Animal Care Committee of Fukuoka Dental College (approval number No. 20017).

Cell culture

Mouse immortalized stem cells isolated from the dental apical papilla tissue of lower incisor teeth (iSCAP: Applied Biological Materials Inc., Vancouver, Canada) were employed in this study. These cells expressed many MSC markers and had multipotent properties to differentiate into osteogenic/odontogenic, chondrogenic, and adipogenic lineages.[21] The cells were cultured in Prigrow III medium (Applied Biological Materials Inc.) containing 10% fetal bovine serum (Corning Inc., Corning, NY, USA) and 1% penicillin/streptomycin solution (Sigma) at 37 °C in a 5% CO_2 atmosphere.

For odontogenic differentiation, the cells were seeded at 80% confluence and cultured in Osteoblast-Inducer Reagent (Takara Bio Inc., Shiga, Japan) containing 2% ascorbic acid, 0.4% hydrocortisone, and 0.4% β-glycerophosphate. S1P (1 μM) or BMP-9 (1 ng/ml) was introduced 24 h prior to the initiation of odontogenic differentiation, with the medium replaced every 3 days thereafter.

Real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using RNAiso Plus (Takara Bio Inc.). Thereafter, 500 ng of single-stranded cDNA was synthesized using the PrimeScript RT Master Mix kit (Takara Bio Inc.). RT-qPCR was performed on a StepOne system (Thermo Fisher Scientific Inc., Waltham, MA, USA) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Inc.). GAPDH was used as the internal control. The primers used in the experiment were synthesized based on sequences obtained from the GenBank database (Table 1).

Enzyme-linked immunosorbent assay (ELISA)

The levels of the secreted proteins in the culture supernatant were measured using ELISA based on the manufacturer's protocol. Mouse DSPP ELISA Kit (Bioassay Technology Laboratory, Shanghai, China), DMP1 ELISA Kit (Abcam PLC., Cambridge, UK), and MEPE ELISA Kit (ViroQuest Corporation. Osaka, Japan) were employed. The optical density was measured using an Infinite F50 Plus (Tecan Group Ltd., Männedorf, Switzerland) at an absorbance of 450 nm.

Alizarin red S staining

Following a 4-week incubation in an odontogenic induction medium, the cells were washed thrice with phosphate-buffered saline (PBS), fixed with methanol (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) at 4 °C for 20 min, washed three times with purified water, and stained with Alizarin Red S (Cosmo Bio Co., Ltd., Tokyo, Japan) for 30 min at room temperature. The cells were then rinsed with purified water and treated with 5% formic acid (PG Research Inc., Tokyo, Japan), which served as a dissolving solution for calcified nodules, to extract the pigment. The absorbance of each eluate was quantified at 405 nm using a 1420 Multilabel Counter ARVO MX (PerkinElmer, Inc., Waltham, MA, USA).

Oil red O staining

Lipid droplet formation was initiated with Adipoinducer Reagent (Takara Bio Inc.) containing an adipogenic cocktail of 10 μg/ml insulin, 2.5 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. Following a 2-day incubation in adipogenic medium, the medium was replaced with maintenance medium containing 10 μg/ml insulin. The cells were cultured for an additional 2 days, fixed for 20 min at room temperature using PBS containing 10% paraformaldehyde, and stained with a solution composed of 1.8 mg/ml Oil Red O (Sigma) and 60% (vol/vol) isopropanol (Fujifilm Wako Pure Chemical Corporation) for 60 min at 37 °C. Thereafter, the cells were washed with 70% ethanol (Fujifilm Wako Pure Chemical Corporation) and distilled water; the dye was eluted using 100% isopropanol. The absorbance of each eluate at 540 nm was measured using 1420 Multilabel Counter ARVO MX. Images were captured using a fluorescence microscope (BZ-X710: Keyence Co., Osaka, Japan).

Statistical analyses

The results are presented a standard error of the mean (SEM). Significance was determined using Student's t-test (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA). A P value < 0.05 was considered to indicate statistical significance.

Results

Expression of sphingosine-1-phosphate receptor 1-positive cells around the periapical area of immature rat molars and at the dentin-pulp interface of the root canal wall

First, we investigated the expression of S1PR1-positive cells around the root apex of immature rat molars. S1PR1-

Table 1 RT-qPCR primers.

GenBank ID	Target gene	Primer sequence forward/reverse	Size of amplified products (bp)
NM_007901	mouse S1PR1	5′- TTCTCATCTGCTGCTTCATCATCC -3′ 5′- GGTCCGAGAGGGCTAGGTTG -3′	117
NM_010333	mouse S1PR2	5′- TTACTGGCTATCGTGGCTCTG -3’ 5′- ATGGTGACCGTCTTGAGCAG -3′	107
NM_001146038	mouse Runx2	5′- ACTCTTCTGGAGCCGTTTATG -3’ 5′- GTGAATCTGGCCATGTTTGTG-3′	102
NM_007431	mouse ALP	5′- GTTGCCAAGCTGGGAAGAACAC -3′ 5′- CCCACCCCGCTATTCCAAAC -3′	121
NM_009263	mouse OPN	5′- TACGACCATGAGATTGGCAGTGA -3′ 5′- TATAGGATCTGGGTGCAGGCTGTAA -3′	114
NM_001032298	mouse OCL	5′- GAACAGACTCCGGCGCTA -3′ 5′- AGGGAGGATCAAGTCCCG -3′	227
NM_010080	mouse DSPP	5′- AACTCTGTGGCTGTGCCTCT -3′ 5′- TATTGACTCGGAGCCATTCC -3′	170
NM_016779	mouse DMP1	5′- AGCTGGGCTACATTGCTTTGG -3′ 5′- TCCAGGCTTTGCTACTGTGGAAC -3′	155
NM_053172	mouse MEPE	5′- AGCAAATGCCCAGAGACTAAGCCC -3′ 5′- TGAGGCCCTCTGGCGTCATTCA -3′	69
NM_008084	mouse GAPDH	5′- TGTGTCCGTCGTGGATCTGA -3′ 5′- TTGCTGTTGAAGTCGCAGGAG -3′	150

Sphingosine-1-phosphate receptors (S1PR1 and S1PR2), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCL), dentin sialophosphoprotein (DSPP), dentin matrix phosphoprotein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

positive cells are expressed in both the apical papilla (Fig. 1A and B) and the dentin-pulp interface of the root canal wall (Fig. 1C and D).

Sphingosine-1-phosphate increases the expression of sphingosine-1-phosphate receptor 1 in immortalized stem cells of dental apical papilla

We next examined the impact of S1P on the mRNA expression of the S1P receptors during odontogenic differentiation of iSCAP. In the presence of the odontogenic induction medium, the mRNA expression of S1PR1 gradually increased, with a significant increase of approximately 2-fold obtained on days 7 and 14 with the addition of S1P (Fig. 2A). Although the mRNA expression of S1PR2 increased after reaching its peak on day 7, no discernible effect was observed upon the addition of S1P (Fig. 2B). Conversely, the addition of BMP-9 resulted in a notable increase in the mRNA expression of S1PR1 on day 7; however, such addition did not have a significant effect on the mRNA expression of S1PR2 (Fig. 2A and B). In non-odontogenic induction media, S1PR1 mRNA expression was significantly increased by the addition of S1P on day 14 of culture (Supplementary Fig. 1A). However, BMP-9 addition had no effect on the mRNA expression of either S1PR1 or S1PR2 (Supplementary Fig. 1B).

Sphingosine-1-phosphate promotes odontogenic differentiation of immortalized stem cells of dental apical papilla via sphingosine-1-phosphate receptor 1

Owing to the observed increase in S1PR1 mRNA expression with the addition of S1P, we proceeded to explore the impact of S1PR1-mediated signals on the odontogenic differentiation of iSCAP. Fig. 2 shows the impact of S1P on the

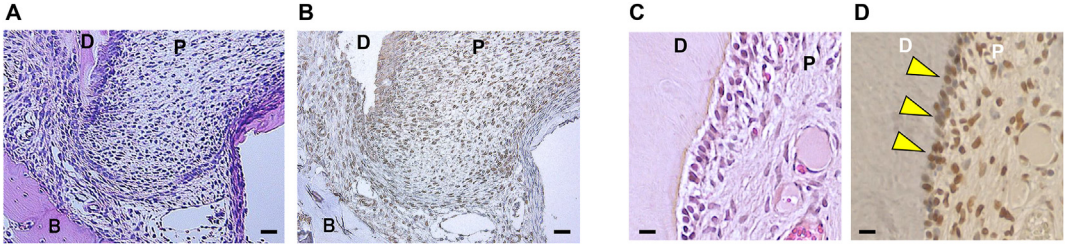

Figure 1 Sphingosine-1-phosphate receptor 1 (S1PR1)-positive cell expression in the mesial root apex of the mandibular first molar in a 5-week-old rat. (A) Hematoxylin and eosin (HE) staining of periapical region. × 100 magnification. Scale bar: 100 μm. B: bone, D: dentin, P: dental pulp. (B) S1R1 immunostaining of periapical region. × 100. (C) HE staining of the root canal wall. × 200. (D) S1R1 immunostaining of the root canal wall. × 200. Arrowhead: S1PR1-positive cells.

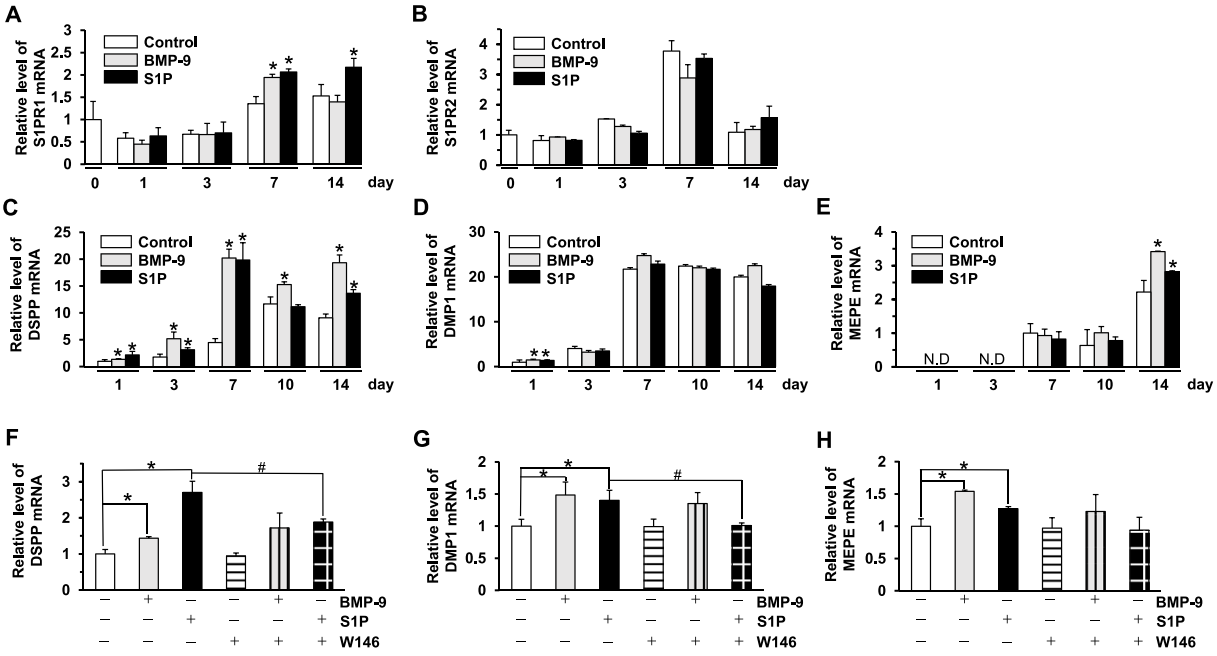

Figure 2 Effects of sphingosine-1-phosphate (S1P) on the mRNA expression of S1P receptors (S1PR1 and S1PR2) and odontogenic differentiation marker genes including dentin sialophosphoprotein (DSPP), dentin matrix phosphoprotein 1 (DMP1), and matrix extracellular phosphoglycoprotein (MEPE) in iSCAP. (A–E) A total of 2.0×10^5 cells was seeded in a 60 mm dish and treated with S1P (1 μM) or bone morphogenetic protein-9 (BMP-9; 1 ng/ml). The cells were cultured for 24 h and then maintained for 1–14 days in odontogenic induction medium. (F–H) After the cells were seeded and cultured in serum-free medium for 24 h, an S1PR1 inhibitor (W146; 10 μM) was applied for 30 min. Thereafter, the cells were cultured either for 1 day (F: DSPP, G: DMP1) or 14 days (H: MEPE) in an odontogenic induction medium. The mRNA level of each gene was quantified via RT-qPCR. Values are expressed as SEM (n = 3). N. D.: Not detected. $^*P < 0.05$ vs control, $^{\#}P < 0.05$ vs S1P based on the Student's t-test.

mRNA expression of odontogenic differentiation marker genes, such as DSPP, DMP1, and MEPE. The expression of the early differentiation marker, DSPP, significantly increased on days 1, 3, 7, and 14 following the addition of S1P. Moreover, the expression of DMP1 significantly increased on day 1 while that of the late differentiation marker, MEPE, significantly increased on day 14 compared with the expression of the control (Fig. 2A–C). The addition of BMP-9 led to a significant increase in the expression of DSPP on days 1 to 14, DMP1 on day 1, and MEPE on day 14 (Fig. 2A–C). We also explored the effect of S1P on the mRNA expression of osteo/odontogenic differentiation markers, including Runx2, alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCL), in cells cultured in non-odontogenic induction medium. The expression level of ALP was increased by the addition of S1P and BMP-9, and that of OCL, was increased by the addition of BMP-9. No clear differences were observed for Runx2 or OPN (Supplementary Fig. 2). The S1PR1 inhibitor, W146, was subsequently used to investigate the influence of S1PR1 signals on the enhanced expression of the odontogenic differentiation marker mRNA. DSPP and DMP1 were assessed on day 1 (Fig. 2D and E), where significant differences were observed, while MEPE was examined on day 14 (Fig. 2F). The increase in DSPP and DMP1 mRNA expression caused by S1P was significantly suppressed by S1PR1 inhibition, and MEPE also tended to be suppressed (Fig. 2D–F). However, BMP-9-induced increase in the expression of these differentiation marker mRNAs was not affected by S1PR1 inhibition (Fig. 2D–F).

Fig. 3 shows the effects of S1P on the secreted amounts of DSPP, DMP1, and MEPE. Compared to that observed with the control, significant increases in DSPP were noted on days 3 and 7 following the addition of S1P, while significant increases were observed on day 1 for DMP1 and on day 7 for MEPE (Fig. 3). Conversely, with the addition of BMP-9, a notable increase in DSPP was observed on day 7 (Fig. 3A). DMP1 exhibited an increasing trend on days 1, 3, and 7, while MEPE exhibited an increasing trend on days 7 and 10; however, no statistically significant difference was observed (Fig. 3B and C). Further, the S1P-induced increases in the secretion of DSPP (days 3 and 7), DMP1 (day 1), and MEPE (day 7) were significantly suppressed by S1PR1 inhibition. However, in the BMP-9 supplemented group, no effect of S1PR1 inhibition was observed (Fig. 3).

Sphingosine-1-phosphate promotes mineralization and suppresses lipid droplet formation via sphingosine-1-phosphate receptor 1

We examined mineralization in iSCAP. The addition of S1P resulted in a significant increase in mineralization compared to that observed with the control. Notably, a similar significant calcifying effect was observed with the addition of BMP-9 (Fig. 4A). This promotion of mineralization by S1P was significantly suppressed by the inhibition of S1PR1; however, when induced by BMP-9, this effect was not affected by S1PR1 inhibitor (Fig. 4A). To validate the pluripotency of iSCAP, we investigated lipid droplet

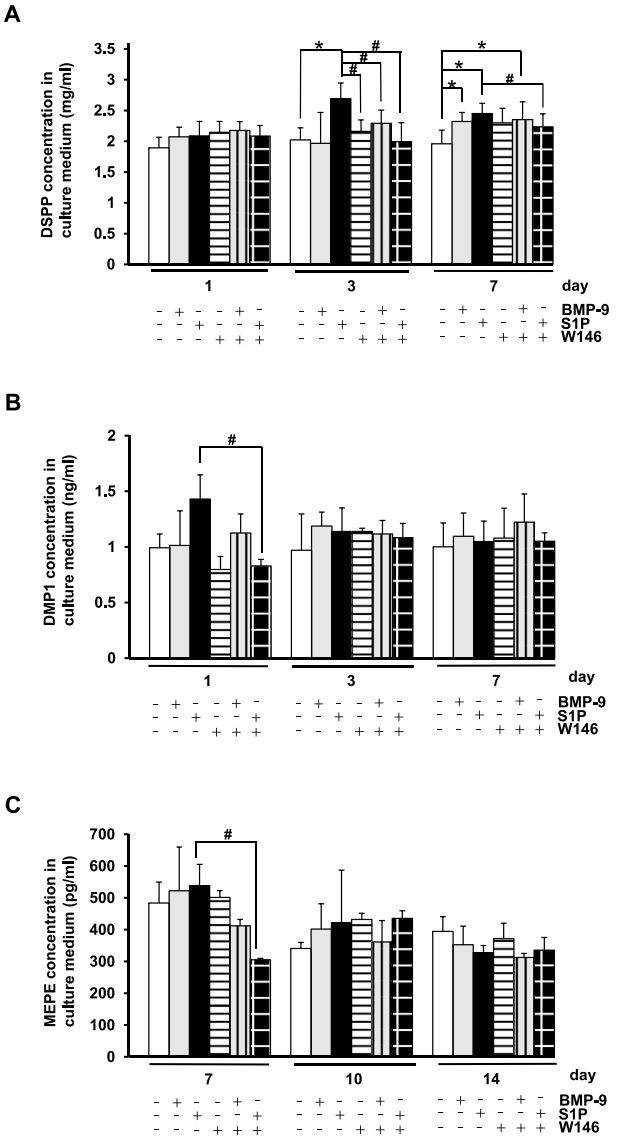

Figure 3 Increased expression of secreted odontogenic differentiation marker proteins through sphingosine-1-phosphate receptor 1 (S1PR1). Effects of sphingosine-1-phosphate (S1P) and bone morphogenetic protein-9 (BMP-9) on the secreted protein levels of odontogenic differentiation markers including dentin sialophosphoprotein (DSPP), dentin matrix phosphoprotein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE) in iSCAP. A total of 2.0×10^5 cells was seeded in a 60 mm dish, treated with S1P (1 μM) or BMP-9 (1 ng/ml), and cultured for 24 h. Thereafter, the cells were cultured for 1—7 days (A: DSPP, B: DMP1) or 7—14 days (C: MEPE) in an odontogenic induction medium. In terms of the S1PR1 inhibitor, after the cells were seeded and cultured in serum-free medium for 24 h, the inhibitor (W146; 10 μM) was applied for 30 min. Thereafter, the cells were cultured in odontogenic induction medium for the indicated period. Values are expressed as SEM ($n = 3$). *$P < 0.05$ vs control, $^{\#}P < 0.05$ vs S1P based on the Student's t-test.

formation through Oil Red O staining in the context of adipogenic differentiation owing to its reciprocal relationship with bone cells. The addition of S1P significantly suppressed lipid droplet formation compared to that observed under control conditions (Fig. 4B). In addition, lipid droplet formation was observed upon S1PR1 inhibition. However, the addition of BMP-9 had no effect on lipid droplet formation. Similarly, the inhibition of S1PR1 had no effect on lipid droplet formation (Fig. 4B).

Discussion

A high prevalence of S1PR1-positive cells was observed around the apical papilla of immature rat molars and at the dentin-pulp interface, particularly in the region occupied by odontoblasts. These findings suggest that S1PR1 may play a role in dentin formation. On the other hand, the intracellular expression levels of S1PR fluctuate in response to diverse extracellular stimuli, including inflammatory signals.[9] Therefore, a further investigation is required into the relationship between S1PR1 and other S1PR signaling pathways such as S1PR2 in dentin formation under different physiological conditions. Regarding the effect of S1P signaling on odontogenic differentiation of iSCAP, our findings indicate that the S1PR1 signaling might be involved in the increase in expression of DSPP, DMP1, and MEPE. In particular, the mRNA levels of DSPP, which is involved in the early stages of dentin formation, was significantly increased by the addition of S1P. Loss of DSPP is associated with a reduction in dentin thickness, an enlarged pulp cavity, hypomineralization, and extensive pulp exposure.[23] Furthermore, previous studies have demonstrated that the teeth of DSPP knockout mice are similar to those observed in individuals with human dentinogenesis imperfecta type III.[24] As DSPP is associated with the highest expression among the NCPs constituting the dentin matrix,[5] the principal role of S1P in odontogenic differentiation may involve its contribution to mineralization of the dentin matrix by facilitating DSPP expression. In this study, the mRNA and secreted protein levels of DMP1 markedly increased the day after S1P addition. This increase was also significantly suppressed by the inhibition of S1PR1. Although the expression of DMP1 was not markedly affected by S1P compared to DSPP, DMP1 shares a relatively similar structure with DSPP. Consequently, some of its expression may be influenced by S1PR1. The expression of DMP1 is regulated by the same Wnt/β-catenin canonical signal as DSPP.[25] Conversely, MEPE expression, the late differentiation marker, was not found on day 1, but detectable from day 7, and the effects of S1P were not as pronounced in terms of the mRNA and secreted protein levels as those obtained for DSPP. The quantity of secreted MEPE protein in this study was approximately 1/4000 that of DSPP, suggesting that this discrepancy may serve as a contributing factor.

Based on clinical reports, the hard tissue formed at the root apex following pulp revascularization is identified as either bone or cementum.[3,26] As PDLSCs and BMSCs are also present around the root apex, osteogenic differentiation by these stem cells that have invaded the root canal may be a contributing factor to the formation of ectopic hard tissue.[27] In addition, DSPP may be involved in bone formation;[28] hence, the identification of genes pivotal in regulating differentiation into dentin and bone holds

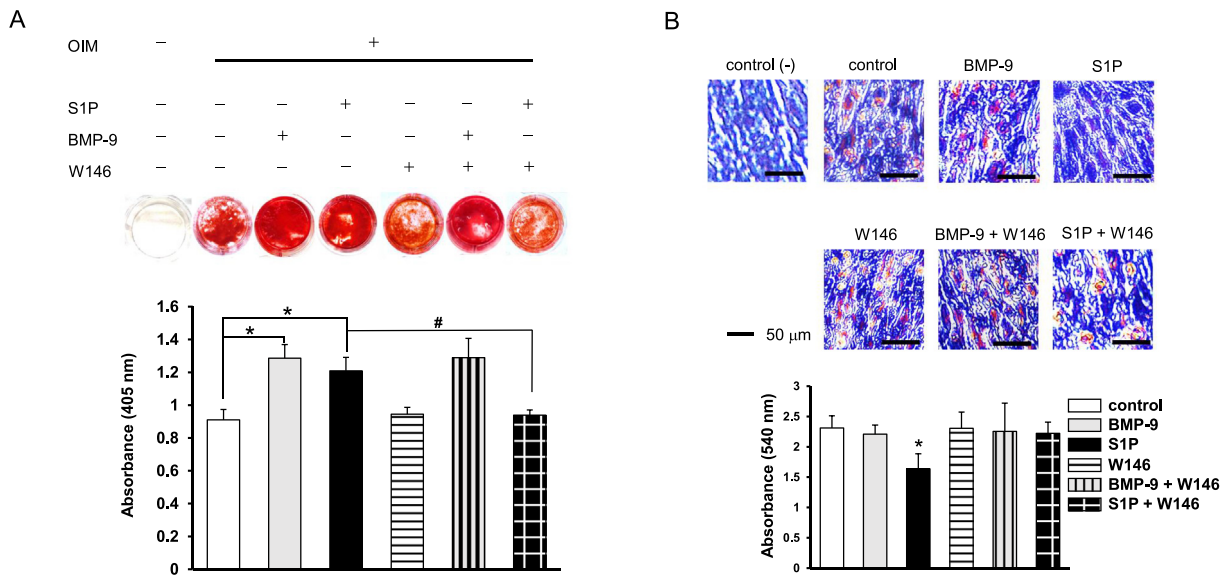

Figure 4 Effect of sphingosine-1-phosphate receptor 1 (S1PR1) signaling on mineralization and lipid droplet formation. (A) Evaluation of mineralization via Alizarin red S staining. A total of 1.8×10^4 cells was seeded in a 24-well plate and then treated with sphingosine-1-phosphate (S1P; 1 μM) or bone morphogenetic protein-9 (BMP-9; 1 ng/ml). The cells were cultured for 24 h and then maintained for 28 days in an odontogenic induction medium (OIM). In terms of the S1PR1 inhibitor, after the cells were seeded and cultured in serum-free medium for 24 h, the inhibitor (W146; 10 μM) was applied for 30 min. Thereafter, the cells were cultured in OIM. Values are expressed as SEM (n = 3). $^*P < 0.05$ vs control, $^{\#}P < 0.05$ vs S1P based on the Student's t-test. (B) Evaluation of lipid droplet formation via Oil red O staining. A total of 1.8×10^4 cells were seeded in a 24-well plate and then treated with S1P (1 μM) or BMP-9 (1 ng/ml). The cells were cultured for 2 days with adipogenic induction medium and then maintained for 2 days. In terms of the S1PR1 inhibitor, after the cells were seeded and cultured in serum-free medium for 24 h, the inhibitor (W146; 10 μM) was applied for 30 min × 20. Values are expressed as SEM (n = 3). $^*P < 0.05$ vs control based on the Student's t-test.

significance as a strategy for inducing dentin regeneration. In this study, the addition of S1P increased ALP and OCL expression in iSCAP, but did not affect Runx2 and OPN expression. On the other hand, we have previously reported increased expression of Runx2 and OPN in S1P-induced osteogenic differentiation.[12] Therefore, it may be possible that these two genes are candidates for specific regulators of odontogenic and osteogenic differentiation. However, it has been demonstrated that increased expression of Runx2 by β-catenin and TGF-β1 in DPSCs promotes odontogenic differentiation.[29,30] Further, there are also reports that increased expression of OPN by Wnt5a in human dental papilla cells[31] and histone acetyltransferase p300 in dental pulp cells[32] promotes odontogenic differentiation. It is currently unclear whether this discrepancy is cell specific, but future works are needed to identify specific factors or master genes required for commitment to odontogenic differentiation.

On the generation of S1P, Sphk1 is present in the cytoplasm and produces S1P when phosphorylated, part of which is excreted out of the cell and stimulates the S1P receptor, thereby exhibiting autocrine/paracrine effects.[9] On the other hand, Sphk2 produces S1P by binding to histone H3 in the nucleus. This S1P binds to histone deacetylase and suppresses its activity, resulting in an increase histone acetylation, and thereby promoting transcriptional activity.[33] This suggests that S1P is involved in the regulation of gene expression by epigenetic mechanisms in response to environmental factors including pathological conditions. In dentin formation, increased histone acetylation has been reported to promote p300-and histon deacetylase3-regulated odontogenic differentiation.[34] Further studies are needed on the relationship between epigenetic mechanisms and dentinogenesis in addition to the intracellular targets of S1P.

In this study, BMP-9 was found to promote odontogenic differentiation marker expression and mineralization to the same extent as S1P. However, as the inhibition of S1PR1 did not exert an impact, the regulation of odontogenic differentiation-related gene expression by BMP-9 signaling may be S1PR1 signal-independent, or alternatively, secreted BMP-9 via S1PR1 signaling could act in an autocrine manner. According to a recent report, BMP-9 activates PI3K/Akt/MDM2 signaling in periosteal-derived stem cells, thereby inducing osteogenic differentiation through the promotion of p53 expression.[35] This study also showed that BMP-9 exhibits a differentiation-promoting effect equal to or surpassing that of BMP-2. On the contrary, the crucial difference between BMP-9 and S1P is the suppression of adipogenic differentiation of iSCAP by S1P, but not by BMP-9. These findings align with the reported inhibitory effect of the S1PR1 signaling on adipogenic differentiation of mesenchymal stem cells[14] and the adipogenic differentiation-promoting effect of the BMP-9 signaling on iSCAP.[21] Recently, it has been reported that BMP-9 is expressed at protein and mRNA levels in human dental pulp and that BMP-9-positive cells are expressed in the odontoblast layer compared to the central part of the pulp.[36]

Therefore, the mechanism by which BMP-9 signaling promotes odontogenic differentiation needs to be elucidated in the future.

In humans, S1P exists primarily in plasma at nM levels, and can be produced by osteoblasts and osteoclasts.[37,38] On the other hand, BMP-9 is present in serum and plasma at 2—12 ng/ml.[39] In this study, S1P and BMP-9 were added exogenously. Considering the S1P and BMP-9 levels achieved in vivo, S1P must be locally and exogenously applied when used in regenerative endodontic therapy. Currently, we have begun preparation and analysis of a regenerative endodontics animal model[27] by placing S1P in the root canal.

In conclusion, S1P/S1PR1 signaling significantly increased the expression of DSPP, DMP1, and MEPE, resulting in the promotion of odontogenic differentiation of iSCAP. In addition, S1PR1-positive cells were expressed in the apical papilla of immature rat molars and in the dentin-pulp interface where odontoblast-like cells exist. Thus, S1PR1 has the potential to be a target for regenerative endodontic therapy.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgements

This research was supported by JSPS Grants-in-Aid for Scientific Research JP20K9969, JP23K09193, JP21H03117, JP20K9967, and JP21K09927. We would like to thank Prof. Yuichiro Noiri (Niigata University Graduate School of Medical and Dental Sciences) for his advice and Editage for their English language editing services.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2024.02.004.

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