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Article

Biological Effects of Amelogenin Exon 5 Encoded Peptide from Enamel Matrix Derivative in Human Dental Pulp Cells

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Abstract: Enamel matrix derivative (EMD) is used for periodontal tissue regeneration therapy, and can induce mineralization in dental pulp cells (DPCs). We designed a synthetic peptide (SP) derived from the response of cells to EMD, and investigated the effect of the SP on potentiating osteogenesis in DPCs, which have a critical role of dental pulp homeostasis. DPCs were treated with 0, 10, 100, or 1000 ng/mL SP to determine its effect on cell proliferation, cell migration, cell differentiation, and mineralization. We then examined the molecular effects of the SP, focusing on changes in the mitogen-activated protein kinases (MAPK) signaling pathway in these cells. The SP significantly promoted DPC proliferation and migration. Cultures treated with the SP also showed an enhanced expression of markers of osteogenic differentiation and mineralization. The SP also induced the activation of MAPK signaling pathway components. These results suggest that our SP could promote the dental pulp tissue repair by hard tissue formation and the mineralization through activating MAPK signaling pathway. This study provides the first evidence that SP might be a new material for dental pulp tissue treatment.

Keywords: Emdogain; amelogenin; dental pulp cells; cell differentiation; cell migration; mineralization

1. Introduction

Dental caries, tooth fracture, and other types of dental trauma require measures that can repair the tooth and dental pulp. Direct pulp capping and partial pulpotomy treatments are used to seal the exposed dental pulp, using materials that not only protect the pulp tissue but induce hard tissue formation for repair and maintenance [1-5]. After direct pulp capping and pulpotomy, resident dental pulp cells (DPCs) are influenced by the choice of dental material used for treatment. This knowledge led to the design and introduction of new, bioactive agents in pulp capping materials that can accelerate and improve the repair process.

Previous studies have shown that enamel matrix derivative (EMD)—an extract of porcine fetal tooth material—can enhance the proliferation and mineralization of dental pulp cells (DPCs) [6, 7]. EMD has been used for periodontal tissue regenerative surgery, and evidence suggests that EMD can induced hard tissue formation, such as new cementum and bone tissue [8, 9]. We previously showed that subcutaneous injections of EMD on the backs of rats can induce cartilage-like tissue formation and eosinophilic round bodies (ERBs) [10]. We further analyzed these ERBs using MALDI-TOF, and found fragments of the exon 5 of amelogenin, a protein involved in the production of enamel.

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We synthesized a 7-amino acid (WYQNMIR) peptide based on these fragments found in vivo, and we tested whether the SP would behave similarly to EMD and induce cementum and bone-like tissue. We found that the SP could induce hard tissue formation in periodontal artificial defects in rats [11, 12]. Moreover, we found that the SP could promote proliferation of human periodontal ligament (PDL) fibroblasts [13], and enhance osteogenic differentiation of human mesenchymal stem cells (MSCs) and PDL stem cells [14-16].

Previous studies have shown that EMD induces the production of an antibody [17], and it is generally recognized that peptides longer than 10 residues or those over 5 kDa can function as an antigen [18, 19]. Given that our synthetic peptide was only 7 amino acids (1,118 Da), it is unlikely to be at risk of producing an antibody.

Mitogen-activated protein kinases (MAPKs) are essential regulators of cell proliferation and differentiation in PDL cells and DPCs [20-22]; however, the mechanism of action of the SP in periodontal regeneration remains unclear. It is also unclear whether the SP would influence dental pulp stem cells (DPSCs), which have a critical role of dental pulp homeostasis. Therefore, the aim of this study was to investigate the effect of the SP on the proliferation, differentiation, and mineralization of human DPSCs, and to test whether the SP acts through the MAPK signaling pathway.

2. Results

2.1. Cell proliferation

We first tested varying concentrations of the SP on DPC cells to determine an effective concentration. We found that the SP significantly promoted DPC proliferation at 1, 3 and 7 days (Fig. 1, *p < 0.05), with a concentration of 100 ng/ml generating the highest change in cell proliferation among the tested concentrations. From these results, we chose 100 ng/mL SP as the optimal concentration for subsequent experiments.

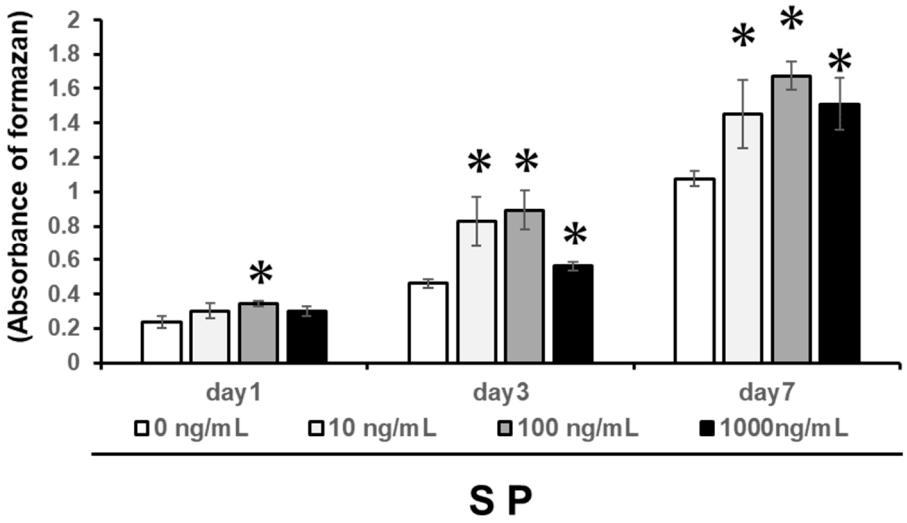

Figure 1. Effect of the synthetic peptide (SP) on cell proliferation on dental pulp cells (DPCs). DPCs were treated with 0, 10, 100, or 1000 ng/ml SP diluted in 100 μl of culture medium. Cell proliferation was measured on days 1, 3, and 7. Significant differences (*p> 0.05) were determined as compared with the control (no SP, 0 ng/mL).

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2.2. Cell migration

Migration was tested using a modified Boyden chamber assay. As shown Fig. 1B, cell migration in the experimental group (0% FBS with SP 100 ng/mL) and in the 10% FBS group (positive control) was greater than that in the negative control group (0% FBS without SP).

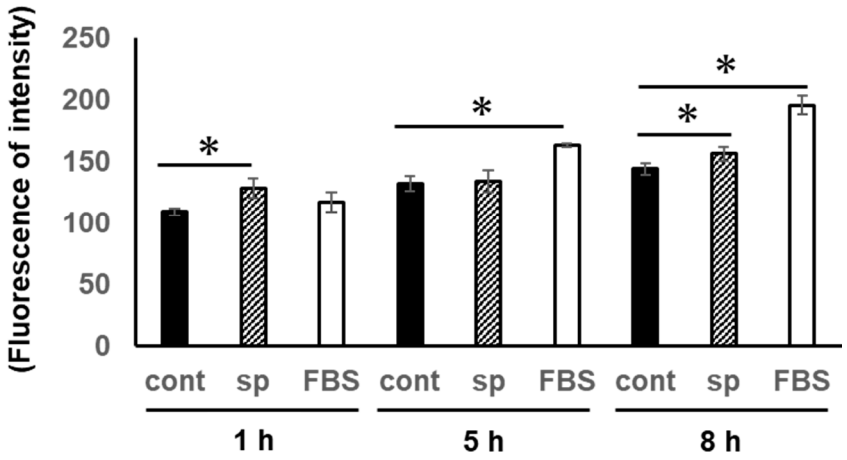

Figure 2. Effects of the SP on cell migration (*p>0.05).

2.3. Wound healing

The effect of the SP on wound repair was evaluated using a wound healing assay kit. As shown in Fig. 3A and 3B, wound healing was significantly faster in the cultures treated with the SP as compared with the control group.

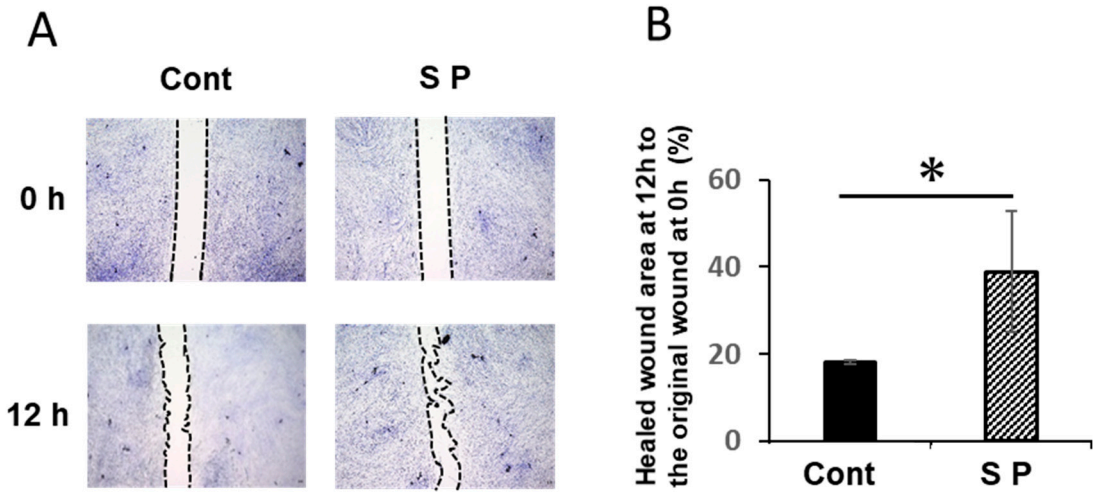

Figure 3. Effects of the SP on wound healing. Wound healing was measured after 0 and 12 h (Scale bar, 200 μm). (A) The change in the wound area is presented as the ratio of the final to initial wound sizes. (B) Quantitative data showing the wounded area. *p>0.05.

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2.4. ALP activity and OCN production

ALP activity and OCN production in the SP-treated group were significantly increased compared with cells treated with osteogenic media only (Osteo group; Fig. 4A, B; $p < 0.05$).

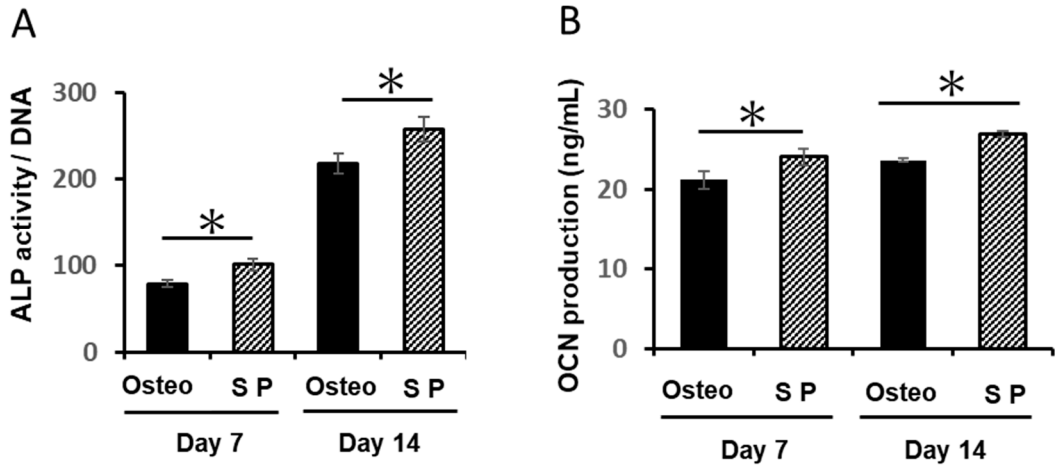

Figure 4. Effect of the synthetic peptide (SP) on dental pulp cell (DPC) osteogenic differentiation and mineralization. Confluent DPCs were treated with osteogenic medium (Osteo) with or without 100 ng/ml SP for 7 and 14 days (*P > 0.05), and tested for changes in alkaline phosphatase (ALP) activity and osteocalcin (OCN) production. (4A and B)

2.5. Quantitative Real-Time PCR Analysis of Osteogenesis-Related Gene Expression

The mRNA expression levels of Col1A1, Runx2, and ON were all significantly enhanced in the SP group as compared to the Osteo group at both time points (Fig. 5A, B, C) (*p < 0.05).

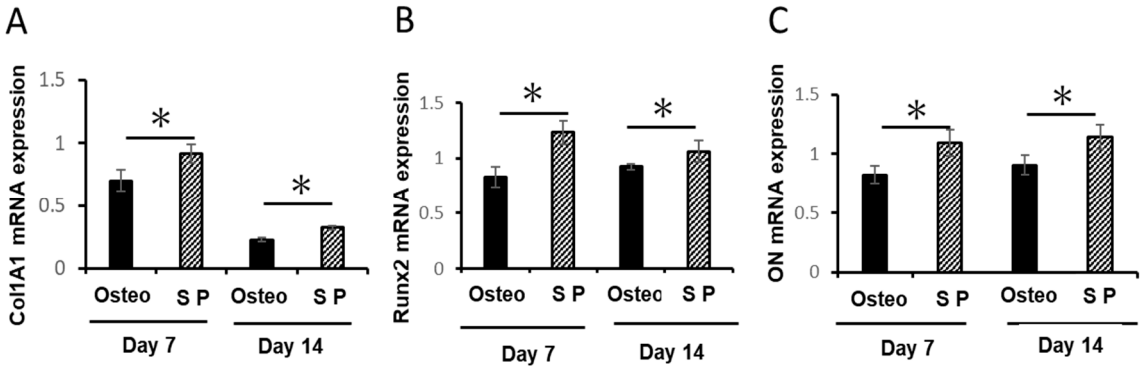

Figure 5. Effects of the SP on mRNA expression of (A) collagen 1-alpha 1 (COL1A1), (B) Runx2, and (C) osteonectin (ON). The mRNA levels were analyzed by quantitative RT-PCR (*p > 0.05).

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2.6. Alizarin red staining

Calcified nodules stained with Alizarin Red were larger in the SP-treated group than those in the Osteo group (Fig. 6).

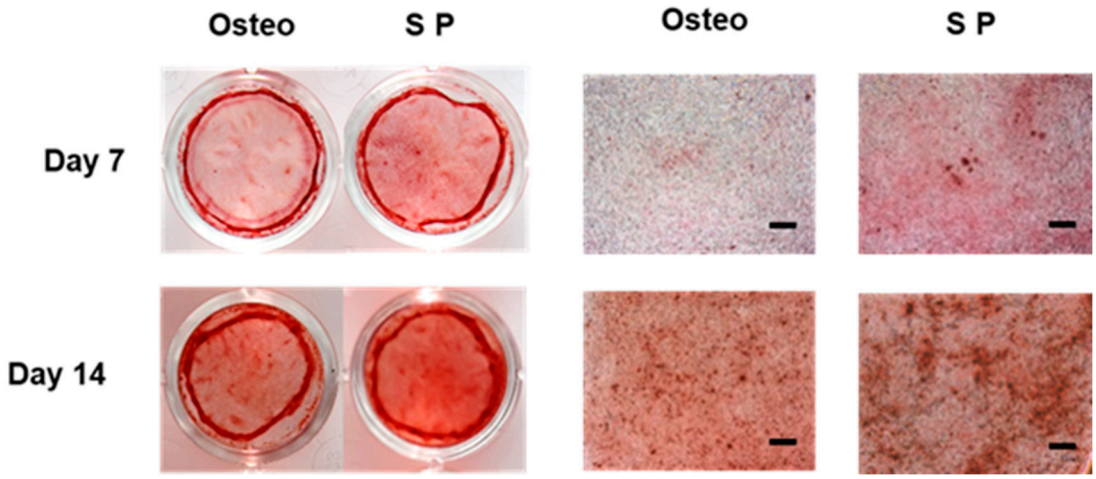

Figure 6. Effect of the SP on the mineralized nodule formation, as measured with Alizarin Red staining. Scale bar = 100 μm.

2.7. Calcium and phosphate deposition in the extracellular matrix

Calcium deposition in the SP-treated group was significantly promoted at both days 7 and 14 as compared with the Osteo group (Fig. 7A; *p < 0.05), as was the Ca/P ratio at day 7 (Fig. 7B; *p < 0.05).

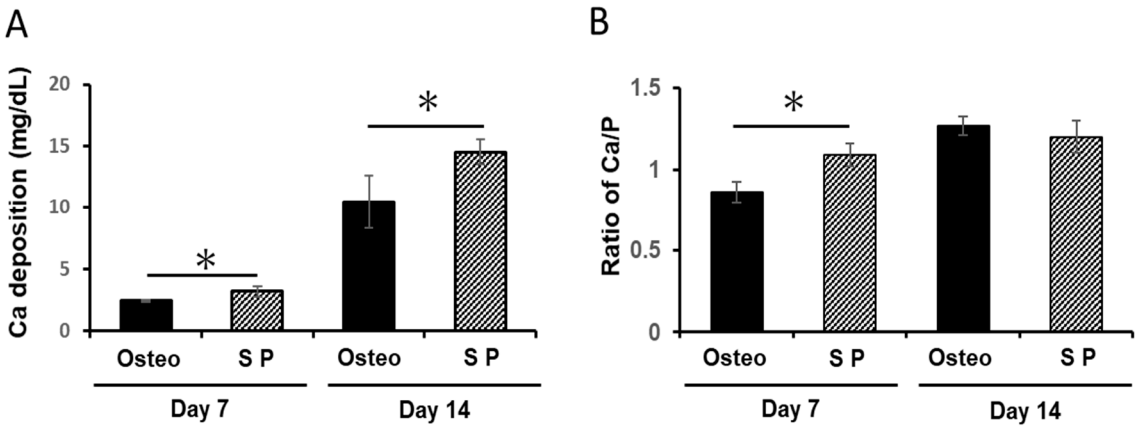

Figure 7. (A) calcium deposition, and (B) phosphate deposition (ratio of Ca/P) in the extracellular matrix. (*p > 0.05)

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2.8. The activation of the MAPK signaling pathway

The SP activated the protein expression levels of phospho-ERK, phospho-JNK, and phospho-p38, with the greatest change evident at SP (Fig. 8A). The ratio of phosphor-ERK 1/2 intensity. The expression levels of (phosphor-ERK 1/2) (ERK 1/2) were increased at 60 min (Fig. 8B). The ratio of phosphor-JNK intensity. The expression levels of (phosphor-JNK) (JNK) were increased at 30 min (Fig. 8C). (D) The ratio of phosphor-p-38 intensity. The expression levels of (phosphor-p-38) (p-38) were increased at both 30 and 60 min (Fig. 8D).

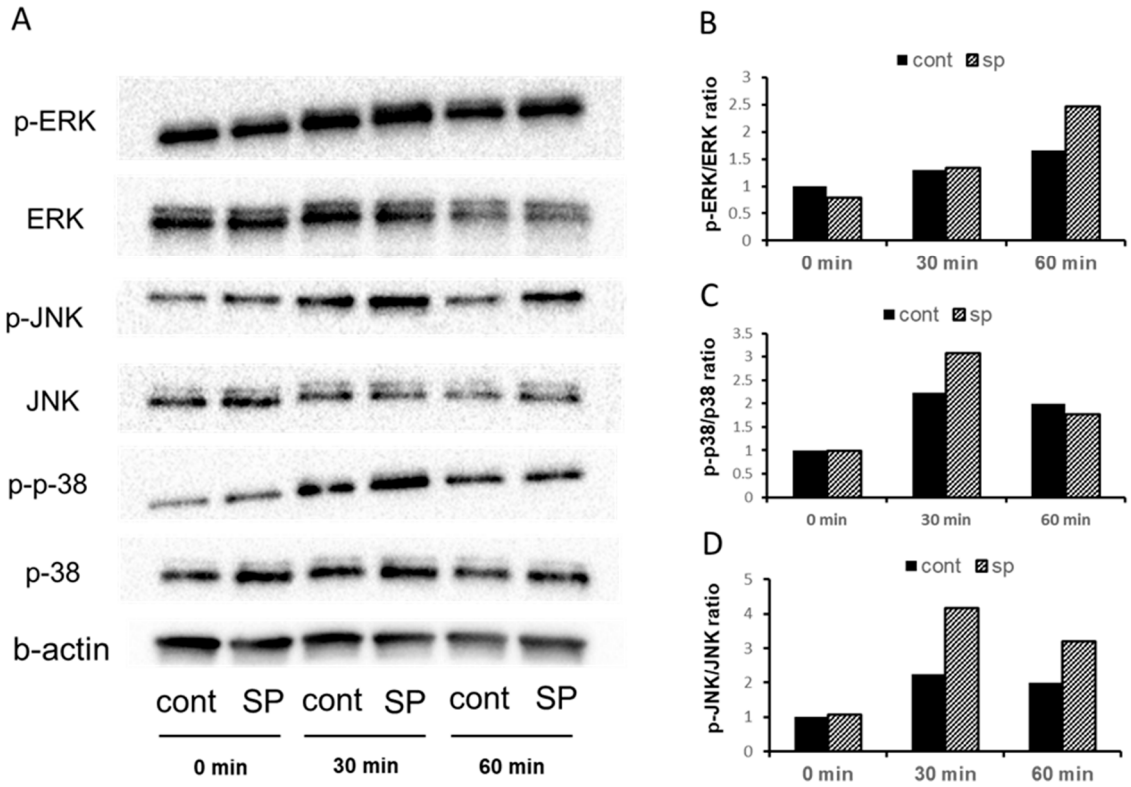

Figure 8. Effect of the synthetic peptide (SP) on the activation of MAPK signaling pathway components. Dental pulp cells were cultured in medium with or without 100 ng/ml SP for 0, 30, or 60 min. Protein expression was evaluated by immunoblotting analysis. (A) Protein expression of ERK 1/2, JNK, p-38, phosphor-ERK 1/2, phosphor-JNK, and phosphor-p-38. (B) The ratio of phosphor-ERK 1/2 intensity. The expression levels of (phosphor-ERK 1/2) (ERK 1/2) were increased at 60 min. (C) The ratio of phosphor-JNK intensity. The expression levels of (phosphor-JNK) (JNK) were increased at 30 min. (D) The ratio of phosphor-p-38 intensity. The expression levels of (phosphor-p-38) (p-38) were increased at both 30 and 60 min.

3. Discussion

EMD and other enamel matrix proteins promote the proliferation of dental tissue cells, such as PDL cells [16,22] and dental pulp cells. In our previous study, we showed that the SP derived from the response of cells to EMD can also promote PDL cell [24] and BMSC [14] proliferation. However, the effects of the SP on DPCs had yet to be tested. In the present study, we show that the SP promotes DPC proliferation at 100 ng/mL, similar to the concentration found to be optimal for PDL cells [24] and PDL stem cells. [16] Therefore, we further investigated the effects of 100 ng/mL SP on osteogenic differentiation and mineralization using this concentration.

Cell migration is necessary for homoeostatic tissue maintenance and the regeneration of injured tissues. The promotion of wound healing in dental pulp tissue is a key determinant of the success of endodontic therapy. We found that the SP promoted the migration of DPCs using a transwell

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chamber assay and a wound healing assay. Therefore, we conclude that the SP can promote the migration of cells that will contribute to dental pulp tissue repair and regeneration.

ALP and OCN are considered to be markers of osteogenesis phenotype. [25, 26] In the present study, we found that the SP promoted ALP activity and OCN production, which is reminiscent of the effect of EMD in hard tissues and the effect of SP in human MSCs [14] and PDLSCs. [16] Therefore, the SP similarly can induce the differentiation of DPCs. Runx2, ON, and COL1A1 are essential factors required during the early stages of osteogenic differentiation. [27-29] COL1A1 in particular has an important role in the formation of new hard tissue. [30] Previous work has shown that EMD can promoted the expression of all three genes in human osteoblasts and human DPCs. [31] Similarly, the SP can promote the expression of COL1A1, Runx2 and ON in human PDLSCs, [16] and promotes the expression of type I collagen after injection into artificial periodontal defect sites. [12] In the present study, we found that the SP promoted the mRNA expression of Runx2, ON, and COL1A1 in DPCs, suggesting that the SP enhances osteogenic differentiation in DPCs during the early stages of differentiation.

Previous work has shown that EMD promotes the mineralization of DPCs, [31] and that the SP can also promote PDLSCs mineralization. [16] Here, we qualitatively and quantitatively determined changes in mineralization in response to treatment with the SP. We found that the SP induced more mineralized nodule formation and calcium deposition on days 7 and 14 as compared with cultures without the SP. Furthermore, the SP promoted the Ca/P ratio at 7 days of culture as compared with control conditions. The theoretical Ca/P ratio of 1.67 is indicative of stoichiometrically pure hydroxyapatite. [32] Therefore, our findings suggest that the SP promotes the formation of high-quality mineralized nodules in DPCs at the early stages of mineralization. Our results suggest that the SP can be used to promote the formation of numerous, high-quality, mineralized nodules in DPCs for earlier protection to the dental pulp tissue.

As shown in Fig. 8, the SP induced the activation of MAPK components in human DPCs. It has been suggested that EMD and the other amelogenin peptides can regulate cellular functions through MAPK signaling. [21, 22] Therefore, our findings suggest that the SP might also regulate cellular function through MAPK signaling as well as EMD. However, the detailed molecular mechanism of action of the SP remains unclear and requires further clarification in future studies.

4. Materials and Methods

4.1. Cell culture

Human DPCs were purchased from Lonza (Tokyo, Japan). According to manufactures protocol, the DPCs have the undifferentiated ability. DPCs were incubated in normal culture medium containing DMEM supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies; Grand Island, NY, USA), 500 U/mL penicillin and 500 μg/mL streptomycin (Nacalai Tesque; Kyoto, Japan). DPCs were seeded into T75 culture dishes (Falcon BD; Franklin Lakes, NJ, USA) and incubated at 37°C in 5% CO2. DPSCs at passage 3 to 4 were used for experimentation. For differentiation assays, cells were cultured in medium containing 50 μM L-ascorbic acid 2-phosphate (Nacalai), 10 mM β-glycerophosphate (Wako Pure Chemical Industries Ltd.; Tokyo, Japan), and 10 nM dexamethasone (Wako Pure Chemical Industries Ltd.), hereafter referred to as osteogenic medium.

4.2. Cell proliferation assay

DPCs were seeded in 96-well plates at 2×10^3 cells/well in normal culture medium. After 24 h, the medium was replaced with normal culture medium containing varying concentrations of the SP (10, 100, or 1000 ng/mL and without SP), and DPCs were cultured for 1, 3 or 7 days. Cell proliferation was determined by measuring the amount of formazan using the Cell Count Reagent SF (Nacalai). The absorbance was measured at 450 nm, and data were analyzed with the SoftMax Pro software (Molecular Devices; Sunnyvale, CA, USA).

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4.3. Transwell migration assay

A modified Boyden chamber assay was performed using 24-well microchemotaxis chambers (Fluoroblock insert system; Falcon). DPCs were cultured with 4 μM Calcein AM solution (Dojindo Laboratory, Kumamoto, Japan) for 30 min at 37°C. Cells were then trypsinized, washed in medium, and resuspended in serum-free medium to a final concentration of 2.5×10^4 cells/500 μL. The cell suspension was then added to the upper chamber of a cell culture insert, and 750 μL of medium containing the SP (0, 100 ng/mL) or medium containing 10% FBS as a positive control was added to the lower chamber. The upper and lower wells were separated by a 3.0-μm pore size HTS FluoroBLock Insert (Falcon). Cell migration was observed for 1, 5, and 8 h. The number of DPCs that passed through the filter to the lower chamber was evaluated using a fluorescence plate reader at 485 nm/530 nm excitation/emission.

4.4. Wound healing assay

In vitro wound healing assays were performed using a wound repair assay kit (Ibidi GmbH, Am Klopterspitz19, Martinsried, Germany). DPCs were seeded at 3.5×10^4 cells/70μL into a cell culture insert. After confluence, the culture insert was lifted to replace the media with serum-free medium containing the SP (0, 100 ng/mL). The culture insert was then replaced into the media, and a wound approximately 500 μm wide was created. DPCs were cultured for a further 12 h, then fixed with 70% ethanol (Nacalai) for 10 min, and stained with 0.1% crystal violet (Merck Millipore, Darmstadt, Germany) for 5 min at room temperature. Pictures of each wound were taken at 0 h and 12 h with a BZ-II all-in-one fluorescence microscope (Keyence Corporation; Osaka, Japan). The images were used to measure the denuded area by Image J. Data are presented as the percentage of the healed wound area at 12 h as compared with the initial wound at 0 h.

4.5. ALP activity and measurement of OCN

DPCs were cultured with osteogenic medium for 7 or 14 days, and then washed with PBS and lysed with 300 μL of 0.2% Triton X-100 (Sigma-Aldrich). ALP activity was measured using a 1-step pNPP substrate (Pierce Biotechnology Inc.; Rockford, IL, USA). ALP activity was normalized to the amount of DNA in the cell lysate. The DNA content was measured using the PicoGreen dsDNA Assay kit (Invitrogen; Paisley, UK). Data were analyzed with the SoftMax Pro software. The cultured supernatant (at 7 and 14 days) was collected to quantify OCN levels using an ELISA kit (Takara Inc.; Shiga, Japan).

4.6. Quantitative Real-Time PCR

DPCs were cultured with osteogenic medium for 7 or 14 days. Total cellular RNA was extracted using a kit, and 10 μL of RNA from each sample were reverse transcribed into cDNA using a kit (PrimeScript Reagent kit; Takara). Gene expression was evaluated using a real-time PCR assay (TaqMan gene expression assay; Applied Biosystems, Thermo Fisher Scientific; Waltham, MA, USA). The mRNA expression levels of collagen type 1 alpha 1 (Col1A1; Hs00164004_m1) osteonectin (ON; Hs00213568_m1), and Runt-related transcription factor 2 (Runx2; Hs01047973_m1) were determined by quantitative real-time PCR according to standard protocols.

4.7. Extracellular matrix mineralization

DPCs were cultured with osteogenic medium for 7 or 14 days, using normal culture medium as a negative control. For measurements of calcium production, DPCs were dissolved with 10% formic acid, and calcium deposition (Ca) in the extracellular matrix was measured using a Calcium E test kit (Wako). The amount of phosphate was quantified using a P test kit (Bio Assay Systems; Hayward, CA, USA).

For qualitative histology, other cultures of DPCs at days 7 and 14 were washed with PBS and fixed with 70% ethanol (Nacalai) for 10 min. Cells were then stained with 1% Alizarin Red S for 5 min at room temperature. Calcified nodules were imaged with an BZ-II all-in-one fluorescence microscope (Keyence).

4.8. Western blot analyses

Adherent DPCs were cultured for 0, 30, and 60 min in the presence of the SP. Total protein was extracted using a buffer solution (RIPA buffer, Thermo Fisher Scientific, Rockford, IL) supplemented with a protease inhibitor cocktail. Total protein concentrations were measured using a BCA Protein Assay kit (Pierce Biotechnology). Protein samples were electrophoresed on 12.5% SDS gels, and transferred onto polyvinylidene difluoride membranes. The membranes were treated with blocking solution (Blocking One, Nacalai) and then incubated for 1 h at room temperature with primary antibodies (ERK, phospho-ERK, JNK, phospho-JNK, p38, and phospho-p38). Membranes were then washed and incubated with secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using a chemiluminescence kit (Nacalai) and signals were analyzed with the ChemiDoc MP System (BioRad).

4.9. Statistical Analysis

A one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used to determine significance. P values < 0.05 were considered significant.

5. Conclusions

We found that the amelogenin exon5 encoded peptide derived from EMD can promote the proliferation, migration, differentiation and mineralization of DPCs at first time. Our findings suggest that the SP might be a useful agent for dental pulp repair.

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Author Contributions: Hirohito Kato and YoichiroTaguchi conceived and designed the experiments; Hirohito Kato, Masahiro Noguchi, Kazutaka Imai, Ruan Yaru, and Saitatsu Takahashi performed the experiments; Hirohito Kato, Yoichiro Taguchi, Daisuke Kimura and Makoto Umeda analyzed the data; Kazuya Tominaga, Reiko Taguchi, Muneyasu Shida, Hiroshi Maeda and Akio Tanaka contributed reagents/materials/analysis tools; Hirohito Kato and Yoichiro Taguchi wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

EMD	enamel matrix derivative
DPCs	dental pulp cells
SP	synthetic oligo peptide
MAPK	mitogen-activated protein kinases
ERBs	eosinophilic round bodies
PDL	periodontal ligament
MSCs	mesenchymal stem cells
ALP	alkaline phosphatase
OCN	osteocalcin
Osteo	osteogenic medium
Col1A1	collagen 1-alpha-1
ON	osteonectin

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