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The effects of amelogenin exon 5 encoded peptide from enamel matrix derivative enhances in odontoblast like cells, KN-3 cells

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Featured Application: Amelogenin exon 5 could be a potential for applicate to the dental pulp capping.

Abstract: Enamel matrix derivative (EMD) is used for periodontal tissue regeneration therapy. We designed a synthetic amelogenin peptide (SP) derived from EMD, and have previously investigated the biological function of SP. However, it is unknown whether SP affects odontoblastic differentiation. In the present study, we investigated the effects of SP in odontoblast-like cells, KN-3 cells. KN-3 cells were treated with SP (0, 1, 10, 100, or 1000 ng/mL) and then cultured for 3, 8, 24, or 48 hours, in order to determine the effects of SP on cell proliferation and detect its optimum concentration. To investigate the effect of SP on odontogenic differentiation, KN-3 cells were treated with SP in odontogenic differentiation medium cultured for 3 or 7 days. Odontogenic differentiation was performed by measuring alkaline phosphatase (ALP) activity, the mRNA expression of dentin sialophosphoprotein (DSPP), the formation of calcified nodules, and calcium deposition into the extracellular matrix. The addition of SP significantly promoted KN-3 cell proliferation; a concentration of 100 ng/ml generated the greatest change in cell proliferation. SP also showed increased expression of markers of odontogenic differentiation and mineralization. These results suggest that SP, derived from EMD, could be a potential for applicate to the dental pulp capping.

Keywords: Emdogain. Amelogenin. Odontoblast. Differentiation. Mineralization.

1. Introduction

Enamel matrix derivative (EMD) can induce the formation of hard tissue, such as alveolar bone and cementum tissue [1,2]. EMD is widely used in periodontal tissue regeneration and bone regeneration. We previously showed that subcutaneous injections of EMD on the backs of rats can induce cartilage-like tissue formation and eosinophilic round bodies (ERBs) [3]. We further analyzed these ERBs by using MALDI-TOF, and found fragments of exon 5 of amelogenin.

We synthesized a 7-amino acid (WYQNMIR) peptide based on these fragments and tested whether the synthetic peptide (SP) would behave similarly to EMD [4]. We found that the SP could

induce hard tissue formation in artificial periodontal defects in rats [5,6]. Moreover, we found that SP could promote the proliferation of human periodontal ligament (PDL) fibroblasts [7] and enhance osteogenic differentiation of human mesenchymal stem cells (MSCs) [8-10]. These findings also could help to clarify the biological functions of amelogenin exon 5.

EMD has been reported to induce the production of anti-EMD antibodies in the host [11]; it is generally recognized that only peptides of greater than approximately 10 residues (or > 5 kDa) can function as antigens [12,13]. Thus, because SP is only seven amino acids in length and 1,118 Da in molecular mass, it exhibits very little risk of eliciting an immunological response.

Dental caries, tooth fractures, and other types of dental trauma induce tooth loss. Direct pulp treatment requires materials that protect the pulp tissue but induce hard tissue formation, in order to repair and maintain dental pulp tissues [14-19]. This requirement has led to the design and introduction of new, bioactive agents for dental pulp tissue engineering materials.

To investigate the mechanisms of dental pulp regeneration, a rat clonal odontoblast-like cell line, KN-3, has been established by Prof. Kitamura and Prof. Nishihara [20]. KN-3 cells showed high levels of alkaline phosphatase (ALP) activity, dentin sialophosphoprotein (DSPP) expression, and the ability to form calcium nodules [21]. KN-3 cells have also been used as an authentic control to study the differentiation of induced pluripotent stem (iPS) cells and embryonic stem (ES) cells into odontoblast-like cells [22,23]

The establishment of KN-3 cells has clarified the responses of odontoblasts in vitro and provided a means to explore the mechanisms of wound healing and regeneration of the dentin-pulp complex, in combination with in vivo studies.

However, the effects of SP on odontoblastic activity have not yet been investigated. In the present study, we evaluated the odontogenic effects of SP in KN-3 cells.

2. Materials and Methods

2.1. Cell culture

The rat clonal odontoblast-like cell line, KN-3, was kindly provided by Prof. Chiaki Kitamura (Kyushu Dental College, Kitakyushu, Japan) and Prof. Tatsuji Nishimura (Kyushu Dental College). KN-3 cells were maintained as described previously.²⁰ KN-3 cells were incubated in normal culture medium comprising α -MEM supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies; Grand Island, NY, USA), 500 U/mL penicillin, 500 μ g/mL streptomycin, and 25 μ g/mL amphotericin B (Nacalai Tesque; Kyoto, Japan). For differentiation assays, KN-3 cells were cultured in medium containing 50 μ M L-ascorbic acid 2-phosphate (Nacalai) and 10 mM β -glycerophosphate (Wako Pure Chemical Industries Ltd.; Tokyo, Japan).

2.2. Cell proliferation assay

KN-3 cells 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 SP (0, 1, 10, 100, or 1000 ng/mL); KN-3 cells were cultured for 3, 8, 24, or 48 hours. Cell proliferation was determined by measuring the amount of formazan with 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).

2.3. Morphological analysis

KN-3 cells were cultured in normal culture medium containing varying concentrations of SP (0, 1, 10, 100, or 1000 ng/mL) for 48 hours. Images were obtained with a BZ-II all-in-one fluorescence microscope (Keyence Corporation, Osaka, Japan).

2.4. Alkaline phosphatase (ALP) activity assay

KN-3 cells were cultured in osteogenic medium for 3 or 7 days, then washed with PBS and lysed with 300 µL of 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). ALP activity was measured by using a 1-step pNPP substrate (Pierce Biotechnology Inc.; Rockford, IL, USA). ALP activity was normalized to the quantity of DNA in the cell lysate. The DNA content was measured by using the PicoGreen dsDNA Assay kit (Invitrogen; Paisley, UK). Data were analyzed with the SoftMax Pro software.

2.5. Extracellular matrix mineralization

KN-3 cells were cultured in osteogenic medium for 7 days. For measurements of calcium production, KN-3 cells were dissolved with 10% formic acid; then, calcium deposition (Ca) in the extracellular matrix was measured by using a Calcium E test kit (Wako). For qualitative histology, other cultures of KN-3 cells at days 7 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).

2.6. Quantitative Real-Time PCR

KN-3 cells were cultured in differentiation medium for 1, 3, 6, or 12 hours. Total cellular RNA was extracted by using a kit; then, 10 µL of RNA from each sample were reverse transcribed into cDNA by using a kit (Prime Script Reagent kit; Takara, Kyoto, Japan). Gene expression was evaluated by using a real-time PCR assay (TaqMan Gene Expression Assay; Applied Biosystems, Thermo Fisher Scientific; Waltham, MA, USA). The mRNA expression levels of dentin sialoprotein (DSPP; Rn02132391_s1) were determined by quantitative real-time PCR, in accordance with standard protocols.

2.7. Immunofluorescence staining

KN-3 cells were fixed in cold 70% ethanol for 10 min at -20°C, treated with 0.2% Triton X-100 in phosphate-buffered saline (PBS), blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and then incubated with mouse anti-rat DSPP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. After cells were washed with PBS, they were incubated for 60 min at room temperature with a fluorescence-labeled secondary anti-mouse polyclonal IgG antibody (Santa Cruz). The samples were washed with PBS and stained with DAPI solution (Dojindo Laboratory, Kumamoto, Japan). The negative control was treated with PBS, in place of a primary antibody. Images were obtained with a BZ-II all-in-one fluorescence microscope (Keyence Corporation).

2.8. Statistical Analysis

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

3. Results

3.1. Cell proliferation

We first tested varying concentrations of the SP on KN-3 cells to determine an effective concentration. We found that SP (100 ng/mL) significantly promoted KN-3 proliferation at 8, 24, 48, and 72 hours (Fig. 1A, $P < 0.05$). From these results, we chose 100 ng/mL SP as the optimal concentration for subsequent experiments.

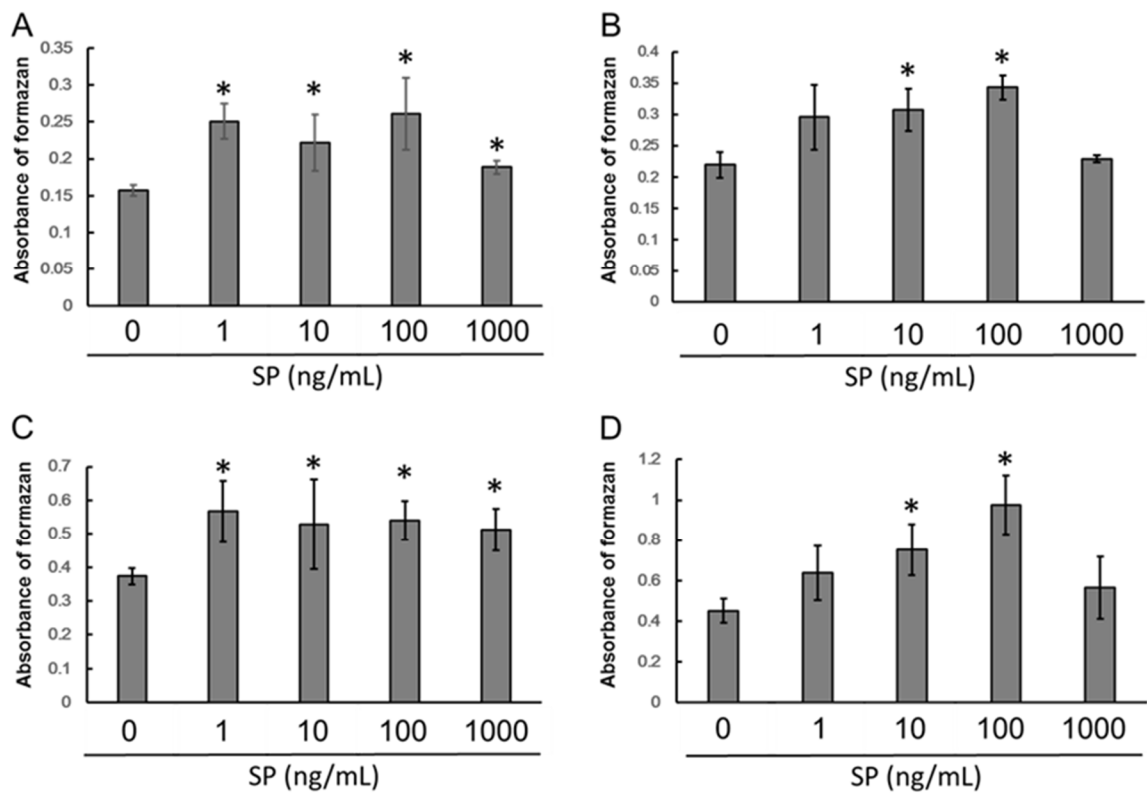


Figure 1. Effect of synthetic peptide (SP) on KN-3 cell proliferation. KN-3 cells were treated with 0, 1, 10, 100, or 1000 ng/ml SP diluted in 100 μ l culture medium. Cell proliferation was measured for 3, 8, 24, or 48 h. Significant differences ($*P < 0.05$) were determined in comparison with the control (SP, 0 ng/mL).

3.2. Cell morphology

KN-3 cells treated with SP (0 ng/mL) exhibited round-shaped morphology. On the other hand, KN-3 cells treated with SP (100 ng/mL) exhibited spindle-shaped morphology.

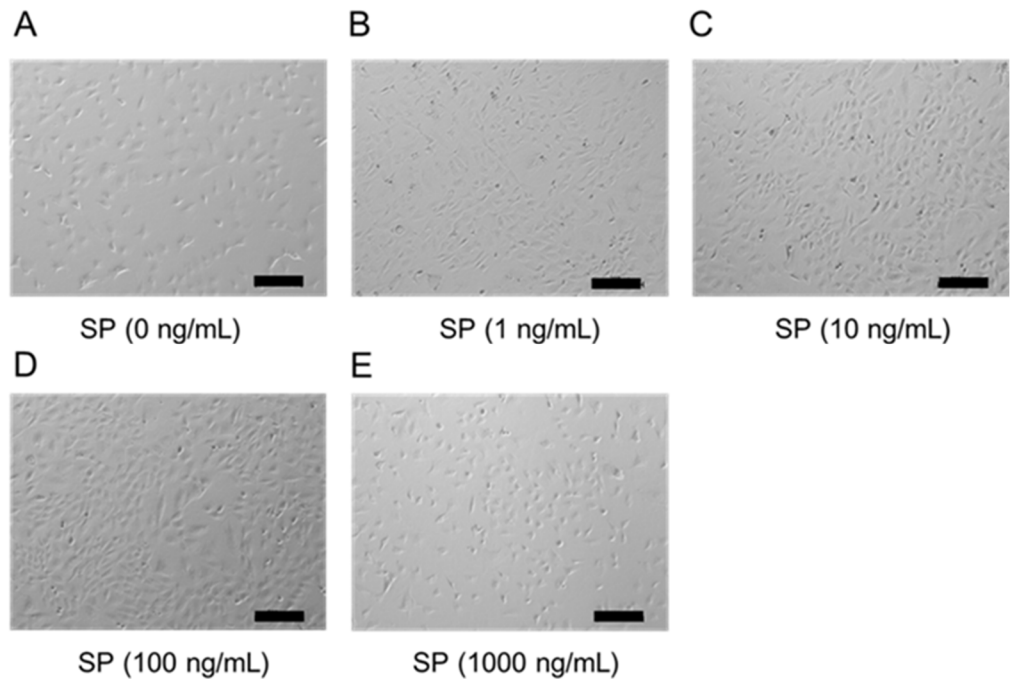


Figure 2. Effect of SP on cell morphology in KN-3 cells. Phase-contrast microphotographs of KN-3 cells treated with SP (0, 1, 10, 100, or 1000 ng/mL) for 48 h. Scale bar = 200 μ m

3.3. ALP activity

ALP activity in the SP-treated group was significantly increased after 7 days, compared with cells solely treated with differentiation media (Fig. 3A; $P < 0.05$).

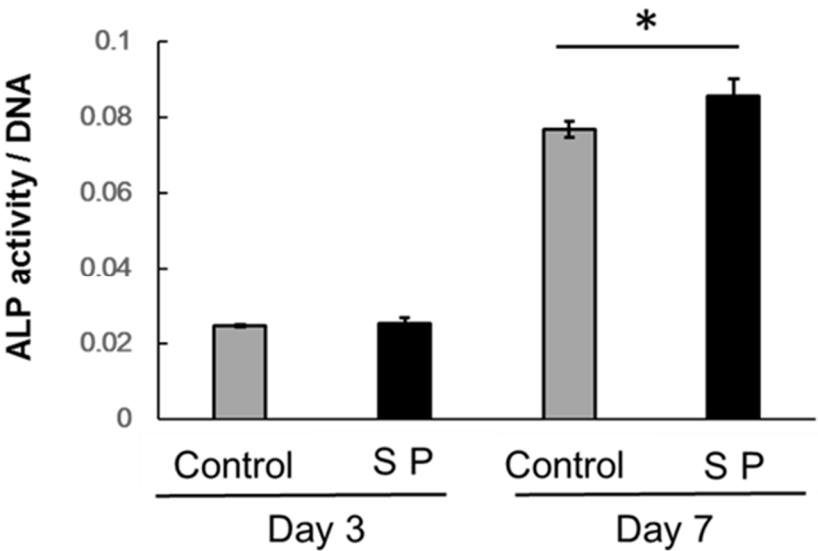


Figure 3. Effect of SP on alkaline phosphatase (ALP) activity in KN-3 cells cultured in differentiation medium. Confluent KN-3 cells were treated with osteogenic medium, with or without 100 ng/ml SP, for 3 or 7 days ($*P > 0.05$); changes in ALP activity were investigated.

3.4. Extracellular matrix mineralization

Calcified nodules stained with Alizarin Red were larger in the SP-treated group than in the Control group (Fig. 3B). Calcium deposition in the SP-treated group significantly increased after 7 days, compared with the Control group (Fig. 3C; $P < 0.05$)

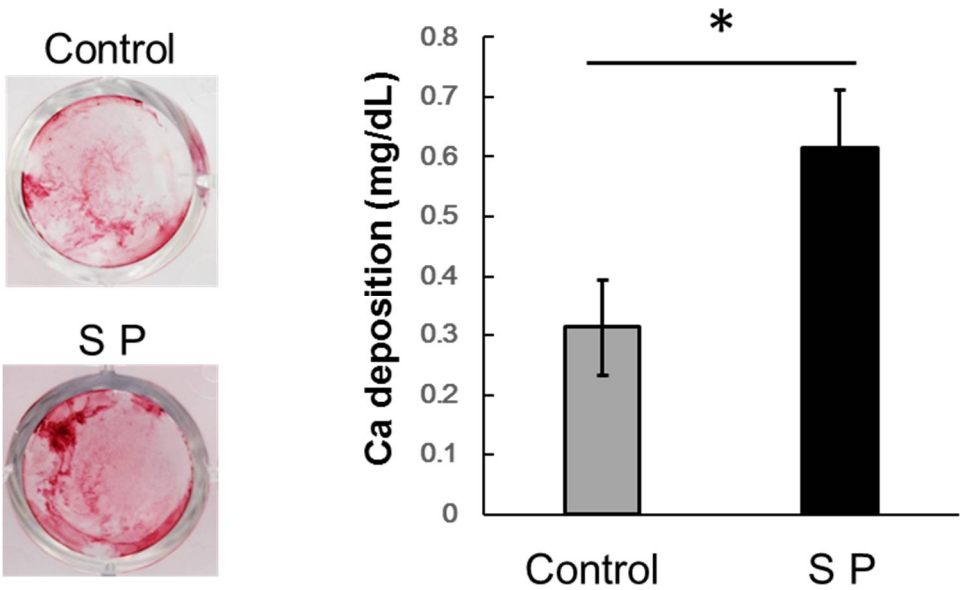


Figure 4. Effect of SP on mineralized nodule formation, as measured with (A) Alizarin Red staining, and (B) extracellular matrix calcium deposition.

3.5 mRNA expression of DSPP

mRNA expression of DSPP was significantly enhanced in the SP group, compared with the Control group, at 1, 3, and 5 hours ($P < 0.05$).

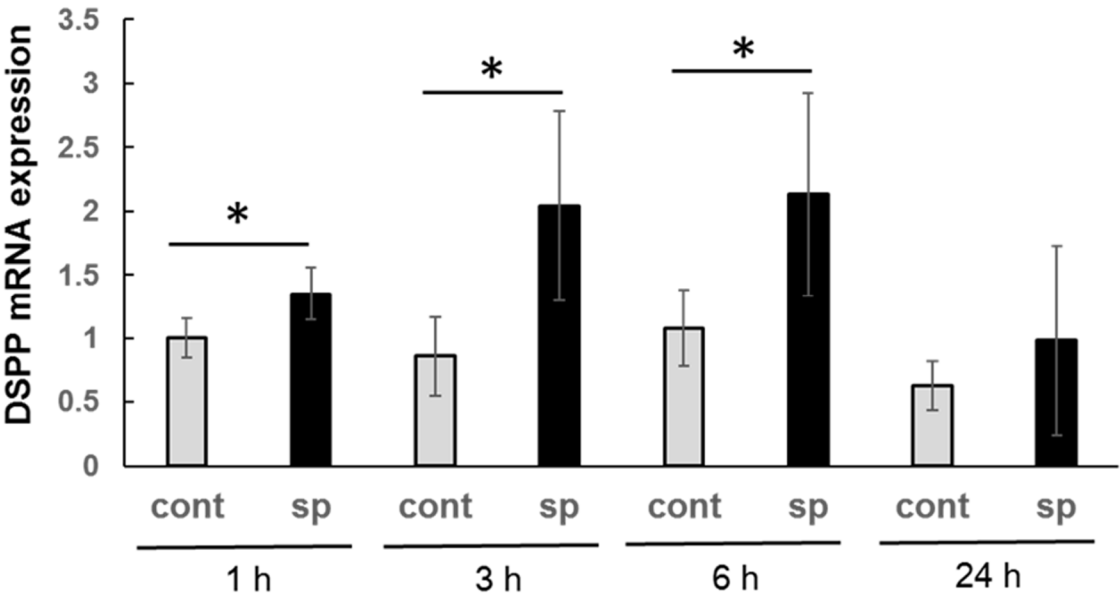


Figure 5. Effect of SP on odontoblastic differentiation in KN3 cells. Quantitative RT-PCR analysis of DSPP mRNA expression in KN3 cells treated with or without SP (100 ng/ml) at 1, 3, 6, and 24 h.

3.6 Immunofluorescence expression of DSPP

Sigillary to mRNA expression, the fluorescent intensity of DSPP by immunofluorescence staining was enhanced in the SP group, compared with the Control group.

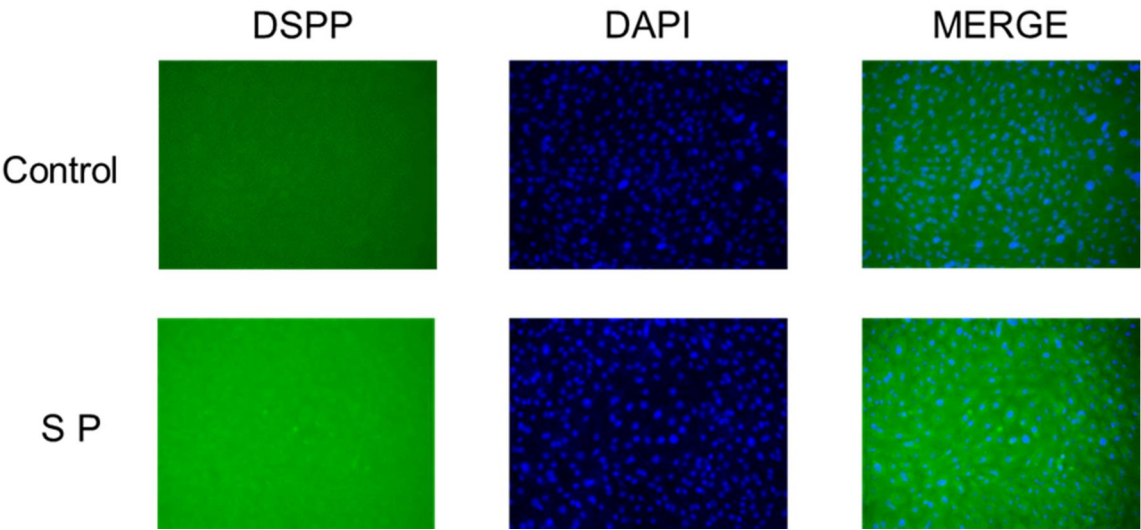


Figure 6. KN-3 cells treated with or without SP were incubated with anti-DSPP monoclonal antibody. The samples were then incubated with a fluorescence-labeled secondary antibody. The samples were washed with PBS and stained with DAPI solution. Images were obtained with a fluorescence microscope. Scale bar = 100 μ m.

4. Discussion

Many studies have used primary dental pulp cells, existing pulp cell lines, and a few odontoblastic cell lines. In the present study, we found that SP promotes cell proliferation, expression of odontogenic differentiation, and mineralization in KN-3 cells.

EMD and amelogenin peptide promote the proliferation of dental tissue cells, such as PDL cells and bone marrow stromal cells (BMSCs) [24,25]. In a previous study, we showed that SP, derived from EMD, can also promote cell proliferation of human BMSCs [8]. However, the effects of SP on proliferation in odontoblasts was not previously investigated.

In the present study, we showed that SP promotes KN-3 proliferation at 100 ng/mL, similar to the optimal concentration for PDL stem cells [10]. Therefore, we further investigated the effects of 100 ng/mL SP on odontogenic differentiation and mineralization.

ALP is considered a marker of odontogenesis phenotype [26]. In the present study, we found that SP promoted ALP activity, which is similar to the effect of EMD in hard tissues and the effect of SP in KN-3 cells. Therefore, SP can also induce odontogenic differentiation in KN-3 cells.

We quantified calcium levels in cultures of KN-3 cells by using alizarin red staining and measurement of extracellular calcium deposition. Alizarin red staining is used for the detection of mineralized nodules formed by cells of odontogenic lineages [27], which are indicative of the calcification of bone and dentin matrix. We qualitatively and quantitatively determined changes in mineralization in response to treatment with SP. We found that SP induced greater mineralized nodule formation and calcium deposition in cultures treated for 7 days, compared with untreated cultures. Our previous study showed that SP can also promote mineralization of PDL stem cells [16]. Our results suggest that SP can be used to promote the formation of mineralized nodules in KN-3 cells as the dentin formation for earlier protection of dental pulp tissue.

We also investigated whether SP has effects on the expression of odontoblast specific molecules in KN-3 cells and found that it impacted the levels of DSPP, which is a noncollagenous dentin matrix protein that is known as an early stage marker of odontoblastic differentiation [28,29]. A previous study revealed that KN-3 cells cultured for 1–12 h expressed high levels of DSPP [30]; this indicated that KN-3 cells are precursor cells, which can differentiate into odontoblast-like cells. In the present study, we found that SP enhanced expression of DSPP in KN-3 cells, indicating upregulation of odontoblastic differentiation.

5. Conclusions

We found that SP, an amelogenin peptide derived from EMD, can promote the proliferation, odontogenic differentiation, and mineralization of KN-3 cells in vitro. Our findings suggest that SP might provide a new biomaterial for dental pulp capping. Moreover, the present study partially clarified the function of amelogenin exon 5 in odontogenesis.

Author Contributions:

Conceptualization, H.K. and T.Y.; Methodology, I.K., Y.R., M.N., Y.T. and Y.C.; Writing—original draft preparation, H.K. and T.Y.; Data curation, M.S. and R.T.; Supervision, H.M. and T.K.; Project administration, Y.T. and M.U.; funding acquisition, A.T.”

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

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