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Original Article

Wnt signaling pathway involves in simvastatin-induced mineralization of murine odontoblast-like cells

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Abstract: Objective: The present study aimed to investigate the effect of simvastatin on the mineralization of murine odontoblast-lineage cells (OLC) and the role of Wnt signaling pathway. Methods: Cell proliferation was examined after treatment with simvastatin at different concentrations (10^{-7}, 10^{-6} and 10^{-5} mol/L). Alkaline phosphatase (ALP) and alizarin red S (ARS) staining were carried out after the application of 10^{-6} mol/L simvastatin at 7 and 28 days. The mRNA and protein expressions of odontoblastic markers including dentin matrix phosphoprotein-1 (DMP-1), dentin sialophosphoprotein (DSPP), runt-related gene-2 (Runx2) and dentin sialoprotein (DSP) were examined by q-RT PCR and western blot assay. The Wnt signaling pathway associated factors including typical transcription factor of canonical Wnt/β-catenin signaling (Lef-1), Wnt10a and Wnt11 were also investigated. Results: The cell growth was significantly suppressed after 10^{-5} mol/L simvastatin treatment while no effects at 10^{-7} and 10^{-6} mol/L. OLCs showed enhanced ALP production, increased mineralization and elevated odontoblastic capability after 10^{-6} mol/L simvastatin exposure. Furthermore, the expressions of typical transcription factor of canonical Wnt/β-catenin (Lef-1), Wnt10a and Wnt11 significantly increased after 10^{-6} mol/L simvastatin treatment. Conclusion: These findings suggest that simvastatin could promote the mineralization of OLCs, which might mediate by the Wnt signaling pathway.

Keywords: OLCs, mineralization, simvastatin, Wnt signaling pathway

Introduction

Odontoblast is the neural crest-derived cell lineage, which is essential for dentinogenesis. However, the isolation and primary culture of odontoblasts *in vitro* are technically difficult due to their anatomical structure, although the culture of odontoblasts is crucial for the investigation of mechanisms underlying the dentinogenesis and tooth development. In 2006, spontaneously immortalized odontoblast-lineage cell was reported by Arany et al. [1]. These odontoblast-lineage cells have already been chosen for various experiments in that they display many phenotypic properties similar to those of immature odontoblasts, which have been proven to possess the ability to differentiate into functional odontoblasts in tooth regeneration [2, 3].

Statin is a potent lipid-lowering drug that has been widely used for more than 20 years as a first-line drug against dyslipidemia with favorable safety. It has pleiotropic effects including anti-inflammation [4], angiogenesis induction [5] and osteogenesis induction [6-9]. Additionally, statin may also affect the dentinogenesis [10]. A recent retrospective and case-control study reported that the systematic statin could induce dental pulp chamber calcification [11]. Thus, dentists should be aware of the patient history of systematic medication with statin before the endodontic treatment (root canal therapy). Furthermore, several studies have shown that simvastatin could induce the odontogenic differentiation of human dental pulp stem cells *in vitro* and *in vivo* by up-regulating dentin sialophoprotein (DSPP) mRNA expression and accelerating mineralized nodule for-

Table 1. Primers used in real-time PCR

Genes	Forward	Reverse
GAPDH	CGGCAAGTTCAACGGCACAG	CGCCAGTAGACTCCACGACAT
DSPP	TCGGTTACCGGTTGACATGG	GCAGG CCCTGTTTCCTCAT
Runx2	GCCCTGGTGTTTAAATGGTT	TGCTTGCAGCCTTAAATGAC
DMP1	ACAGCCTGAACACATTCTCC	ATGTTCTTGG GACGGATGTC
Lef-1	TCACTGTCAGGCGACACTTC	TGAGGCTTCACGTGCATTAG
Wnt11	TTCCGATGCTCCTATGAAGG	CCCCATGGCATTTACACTTC
Wnt10a	GCGCTCCTGTTCTTCCTACT	GTCAGGCACACTGTGTTGG

mation [10]. However, the mechanism of simvastatin-induced odontogenic differentiation has remained unclear.

The Wnt family of secreted proteins in mammals consists of 19 members that can trigger various cellular responses through several distinct pathways [12]. The expressions of various Wnt family members and Wnt-related molecules in the tooth germ have previously been reported to play an essential role in the activation of the odontogenic mesenchyme during early tooth development [13-15]. Wnt10a and Wnt11 have been reported to promote the differentiation of odontoblast. Moreover, Wnt11 is detectable in rat dental pulp, and particularly higher expression in the odontoblast layer [16-19]. Although Wnt signaling pathway is known as a pivotal regulator of tooth development, the relationship between simvastatin and Wnt signaling pathway activation has not been fully understood. Previous studies demonstrate that the expression of Wnt signal-related genes significantly increase after simvastatin treatment during the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cell (BMSC) and mouse embryonic stem cell (ESC) [20, 21]. However, the involvement of Wnt-related molecules in simvastatin-induced odontogenic differentiation has not been evaluated.

We hypothesize that Simvastatin could promote the odontogenic differentiation of OLCs, and Wnt signaling pathway might exert an essential role in this process. To verify our hypothesis, simvastatin was used in the odontogenic differentiation of OLCs, the odontogenic markers and Wnt related molecules were examined by qRT-PCR and western blot. The results showed that simvastatin could enhance mineralization of OLCs and up-regulate the Wnt associated factors. We speculated that the Wnt signaling pathway might involve in this process.

Materials and methods

Cells and cell culture

Murine OLCs were kindly provided by Prof S. Arany (Department of Biochemistry, Akita University School of Medicine, Akita, Japan). OLCs were cultured in α-modified minimum essential medium (α-MEM, Gibco, USA) containing 2 mmol/L GlutaMAX (Gibco), 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin; Gibco, USA). OLCs were maintained at 37°C in a humidified chamber with 5% CO_2 in air, and the medium were refreshed every 2 days. When the cell confluence reached 60%, the medium was refreshed with mineralized medium containing 90% (v/v) of α-MEM, 50 mg/ml phosphate ester of ascorbic acid (Sigma, USA), 10 mmol/L β-glycerophosphate (Sigma, USA), 10 nmol/L dexamethasone (Sigma, USA) and 10% (v/v) FBS, and then cells were maintained for 1-28 days, and the medium was refreshed every 2 days. Subsequently, cells were exposed to simvastatin (10^{-6} mol/L; Merck, USA) for the periods indicated. Simvastatin was dissolved in ethyl alcohol immediately before use, and the final concentration of ethyl alcohol was not higher than 0.1% (v/v).

Evaluation of cell proliferation

OLCs were seeded onto 96-well plates at 2.0×10^3 cells/well and grown in growth medium for 24 h. The medium was refreshed with growth medium supplemented with simvastatin (10^{-7}, 10^{-6} and 10^{-5} mol/L). At 1, 3, 5 and 7 days, the MTT assay was carried out to detect the viable cells using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, Beijing, PR China). Absorbance was measured with a microplate reader (Bio Rad, Hercules, CA) at 490 nm. Detection was done at 5 well/group, and average was obtained.

Alizarin red S (ARS) and alkaline phosphatase (ALP) staining

To investigate the effect of simvastatin on the mineralization of OLCs, three groups of OLCs were cultured for 28 days cells were divided into blank group (cells were cultured in growth

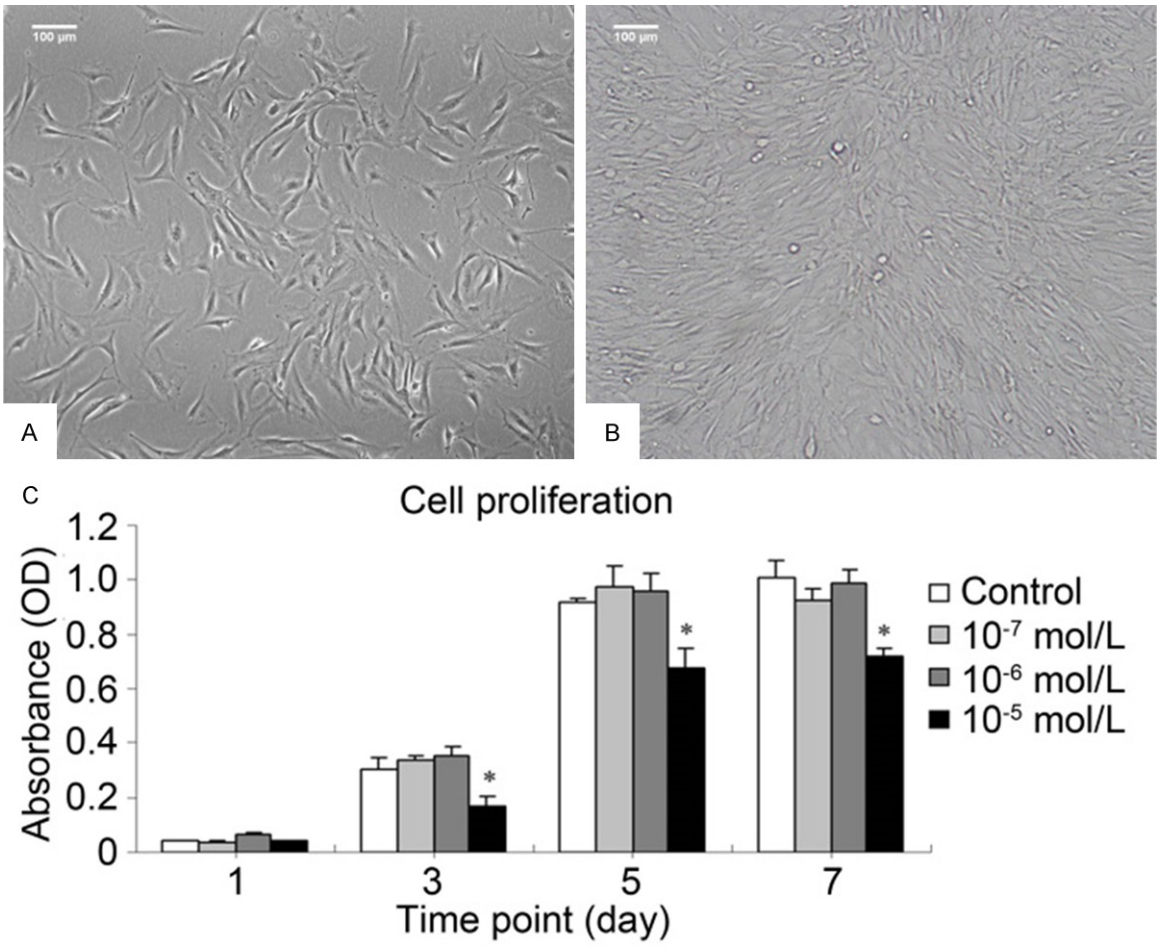

Figure 1. Effect of simvastatin at different concentrations on the proliferation of OLCs. A. Morphology of OLCs. B. Phase contrast microscopic image of OLC cells at 3 days (Scale bar, 100 μm). C. The cell proliferation in different concentration of 10^{-7} mol/L, 10^{-6} mol/L and 10^{-5} mol/L simvastatin was tested by MTT assay at day 1, 3, 5 and 7. (n = 5, t-test; *P<0.05).

medium), positive control group (cells were cultured in odontogenic medium), simvastatin group (cells were cultured in odontogenic medium supplemented with 10^{-6} mol/L simvastatin). Cells were maintained for 28 days, harvested, washed twice with phosphate-buffered saline (PBS, Hyclone, USA) and then fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were stained with 0.1% alizarin red S (Sigma, USA) in distilled water (pH = 4.13) for 30 min at 37°C in dark. Several washes in deionized water were done to remove unbound dye. For ALP staining, cells were harvested on day 7 and stained using a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology, Beijing, PR China) for 30 min at 37°C in dark. Then, cells were washed 3 times with distilled water (1 min for each) and observed under a phase contrast microscope. For quantification, ALP positive staining area and mineralized nodules were analyzed with software of image J (2.0).

Quantitative RT-PCR

Total cellular RNA was extracted from OLCs by using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Approximately 1 μg total RNA waws reverse transcribed into cDNA using a PrimeScript™ RT Reagent Kit containing gDNA Eraser (TaKaRa, Japan). Odontogenesis and Wnt-related molecules were evaluated in the OLCs on the basis of the expression of specific genes (Dentin Matrix Phosphoprotein-1; DMP-1, Dentin Sialophosphoprotein; DSPP and Runt-related Gene-2; Runx2, Lef-1, Wnt10a and Wnt 11). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Quantitative RT-PCR was performed by using an

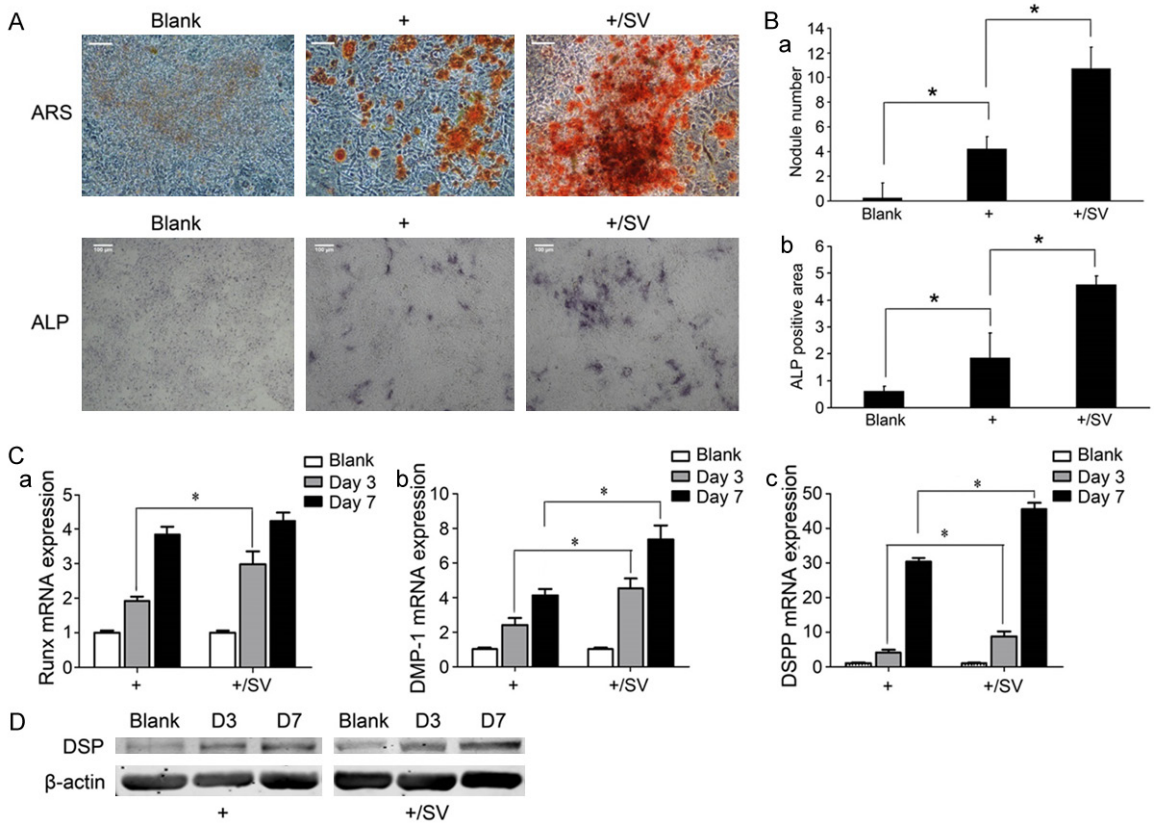

Figure 2. Effect of simvastatin on the odontogenic differentiation of OLCs. A. ARS and ALP staining of OLCs in different groups (blank control: Blank; odontogenic medium [OM]: positive control (+) and odontogenic medium supplemented with 10^{-6} mol/L simvastatin [OM+SV]: simvastatin group). (Scale Bar = 100 μm). SV, simvastatin. Ba. Nodule number after ARS (n = 5). Bb. Positive ALP staining area (n = 5). C. mRNA expressions of Runx2, DMP-1 and DSPP in three groups on days 3 and 7 (RT-PCR) (n = 5, **P*<0.05). D. Protein expression of DSP in different groups (Western blot assay). On day 7, the relative gray value of DSP in +/SV group (0.590±0.045) was significantly higher than that in + group (0.482±0.026) (P<0.05).

ABI 7500 Real-time PCR System (Applied Biosystems, USA) with SYBR® Premix Ex TaqTM II (TaKaRa, Japan). The samples were subjected to 40 cycles of denaturation at 95°C for 15 sec, annealing at 64°C for 20 sec and extension at 72°C for 25 sec using specific primers (**Table 1**). The relative gene expression was calculated with $2^{-\Delta\Delta Ct}$ method. The mean Ct value of target gene was normalized to that of GAPDH to obtain a ΔCt value, which was then used to normalize the expression of target gene in control group to obtain ΔΔCt value.

Western blot assay

On days 0, 3, and 7, OLCs in different groups were washed 3 times with ice-cold PBS, and then lysed using a protein extraction kit (Piece, USA). Protein concentrations were detected using a BCA protein assay kit (Pierce, Thermo, USA). Protein samples (25 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore Corporation, USA). The membranes were washed with TBST (10 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, and 0.05% Tween-20), blocked with 5% skim milk for 2 h, and incubated with corresponding primary antibody (rabbit anti-mouse Lef-1; rabbit anti-mouse Wnt10a; rabbit anti-mouse Wnt11; Abcam, Burlingame, CA; DSP antibody, Santa Cruz Biotechnology, USA) at 1:200 and rabbit anti-mouse β-actin antibody at 1:2000 (Santa Cruz Biotechnology, USA) overnight at -4°C. The membrane was washed, and the secondary antibody (goat anti-rabbit immunoglobulin G; LiCor Co., USA) was added at 1:5000, followed by incubation for 2 h at room temperature. The membranes were washed, and visualization was done with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, USA) and the membranes were exposed to X-ray film (Eastman Kodak, USA).

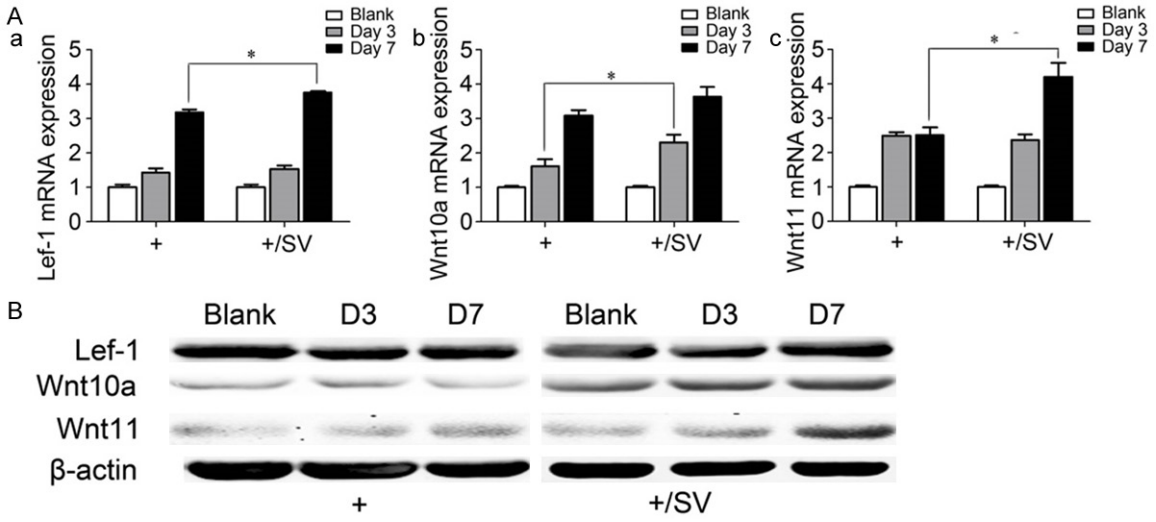

Figure 3. Effect of simvastatin on the Wnt-signaling pathway related molecules during odontogenic differentiation of OLCs. Blank: blank control; +: positive control; +/SV: 10^{-6} mol/L simvastatin group. A. mRNA expressions of Lef-1, Wnt10a and Wnt11 in three groups on days 3 and 7 (RT-PCR). (n = 5, **P*<0.05). B. Protein expressions of Lef-1, Wnt10a and Wnt 11 in three groups on days 3 and 7. On day 7, the relative gray value of Lef-1, Wnt10a and Wnt11 in +/SV group (1.286±0.090, 0.667±0.086 and 0.724845±0.161) was significantly higher than those in + group (1.006±0.07, 0.169±0.060 and 0.384214±0.081) (P<0.05). In +/SV group, the relative gray value of Wnt10a on day 3 (0.432875±0.069) was significantly higher than that on day 7 (0.311732±0.050) (P<0.05).

Statistical analysis

Statistical analysis was performed with SPSS version 19.0. *t*-test for comparison between groups. A value of *P*<0.05 was considered statistically significant.

Results

Effect of Simvastatin on cell proliferation of OLCs

The morphology of OLCs is shown in **Figure 1A**. OLCs are uniformly elongated (**Figure 1A**), and tend to be swirling-like and wave-like before reaching 100% confluence (**Figure 1B**). Simvastatin at 10^{-7} mol/L and 10^{-6} mol/L had no effect on the cell proliferation within 7 days. However, simvastatin in 10^{-5} mol/L significantly suppressed the cell proliferation on days 3, 5 and 7. Therefore, simvastatin at 10^{-6} mol/L was used in subsequent experiments to examine the effects of simvastatin, which was in accordance with previous study [10].

Simvastatin promotes the mineralization of OLCs

After 7-day and 28-day culture, no obvious ALP positive staining area and mineralized nodules were observed in blank control group compared to the positive control (+). When addition of 10^{-6} mol/L simvastatin into odontogenic medium (+/SV), larger ALP positive area and more mineralized nodules were found, as compared to positive control (+). (**Figure 2A**). Quantification confirmed that simvastatin increased the mineralization of OLCs (**Figure 2B**). Moreover, the mRNA expressions of several odontoblastic markers were significantly increased in the simvastatin group (**Figure 2C**). The protein expression of DSP was markedly up-regulated in response to simvastatin treatment on day 7 (**Figure 2D**).

Involvement of Wnt signaling pathway in simvastatin-induced mineralization

In order to explore whether Wnt signaling pathway is involved in the simvastatin-induced mineralization, the mRNA and protein expressions of Wnt-related molecules (Lef-1: typical transcription factor of canonical Wnt/β-catenin signaling; Wnt10a: a protein involved in canonical Wnt/β-catenin signaling pathway; Wnt11: a protein associated with both canonical and non-canonical Wnt signaling pathways) on days 3 and 7. qRT-PCR showed that the mRNA expressions of Lef-1, Wnt10a and Wnt11 were significantly higher in simvastatin group (+/SV) than positive control group (+) after odontogenic differentiation (**P*<0.05; **Figure 3Aa-c**). Western blot assay confirmed it.

Discussion

Odontoblast is one of the important cell types involved in dentinogenesis. OLC is an ideal model for odontoblast because of their similar characteristics to the immature odontoblasts. OLCs could be successfully differentiated into odontoblast-like cells showing several phenotypes of mature odontoblasts, such as increased ALP production, odontogenic gene (DSPP and DMP-1) mRNA expression, and mineralized nodule formation [10, 22]. ALP is an early indicator of odontoblast differentiation [5]. ARS staining was used to identify the mineralized deposits in the extracellular matrix. DSPP and DMP-1 are specific markers for mature odontoblasts, and Runx2 has been identified as essential for the later stage of tooth formation, is intimately involved in the development of calcified tooth tissue [23]. In our present study, OLCs were employed to investigate the mechanism of simvastatin-induced odontogenic differentiation and ALP, DSPP, DMP-1 and Runx2 were chosen as differentiation makers of odontoblasts.

Odontogenic differentiation can be stimulated by odontoinductive factors (such as dexamethasone, ascorbic acid, and β-glycerophosphate) and growth factors (such as bone morphogenetic protein-2) [19]. It was reported that simvastatin could promote the odontogenic differentiation of tooth-derived cells, such as human dental stem cells (hDPSCs) [10], human dental pulp cells (hDPCs) [5, 22, 24]. In our study, the results showed that the mRNA and protein expressions of DSPP, DMP-1, Runx2 and DSP were upregulated, suggesting simvastatin could enhance the odontogenic differentiation of OLCs.

Wnt signaling pathway closely associated with the tooth initiation and development. The odontogenic differentiation could be promoted by simvastatin. Therefore, we speculate that the odontogenic differentiation of OLCs promoted by simvastatin might be mediated by Wnt signaling pathway. Although Wnt signaling pathway is a critical regulator of tooth development and dentinogenesis, the effect of simvastatin on the Wnt signaling pathway activation remain unclear. Wnt protein initiates intracellular cascades via binding to membrane receptor complexes (including low-density lipoprotein receptor-related protein (LRP) 5/6 and its co-receptors) and frizzled (Fz) G-protein-coupled receptor [25]. Lef, the downstream transcription factors of canonical Wnt signaling pathway, triggers the expression of multiple genes. At Embryo 12 (E12), Lef-1 mRNA expression is observed in the dental epithelium and the underlying presumptive dental mesenchyme [15, 26], whereas mice lacking Lef-1 exhibit an arrest of tooth development at the bud stage [27]. Furthermore, Lef-1 induces DSPP expression by binding to its promoter region [28]. Thus, Lef-1 is important transcription factor in dentinogenesis. In our study, we found Lef-1 was significantly up-regulated, suggesting that Lef-1 play an important role in the odontogenic differentiation of OLCs promoted by simvastatin. We speculated that canonical Wnt signaling pathway involved in this process enhanced by simvastatin. Wnt10a, a canonical Wnt family member, is related to the odontoblast differentiation and cusp morphogenesis through regulating DSPP mRNA expression [16]. In the present study, the expression of Wnt10a was examined during this process, which showed upregulating. Therefore, we infer canonical Wnt signaling pathway might mediate the simvastatin-induced odontogenic differentiation by the important transcription factor of Lef-1. It is well known that Wnt signaling is consist of canonical and non-canonical. Although in the present study we found the canonical Wnt signaling was involved, it is necessary to test the role of non-canonical Wnt signaling in this process. Wnt11, a member of Wnt signaling pathway, is associated with non-canonical signaling pathways [29] and has been reported to be involved in BMP2-induced odontoblast differentiation [19]. Our results revealed that the expression of Wnt11 was increased, suggesting the non-canonical might also involved. It is indicated that Simvastatin promotes the osteogenic differentiation via canonical Wnt/β-catenin pathway [20] in mouse embryonic stem cells (mESCs), while it is via both canonical and non-canonical Wnt/β-catenin signaling pathways [21] in rat bone marrow-derived mesenchymal stem cells (rBMSCs). Our results suggest that the Wnt signaling pathway is involved in the statin-induced odontogenic differentiation of OLCs. However, further studies are necessary to evaluate whether canonical or non-canonical Wnt/β-catenin signaling pathway plays a dominant role in this process.

Here, our results revealed that simvastatin promotes odontogenic differentiation. Considering

the pleiotropic effects of simvastatin (anti-inflammatory and angiogenic effects) [4, 5], our findings indicate that simvastatin might be an ideal pulp-capping material [10].

In summary, our study demonstrates that simvastatin at an appropriate concentration could enhance the odontogenic differentiation of OLCs which might mediated by either canonical or non-canonical Wnt/β-catenin signaling pathways. These findings might provide new insight into dental treatment that simvastatin may be used as a dental capping material.

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Disclosure of conflict of interest

None.

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References

[1] Arany S, Nakata A, Kameda T, Koyota S, Ueno Y and Sugiyama T. Phenotype properties of a novel spontaneously immortalized odontoblast-lineage cell line. Biochem Biophys Res Commun 2006; 342: 718-724.

[2] Arany S, Nakata A, Kameda T, Koyota S, Ueno Y and Sugiyama T. Phenotype properties of a novel spontaneously immortalized odontoblast-lineage cell line. Biochem Biophys Res Commun 2006; 342: 718-724.

[3] Wang Z, Ma F, Wang J, Zhou Z, Liu B, He X, Fu L, He W and Cooper PR. Extracellular Signal-regulated Kinase Mitogen-activated Protein Kinase and Phosphatidylinositol 3-Kinase/Akt Signaling Are Required for Lipopolysaccharide-mediated Mineralization in Murine Odontoblast-like Cells. J Endod 2015; 41: 871-876.

[4] Kwak B, Mulhaupt F, Myit S and Mach F. Statins as a newly recognized type of immunomodulator. Nat Med 2000; 6: 1399-1402.

[5] Min KS, Lee YM, Hong SO and Kim EC. Simvastatin promotes odontoblastic differentiation and expression of angiogenic factors via heme oxygenase-1 in primary cultured human dental pulp cells. J Endodont 2010; 36: 447-452.

[6] Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M and Gutierrez G. Stimulation of bone formation in vitro and in rodents by statins. Science 1999; 286: 1946-1949.

[7] Ayukawa Y, Yasukawa E, Moriyama Y, Ogino Y, Wada H, Atsuta I and Koyano K. Local application of statin promotes bone repair through the suppression of osteoclasts and the enhancement of osteoblasts at bone-healing sites in rats. Oral Surg Oral Med Oral Pathol Oral Radiol Endodontol 2009; 107: 336-342.

[8] Kaji H, Naito J, Inoue Y, Sowa H, Sugimoto T and Chihara K. Statin suppresses apoptosis in osteoblastic cells: role of transforming growth factor-beta-smad3 pathway. Hormone Metabol Res 2008; 40: 746-751.

[9] Ruan F, Zheng Q and Wang J. Mechanisms of bone anabolism regulated by statins. Biosci Rep 2012; 32: 511-519.

[10] Okamoto Y, Sonoyama W, Ono M, Akiyama K, Fujisawa T, Oshima M, Tsuchimoto Y, Matsuka Y, Yasuda T, Shi S and Kuboki T. Simvastatin Induces the Odontogenic Differentiation of Human Dental Pulp Stem Cells In Vitro and In Vivo. J Endodont 2009; 35: 367-372.

[11] Pettiette MT, Zhong S, Moretti AJ and Khan AA. Potential Correlation between Statins and Pulp Chamber Calcification. J Endodont 2013; 39: 1119-1123.

[12] Logan CY and Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004; 20: 781-810.

[13] Lena Sarkar and Sharpe PT. Expression of Wnt signalling pathway genes during tooth development. Mechan Dev 1999; 85: 197-200.

[14] Suomalainen M and Thesleff I. Patterns of Wnt Pathway Activity in the Mouse Incisor Indicate Absence of Wnt/beta-Catenin Signaling in the Epithelial Stem Cells. Dev Dynam 2010; 239: 364-372.

[15] Chen J, Lan Y, Baek JA, Gao Y and Jiang R. Wnt/beta-catenin signaling plays an essential role in activation of odontogenic mesenchyme during early tooth development. Dev Biol 2009; 334: 174-185.

[16] Yamashiro T, Zheng L, Shitaku Y, Saito M, Tsubakimoto T, Takada K, Takano-Yamamoto T and Thesleff I. Wnt10a regulates dentin sialo-

phosphoprotein mRNA expression and possibly links odontoblast differentiation and tooth morphogenesis. Differentiation 2007; 75: 452-462.
[17] Peng L, Ren LB, Dong G, Wang CL, Xu P, Ye L and Zhou XD. Wnt5a promotes differentiation of human dental papilla cells. Int Endodont J 2010; 43: 404-412.
[18] Lin M, Li L, Liu C, Liu H, He F, Yan F, Zhang Y and Chen Y. Wnt5a Regulates growth, patterning, and odontoblast differentiation of developing mouse tooth. Dev Dynam 2011; 240: 432-440.
[19] Koizumi Y, Kawashima N, Yamamoto M, Takimoto K, Zhou M, Suzuki N, Saito M, Harada H and Suda H. Wnt11 expression in rat dental pulp and promotional effects of Wnt signaling on odontoblast differentiation. Congen Anomal 2013; 53: 101-108.
[20] Qiao LJ, Kang KL and Heo JS. Simvastatin promotes osteogenic differentiation of mouse embryonic stem cells via canonical Wnt/beta-catenin signaling. Mol Cell 2011; 32: 437-444.
[21] Zhang L, Zhang L, Tian F, Han D, Niu J and Liu X. Effect of simvastatin on mRNA expressions of some components of Wnt signaling pathway in differentiation process of osteoblasts derived from BMSCs of rats. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2009; 23: 1371-1375.
[22] Karanxha L, Park SJ, Son WJ, Nor JE and Min KS. Combined effects of simvastatin and enamel matrix derivative on odontoblastic differentiation of human dental pulp cells. J Endodont 2013; 39: 76-82.
[23] Camilleri S and McDonald F. Runx2 and dental development. Eur J Oral Sci 2006; 114: 361-373.
[24] Min KS, Lee YM, Hong SO and Kim EC. Simvastatin promotes odontoblastic differentiation and expression of angiogenic factors via heme oxygenase-1 in primary cultured human dental pulp cells. J Endod 2010; 36: 447-452.
[25] Chen J, Lan Y, Baek JA, Gao Y and Jiang R. Wnt/beta-catenin signaling plays an essential role in activation of odontogenic mesenchyme during early tooth development. Dev Biol 2009; 334: 174-185.
[26] Liu F, Chu EY, Watt B, Zhang Y, Gallant NM, Andl T, Yang SH, Lu MM, Piccolo S, Schmidt-Ullrich R, Taketo MM, Morrisey EE, Atit R, Dlugosz AA and Millar SE. Wnt/beta-catenin signaling directs multiple stages of tooth morphogenesis. Dev Biol 2008; 313: 210-224.
[27] Kratochwil K, Galceran J, Tontsch S, Roth W and Grosschedl R. FGF4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Lef1(-/-) mice. Genes Dev 2002; 16: 3173-3185.
[28] Yokose S and Naka T. Lymphocyte enhancer-binding factor 1: an essential factor in odontoblastic differentiation of dental pulp cells enzymatically isolated from rat incisors. J Bone Miner Metab 2010; 28: 650-8.
[29] Mikels AJ and Nusse R. Wnts as ligands: processing, secretion and reception. Oncogene 2006; 25: 7461-7468.