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Histone acetylation regulates osteodifferentiation of hDPSCs via DSPP

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1. ABSTRACT

Dental pulp stem cells (DPSCs) are a unique population of precursor cells isolated from postnatal human dental pulp, with the ability to regenerate a reparative dentin-like complex. We examined the regulation of odontoblast-like differentiation of DPSCs by histone acetylation. Western blot analysis showed that histone H3 acetylation was strongly induced in osteodifferentiation medium. Inhibition of histone acetyltransferase by garcinol reversed osteodifferentiation and mineral formation. Real-time polymerase chain reaction assay indicated that the dentin sialophosphoprotein (DSPP) gene, which is mainly expressed in odontoblasts and preameloblasts in teeth and plays an important role in tooth function, was also down-regulated in garcinol-treated cells. Moreover, lentivirus-mediated knockdown of *DSPP* in human DPSCs was associated with significant inhibition of mineral formation, but not osteoblast differentiation. In conclusion, the results of this study suggest that DSPP positively affects mineral formation, and that odontoblast-like differentiation and maturation of DPSCs can be regulated by histone acetylation of the *DSPP* gene.

2. INTRODUCTION

Human dental pulp stem cells (hDPSCs) constitute a unique mesenchymal stem cell (MSC) population that has been isolated from adult tooth pulp and characterized in terms of its self-renewal capability, multi-lineage differentiation, and clonogenic efficiency (1,2). The most striking feature of DPSCs is their ability to regenerate a dentin-pulp-like complex composed of a mineralized matrix with tubules lined with odontoblasts, and fibrous tissue containing blood vessels, in an arrangement similar to the dentin-pulp complex found in normal human teeth. Isolated DPSCs can be induced to differentiate into odontoblast-like cells and form calcified nodules by stimulation with dexamethasone, β-glycerophosphate and inorganic phosphate, and a dentin-like mineral structure, which is important for the dental repair process, can be observed *in vitro*(3,4). However, although hDPSCs show great promise for tooth repair and dentin regeneration, the molecular mechanisms regulating hDPSCs remain poorly understood.

DSPP is a bicistronic transcriptional unit encoding two proteins, dentine sialoprotein (DSP) and

dentine phosphoprotein (DPP)(5-7), the expression of which is restricted mainly to developing teeth(8-10). *DSPP*-null mice show tooth defects similar to human dentinogenesis imperfecta III, with enlarged pulp chambers, increased width of the predentin zone, hypomineralization, and pulp exposure(11). However, the role of *DSPP* in human DPSCs had not yet been examined. Epigenetic modifications of chromatin are of increasing interest as a molecular connection between genes and the environment. In mammalian cells in particular, chromatin structure can be regulated by environmental factors such as diet, chemicals and pathogens(12). The nucleosome, which represents the basic repeat unit of chromatin, consists of two copies each of the histones H2A, H2B, H3, and H4 wrapped in 147 bp of DNA(13). In addition to DNA methylation, post-translational modifications (PTMs) of the N-terminal amino acids of histones play an important role in affecting chromatin structure and modulating gene transcription(14-17). Epigenetics refers not only to heritable changes, including those conferred mitotically or meiotically, that occur without changes in the DNA sequence, but also refer to the structural adaptation of chromosomal regions(18). Epigenetic transmission can thus be controlled by both DNA methylation and histone PTMs(19). Essentially, the chromatin status of a gene promoter plays a role in determining how that gene is affected by various stimuli. Histone PTMs have varying effects on gene expression depending on the position, type of modification (acetylation, methylation, phosphorylation, or ubiquitylation), and also the degree of methylation (mono-, di-, or tri-), through recognition by key chromatin-modifying proteins (14-17). In general, histone H3 lysine 4-trimethylation (H3K4me3) and H3 K9-acetylation (H3K9Ac) are associated with promoters and active genes, while H3K9me2, H3K9me3 and H3K27me3 are generally associated with repressed genes, and H4K16Ac may control chromatin structure through interactions with key proteins(14-17,20).

In the present study, we investigated the roles of dentin sialophosphoprotein (DSPP) and epigenetic modification in the regulation of DPSC differentiation and mineralization.

3. MATERIALS AND METHODS

3.1. Cell culture and lentiviral infection

DPSCs were isolated from human adult third molars as described previously (1). DPSCs were cultured in α-modified Eagle's medium (α-MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal calf serum (FCS; Invitrogen), 10 mM L-ascorbic acid 2-phosphate (AA), 2 mM L-glutamate, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2, as reported previously(1). To generate DPSCs with stable knockdown of *DSPP*, we generated a vector containing a small hairpin (sh)RNA sequence specific to *DSPP*, and generated viruses in Lenti-X-293T cells (Clontech). DPSCs were infected with lentiviruses expressing ZsGreen (to determine transfection efficiency), pLVX-*DSPP*-shRNA, or vector control in the presence of 6 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). At 36 h after infection, cells were selected with puromycin (1 μg/mL) for 10 days. Resistant clones were pooled, and cells expressing *DSPP*-shRNA were confirmed by Western blot analysis. For growth rate analysis, 100,000 cells were plated onto 15 × 60-mm plates, and plates were trypsinized at 24, 48, and 72 h. Triplicate cell counts were performed with MTT assays to determine viable cells.

3.2. Alkaline phosphatase (ALP) activity

DPSCs were induced to differentiate in α-MEM supplemented with 10 mM β-glycerophosphate, 10 nM dexamethasone, and 1.8 mM KH_2PO_4. Induced cells were rinsed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. One milliliter per well of substrate solution was added, and the plate was covered with foil and incubated at 37°C for 30 min. The substrate solution contained 12 mg fast blue BB salt, 4 mg naphthol AS-TR phosphate, and 0.15 mL dimethylformamide (DMF) in 15 mL Tris-HCl (pH 9.6). The naphthol was first dissolved in the DMF before being mixed with the Tris-HCl. The mix was filtered, and 50 μL $MgCl_2$ was added. Quantification of the staining density was performed using Scion Image software (Scion Corp., Frederick, MD, USA).

3.3. Alizarin red staining

Induced cells were rinsed with PBS and fixed in 70% ethanol for 1 h at 4°C. Cells were stained with 40 mM Alizarin red, pH 4.2, at room temperature for 10 min on a shaker. Cells were rinsed five times with water to remove unbound Alizarin red. PBS was added for an additional 15 min for further reduction of non-specific staining. Quantification of the staining intensity was performed using Scion Image software.

3.4. Western blot analysis

Cells underwent lysis in RIPA buffer (10 mM Tris-HCL, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM -glycerophosphate, 50 mM sodium fluoride). The samples were separated on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were treated with 5% milk for 2 h and then incubated with primary antibodies overnight. The immune complexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI, USA) and visualized with SuperSignal reagents (Pierce, Rockford, IL, USA). Primary antibodies were purchased from the following commercial sources: monoclonal antibodies against acetyl-histone H3 (1:500; University of Iowa, Iowa City, IA, USA); monoclonal antibodies against total histone H3 (1:1000; Covance, Princeton, NJ, USA); polyclonal antibodies against DSPP (NIDCR/NIH, Bethesda, MD, USA); and monoclonal antibodies against β-actin (1:7500; Sigma-Aldrich).

3.5. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from cells by treatment with TRIzol reagent (Invitrogen). Total RNA (2–5 μg) was converted to cDNA using a SuperScript First Strand

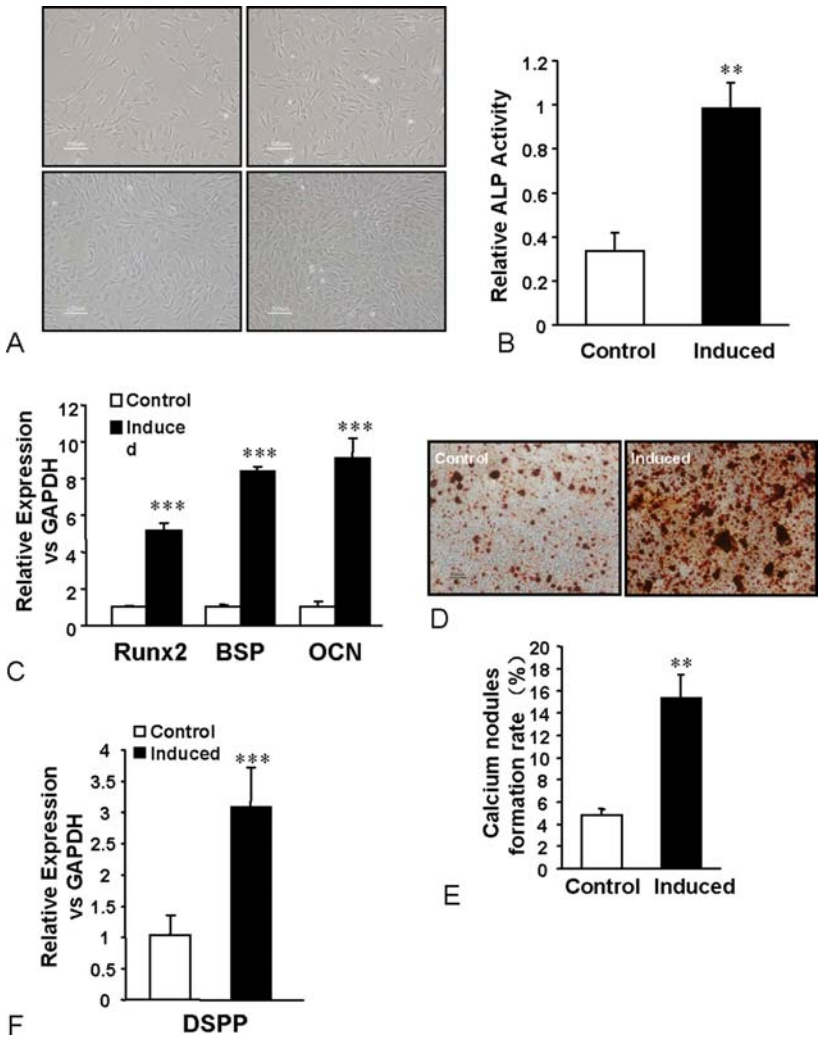

Figure 1. Morphology and characterization of cultured dental pulp stem cells (DSPCs). (A) Cell morphology. Cells within each colony were characterized by a typical fibroblast-like morphology analogous to the progeny of human bone marrow CFU-Fs. (B) Alkaline phosphatase activity in osteo-induced DPSCs. (C) Osteodifferentiation-related gene expression. (D) Mineral formation in DPSCs in osteodifferentiation medium; (E) Formation rate of calcium nodules stained by Alizarin red. (F) *DSPP* expression in induced and control DPSCs determined by real-time PCR. Numbers indicate formation rate of the calcium nodules. Values are means ± S.E.M. **P<0.01, ***P<0.001 vs. control group. Experiments were performed in duplicate for three separate experiments.

Synthesis kit (Invitrogen). The resulting cDNA was diluted 1:20 and used for 20-μL reactions containing SYBR Green Master Mix, dNTPs, primers, and Platinum Taq DNA polymerase (Invitrogen).Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was quantified simultaneously as an internal control. All experiments were run in triplicate and repeated at least twice to confirm the results. Primers for human bone sialoprotein (BSP), human-Runx2 and human osteocalcin (OCN) were self-designed.

3.6. Statistical analysis

Continuous values are expressed as mean ± standard deviation (SD) based on at least five (activities of LDH and CK-MB) or three (infarct area, apoptosis index, and Western blotting analysis) independent experiments. Differences in means between groups were tested by one-way analysis of variance (ANOVA). P values <0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

4. RESULTS

4.1. Isolation and characterization of hDPSCs

Dental pulp tissues were isolated from third molars. When seeded in culture dishes, the cells within each colony were characterized by a typical fibroblast-like morphology (Figure 1A) analogous to the progeny of human bone marrow CFU-F. The cells reached 90% confluency in 8 days, and were then cultured in medium promoting osteo/odontoblastic differentiation. After 15 days in culture, they became ALP positive and produced calcified matrix, as judged by the results of Alizarin red

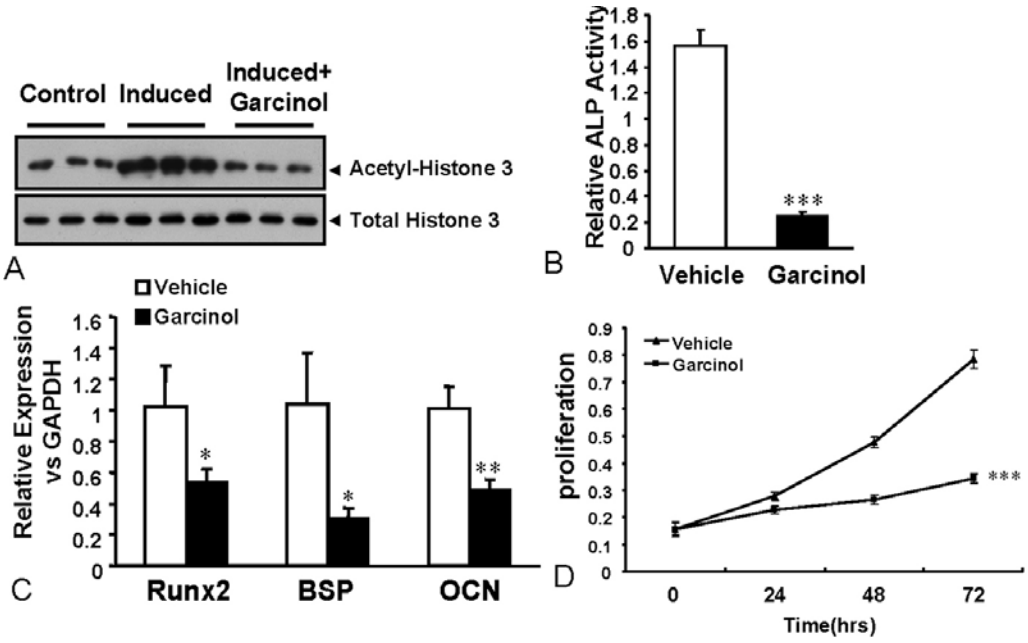

Figure 2. Effect of inhibition of histone H3 acetylation on human dental pulp stem cell (hDPSC) osteodifferentiation. (A) Western blot assay of acetyl-histone H3 in control, osteo-induced, and garcinol-treated hDPSCs. (B) Alkaline phosphatase activity in garcinol-treated and control hDPSCs. (C) Osteo-related gene expression in control and garcinol-treated hDPSCs. (D) Proliferation of control and garcinol-treated hDPSCs. Values are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle group. Experiments were performed in duplicate for three separate experiments.

staining (Figure 1B, D). The expression of the osteoblastic differentiation markers Runx2, BSP, and OCN were confirmed by real-time PCR and compared between the induced and control groups. The results indicated that hDPSCs isolated from developing third molars had differentiation potential comparable to that previously reported for later stages.

4.2. Inhibition of histonH3 acetylation suppresses hDPSC osteodifferentiation

hDPSCs were cultured in osteoinduction medium with or without the histone acetyltransferase (HAT) inhibitor garcinol. Protein and RNA from the cells were collected and analyzed. The results of western blotting showed that acetyl-histone H3 was significantly up-regulated by osteoinduction (Figure 2A). However, osteodifferentiation of hDPSCs was significantly down-regulated by the addition of garcinol. These results were supported by ALP activity and osteo-marker expression (Figure 2B, C). MTT assays revealed that the proliferation of hDPSCs was also down-regulated by the addition of garcinol (Figure 2D).

4.3. Inhibition of histone H3 acetylation suppresses hDPSC mineral formation

The effect of garcinol on mineral formation was determined by Alizarin red staining and calcium quantification. Garcinol treatment significantly inhibited calcium-nodule formation and calcium quantity (Figure 3A, B, C). Furthermore, real-time PCR revealed significant down-regulation of *DSPP* by garcinol, compared to the control group (Figure 3D).

4.4. DSPP knock-down does not affect osteoblastic differentiation

To identify the role of *DSPP* in osteoblastic differentiation, hDPSCs were infected with lentiviruses expressing *DSPP*-shRNA (Figure 4A). Stable cell lines were established by fluorescence-activated cell sorting sorting and confirmed by western blot analysis (Figure 4B). The cells were then cultured in osteoinduction medium. There were no significant differences in ALP activity and osteo-differentiation marker expression between the *DSPP*-shRNA and control groups (Figure 4C, D). There were no differences in cell proliferation at 24 and 48 h, but the number of *DSPP*-knockdown cells was significantly lower in the *DSPP*-shRNA group, compared to the control group at 72h (Figure 4E). The higher proliferation rate at 72 h may be attributed to the fact that, under physiological conditions, damage to the dental pulp results in activation and proliferation of resting DSPCs.

4.5. DSPP knockdown inhibits mineral formation

To identify the role of *DSPP* in osteoblastic maturation, induced cells were stained with Alizarin red and calcium nodules were quantified (Figure 5A,B). Alizarin red staining and calcium-nodule formation were significantly reduced in the *DSPP*-knockdown group. This result was confirmed by measurement of relative calcium quantities (Figure 5C).

5. DISCUSSION

Human adult bone marrow MSCs represent one of the most promising adult stem cell populations for bone

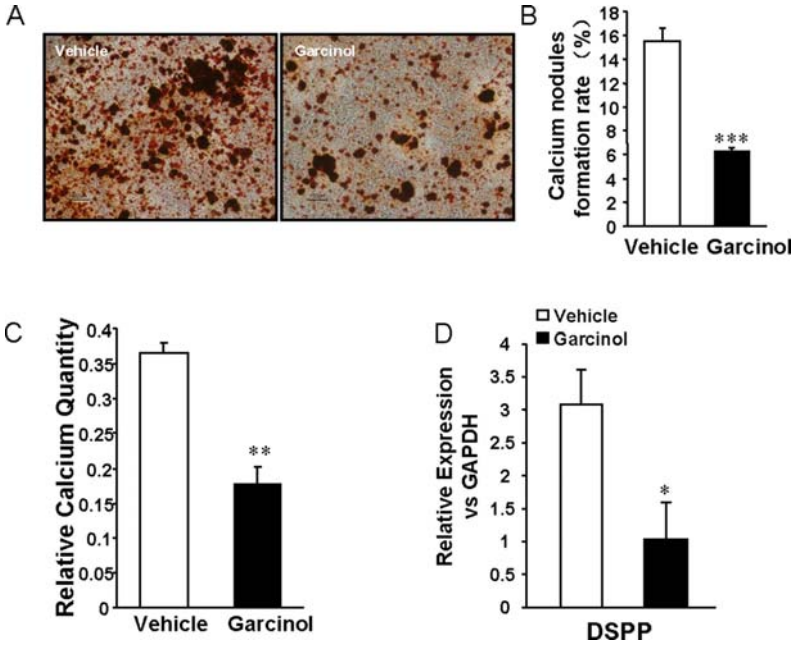

Figure 3. Effect of inhibition of histone H3 acetylation on human dental pulp stem cell (hDPSC) mineral formation. (A) Calcium nodules in control and garcinol-treated hDPSCs assayed by Alizarin red. (B) Rate of calcium-nodule formation in control and garcinol-treated hDPSCs. (C) Calcium content in control and garcinol-treated hDPSCs. (D) *DSPP* expression in control and garcinol-treated hDPSCs. Values are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle group. Experiments were performed in duplicate for three separate experiments.

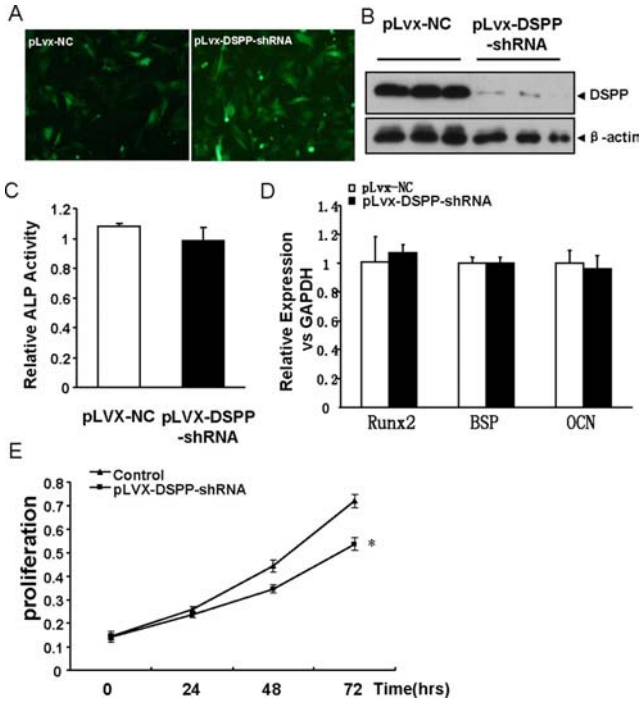

Figure 4. Proliferation and osteo-differentiation in *DSPP*-knockdown human dental pulp stem cells (hDPSCs). (A) ZsGreen expression in pLVX-*DSPP*-shRNA and negative control cells (NC) cells. (B) Western blot assay of DSPP in pLVX-*DSPP*-shRNA and NC cells. (C) Alkaline phosphatase activity in pLVX-*DSPP*-shRNA and NC cells. (D) Osteo-related gene expression in pLVX-*DSPP*-shRNA and NC cells. (E) Proliferation of pLVX-*DSPP*-shRNA and NC cells. Values are means ± S.E.M. *P<0.05, vs. NC group. Experiments were performed in duplicate for three separate experiments.

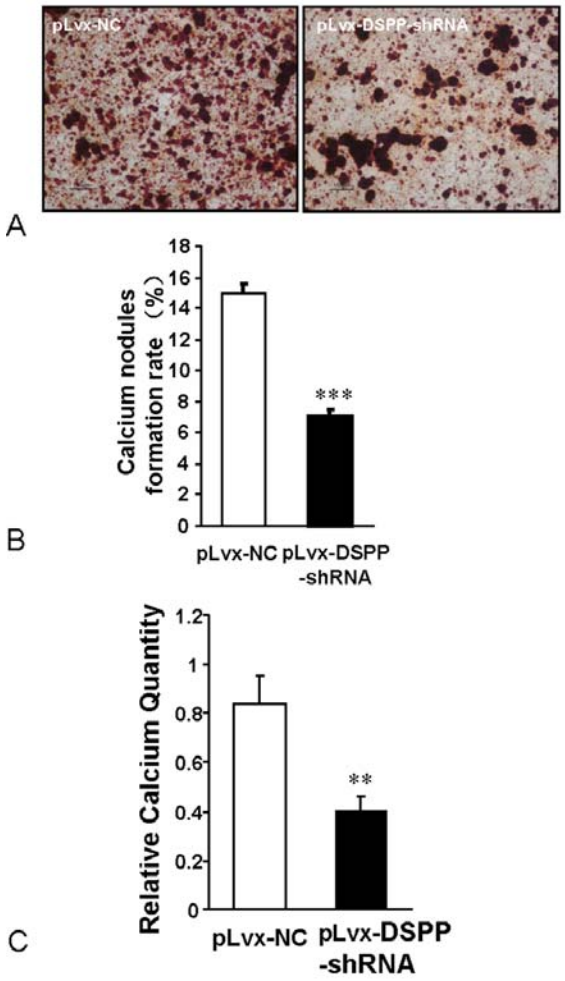

Figure 5. Mineral formation in *DSPP*-knockdown human dental pulp stem cells (hDPSCs). (A) Calcium-nodule formation in pLVX-*DSPP*-shRNA and negative control (NC) cells. Rate of calcium-nodule formation in pLVX-*DSPP*-shRNA and NC cells (B). (C) Relative calcium quantities in pLVX-*DSPP*-shRNA and NC cells. Values are means ± S.E.M. **P<0.01, ***P<0.001, vs. NC group. Experiments were performed in duplicate for three separate experiments.

regeneration and repair. Several important discoveries have recently suggested that the induction of acetylation modification may help to improve bone or dental tissue engineering (21-23). The results of the current study demonstrated that acetylation of histone H3 was up-regulated in osteo-induced hDPSCs, while abrogation of acetylation with a specific HAT inhibitor attenuated osteo-differentiation and inhibited mineral formation in DPSCs. Acetylation can affect signaling pathways and thereby alter cell fate and function. mRNA splicing, transport, integrity and translation, and protein activity, localization, stability and interactions are all regulated by acetylation. Acetylation can thus interfere with every step of the regulatory processes from signalling to transcription to protein degradation. This study showed that H3 acetylation modification regulates osteoblast-like differentiation and mineral formation in hDPSCs, partly via the *DSPP* gene. These results indicate an important role for *DSPP* in promoting mineral formation, and suggest that suppression of H3-acetylation can inhibit osteoblast-like differentiation and mineral formation in DPSCs. However, the detailed molecular mechanisms have not yet been explored, and further studies are required to establish the effects of targeting acetylation modification *in vivo* on mineralized tissue regeneration.

We further investigated the key factors affecting osteodifferentiation. Histone deacetylase inhibitors (HDIs) are currently undergoing phase I and II clinical trials as anticancer agents (24,25), and some HDIs are also commonly prescribed as treatments for epilepsy and bipolar disorders. However, despite their systemic administration, the effects of HDIs on osteoblasts and bone formation have not been extensively examined. Schroeder *et al.* investigated the effects of HDIs on osteoblast proliferation and differentiation and demonstrated that they promoted osteoblast maturation (26). The results of the current study provide the first evidence that osteoblast-like differentiation

of DPSCs was attenuated by inhibition of histone 3 acetylation, which was consistent with the results of the previous study. Several groups have recently demonstrated the importance of acetylation modification in skeletal development (27).

DSPP is critical for proper mineralization of tooth dentin, and has been implicated in the etiologies of dentin dysplasia type II and dentinogenesis imperfecta (types II and III) (11,28,29). These autosomal dominant disorders affect about one in every 6000–8000 individuals. There is much interest in the roles and mechanisms of DSPP-derived proteins. DSPP represents 90% of the non-collagenous proteins in dentin. DPSCs stably expressing *DSPP*-shRNA were established, and down-regulation of DSPP was confirmed by Western blot analysis. Proliferation of the *DSPP*-knockdown cell line was significantly lower than that of the control at 72 h, possibly because *DSPP* knockdown change the intracellular signaling pathway and lead the cells to low proliferation state. Our results also suggest that DSPP can inhibit mineral formation but not osteodifferentiation in hDPSCs. Mineral formation is the first step of osteodifferentiation, and these two processes represent related but separate steps. Our results suggest that DSPP may only be involved in mineral formation.

In conclusion, this study provides compelling evidence to suggest that acetylation of DSPC might improve the efficiency of DSPCs transplantation in the dental repair process, and combined acetylation treatment and DSPC transplantation thus represents a promising treatment for use in clinical practice.

6. ACKNOWLEDGEMENTS

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Abbrevations: DPSC, dental pulp stem cell; ALP, alkaline phosphatase; BSP, bonesialoprotein; MSC, mesenchymal stem cell; alpha-GP,_ alpha -glycerophosphate; APC, adenomatous polyposiscoli; FCS, fetal calfserum; AA, L-ascorbic acid 2-phosphate; alpha -MEM,_ alpha -modified Eagle's medium; PBS, phosphatebufferedsaline; ON,osteonectin; OPN, osteopontin.

Key Words: DSPP, Acetylation Modification, Dental Pulp Stem Cell, Osteo-Differetiation , Mineralization

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