Hey1 functions as a positive regulator of odontogenic differentiation in odontoblast-lineage cells

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Abstract. Substantial evidence has indicated that Notch and bone morphogenetic protein (BMP) signaling may regulate odontoblastic differentiation. Hairy/enhancer-of-split related with YRPW motif 1 (Hey1), a downstream target gene of Notch and BMP signaling, is expressed in dental pulp tissues and has been demonstrated to be responsible for osteoblast mineralization. The aim of this study was to investigate the effects of Hey1 on odontoblast differentiation. The results of the study demonstrated that Hey1 expression in odontoblast-lineage cells (OLCs) was upregulated by stimulation of osteoblastic/odontoblastic differentiation medium containing ascorbic acid, β-glycerol phosphate and dexamethasone. Furthermore, stable Hey1-overexpressing cells expressed higher levels of dentin sialophosphoprotein (DSPP) and exhibited higher mineralization capabilities following stimulation by differentiation medium. Furthermore, RNA interference-mediated knockdown of Hey1 downregulated the expression levels of DSPP in OLCs stimulated by differentiation medium. Taken together, the findings indicate that Hey1 may be a positive regulator of odontoblastic differentiation. The present study broadens the understanding of odontoblast differentiation and biomineralization.

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Introduction

Odontoblasts are neural crest-derived, highly differentiated cells aligned in a single layer at the periphery of the dental pulp. The main function of these cells is to form dentin, the largest part of the hard tissue in teeth. Following differentiation from dental papilla mesenchymal cells, functional odontoblasts synthesize and secrete collagenous and non-collagenous matrix proteins that are essential for mineralized dentin formation (1).

The differentiation of odontoblasts is a complex process regulated by reciprocal epithelium-mesenchyme interactions. A number of signaling factors are reported to be involved in this process, including Notch, bone morphogenetic protein (BMP), Wnt and transforming growth factor-β (TGF-β). Notch signaling is an evolutionarily conserved pathway that is responsible for the control of cell fate through local cell-cell interactions (2). It has been well documented that during tooth development, Notch receptors and ligands are expressed in dental epithelium, dental papilla mesenchyme, ameloblast or odontoblast at different stages of tooth germ development (3,4). Additionally, in the pulp of injured teeth, the expression of Notch receptors and the Delta-1 ligand is significantly upregulated (5,6). These results suggest that Notch signaling is involved in primary and reparative dentinogenesis. Further evidence has demonstrated that Notch signaling has a critical role in dental pulp stem cell (DPSC) differentiation into odontoblasts *in vitro* (7,8). BMP signaling is also a potent regulator of odontoblast differentiation. As one of the strongest signals stimulating biomineralization, BMP-2 has been identified to be required for odontoblast differentiation *in vivo* and *in vitro* (9-11). Additionally, *in vitro* studies have demonstrated that BMP-2 gene transfection enhances the odontogenic differentiation of DPSC and stem cells from apical papilla (12,13).

Hairy/enhancer-of-split related with YRPW motif 1 (Hey1), also known as CHF2, HRT1, Herp2 or Hesr1, is a member of the basic helix-loop-helix family. Hey1 was first characterized as a downstream effector of canonical Notch signaling (14), and further investigations indicated that Hey1 was also induced by TGF-β/BMP signaling independently of Notch (15,16). Numerous studies have demonstrated that Hey1 is responsible for the development of various tissues, including bone, nerve, heart, muscle and vascular tissues (17-21). Our preliminary study demonstrated that Hey1 was expressed in dental pulp tissues and may affect dentin sialophosphoprotein (DSPP) expression during odontogenesis (22). In addition, substantial evidence has demonstrated the regulatory roles of Hey1 in mineralization (23,24). However, it remains unclear whether Hey1 regulates odontoblastic differentiation.

In this study, the effects of Hey1 on the differentiation of odontoblasts were investigated in an odontoblast-lineage cell line (OLC) (25,26). The expression of Hey1 in OLCs was first observed during odontogenic differentiation. Subsequently, a plasmid encoding the full-length sequence of Hey1 or Hey1-silencing short hairpin RNA (shRNA) were transfected into OLCs to compare the differentiation and mineralization capabilities of cells expressing different levels of Hey1. The findings suggested that Hey1 has an important role in odontoblastic differentiation.

Materials and methods

Cell culture and differentiation induction. OLC cell line was provided by Professor S. Arany (Department of Biochemistry, Akita University School of Medicine, Akita, Japan). It is a murine spontaneously immortalized cell line which was developed by Arany *et al* (25). OLCs were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; both from Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. For differentiation induction, cells were plated at a density of $2x10^4$ cells/cm^2 in 6-well plates and were cultured until they reached 80% confluence. Cells were in serum-free α-MEM for 24 h to be synchronized, then in osteoblastic/odontoblastic differentiation medium [α-MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid (AA), 10 mM β-glycerol phosphate (β-GP) and 10^{-8} M dexamethasone (DEX)] (27,28).

Establishment of stable Hey1-overexpressing cell lines. The plasmid encoding the full-length sequence of mouse Hey1 with a C-terminal His-tag, obtained from Dr Nobuyuki Kawashima (Department of Endodontics and Dental Pulp Biology, Tokyo Medical and Dental University, Tokyo, Japan), was constructed using eukaryotic expression vector pEF-Dest51 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was named pEF-Hey1. Following confirmation by DNA sequencing, pEF-Hey1 was transfected into OLCs using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following 24 h of transfection, the cells were subcultured at 1:12 for another 24 h and selected in growth medium containing 4 μg/ml blasticidin (Invitrogen; Thermo Fisher Scientific, Inc.). Single cell isolation was performed using 96-well plates. These single cell clones were amplified in blasticidin selection medium and then processed for selection by reverse-transcription polymerase chain reaction (RT-PCR) and western blot analyses. Empty pEF-Dest51 vector was transfected into OLCs as a mock negative control.

Construction and transient transfection of shRNA expression vectors targeting Hey1. A mouse Hey1-targeting sequence (5'-TGAAGGACTCGATGCCTCCGA-3') was designed using Invitrogen's online RNAi designer and was verified using BLAST to avoid off-target gene silencing. Two pairs of oligonucleotides coding shRNA, one pair containing mouse Hey1-targeting sequence and the other containing a scrambled sequence (5'-GTT CTC CGA ACG TGT CAC GT-3') with no significant similarity to any mouse gene sequences, were synthesized. Pairs of oligonucleotides were annealed and inserted into the shRNA expression vector pGPU6/GFP/Neo (Shanghai GenePharma Co., Ltd., Shanghai, China). Transient transfections into OLCs were performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to yield OLC/Hey1-knockdown (KD) and OLC/pGP-Mock.

RNA preparation and RT-quantitative PCR (RT-qPCR). Total RNA was extracted from OLCs using TRIzol reagent (Thermo Fisher Scientific, Inc.) and was quantified by spectrophotometry using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Total RNA (1 μg) was reverse transcribed into cDNA using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's guidelines. qPCR was performed on 1 μl of cDNA in a 20 μl reaction with SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Dalian, China) using the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sense and antisense primers were as follows: 5'-CGACGAGACCGAATC AATAAC-3' and 5'-CAAACTCCGATAGTCCATAGCC-3' for Hey1; 5'-AGCATCAAGAATAGCACCAACC-3' and 5'-CCC ATCAGTATCATCCAAACCT-3' for DSPP; 5'-GACCCCTTC ATTGACCTCA-3' and 5'-GCTCCTGGAAGATGGTGA-3' for GAPDH. The protocol for the qPCR reactions consisted of an initial denaturation step (95˚C for 3 min), followed by 45 cycles of denaturation (95˚C for 10 sec), annealing (55˚C for 10 sec), and extension (72˚C for 20 sec). GAPDH was used as the housekeeping gene for template normalization. The relative expression level of mRNA was calculated using $2^{-\Delta\Delta Cq}$ analysis (29). All RT-qPCR reactions were performed in triplicate.

Western blot analysis. The cells were washed with cold phosphate-buffered saline and lysed on ice using radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) containing 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 4˚C for 20 min at 13,500 x g, and the total protein content of the supernatant was collected. Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The protein samples were mixed with 5X loading buffer and then were boiled for denaturation. Protein extracts (30 μg) from each sample were subjected to 8% SDS-PAGE and were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were

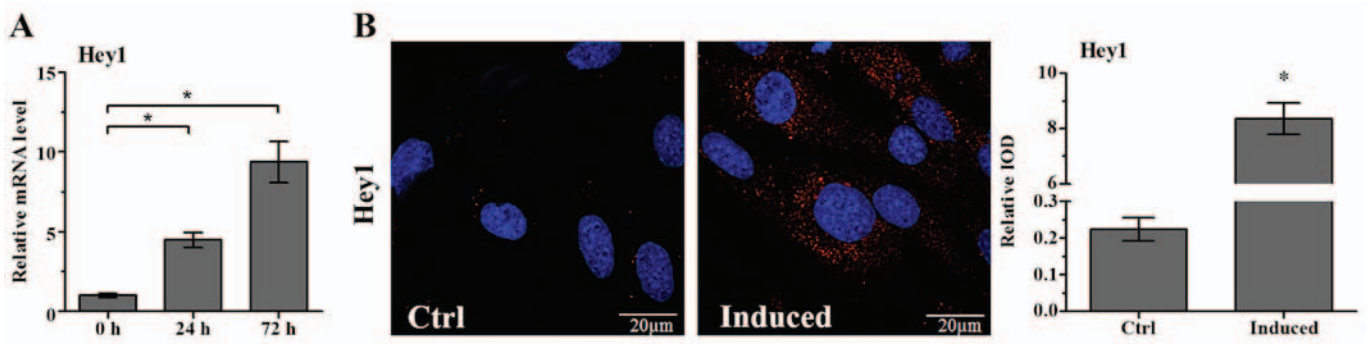

Figure 1. Odontoblastic differentiation medium induced an increase in Hey1 expression in OLCs. (A) Hey1 mRNA levels in OLCs cultured in differentiation medium were evaluated by reverse transcription-quantitative polymerase chain reaction on the 1st and 3rd days after induction. (B) Hey1 expression in protein levels in OLCs cultured by normal or differentiation mediums for 5 days was determined by immunofluorescence staining. Data are presented as the mean ± standard deviation. *P<0.05. Hey1, hairy/enhancer-of-split related with YRPW motif 1; OLCs, odontoblast-lineage cells; IOD, integrated optical density.

blocked in 5% BSA for 2 h at 37˚C and were incubated with rabbit anti-His-probe (cat. no. sc-803; 1:500), anti-dentin sialoprotein (DSP; cat. no. sc-33587; 1:200) both from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) or anti-β-actin (cat. no. ab8227; 1:2,000; Abcam, Cambridge, UK) primary antibodies overnight at 4˚C. The membranes were then rinsed in TBS-Tween and incubated with an HRP-conjugated goat anti-rabbit secondary antibody (cat. no. AP307P, 1:5,000; EMD Millipore) for 1 h, and then detected using an enhanced chemiluminescence system (GE ImageQuant 350; GE Healthcare, Piscataway, NJ, USA). Semi-quantitative analyses of the bands were performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Immunofluorescence staining. OLCs in 35 mm glass bottom dishes were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized in 0.1% Triton X-100 for 5 min, and blocked with 2% goat serum (cat. no. C0265; Beyotime Biotechnology, Shanghai, China) at 37˚C for 1 h. Cells were then incubated with rabbit anti-Hey1 (cat. no. ab22614; 1:50; Abcam) or anti-DSP (cat. no. sc-33587; 1:50; Santa Cruz Biotechnology, Inc.) primary antibody at 4˚C overnight. Finally, cells were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (cat. no. A11037; 1:400; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 1 h, and nuclei were stained with 2 μg/ml DAPI (cat. no. C1002; Beyotime Biotechnology) for 5 min at room temperature. Fluorescence was examined using a FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan). Fluorescence intensity was determined with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Mineralization assay. Mock OLCs and stable Hey1-overexpressing cells were plated in 6-well plates and cultured by osteoblastic/odontoblastic differentiation medium, respectively. Media were collected every 4 days for determination of alkaline phosphatase (ALP) activity using an Alkaline Phosphatase assay kit (Jiancheng Bioengineering Institute, Nanjing, China) as described in a previous study (30). In brief, 20 μl of cell culture medium mixed with 1 ml of reaction solution containing 18 mM 4-nitrophenyl phosphate and 0.5 M 2-amino-2-methyl-1-propanol was incubated in the dark for 15 min at 37˚C. ALP activity was quantified by measuring the absorbance values of the reaction solution at 405 nm using an absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). After culture for 32 days, cells were rinsed with distilled water and fixed with 4% paraformaldehyde for 30 min at room temperature. Mineralized deposits were then stained with 40 mM alizarin red S (cat. no. A5533; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. The excess dye was removed by washing 3 times with distilled water. Red stain of mineralized deposits was observed by a light microscope (Olympus Corporation).

Statistical analysis. All values presented are expressed as the mean ± standard deviation. One-way analysis of variance was used to analyze the differences between the groups. The differences between groups were detected with post hoc Student-Newman-Keuls tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Odontoblastic differentiation medium induces an increase in Hey1 expression. Hey1 mRNA levels in the OLCs were significantly increased on the day 1 and 3 of culture in osteoblastic/odontoblastic differentiation medium containing AA, β-GP and DEX (Fig. 1A). Additionally, immunofluorescence staining was performed to evaluate the protein levels of Hey1 during differentiation induction. There was almost no positive staining in the untreated OLCs. However, the expression of Hey1 protein was observed in OLCs following stimulation with differentiation medium for 5 days (Fig. 1B).

Stable Hey1-overexpressing cells line was established. To investigate the effects of Hey1 on the differentiation of odontoblasts, pEF-Hey1 and the control vector pEF-Dest51 were transfected into OLCs. Following pEF-Hey1 transfection, four single cell clones that were resistant to blasticidin were amplified. RT-PCR demonstrated that Hey1 mRNA expression was barely detected in normal OLCs (untransfected) or mock-transfected cells (transfected with empty pEF-Dest51), whereas the four single cell clones transfected with pEF-Hey1 expressed much higher levels of Hey1 mRNA (Fig. 2A). Western blot

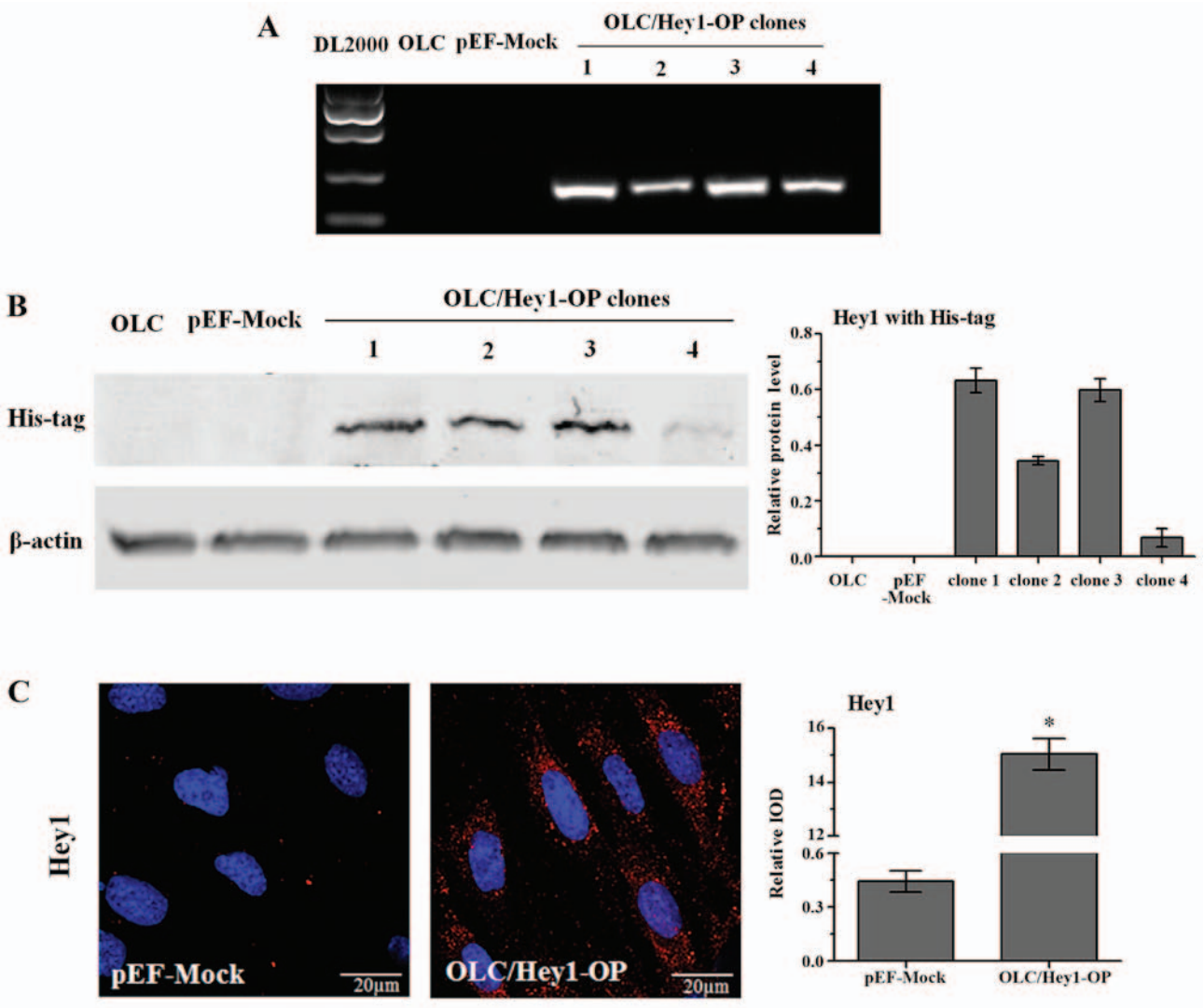

Figure 2. Hey1-overexpressing cell line OLC/Hey1-OP was established. (A) pEF-Hey1 and empty pEF-Dest51 were individually transfected into OLCs, and four resistant cell clones were isolated. Four Hey1-OP clones expressed much higher levels of Hey1 mRNA than the OLCs and mock cells. (B) Among the four clones, clone one synthesized the highest levels of Hey1 protein. (C) Immunofluorescence staining for Hey1 protein in OLC/pEF-Mock and OLC/Hey1-OP. Data are presented as the mean ± standard deviation. *P<0.05. Hey1, stable hairy/enhancer-of-split related with YRPW motif 1; OLC, odontoblast-lineage cell; OP, overexpression.

analysis with an anti-His-tag antibody was performed to verify the effectiveness of the plasmid to induce Hey1 overexpression. Among the four clones, clone one synthesized the highest levels of Hey1 protein (Fig. 2B) and was designated as OLC/Hey1-OP for further investigations. Immunofluorescence staining with an anti-Hey1 antibody further confirmed that the OLC/Hey1-OP cell line expressed a higher level of Hey1 protein compared with mock transfection cells (Fig. 2C).

Stable Hey1-overexpressing cells line exhibit increased differentiation capabilities. The differentiation capabilities between the stable Hey1-overexpressing and mock cell lines were compared. The results of RT-qPCR demonstrated that overexpressing Hey1 alone did not affect the mRNA levels of DSPP, the odontoblastic differentiation marker, in OLCs (P>0.1). However, following culture in differentiation medium for 3 days, OLC/Hey1-OP expressed a significantly higher mRNA level of DSPP than the mock cells (Fig. 3A). Since DSPP is a large protein that can be specifically cleaved into two fractions, DSP and dentin phosphoprotein, an antibody against the DSP portion of DSPP was used to detect protein expression of the full-length DSPP. The results of western blot analysis and immunofluorescence staining further revealed that Hey1 overexpression increased DSPP protein expression in OLCs when the cells were induced by differentiation medium for 7 days (Fig. 3B and C). Furthermore, when cultured in differentiation medium, the ALP activities of the OLC/Hey1-OP cells were much higher than that of the mock cells (Fig. 4A). Alizarin red S staining revealed that the OLC/Hey1-OP cells formed more and larger mineralized nodules than the mock cells (Fig. 4B).

Transient Hey1 knockdown inhibited OLC differentiation. To further determine whether Hey1 is critical for odontoblastic differentiation, a Hey1-targeting shRNA expression vector was constructed, and transient transfection into OLCs was performed. To evaluate the efficiency of the RNA interference in silencing Hey1 expression, the mRNA and protein expression levels of Hey1 in transfected OLCs and mock cells were measured using

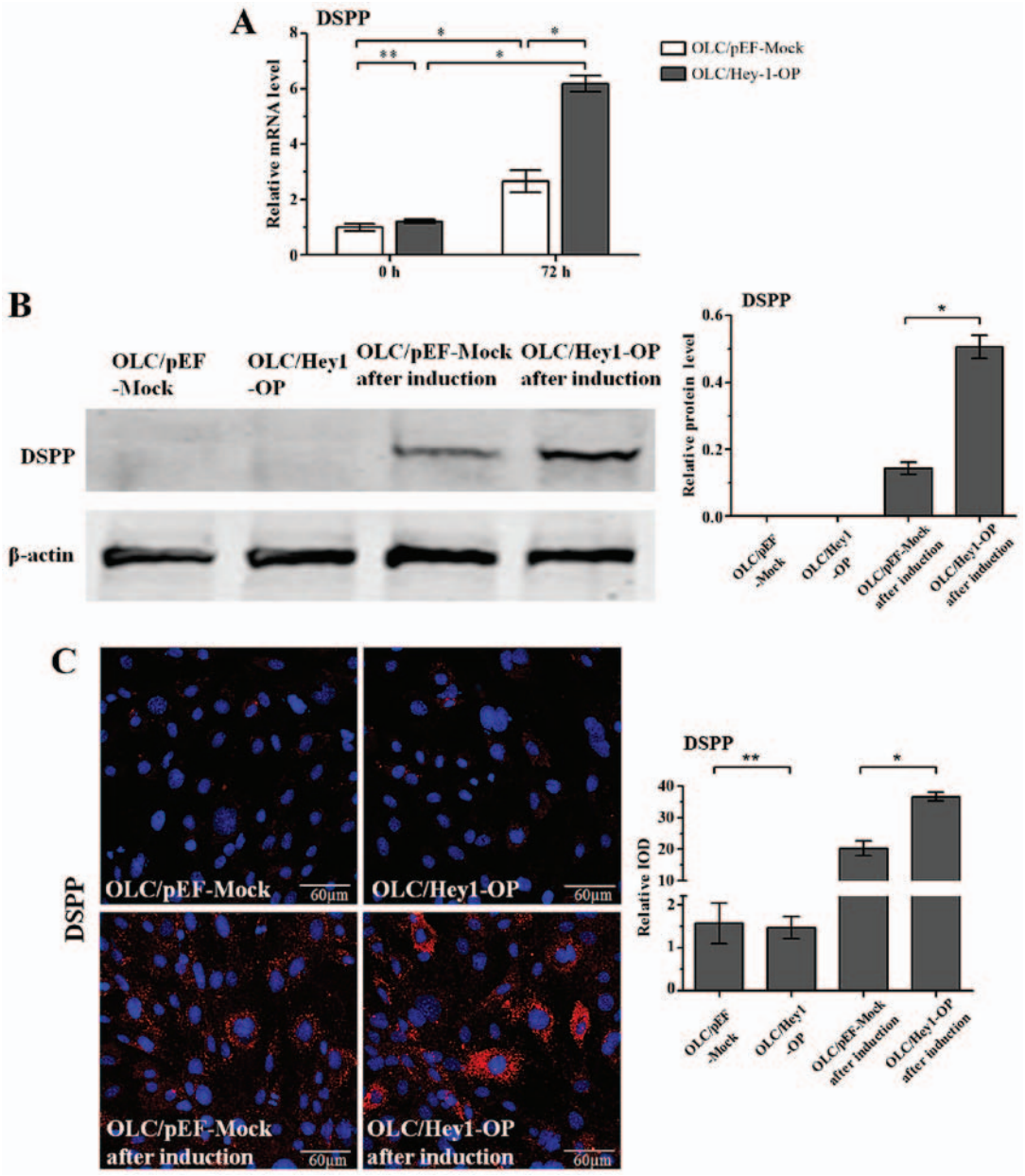

Figure 3. Stable Hey1-overexpressing cell line expressed a higher level of DSPP following culture in differentiation medium. (A) mRNA levels of DSPP gene in OLC/pEF-Mock and OLC/Hey1-OP cultured by differentiation medium for 3 days. Protein levels of DSPP in OLC/pEF-Mock and OLC/Hey1-OP cultured by differentiation medium for 7 days, determined by (B) western blotting and (C) immunofluorescence staining. Data are presented as the mean ± standard deviation. *P<0.05 and **P>0.1. DSPP, dentin sialophosphoprotein; Hey1, hairy/enhancer-of-split related with YRPW motif 1; OP, overexpression; OLC, odontoblast-lineage cell; IOD, integrated optical density.

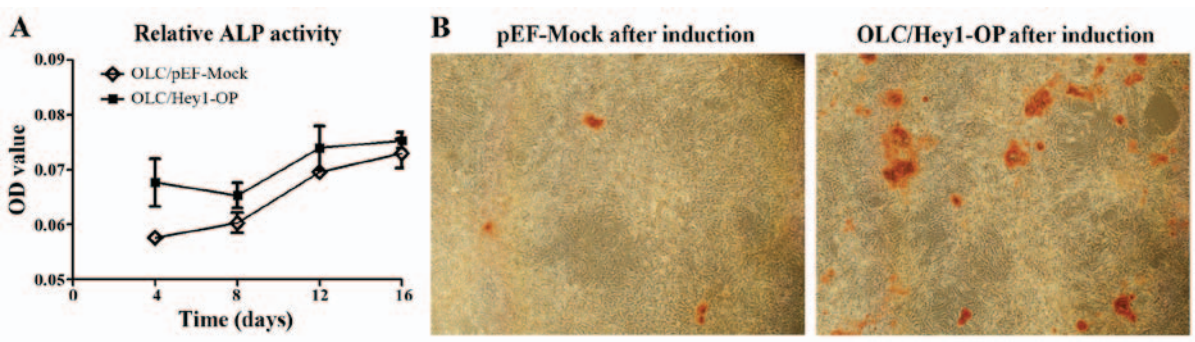

Figure 4. Hey1 overexpression promoted ALP activity and OLC mineralization. (A) The ALP activities secreted into the medium by OLC/pEF-Mock and OLC/Hey1-OP during differentiation induction. (B) Alizarin red S staining of OLC/pEF-Mock and OLC/Hey1-OP cultured in differentiation medium for 32 days. Hey1, hairy/enhancer-of-split related with YRPW motif 1; OP, overexpression; ALP, alkaline phosphatase; OLC, odontoblast-lineage cell; OD, optical density.

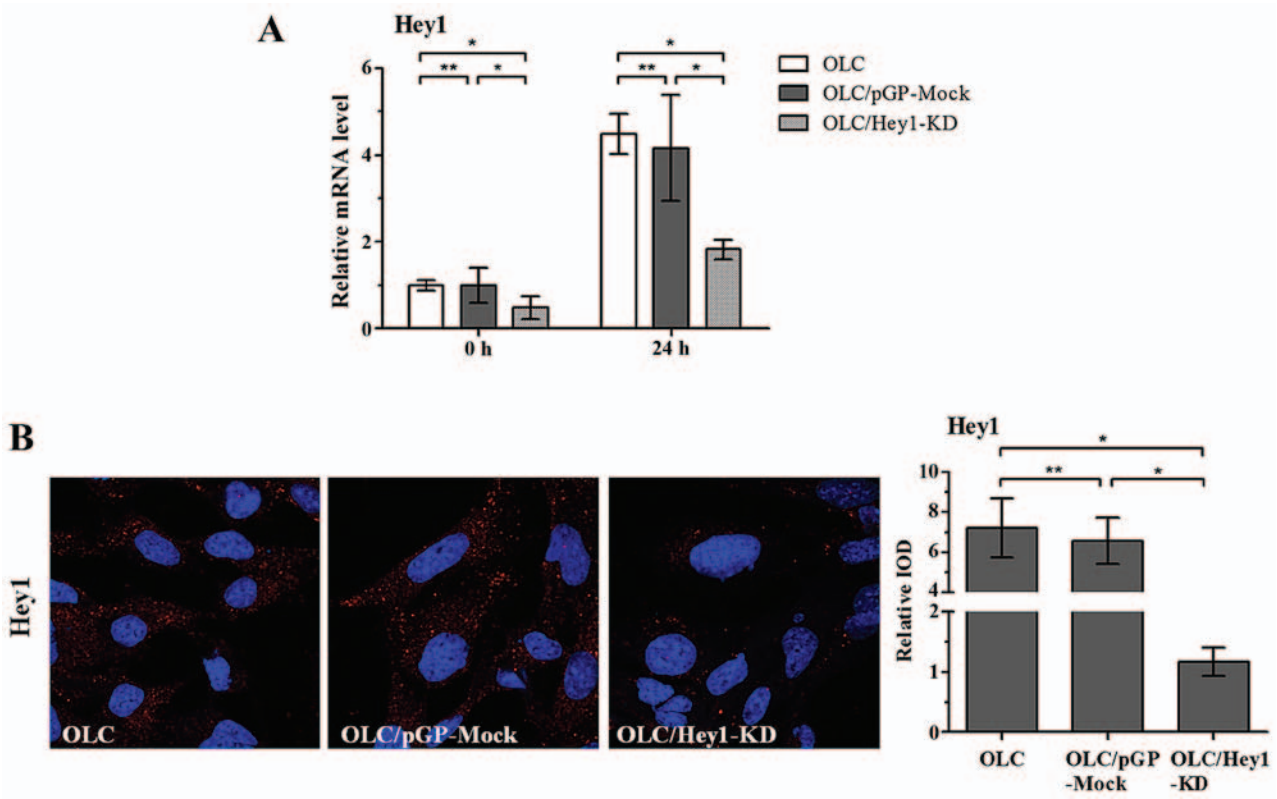

Figure 5. Short hairpin RNA-mediated inhibition of Hey1 expression. (A) mRNA levels of Hey1 in OLCs, OLC/pGP-Mock and OLC/Hey1-KD cultured in differentiation medium for 24 h. (B) Immunofluorescence staining for Hey1 protein in OLCs, OLC/pGP-Mock and OLC/Hey1-KD cultured in differentiation medium for 5 days. Data are presented as the mean ± standard deviation. *P<0.05 and **P>0.1. Hey1, hairy/enhancer-of-split related with YRPW motif 1; OLC, odontoblast-lineage cells; KD, knockdown; IOD, integrated optical density.

RT-qPCR and immunofluorescence staining, respectively, following stimulation in differentiation medium. OLC/Hey1-KD cells expressed lower levels of Hey1 mRNA and protein than OLC/pGP-Mock cells (P<0.05; Fig. 5). Further investigations revealed that DSPP expression was decreased in OLC/Hey1-KD cells compared with the mock cells following differentiation induction (Fig. 6). Following culturing in differentiation medium for 3 days, the mRNA level of DSPP in OLC/Hey1-KD cells was significantly lower than OLC/pGP-Mock cells (Fig. 6A). Furthermore, after culture in differentiation medium for 7 days, DSPP protein expression in OLC/Hey1-KD cells was significantly lower than in OLC/pGP-Mock cells (Fig. 6B and C).

Discussion

Differentiation of the progenitor cells derived from dental pulp tissue into odontoblasts has a vital role in the dentinal regeneration process. Elucidating the underlying mechanisms will facilitate the development of therapeutic approaches for injured dentin-pulp complex. Numerous methods have been used to induce odontoblast differentiation *in vitro*. The application of osteoblastic/odontoblastic differentiation medium (containing AA, β-GP and DEX) has been demonstrated to be an effective method and has been adopted in numerous studies (7,8,27,28,31-33). Previous *in vitro* studies have demonstrated the involvement of Notch and BMP signaling in odontoblast differentiation induced by AA + β-GP + DEX (7,8,32,33). However, the underlying mechanisms remain unclear. The present study revealed that Hey1 expression in odontoblast-lineage cells was significantly upregulated by AA + β-GP + DEX stimulation, suggesting that Hey1 may be involved in their differentiation. Because Hey1 has been reported to regulate osteoblast differentiation and matrix mineralization (23,24,30), it is assumed that Hey1 may have an important role in odontoblast differentiation. To verify this hypothesis, Hey1 overexpression and knockdown models were established *in vitro* to investigate the effects of Hey1 on odontoblast differentiation.

DSPP, a non-collagenous protein that is predominantly expressed in odontoblasts or dentin, was demonstrated to be critical for dentin mineralization. DSPP is synthesized and secreted by differentiated odontoblasts and is regarded as a marker of odontogenic differentiation (34,35). In the present study, OLCs cultured in differentiation medium exhibited increased expression of DSPP, a result is consistent with previous *in vitro* studies. Furthermore, the results revealed that overexpression of Hey1 did not directly upregulate DSPP expression in OLCs but enhanced the upregulatory effect of AA + β-GP + DEX stimulation on DSPP expression. Previous studies have demonstrated that Hey1 not only regulated downstream targets as a transcriptional repressor, but also functioned through interaction with other transcription factors (36,37). Similarly, it has been shown that Hey2 overexpression alone is not sufficient to induce strong changes in downstream gene expression, but needs additional cofactors (38). Therefore, according to the results of the present study, Hey1 may regulate DSPP expression indirectly by interacting with other cofactors activated in the process of odontoblastic differentiation; this requires further investigations.

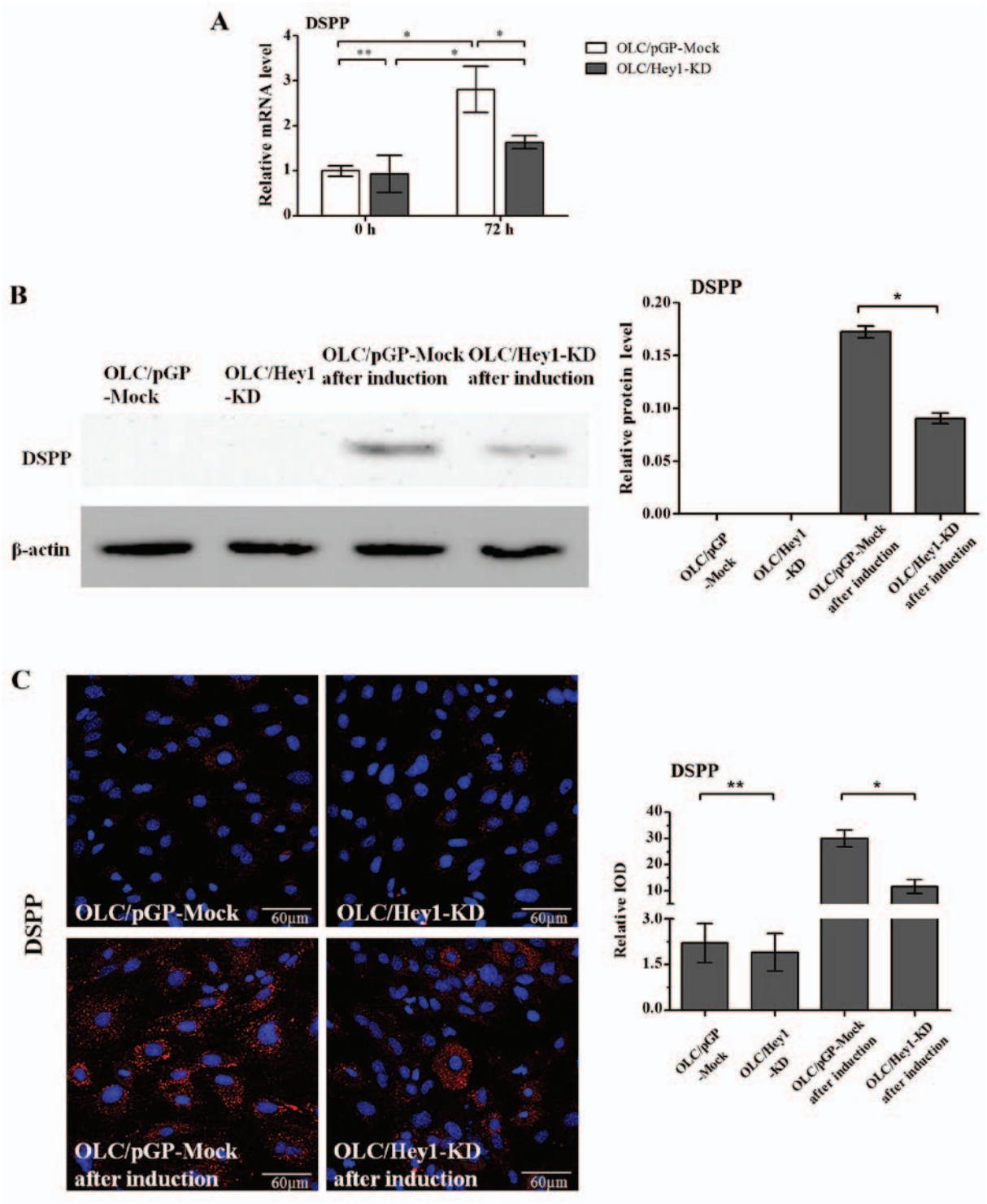

Figure 6. Hey1 KD diminished DSPP expression induced by differentiation medium. (A) mRNA levels of DSPP gene in OLC/pGP-Mock and OLC/Hey1-KD cultured in differentiation medium for 3 days. Protein levels of DSPP in OLC/pGP-Mock and OLC/Hey1-KD cultured in differentiation medium for 7 days as determined by (B) western blotting and (C) immunofluorescence staining. Data are presented as the mean ± SD. *P<0.05 and **P>0.1. Hey1, hairy/enhancer-of-split related with YRPW motif 1; DSPP, dentin sialophosphoprotein; OLC, odontoblast-lineage cell; KD knockdown; IOD, integrated optical density.

OLCs in which Hey1 was exogenously overexpressed exhibited upregulation of ALP activity and increased nodule formation. These results indicate that Hey1 may be a positive regulator of odontoblast cell mineralization. These findings are consistent with previous studies showing that Hey1 enhanced osteogenic differentiation and mineralization of mesenchymal stem cells induced by BMP-9 or BMP-7 (23,39). However, Zamurovic *et al* (24) reported that Hey1 inhibited mineralization of the MC3T3 cell line. This discrepancy may result from differences in the source of cells. Certain transcription factors, such as runt related transcription factor 2 and nuclear factor erythroid 2-related factor 1, have demonstrated different effects on the differentiation of odontoblasts and osteoblasts (40,41). The function of Hey1 may also be dependent on cell type.

An *in vivo* study demonstrated that Hey1 single knockout mice exhibited no major developmental or obvious functional impairments. However, the dental phenotypes of Hey1-deficient mice have not been analyzed (42). The present *in vitro* study revealed that RNA interference-mediated knockdown of Hey1 expression led to decreased DSPP expression compared with the control mock cells following differentiation induction, suggesting that Hey1 is critical for odontoblastic differentiation. Previous *in vitro* studies demonstrated that knockdown of Hey1 promoted myogenesis and inhibited osteogenic differentiation (23,43). Taken together, Hey1 may participate in the regulation of cell fate.

In conclusion, the present study demonstrated that Hey1 was involved in the differentiation of odontoblast-lineage cells. Hey1 overexpression increased DSPP expression during odontoblastic differentiation and further augmented mineralization of OLCs. Additionally, knockdown of Hey1 diminished DSPP expression induced by odontoblastic differentiation medium. The findings of the current study indicate that Hey1 functions as a positive regulator of odontogenic differentiation. This study broadens our understanding of odontoblast differentiation and biomineralization.

Acknowledgements

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