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CPNE7, a preameloblast-derived factor, regulates odontoblastic differentiation of mesenchymal stem cells

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ABSTRACT

Tooth development involves sequential interactions between dental epithelial and mesenchymal cells. Our previous studies demonstrated that preameloblast-conditioned medium (PA-CM) induces the odontogenic differentiation of human dental pulp cells (hDPCs), and the novel protein Cpne7 in PA-CM was suggested as a candidate signaling molecule. In the present study, we investigated biological function and mechanisms of Cpne7 in regulation of odontoblast differentiation. Cpne7 was expressed in preameloblasts and secreted extracellularly during ameloblast differentiation. After secretion, Cpne7 protein was translocated to differentiating odontoblasts. In odontoblasts, Cpne7 promoted odontoblastic markers and the expression of Dspp *in vitro*. Cpne7 also induced odontoblast differentiation and promoted dentin/pulp-like tissue formation in hDPCs *in vivo*. Moreover, Cpne7 induced differentiation into odontoblasts of non-dental mesenchymal stem cells *in vitro*, and promoted formation of dentin-like tissues including the structure of dentinal tubules *in vivo*. Mechanistically, Cpne7 interacted with Nucleolin and modulated odontoblast differentiation via the control of *Dspp* expression. These results suggest Cpne7 is a diffusible signaling molecule that is secreted by preameloblasts, and regulates the differentiation of mesenchymal cells of dental or non-dental origin into odontoblasts.

1. Introduction

During tooth morphogenesis, numerous signaling molecules and transcription factors mediate odontoblast differentiation and dentin formation through sequential epithelial-mesenchymal interactions [1]. Dentin is formed by odontoblasts, which are derived from dental papilla ectomesenchymal cells. Odontoblast differentiation is induced by signaling molecules and growth factors derived from inner enamel epithelial cells, which are located directly adjacent to the dental papilla [2]. After the epithelial induction, odontoblasts secrete the organic matrix proteins, which consist mainly of type I collagen, dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1), and are then modified in order to initiate mineralization [3–5]. However, the precise molecular mechanisms of odontoblast differentiation mediated through epithelial-mesenchymal interaction are still under investigation.

Previously, we reported that dental epithelium-derived factors present in the conditioned medium of preameloblasts (PA-CM) induce the odontogenic differentiation of dental mesenchymal cells, such as dental pulp stem cells, and promote dentin formation *in vitro* and *in vivo*. Moreover, we identified the protein Cpne7 among those secreted dental epithelium-derived factors, and it has been suggested to be mainly related to odontoblast differentiation [6]. Members of the copine family are ubiquitous calcium-dependent phospholipid-binding proteins and are evolutionally conserved from *Arabidopsis* to *Homo sapiens*. The nine copine genes (CPNE1-9) have been identified. They contain two C2 domains (C2Ds) in the N-terminus and a von Willebrand factor type A domain (vWA) in the C-terminus. The C2Ds of copines were originally identified in conventional protein kinase C (PKC) and are involved in calcium influx [7]. The vWA domain mediates protein–protein interaction [8]. Moreover, it has been demonstrated that copines translocate from cytoplasm into the nucleus (CPNE1, 2, 3, and 7) [9], regulate NF-κB gene expression (CPNE1) [10], and

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interact with the protein nucleolin (CPNE3) [11]. However, there have been few studies about whether Cpne7 can regulate odontoblastic differentiation of mesenchymal cells.

In the present study, we investigated biological function and mechanisms of Cpne7 in regulation of dental and non-dental mesenchymal cell differentiation into odontoblasts via epithelial-mesenchymal interaction. Our findings suggest the novel concept that Cpne7, a dental epithelium-derived protein, plays an essential role in commitment of odontoblasts, odontoblast differentiation, and dentin formation.

2. Materials and methods

2.1. Co-culture system

MDPC-23 cells were provided by Dr. J.E. Nör (University of Michigan, Ann Arbor, MI). ALCs were provided by Dr. T. Sugiyama (Akita University School of Medicine, Akita, Japan). For co-culture of ALCs with MDPC-23 cells, Transwell® permeable supports (Corning Inc., Corning, NY) were used. The ALCs were seeded in the upper compartment of the transwell, and the MDPC-23 cells were seeded in the lower compartment of the dish. When cells reached 80%–90% confluence, the upper compartment was combined with the lower compartment.

2.2. Cell culture

C3H10T1/2 and HEK293 human embryonic kidney cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human BMSCs (PT-2501) were purchased from Lonza (Walkersville, MD). MDPC-23, C3H10T1/2, hBMSCs, and HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Grand Island, NY), and ALCs were cultured in minimum essential medium (MEM; Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) and antibiotic-antimycotic (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO_2. To induce cell differentiation and mineralized nodule formation, confluent cells were treated with induction medium (DMEM containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) for up to 2 weeks.

2.3. Plasmids

Full-length mouse Cpne7 (mCpne7, NM_170684) cDNA, siRNA targeting Cpne7, and pGL3-Dspp vectors were constructed and verified as described previously [6]. Expression vectors encoding full-length human CPNE7, green fluorescent protein (GFP)-CPNE7 (NM_153636), DDK (Flag)-tagged CPNE7 (NM_153636) and Recombinant CPNE7 (NP 705900) were purchased from Origene (Rockville, MD). Control siRNA (sc-37007) and siRNA targeting nucleolin (sc-29230) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analysis

Total RNA was extracted from the cells with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (3 μg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (New England Biolabs, Ipswich, MA). One microliter of the RT product was amplified by PCR using the following primer pairs: Odam (462 bp, forward [F] 5′-atgtcctatgtggttcctgt-3′, reverse [R] 5′-ttatggttctcttaggctatc-3′), Cpne7 (178 bp, [F] 5′-cccgacccattgaccaagtc-3′, [R] 5′-catacacctcaaaccgtagcttc-3′), Mmp-20 (458 bp, [F] 5′-agctgtgagcaactgatgactgga-3′, [R] 5′-acagctagagccaagaacacacct-3′), Gapdh (452 bp, [F] 5′-accacagtccatgccatcac-3′, [R] 5′-tccaccaccctgttgctgt 3′), Dspp (141 bp, [F] 5′-gtgaggacaaggacgaatctga-3′, [R] 5′-cactactgtcactgctgtcact-3′), and Osteocalcin (283 bp, [F] 5′-ccacagccttcatgtccaag-3′, [R] 5′-ggcagagagagaggacaggg-3′). PCR was carried out under conditions of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA) using SYBR Green PCR Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were 40 cycles of 95 °C for 1 min, 94 °C for 15 s, and 60 °C for 34 s. All reactions were run in triplicate, and PCR product levels were normalized to that of the housekeeping gene Gapdh. Relative changes in gene expression were calculated using the comparative threshold cycle (C_T) method.

2.5. Preparation of CM and inactivation of Cpne7

CM was harvested as described previously [6]. Briefly, ALC cells were seeded at 7.5×10^5 cells/100 mM dish. When the cells reached 80% confluence, medium was replaced with differentiation medium. After 3 days of differentiation, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in differentiation medium without FBS for 24 h. The serum-free conditioned medium was harvested and concentrated to 1 μg/μl by ammonium sulfate precipitation. To inactivate Cpne7 in CM, 20 or 40 μg/ml anti-Cpne7 antibodies was mixed with CM for 2 h at 4 °C with rotation.

2.6. Western blot analysis

Western blot analyses were performed as previously described [6]. Briefly, proteins (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Rabbit and affinity-purified polyclonal anti-Odam, anti-Cpne7, and anti-Dsp antibodies were produced as described previously [6,12]. The Flag (F3165) antibody was purchased from Sigma–Aldrich (St. Louis, MO). The Mmp-20 (sc-26926), Lamin B (sc-6216), Osteocalcin (sc-30044), Nucleolin (sc-8031), and Gapdh (sc-25778) antibodies were purchased from Santa Cruz Biotechnology. The Dmp1 antibody (ab103203) was purchased from Abcam (Cambridge, UK). The labeled protein bands were detected using an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK).

2.7. Transient transfection and luciferase assay

HEK293, MDPC-23 or C3H10T1/2 cells were seeded in 12-well culture plates at a density of 1.5×10^5 cells per well. The cells were transiently transfected using Metafectene Pro reagent (Biontex, Martinsried/Planegg, Germany) with the constructs as described above. Construct pGL3-Dspp was cotransfected into MDPC-23 cells with the Cpne7 expression vector or the Cpne7 or Nucleolin siRNA vector. Construct pGL3-Bsp was transfected into C3H10T1/2 cells with rCpne7 treatment. Following the addition of 50 μl luciferin to 50 μl of the cell lysate, luciferase activity was determined using an analytical luminescence luminometer (Promega, Madison, WI) according to the manufacturer's instructions.

2.8. Co-IP assay

After transfection with the indicated plasmid DNA using Metafectene Pro reagent, MDPC-23 cells were washed in PBS, and the cell lysates were prepared by adding 1 ml of co-IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA) supplemented with protease inhibitors. The lysates were incubated at 4°C for 2 h with a 1:200 dilution of antibodies. Thirty microliters of protein A/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology) were added and incubated at 4°C for 1 h with rotation. The immune complexes were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. Following electrophoresis on 10% SDS-PAGE gels, the immunoprecipitates were analyzed by western blotting with anti-Nucleolin.

2.9. ChIP assay

ChIP assays were performed as previously described [13]. Briefly, after transfection with the indicated plasmid DNA using Metafectene Pro reagent, MDPC-23 cells were sonicated. The fragmented chromatin mixture was incubated with anti-Nfic, anti-Nucleolin, anti-Cpne7, or IgG (1:250) on a rotator at 4 °C for 4 h. Thirty microliters of protein A/G PLUS-agarose were added and incubated at 4 °C for 1 h with rotation to collect the antibody/chromatin complexes. The final DNA pellets were recovered and analyzed by PCR using primers that encompass the *Dspp* promoter region (390 bp), mouse Dspp −400 region: forward 5′-gggtcttaaatagccagtcg-3′ and mouse Dspp-10 region: reverse, 5′-ctgagagtggcacactgt-3′. PCR was carried out under conditions of 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

2.10. Animals, tissue preparation, and immunohistochemistry

All experiments using animals followed protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-111013-2). The heads of mice at embryonic day 19 (E19), postnatal day 7 (P7) and day 10 (P10) were decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and processed for immunohistochemistry. Cpne7 expression was detected using an ABC kit (Vector Labs, Burlingame, CA) with rabbit anti-Cpne7 as the primary antibody and a biotin-labeled goat anti-rabbit IgG (1:200, Vector Labs) as the secondary antibody.

2.11. Primary cell culture, in vivo transplantation, and histological analysis

We collected human impacted third molars at Seoul National University Dental Hospital (Seoul, Korea). The experimental protocol was approved by the Institutional Review Board. Informed consent was obtained from the patients. Human DPCs were isolated and used in an *in vivo* transplantation experiment as described previously [6]. The hDPCs (2×10^6) were mixed with 100 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer, Warsaw, IN) alone, or with CM (30 μg), or with Cpne7 antibody-treated CM, or with rCpne7 (5 μg) in an 0.5% fibrin gel, and then transplanted subcutaneously into immunocompromised mice (NIH-bg-nu-xid; Harlan Laboratories, Indianapolis, IN) for 6 weeks. To inactivate Cpne7 in CM, Cpne7 antibody (5 μl) was mixed with CM for 24 h at 4 °C with rotation before *in vivo* transplantation in the Cpne7 antibody-treated CM group. For histomorphometric analysis of newly formed mineralized tissue, hDPCs transfected with the Cpne7 overexpression or inactivation constructs were transplanted with HA/TCP particles for 12 weeks, as described above. To evaluate dentin/pulp-like tissue formation in root canal spaces, hDPCs (2×10^6) were mixed with CM (10 μg) or Cpne7

antibody-treated CM in a 0.5% fibrin gel, and inserted into the empty root canal spaces of the human tooth segments for 12 weeks. Human tooth segments were fabricated according to the protocol described in our previous study [14]. In addition, hBMSCs were also inserted into the empty root canal spaces with rCpne7 (5 μg) or rBmp2 (5 μg; Cowellmedi, Busan, Korea) for 6 weeks.

Samples were harvested and fixed in 4% paraformaldehyde, decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and stained with hematoxylin-eosin (H-E) (Vector Labs) or processed for immunohistochemistry. For immunohistochemistry, proteins were detected with anti-DSP, anti-BSP [6], or anti-NESTIN (MAB353, Millipore) at a dilution of 1:100 as the primary antibody and a biotin-labeled goat anti-rabbit IgG (Vector Labs) as the secondary antibody. The total mineralized area among the regenerated pulp- and dentin-like tissue was analyzed using the LS starter program (Olympus Soft Imaging Solutions, Münster, Germany).

2.12. Statistical analyses

Statistical analyses were carried out using a Student's *t*-test. All statistical analyses were performed using SPSS software ver. 19.0.

3. Results

3.1. Preameloblasts secrete Cpne7 during ameloblast differentiation

The copine family has been shown to be expressed in various mammalian tissues, including brain, heart, lung, liver, and kidney [8]. Previously, Cpne7 was identified among the secretory proteins of preameloblasts [6]. In our first series of experiments, we cultured ameloblast lineage cells (ALCs) for 2 weeks and analyzed Cpne7 expression by RT-PCR and western blotting to examine the expression patterns of Cpne7 mRNA and protein during ameloblast differentiation. Cpne7 was expressed from day 0 in culture, increased on day 4 (early stage of ameloblast differentiation; pre-ameloblast stage), and then declined gradually from days 7–14 (late stage of ameloblast differentiation and mineralization). Odam and Mmp-20, ameloblast differentiation markers, were expressed during ameloblast differentiation (Fig. 1(A and B)), as expected [12]. Furthermore, secretory Cpne7 was clearly detected in ameloblast conditioned medium (CM) from day 4 (preameloblast stage) and then decreased thereafter (Fig. 1(B)). The subcellular localization of Cpne7 was in cytoplasm and nucleus in ALCs (Fig. 1(C)). These results indicate that Cpne7 is secreted into extracellular matrix soon after synthesis by differentiating preameloblasts.

3.2. Cpne7 is translocated from preameloblasts to differentiating odontoblasts

Paracrine signaling is a form of cell communication system whereby signaling molecules are released by cells and act on neighboring cells [15]. During dentinogenesis, coordinated functions between inner enamel epithelium and dental papilla cells are achieved by paracrine signaling via epithelial-mesenchymal interaction [16]. First, we used immunohistochemistry to investigate localization of Cpne7 protein during tooth development and possibility of Cpne7 as a paracrine signaling molecule. At embryonic day 19 (E19), Cpne7 was expressed in inner enamel epithelium and stratum intermedium of the developing mouse mandibular first molar which was at the bell stage of development. At postnatal day 7 (P7), Cpne7 was localized in differentiating odontoblasts; however, Cpne7 was no longer detected in ameloblasts. Subsequently at P10, Cpne7 was clearly detected in the odontoblasts, pre-dentin, and dentinal tubules (Fig. 2(A)). The expression of Cpne7 showed a specific temporospatial distribution pattern in mouse molar during dentinogenesis, indicating the translocation of Cpne7 from preameloblasts to differentiating odontoblasts *in vivo*. To confirm the translocation of secretory Cpne7 from preameloblasts, ALCs in which Flag-tagged CPNE7 was overexpressed were co-cultured with odontoblastic MDPC-23 cells *in vitro*. Flag-tagged CPNE7 was detected in MDPC-23 cells by western blotting (Fig. 2(B)). Moreover, exogenous Cpne7 was seen in MDPC-23 cells by confocal microscopy (Supporting Information Fig. S1). These findings suggest that Cpne7 secreted by preameloblasts is translocated to differentiating odontoblasts in response to epithelial-mesenchymal interaction.

3.3. Cpne7 in differentiating odontoblasts regulates their differentiation into mature odontoblasts in vitro

A spontaneously immortalized and cloned cell line MDPC-23 has been derived from dental papilla of 18–19 fetal day CD-1 mouse, which already induced by underlying inner enamel epithelium [17]. Prior to clarifying the functional role of translocated Cpne7 in differentiating odontoblasts, the expression pattern of endogenous odontoblast Cpne7 was evaluated in

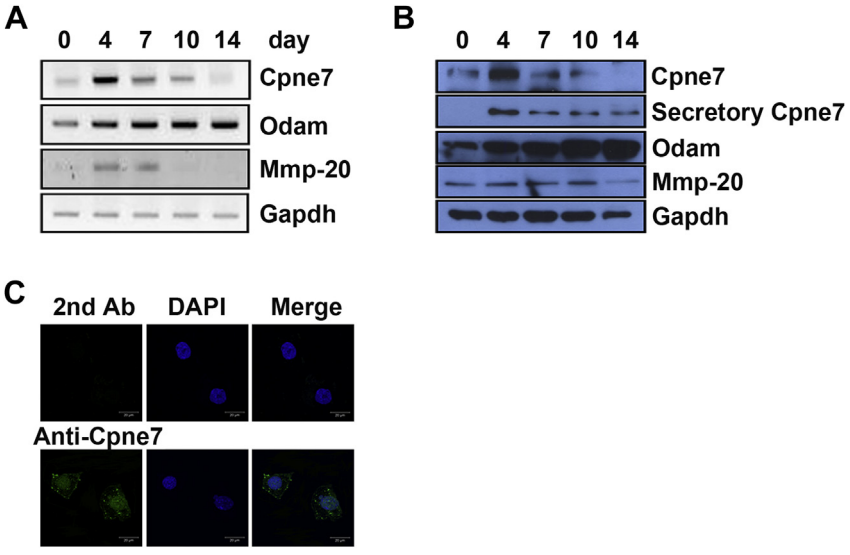

Fig. 1. Cpne7 is expressed during ameloblast differentiation. The ameloblast lineage cell (ALC) line was cultured in differentiation medium for up to 2 weeks. **(A)** The expression of *Cpne7*, *Odam*, and *Mmp-20* mRNA in ALCs was evaluated by RT-PCR. **(B)** The expression of Cpne7, Odam, and Mmp-20 in ALCs on the indicated days was evaluated by western blot analysis. Cpne7, Odam and Mmp-20 were detected in whole cell lysates. Secretory Cpne7 was detected in ALC-CM. **(C)** Localization of Cpne7 in ALCs was examined by immunofluorescence. Scale bars, 20 μm.

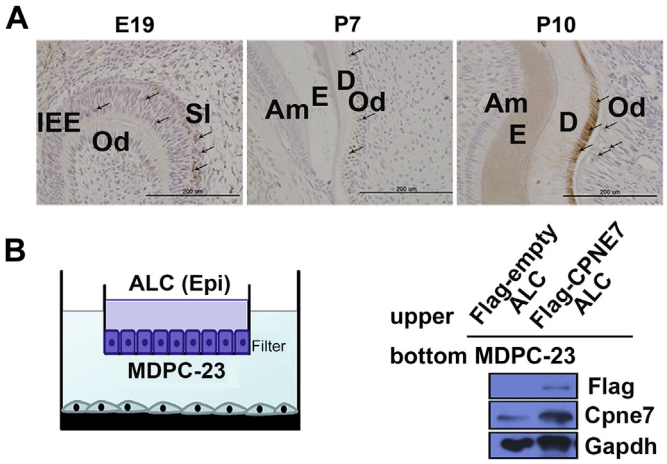

Fig. 2. Cpne7 is translocated from ameloblast to odontoblasts. **(A)** Cpne7 expression was detected by immunohistochemistry during mouse tooth development on embryonic day 19 (E19) and postnatal days 7 (P7) and 10 (P10). Cpne7 protein was localized in the inner enamel epithelium and stratum intermedium at E19; in differentiating odontoblasts at P7; and in dentin, pre-dentin, and differentiated odontoblasts at P10 (arrows). IEE: inner enamel epithelium, SI: stratum intermedium, Od: odontoblast, Am: ameloblasts, E: enamel, D: dentin. Scale bars, 200 μm. **(B)** Schematic diagram of the co-culture system. ALCs transfected with Flag-tagged CPNE7 were seeded in the upper chamber and odontoblastic MDPC-23 cells were seeded in the bottom chamber. Epi: epithelial cells, Mes: mesenchymal cells. Translocation of Flag-tagged CPNE7 synthesized by ALCs to MDPC-23 cells in the co-culture system was detected by western blotting.

odontoblastic MDPC-23 cells. Cpne7 was localized mainly in nuclei of MDPC-23 cells (Fig. 3(A)). The expression levels of Cpne7 protein in differentiating odontoblasts increased from days 0–7 (early to middle stage of odontoblast differentiation), and then decreased from days 10–14 (late stage of odontoblast differentiation and mineralization). The expression of odontoblast differentiation markers, including Dsp and Osteocalcin, increased in odontoblasts from days 7–14, which was later than the increase in Cpne7. Interestingly, secretory Cpne7 was clearly detected in odontoblast conditioned medium from days 10–14, whereas nuclear Cpne7 was mainly localized in differentiating odontoblasts from days 4–10 (Fig. 3(B and C)). These findings provide significant evidences that nuclear Cpne7 in odontoblasts might have functional roles in their differentiation, especially in early to middle stages, and also that the existence of autocrine/paracrine Cpne7 in differentiating odontoblasts of late stage.

To investigate whether Cpne7 influences odontoblast differentiation by upregulating *Dspp*, we measured the expression levels of *Dspp* mRNA and Dsp protein [18], after transfection of MDPC-23 cells with constructs encoding Cpne7 and Cpne7 siRNA. The expression level of Cpne7 was increased by transfection with Cpne7-encoding construct and effectively inhibited by Cpne7 siRNA in MDPC-23 and HEK293 cells, compared with control and cells transfected with control siRNA (Supporting Information Fig. S2, 4B). Cpne7 overexpression significantly increased the expression levels of *Dspp* mRNA and Dsp protein, whereas siRNA-mediated Cpne7 knockdown downregulated them in MDPC-23 cells (Fig. 4(A and B)). To confirm whether exogenous Cpne7 also enhanced *Dspp* expression, we investigated the effects of recombinant CPNE7 (rCPNE7) on *Dspp* expression in MDPC-23 cells. As expected, rCPNE7 treatment also increased the expression levels of *Dspp* mRNA and Dsp protein in MDPC-23 cells (Fig. 4(C and D)). Recombinant Cpne7 treatment enhanced mineralized nodule formation compared to control during odontoblast differentiation *in vitro* (Supporting Information Fig. S3). These data suggest that Cpne7 regulate *Dspp* expression and control odontoblast differentiation and mineralization.

Next approach was taken to confirm the functional consequences of *Cpne7* and *Dspp* gene expression. The Dsp protein was increased in a dose-dependent manner by treatment with PA-CM, but decreased by treatment with increasing concentrations of Cpne7-specific antibody for inactivation (Fig. 4(E)). Moreover, the *Dspp* transcriptional activity was significantly promoted by

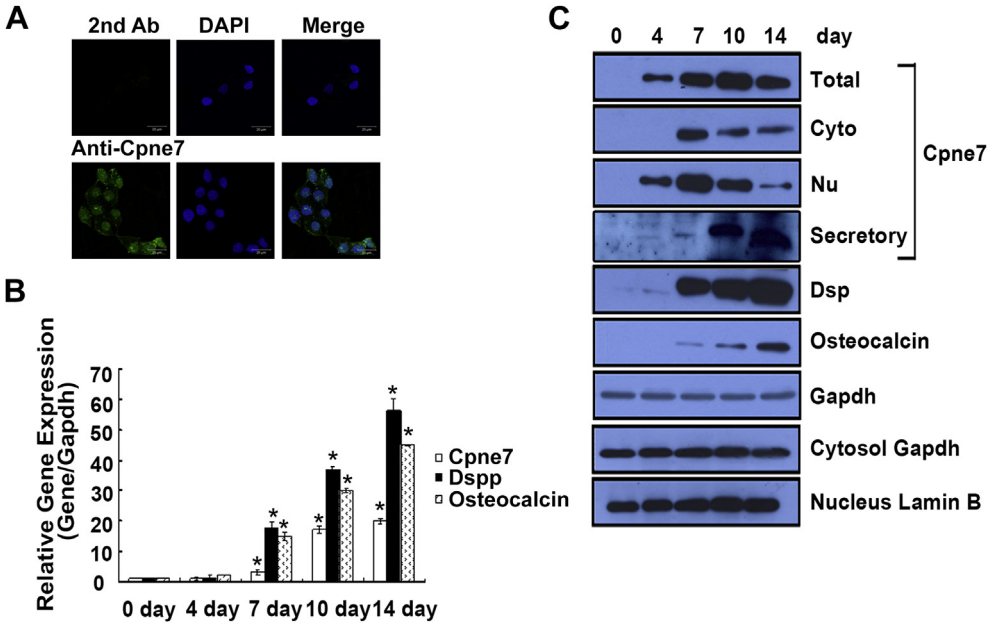

Fig. 3. Cpne7 is expressed during odontoblast differentiation. The odontoblast cell line MDPC-23 was cultured in differentiation medium for up to 2 weeks. **(A)** Localization of Cpne7 in MDPC-23 cells was detected by immunofluorescence. Scale bars, 20 μm. **(B)** Levels of *Cpne7*, *Dspp*, and *Osteocalcin* mRNA were evaluated by quantitative real-time PCR on the indicated days in MDPC-23 cells. All values represent the mean ± standard deviation (SD) of triplicate experiments. $^{*}P < .01$ compared to control. **(C)** Cpne7, Dsp, and Osteocalcin levels in MDPC-23 cells on the indicated days were evaluated by western blot. Cytosolic Gapdh and nuclear Lamin B served as cell fractionation controls. Total: whole cell extract, Cyto: cytosolic fraction, Nu: nuclear fraction.

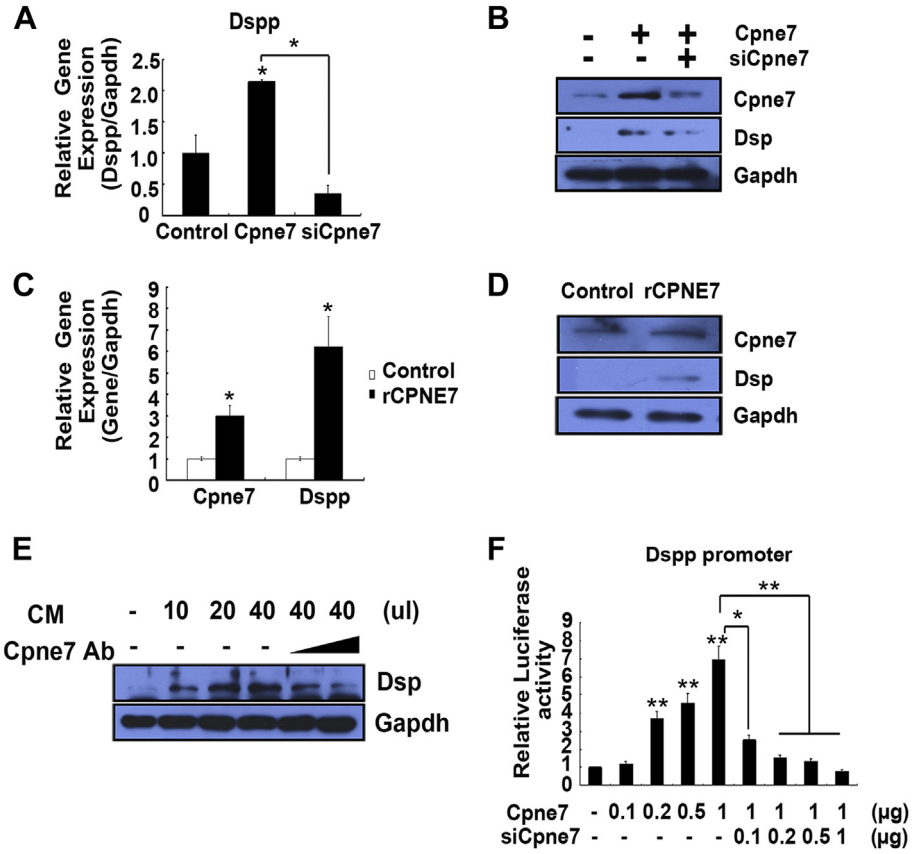

Fig. 4. Cpne7 stimulation and inactivation regulates odontoblast differentiation. **(A, B)** MDPC-23 cells were transfected with Cpne7 overexpression or siRNA construct. **(A)** Expression of *Dspp* mRNA was analyzed by real-time PCR. **(B)** Expression of Cpne7 and Dsp was analyzed by western blotting. **(C, D)** MDPC-23 cells were treated with 100 ng/ml rCPNE7. **(C)** Expression of *Cpne7* and *Dspp* mRNA was evaluated by real-time PCR. **(D)** Expression of Cpne7 and Dsp protein was evaluated by western blotting. **(E)** Expression of Dsp in MDPC-23 cells after inactivation of Cpne7 in CM analyzed by western blotting. **(F)** Transcriptional activity of *Dspp* promoter was evaluated by luciferase assay using overexpression of Cpne7 or Cpne7 siRNA in MDPC-23 cells. All values represent the mean ± SD of triplicate experiments. $^{*}P < .05$, $^{**}P < .01$ compared to control.

overexpression of Cpne7, but suppressed by siRNA-mediated Cpne7 knockdown (Fig. 4(F)). Collectively, these results indicate that Cpne7 regulates odontoblast differentiation and mineralization via control of *Dspp* expression.

3.4. *Cpne7 induces the differentiation of dental mesenchymal cells, such as human dental pulp cells (hDPCs), into odontoblasts and dentin formation in vivo*

To determine the role of Cpne7 in odontoblast differentiation and dentin formation *in vivo*, we transplanted hDPCs into the subcutaneous tissues of immunocompromised mice in the presence of hydroxyapatite/tricalcium phosphate (HA/TCP) under four different conditions, hDPCs-only, hDPCs with PA-CM, hDPCs with PA-CM and Cpne7 antibody, and hDPCs with rCPNE7. Six weeks after transplantation, dentin/pulp-like tissues were formed at the periphery of HA/TCP particles in all groups (Fig. 5(A–H)). PA-CM- or rCPNE7-treated groups exhibited dentin/pulp complex characteristics with odontoblasts more typical than the hDPCs-only group (Fig. 5(E, F and H)). However, in the group treated with Cpne7 antibody-treated PA-CM for inactivation, mineralized tissue formation was barely observed (Fig. 5(F and G)).

Based on the results of *in vitro* experiments shown in Fig. 4, we transplanted hDPCs transfected with Cpne7-encoding, Cpne7 siRNA, or control siRNA constructs into subcutaneous tissue in order to evaluate the effects of the *Cpne7* gene on dentin/pulp-like tissue formation *in vivo*. Twelve weeks after transplantation, hDPCs-only, Cpne7 overexpression, and control siRNA groups showed the generation of dentin-like mineralized tissues at the periphery of HA/TCP particles, whereas the Cpne7 siRNA group revealed little evidence of mineralization (Supporting Information Fig. S4(A–H)). According to the histomorphometric analysis of each group, overexpression of Cpne7 in hDPCs was associated with the highest level of dentin-like mineralized tissue formation *in vivo* and siRNA-mediated knockdown of Cpne7 was associated with the least. There was no significant difference between the hDPCs-only and control siRNA groups (Supporting Information Fig. S4(I)).

The tooth segments, which consist of natural dentinal wall and empty pulp cavity, provide the specific local environment for the regeneration of dentin/pulp-like tissues by dental stem cells [19]. To evaluate dentin/pulp-like tissue formation in root canal spaces, hDPCs were mixed with or without PA-CM or Cpne7 antibody-treated PA-CM, and transplanted into subcutaneous tissue of immunocompromised mice. After twelve weeks of implantation, vascularized pulp-like tissue regenerated inside the root canal spaces in all groups (Fig. 5(I–K)). In the PA-CM-treated group, odontoblast-like cells exhibited a palisade arrangement on the existing dentinal wall, and their cytoplasmic processes, with lengthened nuclei, extended toward existing dentinal tubules (Fig. 5(J, M and P)). In the Cpne7 antibody-treated PA-CM group, however, reparative dentin-like mineralized tissues showing entrapped cells was observed between newly formed pulp-like tissue and the existing dentin. BSP was distinctly expressed in the newly formed reparative dentin area of the Cpne7 antibody-treated PA-CM group compared to hDPCs-only and PA-CM-treated group (Fig. 5(L–N)). However, DSP was clearly detected in the PA-CM

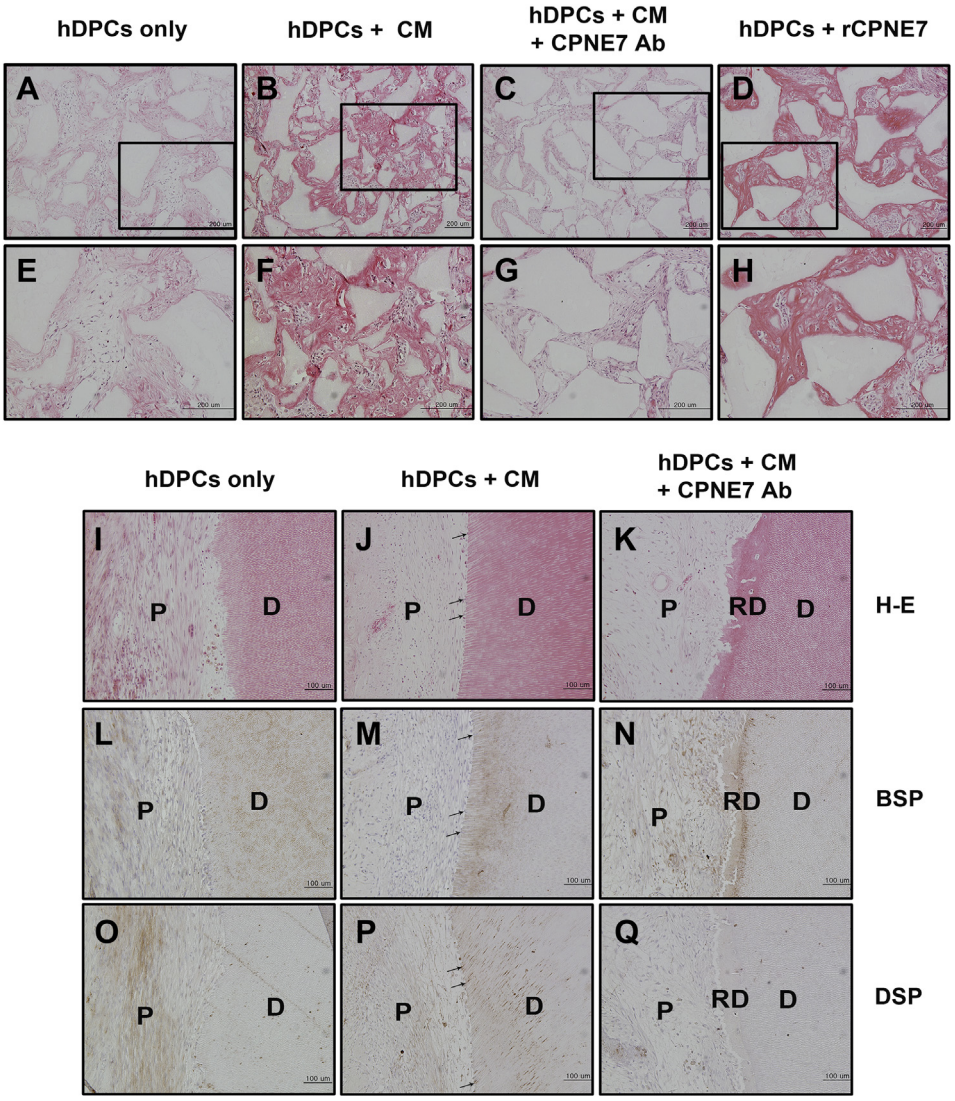

Fig. 5. Histological analysis of the regenerated dentin/pulp complex using human dental pulp cells (hDPCs) *in vivo*. **(A–H)** The hDPCs were mixed with 100 mg HA/TCP particles alone (A, E), or with CM (B, F), CPNE7 antibody-treated CM (C, G), or rCPNE7 (D, H) in a 0.5% fibrin gel and transplanted subcutaneously into immunocompromised mice for 6 weeks. Samples were stained with hematoxylin-eosin (H-E). **(E–H)** Boxed areas in A–D are shown at higher magnification in E-H. Scale bars, 200 μm. **(I–Q)** The hDPCs alone (I, L and O) or together with CM (J, M and P) or Cpne7 antibody-treated CM (K, N and Q) in a 0.5% fibrin gel were inserted into the root canal spaces of the human tooth segments for 12 weeks. Regenerated tissues were stained with H-E (I–K) and immunostained with anti-BSP (L–N) and anti-DSP (O–Q). Arrows in J, M and P indicate regenerated odontoblast-like cells with odontoblastic processes. P: regenerated pulp, D: pre-existing dentin wall, RD: newly formed reparative dentin. Scale bars, 100 μm.

group, but only faint staining was observed in the Cpne7 antibody-treated PA-CM group (Fig. 5(O–Q)).

Taken together, these *in vivo* findings indicate that, among preameloblast-derived factors, Cpne7 plays an important functional role in regeneration of the dentin/pulp complex and induces the odontogenic differentiation of mesenchymal cells of dental origin, such as hDPCs.

3.5. *Cpne7 induces differentiation of mesenchymal cells of non-dental origin into odontoblasts in vivo and in vitro*

Our data showed that Cpne7 induced odontoblastic differentiation of hDPCs and promoted dentin-like tissue formation in subcutaneous tissue and empty pulp cavity (Fig. 5). Therefore, we explored the role of Cpne7 in mesenchymal cells of non-dental origin, which indicates that those cells were not induced by dental epithelium previously. We used C3H10T1/2 cells, which are established from mouse embryonic connective tissue and do not differentiate into odontoblast-like cells without induction [20], and human bone marrow mesenchymal stem cells (hBMSCs). First, we examined the effects of ameloblastic ALC co-culture or Cpne7 overexpression in C3H10T1/2 cells. Cpne7 was barely expressed in both control C3H10T1/2 cells and those cells cultured for 5 days in differentiation medium. However, C3H10T1/2 cells co-cultured with ALCs showed Cpne7, Dmp1 and Dsp expression by western blotting (Fig. 6(A)). Cpne7 and DSPP mRNA expression was also increased after treatment with PA-CM or rCPNE7 (Fig. 6(B)). In addition, Cpne7 overexpression clearly induced Dsp protein expression in C3H10T1/2 cells (Fig. 6(C)). Moreover, the *Dspp* transcriptional activity was more significantly promoted than that of *BSP* by treatment with rCPNE7 (Fig. 6(D)). Next, we extended our studies to *in vivo* experiments using hBMSCs with or without rCPNE7 or rBMP2 proteins in empty root canal spaces for 6 weeks. In the rCPNE7-treated hBMSCs group, these non-dental mesenchymal stem cells differentiated into odontoblast-like cells with odontoblastic cellular processes and dentin-like mineralized

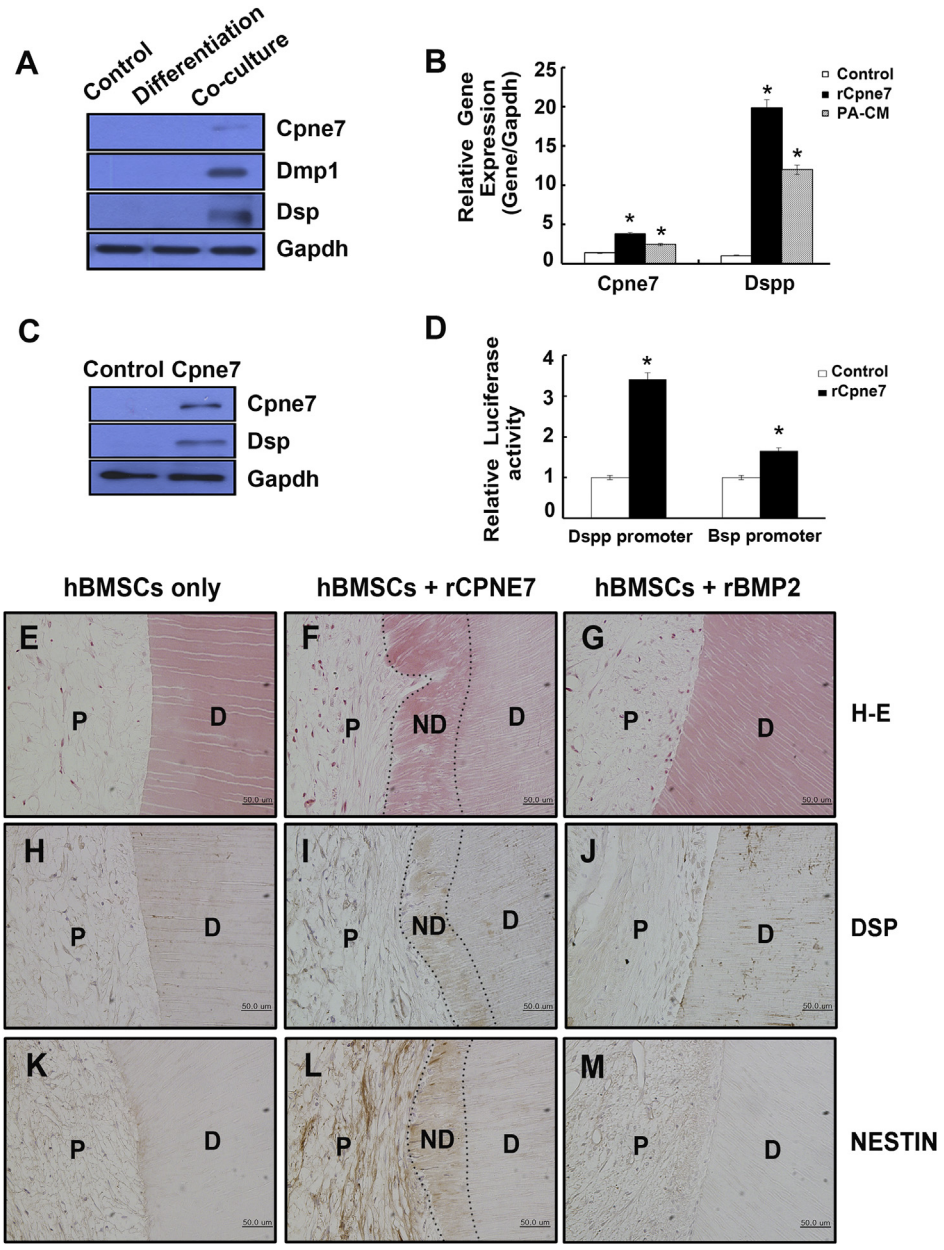

Fig. 6. Effects of Cpne7 on non-dental mesenchymal cells C3H10T1/2 and hBMSCs *in vitro* and *in vivo*. **(A)** C3H10T1/2 cells were cultured in induction medium or in co-culture with ALCs. Expression of Cpne7, Dmp1, and Dsp in C3H10T1/2 cells was evaluated by western blotting. **(B)** Levels of *Cpne7* and *Dspp* mRNA were evaluated by quantitative real-time PCR after 7 days of culture in C3H10T1/2 cells with or without rCPNE7 or PA-CM treatment. All values represent the mean ± standard deviation (SD) of triplicate experiments. $^{*}P < .01$ compared to control. **(C)** C3H10T1/2 cells were transfected with Cpne7 overexpression construct. Expression of Cpne7 and Dsp was analyzed by western blotting. **(D)** Transcriptional activity of *Dspp* and *Bsp* promoter was evaluated by luciferase assay after rCPNE7 treatment in C3H10T1/2 cells. All values represent the mean ± SD of triplicate experiments. $^{*}P < .05$, $^{**}P < .01$ compared to control. **(E–M)** hBMSCs alone (E, H and K) or mixed with rCPNE7 (5 μg; F, I and L) or rBMP2 (5 μg; G, J and M) in a 0.5% fibrin gel were inserted into the root canal spaces of the human tooth segments for 12 weeks *in vivo*. Regenerated tissues were stained by H-E (E–G) and immunostained with anti-DSP (H–J) and anti-Nestin (K–M). Dotted lines in F, I and L indicate the margins of the newly formed dentin, including the structure of dentinal tubules. P: regenerated pulp, D: pre-existing dentin wall, ND: newly formed physiologic dentin. Scale bars, 50 μm.

tissues, including regeneration of the structure of dentinal tubules on the existing dentinal wall (Fig. 6(E–G)). The cellular processes of odontoblast-like cells extended toward the dentinal tubules of newly formed dentin-like tissues, which robustly expressed DSP and NESTIN (Fig. 6(H–M)). On the other hand, no obvious dentin-like structures or DSP or NESTIN expression were observed in hBMSCs-only and rBMP2-treated hBMSCs groups.

Taken together, these findings suggest that Cpne7 from pre-ameloblasts might act as a signaling molecule in the odontogenic induction process and, thus, be capable of programming mesenchymal cells of non-dental origin, as well as those of dental origin, into odontoblast-like cells *in vivo* and *in vitro*.

3.6. *Cpne7 interacts with nucleolin and regulates odontoblast differentiation via the control of Dspp expression*

Our data showed that Cpne7 regulated *Dspp* gene expression in odontoblastic MDPC-23 cells. However, there is no DNA-binding motif in the structure of copine family members [21,22]. Therefore, we further investigated other potential effectors of Cpne7-mediated

Dspp expression because copine family proteins contain two distinctive domains, a phospholipid-binding domain and a protein interaction domain [8]. Nucleolin is a multifunctional protein that is involved in many cellular activities, including cell proliferation, embryogenesis, and cell death [23–26]. Nucleolin plays a functional role in tooth development [27] and interacts with Cpne3 [11].

To provide further evidence for an interaction between Cpne7 and Nucleolin, we performed immunocytochemical and co-immunoprecipitation (co-IP) analyses of MDPC-23 cells. Notably, both endogenous and exogenous Cpne7 proteins showed strong co-localization with Nucleolin protein in nuclei of odontoblasts (Fig. 7(A)) and interacted with Nucleolin (Fig. 7(B)). Next, we investigated whether the Cpne7-Nucleolin complex binds specifically to the *Dspp* promoter. A positive control was designed using the well-characterized nuclear factor I–C antibody on *DSPP* promoter [28]. Chromatin immunoprecipitation (ChIP) assay revealed that Nucleolin bound to the *Dspp* promoter, whereas negative control (pre-immune serum) or Nucleolin siRNA did not precipitate the *Dspp* promoter fragment (Fig. 7(C), Middle panel). Also, Cpne7 remained bound to the *Dspp* promoter (Fig. 7(C), Lower panel). To confirm that Nucleolin is sufficient to interact with the AP-1 site of the *Dspp* promoter, we used a DNA affinity protein-binding assay (DAPA) with biotin-labeled AP-1 site. Nucleolin was associated with the wild-type AP-1 site, but not with the mutated sequence (Supporting Information Fig. S5).

Finally, to further investigate the effect of Nucleolin on Cpne7-mediated *Dspp* transcription, we transfected MDPC-23 cells with Nucleolin siRNA constructs and conducted luciferase reporter assays using a Dspp-responsive reporter. In these cells, Nucleolin level was reduced by Nucleolin siRNA, without alteration of Cpne7 level (Fig. 7(D), Lower panel). Cpne7 stimulated *Dspp* promoter activity and this activation was abolished with the knockdown of Nucleolin (Fig. 7(D), Upper panel). These data indicated that Nucleolin is critical for Cpne7-dependent *Dspp* transcription.

Taken together, the results suggest that Cpne7 as a coregulator is physically associated with Nucleolin protein, and the Cpne7-Nucleloin complex modulates *Dspp* gene transcription.

4. Discussion

Morphogenesis and differentiation of organs are regulated by short-range diffusible signals, commonly referred to as epithelial-

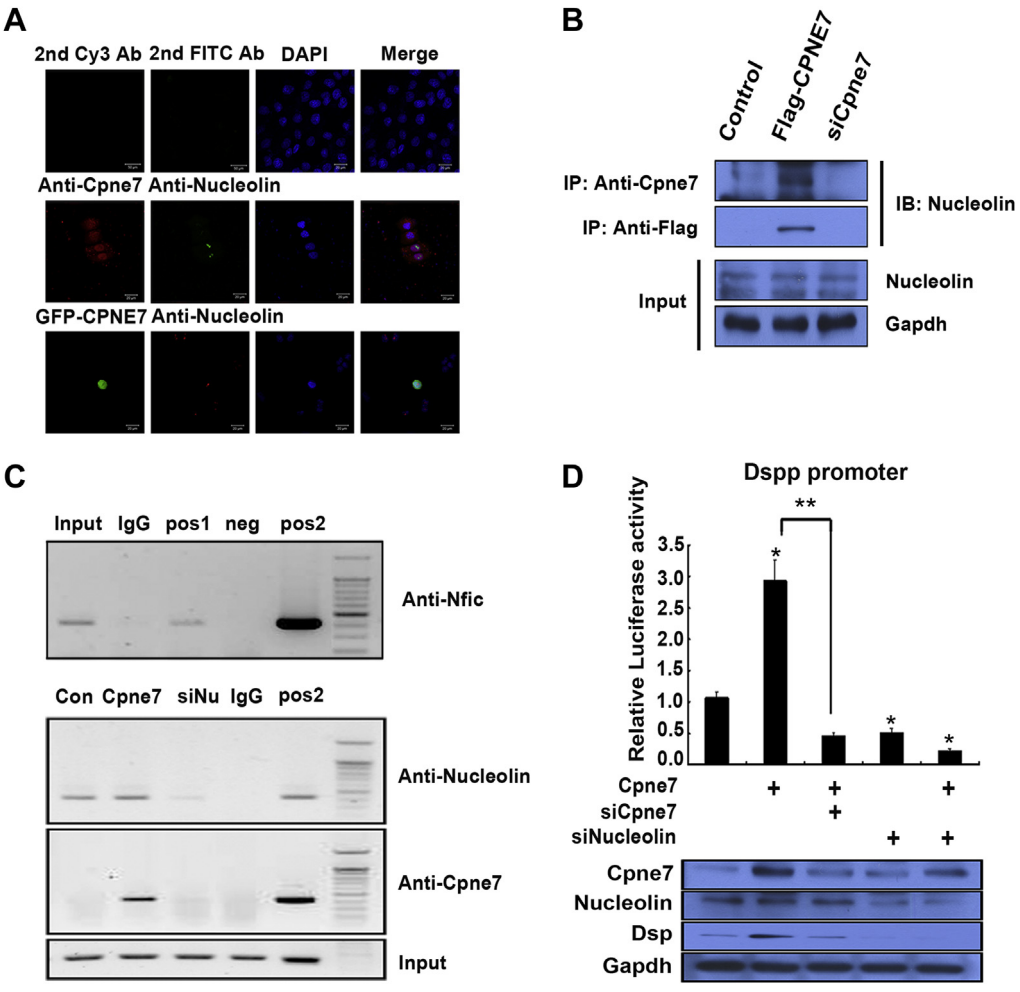

Fig. 7. *Dspp* is regulated by a complex of Cpne7 and Nucleolin. **(A)** Co-localization of Cpne7 and Nucleolin was detected by fluorescence microscopy in MDPC-23 cells (Scale bars: 20 μm). **(B)** MDPC-23 cells were transfected with Flag-tagged CPNE7 expression or Cpne7 siRNA construct. Immunoprecipitated (IP) Cpne7 and the whole cell lysates (Input) were analyzed by western blotting (IB) with anti-Nucleolin antibody. **(C)** Cross-linked chromatin was prepared and immunoprecipitated with pre-immune serum (IgG) or Nfic-specific antibody in MDPC-23 cells (Upper panel). ChIP assays were performed using anti-Nucleolin, anti-Cpne7, or IgG antibodies. Chromatin samples were subjected to PCR analysis using primer pairs spanning the AP-1 site on the *Dspp* promoter. Input: the PCR product of chromatin obtained before immunoprecipitation, IgG: pre-immune serum, pos1: ChIP positive (anti-Nfic), neg: PCR negative, pos2: PCR positive, con: control, siNu: Nucleolin siRNA. **(D)** MDPC-23 cells were cotransfected with the indicated vectors. The transcriptional activity of the *Dspp* promoter was evaluated by luciferase assay. Expression levels of Cpne7, Nucleolin, and Dsp in MDPC-23 cells were evaluated by western blotting. All values represent the mean ± SD of triplicate experiments. $^{*}P < .05$, $^{**}P < .01$ compared to control.

mesenchymal interactions [29]. At the bell stage of tooth development, undifferentiated ectomesenchymal cells become enlarged and are going to differentiate into odontoblasts after induction by dental epithelial cells, which involve paracrine interactions [30]. In this paper, we focus on molecular mechanisms underlying dentinogenesis in the developing crown via epithelial-mesenchymal interactions. First, we observed expression and secretion of Cpne7 only in early stages of ameloblast differentiation, but in early to middle stages of odontoblast differentiation. Second, we demonstrated translocation of Cpne7 from preameloblasts to differentiating odontoblasts. Results of our histological analysis showed that Cpne7 was expressed only in epithelial cells, including inner enamel epithelium and stratum intermedium at E19. At P7 and P10, Cpne7 existed in odontoblasts, pre-dentin, and dentinal tubules, although Cpne7 was no longer expressed in epithelial tissue, continuously. Further, Cpne7 protein was translocated from ameloblasts into odontoblasts in the co-culture system. Therefore, we first identified Cpne7 as a new mediator protein in the molecular mechanism of epithelial-mesenchymal interactions during dentinogenesis in the developing crown. Moreover, we demonstrated that the stage of the shift in Cpne7 localization from the epithelium to the underlying mesenchyme corresponds temporospatially to that of the initiation of odontoblast differentiation.

Next, we explored the function of Cpne7 and how it controls odontoblast differentiation during tooth development. Based on previous reports and our data, Cpne7 mRNA and protein increased during odontoblast differentiation, and stimulation of Cpne7 promotes expression of odontoblast-related genes, including Dspp, Osteocalcin, and Alp [6]. Specifically, we observed that the expression of Dspp (mRNA, protein, and transcriptional activity) was upregulated by Cpne7 overexpression or rCPNE7 treatment, and rCPNE7 promoted mineralized nodule formation *in vitro*. In addition, inactivation of Cpne7 in PA-CM by Cpne7-specific antibody prevented induction of dentin-like mineralized tissue formation in transplantation experiments *in vivo*. Indeed, Cpne7 had a significant effect on formation of new dentin-like structures in mesenchymal cells of both dental and non-dental origin *in vivo*. These findings indicate that Cpne7 is indispensible and also plays important roles in regulating odontoblast differentiation and mineralization.

In the present study, we have used odontoblastic cell line, MDPC-23, to evaluate the expression of Cpne7 in odontoblasts. As a result, endogenous Cpne7 was expressed in MDPC-23 cells from the beginning of the culture without PA-CM induction. It is well known that MDPC-23 cells can differentiate into odontoblasts without dental epithelial induction because the cells were already induced by underlying inner enamel epithelium previously, so that they could express Cpne7. Endogenous Cpne7 expression was enhanced by co-culture with ALCs or rCPNE7 treatment. In addition, Cpne7 was localized in both the cytoplasm and the nucleus of differentiating odontoblasts in early to middle stages, but secreted into extracellular matrix in late stage. These results imply that exogenous Cpne7 enters differentiating odontoblasts, induces endogenous Cpne7 expression, and triggers the autocrine/paracrine secretion of Cpne7 from differentiating odontoblasts similar to that of the Bmp [31]. Once triggered by dental epithelial cell-derived Cpne7, differentiating odontoblasts can secrete Cpne7 by themselves, which acts in autocrine/paracrine manner.

Our *in vivo* transplantation studies establish that Cpne7 induces dentin-like mineralized tissue formation in hDPCs with HA/TCP. The formation of a dentin/pulp-like complex was more extensive in rCPNE7-treated or Cpne7-overexpressing hDPCs groups than in the hDPCs-only group. The upregulation of DSP in the rCPNE7 treatment group indicated that Cpne7 promoted differentiation of hDPCs into cells with more odontogenic characteristics. Moreover, the Cpne7-inactivated CM group showed formation of reparative dentin-like structure containing cells entrapped in the mineralized matrix and expression of BSP, which leads to failure of complete physiological dentin regeneration. Therefore, Cpne7 is critical for the formation and regeneration of a dentin/pulp complex *in vivo*.

Nucleolin is a multifunctional protein and directly or indirectly plays a role in the regulation of cell proliferation, differentiation, and apoptosis. Nucleolin is composed of three domain structures, an acidic histone-like N-terminus, an RNA-binding domain, and an arginine-and-glycine–rich domain. Nucleolin binds various kinds of proteins, such as midkine (MK) in proliferation [32], STAT1 in monocyte differentiation [33], and Fas in apoptosis [34]. Indeed, Nucleolin recognizes sequence-specific DNA-binding sites, including the AP-1 (TGAC/gTCA) and SP-1 (AGCCCA and AGCCCCT) sites, and regulates cPLA2a gene transcription [35,36]. Computer analysis demonstrates that the promoter region of the *Dspp* gene contains various potential binding sites of transcription factors AP-1, AP-2, Runx2, C/EBP, and NF-1 [37]. A previous study showed that Nucleolin is expressed in the inner enamel epithelium, the cusp areas of the dental papilla, and cervical loop during the crown formation stage [27]. In addition, CPNE3 binds Nucleolin through the vWA domain, which is involved in protein–protein interaction in SKBr3 cells [11]. Similarly, our present study showed that Cpne7 and Nucleolin were co-localized and interacted in MDPC-23 cells. In addition, Nucleolin and Cpne7 bound the *Dspp* promoter and regulated *Dspp* gene expression. Based on previous reports and our data, we suggest that the Cpne7-Nucleolin complex might regulate transcriptional activation of the *Dspp* promoter and be responsible for Cpne7-mediated Dspp expression during odontoblast differentiation and mineralization.

5. Conclusions

In conclusion, the expression of Cpne7 showed a temporospatial distribution pattern during dentinogenesis in the developing crown and Cpne7 regulates odontoblast differentiation and dentin formation *in vitro* and *in vivo*. Also, Cpne7 induces differentiation of odontoblast-like cells from mesenchymal cells of dental or non-dental origin. These results establish the role of Cpne7 as a key player in the regulation of mesenchymal stem cell differentiation into odontoblasts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.10.016.

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