치의학박사 학위논문

Role of CPNE7 during Amelogenesis and Odontogenesis

법랑질과 상아질 형성과정에서 CPNE7의 역할

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박 수 진

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Role of CPNE7 during Amelogenesis and Odontogenesis

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ABSTRACT

Role of CPNE7 during Amelogenesis and Odontogenesis

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Odontogenesis is a complex physiological process of tooth development, which involves both ectodermal and mesenchymal components, being the key elements in the development of teeth. In order for the tooth to form, an interactive mechanism between these heterotypic cellular populations is required.

Based on the concept of epithelial-mesenchymal interactions during odontogenesis, Copine-7 (Cpne7), a dental epithelium-derived factor, was identified as a diffusing

signaling molecule for regulation the differentiation of mesenchymal cells of dental or non-dental origin into odontoblasts in the previous study. However, the mechanisms involved in the translocation of Cpne7 from preameloblasts to preodontoblasts and the functions of Cpne7 during odontogenesis have not yet been clarified.

The results from part I study provide confirmation for both the mRNA and protein expression of *Dspp* in differentiating ameloblasts and its expression pattern is similar to that of *Cpne7* during ameloblast differentiation. Moreover, GEO profiles indicate that there is a close correlation between *Cpne7* and *Dspp* expression in various normal human tissues. *Cpne7* overexpression promotes *Dspp* expression, whereas *Dspp* expression is downregulated by *Cpne7* inactivation in early amelogenesis. Mechanism of such regulation is confirmed by findings that CPNE7 binds to the *Dspp* promoter region and regulates its transcription. Taken together, these findings suggest that *Dspp* is synthesized in dental epithelial cells by the control of CPNE7 and its transient expression occurs in early ameloblasts.

The results from part II study strongly suggest a mechanism in which Cpne7 is internalized into preodontoblasts and transported to the nucleus, which implied possible strategies to regulate odontoblast differentiation. Cpne7 acts as a ligand and bounds to its receptor, nucleolin in lipid rafts, and is internalized via caveolae-mediated endocytosis. The Cpne7-nucleolin complex is then translocated to the nucleus of preodontoblasts. Cpne7 increases the formation of primary cilia affecting the expression of Kif3a and Ift88, cilium components. Ift88 promotes up-regulation of Dspp expression. Taken together, I propose that the existence of a Cpne7-nucleolin complex-primary cilia-Dspp pathway

during early odontoblast differentiation.

Collectively, these studies provide the evidence for the roles and underlying mechanisms of Cpne7 on *Dspp* expression during early amelogenesis and ciliogenesis of mesenchymal stem cells during dentinogenesis.

Keywords: Cpne7 · Dspp · Epithelial-mesenchymal interaction · Amelogenesis · Nucleolin · Primary cilia · Odontoblast differentiation

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CHAPTER I. GENERAL INTRODUCTION

Odontogenesis is a complex physiological process of tooth development, which involves both ectoderm-derived epithelial and neural-crest derived mesenchymal components, being the key elements in the development of teeth [1]. In order for the tooth to form, an interactive mechanism between these heterotypic cellular populations is required [2]. At the molecular level, these interactions involve complex signaling networks composed of various signaling molecules, their receptors, and the transcription control systems [3]. However, the molecules and underlying mechanisms involved in the initiation and regulation of odontoblast differentiation have not yet been clarified.

In this paper, I focused on the Copine-7 (Cpne7), a signaling molecule that is secreted by preameloblasts and control of Dspp expression during odontoblast differentiation, and briefly summarized its role in amelogenesis and ciliogenesis of mesenchymal stem cells.

1. Copine-7 (Cpne7), an essential factor on the differentiation of preodontoblasts into odontoblasts

Based on the concept of epithelial-mesenchymal interactions during tooth development, the effects of preameloblast-conditioned medium (PA-CM) on the odontogenic differentiation of human dental pulp cells (hDPCs) have been evaluated and the results showed that dental epithelium-derived factors in PA-CM induced odontogenic differentiation of hDPCs. Among those factors, the novel protein Cpne7 has been

suggested to be mainly related to odontoblast differentiation and dentin formation [4, 5]. Furthermore, Cpne7 protein was translocated to differentiating odontoblasts and to induce the expression of dentin sialophosphoprotein (*Dspp)*, which is a major component of the non-collagenous dentin extracellular matrix and odontoblast differentiation *in vitro* and *in vivo* [5].

Cpne is a ubiquitous family of calcium-dependent phospholipid-binding proteins that is evolutionally conserved from *Arabidopsis* to *Homo sapiens*. The Cpne family of proteins contains nine members with a similar domain structure, namely two N-terminal C2-domains (C2A and C2B) and a C-terminal A-domain (vWA). The C2-domains are thought to be responsible for binding to the inner face of the plasma membrane following increases in intra cellular calcium levels, whereas the A-domain has been suggested to be a protein-binding structure [6-8].

2. Roles in amelogenesis

Ectoderm-derived ameloblasts and ectomesenchyme-derived odontoblasts synthesize and secrete a distinct extracellular matrix for the formation of enamel and dentin, respectively [9, 10]. However, studies by several research groups clearly indicate that *Dspp* is not exclusively expressed in dentin but is also expressed in other tissues, including bone [11], cementum [12], and certain non-mineralized tissues [13]. During enamel formation, *Dspp* is transiently expressed in presecretory ameloblasts when the interface between the enamel and dentin is forming [14-16]. Although *Dspp* is expressed during early amelogenesis, its role and origin in ameloblasts remains unclear.

Immunohistochemical studies have demonstrated that Cpne7 is initially expressed in the dental epithelium and is then translocated to differentiating odontoblasts. However, its expression disappears over time in ameloblasts. Similar to the expression pattern of Cpne7, *Dspp* mRNA and protein are expressed in presecretory ameloblasts but significantly decrease after the secretion of the nonmineralized dentin matrix by odontoblasts. Cpne7 also plays an important role in odontoblast differentiation and dentin formation in human dental pulp cells via the control of *Dspp* expression [5].

Based on prior studies, I hypothesized that the expression of *Dspp* mRNA and protein during early amelogenesis is linked to the expression of Cpne7.

3. Roles in ciliogenesis of mesenchymal stem cells

Primary cilia are single non-motile organelles that typically protrude from the cell surface and are expressed by most mammalian cell types including stem cells [17]. It forms a single organelle consisting of a membrane-bound cylinder surrounding the axoneme, made of 9 microtubule doublets [18]. In many tissues, primary cilia are essential for regulation of cell and tissue development and homeostasis [19, 20] via a variety of signaling pathways, such as Hedgehog and Wnt [19].

In a recent study, the data suggested that primary cilia showing stage- and region-specific morphology are involved in the epithelial-mesenchymal interaction during tooth development [21]. Furthermore, a possible involvement of ciliopathies in tooth defects was approached through the results of *Ofd1* gene mutation in mice that mimic the X-linked oro-facial-digital type I syndrome, encountered in humans, characterized by

missing/supernumerary teeth and enamel hypoplasia [22]. Thus, it has become highly essential to investigate the relation between primary cilia and signaling network involved odontogenesis.

4. Rationale and outline of the thesis experiments

A key purpose of this thesis is to investigate the underlying mechanisms of Cpne7 function in amelogenesis and ciliogenesis of mesenchymal stem cells. To achieve this goal, I performed the studies of 1) the localization of CPNE7 and DSP in developing tooth buds of mouse *in vivo* and their gene expression patterns during early ameloblast differentiation, 2) the mechanisms on translocation of Cpne7 from preameloblasts to preodontoblasts, and 3) the molecular mechanisms responsible for inducing ciliogenesis during odontogenesis.

CHAPTER II. Dentin sialophosphoprotein expression in enamel is regulated by Copine-7, a preameloblast-derived factor

* This Chapter has been largely reproduced from an article published by Park SJ. and Park JC. (2018). Arch Oral Biol., 86:131-137.

I. ABSTRACT

Dentin sialophosphoprotein (*Dspp*) is expressed in odontoblasts and transiently expressed in early ameloblasts. However, the origin of *Dspp* in ameloblasts remains unclear. Our previous studies demonstrated that copine-7 (Cpne7), a molecule that is secreted by the dental epithelium, is expressed in early ameloblasts and is then translocated to differentiating odontoblasts; its expression levels correlate with odontoblast differentiation under the control of *Dspp* expression. The objective of this study is to figure out the relationship between Cpne7 and *Dspp* during amelogenesis. The gene expression patterns of CPNE7 and dentin sialoprotein (DSP) were examined by immunohistochemistry, western blot analysis, and real-time polymerase chain reaction. The effects of Cpne7 on *Dspp* regulation were investigated using luciferase and chromatin immunoprecipitation assays in ameloblastic HAT-7 cells. The gene expression pattern of *Cpne7* was similar to that of *Dspp* during ameloblast differentiation. Moreover, Gene expression omnibus (GEO) profiles indicated that there is a close correlation between *Cpne7* and *Dspp* expression in various normal human tissues. I also confirmed the effects of Cpne7 on the induction of *Dspp* in ameloblastic HAT-7 cells. *Cpne7* overexpression promoted *Dspp* expression, whereas *Dspp* expression was down-regulated by *Cpne7* inactivation. These results suggest that the expression of *Dspp* in early amelogenesis is linked to Cpne7, a preameloblast-derived factor.

II. INTRODUCTION

Tooth organogenesis results from sequential and reciprocal interactions between oral epithelial and ectomesenchymal cells [23]. Ectoderm-derived ameloblasts and ectomesenchyme-derived odontoblasts synthesize and secrete a distinct extracellular matrix for the formation of enamel and dentin, respectively. [9, 10]. During these processes, ameloblasts and odontoblasts are thought to uniquely express native matrix proteins. However, studies by several research groups clearly indicate that dentin sialophosphoprotein (*Dspp*) is not exclusively expressed in dentin but is also expressed in other tissues, including bone [11], cementum [12], and certain non-mineralized tissues [13, 24].

During enamel formation, *Dspp* is transiently expressed in presecretory ameloblasts when the interface between the enamel and dentin is forming [14-16]. Studies using in situ hybridization performed on mouse and rat tooth germs showed that *Dspp* expression can only be detected during early stages of amelogenesis prior to the appearance of secretory ameloblasts associated with extensive enamel matrix production [15]. Immunolocalization studies on developing rat tooth organs have also demonstrated that dentin sialoprotein (DSP) is expressed in presecretory ameloblasts in adjacent pre-dentin and in odontoblasts prior to the initial deposition of mineralized dentin, whereas DSP staining rapidly decreased over time in functional secretory ameloblasts [25]. Recently, the expression of *Dspp* was detected in both presecretory and secretory ameloblasts during the formation of the dentino-enamel junction [26]. Although *Dspp* is expressed

during early amelogenesis, its origin in ameloblasts remains unclear.

Previously, copine-7 (Cpne7) was identified as a factor involved in epithelial-mesenchymal interactions during tooth development [4, 5]. Immunohistochemical studies have demonstrated that Cpne7 is initially expressed in the dental epithelium and is then translocated to differentiating odontoblasts. However, its expression disappears over time in ameloblasts. Similar to the expression pattern of Cpne7, *Dspp* mRNA and protein are expressed in presecretory ameloblasts but significantly decrease after the secretion of the nonmineralized dentin matrix by odontoblasts. Cpne7 also plays an important role in odontoblast differentiation and dentin formation in human dental pulp cells via the control of *Dspp* expression [5].

Based on prior studies, I hypothesized that the expression of *Dspp* mRNA and protein during early amelogenesis is linked to the expression of Cpne7. To prove this hypothesis, I characterized DSP and CPNE7 localization in developing tooth buds of mice *in vivo* and assessed their gene expression patterns during early ameloblast differentiation. I further measured the potential effects of *Cpne7* overexpression and shRNA knockdown on *Dspp* expression in ameloblastic HAT-7 cells.

III. MATERIALS AND METHODS

1. Tissue preparation and immunohistochemistry

All animal experiments followed protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-111013-3). The heads of mice were decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and processed for immunohistochemistry. The expression of CPNE7 and DSP was detected using an ABC kit (Vector Labs, Burlingame, CA, USA) with rabbit anti-CPNE7 and rabbit anti-DSP as the primary antibodies [4, 27] and a biotin-labeled goat anti-rabbit IgG (1:200, Vector Labs) as the secondary antibody.

2. Cell culture

HAT-7 cells, a dental epithelial cell line originating from the cervical loop epithelium of a rat incisor (a generous gift from Dr. H Harada , Department of Oral Anatomy, Iwate Medical University, Yahaba, Iwate, Japan), were grown and maintained in DMEM/F12 (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and antibiotic-antimycotic reagents (Gibco BRL) [28]. To induce ameloblast differentiation, 80-90% confluent cells were cultured in DMEM/F12 supplemented with 5% FBS, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate for up to 1 week.

3. Plasmid construction

Full-length mouse *Cpne7* (*mCpne7*, NM_170684) cDNA and pGL3-*Dspp* vectors

were constructed and verified as described previously [4]. An expression vector encoding DDK (Flag)-tagged *Cpne7* (NM_153636) and *mCpne7* shRNA (sc-142808-SH) were purchased from Origene (Rockville, MD, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

4. Real-time polymerase chain reaction

Total RNA was isolated from HAT-7 cell cultures or mouse tooth germ using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA (3 μg) was reverse transcribed for 1 h at 50 ℃ with 0.5 mg Oligo dT and 1 μL (50 IU) Superscript III enzyme (Invitrogen) in a 20-μL reaction. One microliter of the product was amplified by polymerase chain reaction (PCR) using the following primer pairs: *Dspp*, forward 5′-gtgaggacaaggacgaatct-3′ and reverse 5′-cactactgtcactgctgtca-3′; *Cpne7*, forward 5′-cgggacccattgaccaagtc-3′ and reverse 5′-catacacctcaaaccgtagcttc-3′; *Ameloblastin*, forward 5′-tgagccttgagacaatgagac-3′ and reverse 5′-aaagagttatgcggtgggag-3′; *Amelogenin*, forward 5′-gtcacctctgcatcccatg-3′ and reverse 5′-ttcccgcttggtcttgtc-3′; *Odam*, forward 5′-aacactagagagctttgctggact-3′ and reverse 5′-aggtggtgtctgctgctgagaga-3′; *Gapdh*, forward 5′-aggtcggtgtgaacggatttg-3′ and reverse 5′-tgtagaccatgtagttgaggtca-3′. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) using SYBR GREEN PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were 40 cycles of 95 ℃ for 1 min, 94 ℃

for 15 s, and 60 ℃ for 30 s. All reactions were run in triplicate, and PCR product levels were normalized to those of the housekeeping gene *Gapdh*. Relative changes in gene expression were calculated using the comparative threshold cycle method.

5. Western blot analysis

Cellular proteins (30 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T), and incubated overnight at 4 ℃ with the primary antibody diluted in PBS-T buffer (1:1,000). Affinity-purified rabbit polyclonal anti-CPNE7, anti-DSP, and anti-ODAM antibodies were produced as described previously [4, 27]. The anti-Flag (F3165) antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-Ameloblastin (sc-50534), anti-Amelogenin (sc-365284), and anti-GAPDH (sc-25778) antibodies were purchased from Santa Cruz Biotechnology. After washing, membranes were incubated for 1 h with secondary antibodies. Labeled protein bands were detected using an enhanced chemiluminescence system (Dogen, Cambridge, MA, USA).

6. Transient transfection and luciferase assay

HAT-7 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 the Metafectene Pro reagent (Biontex, Martinsried/Planegg, Germany) with the reporter constructs described above and an

SV40-driven β-galactosidase expression vector as an internal control. The pGL3-*Dspp* vector was cotransfected into HAT-7 cells with either the *Cpne7* expression vector or the *Cpne7* shRNA vector. Following the addition of 50 μL Luciferin to 50 μL cell lysate, luciferase activity was determined using a Luminometer according to the manufacturer's instructions (Promega, Madison, WI, USA). β-galactosidase activity was determined in 96-well plates that were read at 405 nm using an ELISA reader. The luciferase activity was normalized to the β-galactosidase activity.

7. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as previously described [27]. Briefly, after transfection with the Flag-tagged *Cpne7* expression vector using the Metafectene Pro reagent, HAT-7 cells were sonicated. The fragmented chromatin mixture was incubated with anti-Flag antibodies or IgG (1:100) on a rotator at 4 ℃ overnight, then 30 μL protein A/G PLUS-agarose (Santa Cruz Biotechnology) was added and incubated for 2 h at 4 ℃ on a rotator to collect the antibody/chromatin complex. The final DNA pellets were recovered and analyzed by PCR using primers spanning the *Dspp* promoter region (453 bp), rat *Dspp* -393 region, and rat *Dspp* +60 region: forward 5′-tatagacccaggtggggagt-3′ and reverse, 5′-aagacgtccttaccggaatg-3′. PCR was carried out under conditions of 35 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, and 72 ℃ for 1 min. The PCR products were electrophoresed through a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

8. Gene expression profiling

Gene expression data (GSE2361) were obtained from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Publicly available gene expression datasets were downloaded the GEO (accession number GDS1096 to normal tissues of various types) and *Cpne7* and *Dspp* mRNA expression was analyzed using GEO data.

9. Statistical analysis

All quantitative data are presented as the mean ± SD. Statistical differences were analyzed using Student's *t* test (*; $p < 0.05$, **; $p < 0.01$).

IV. RESULTS

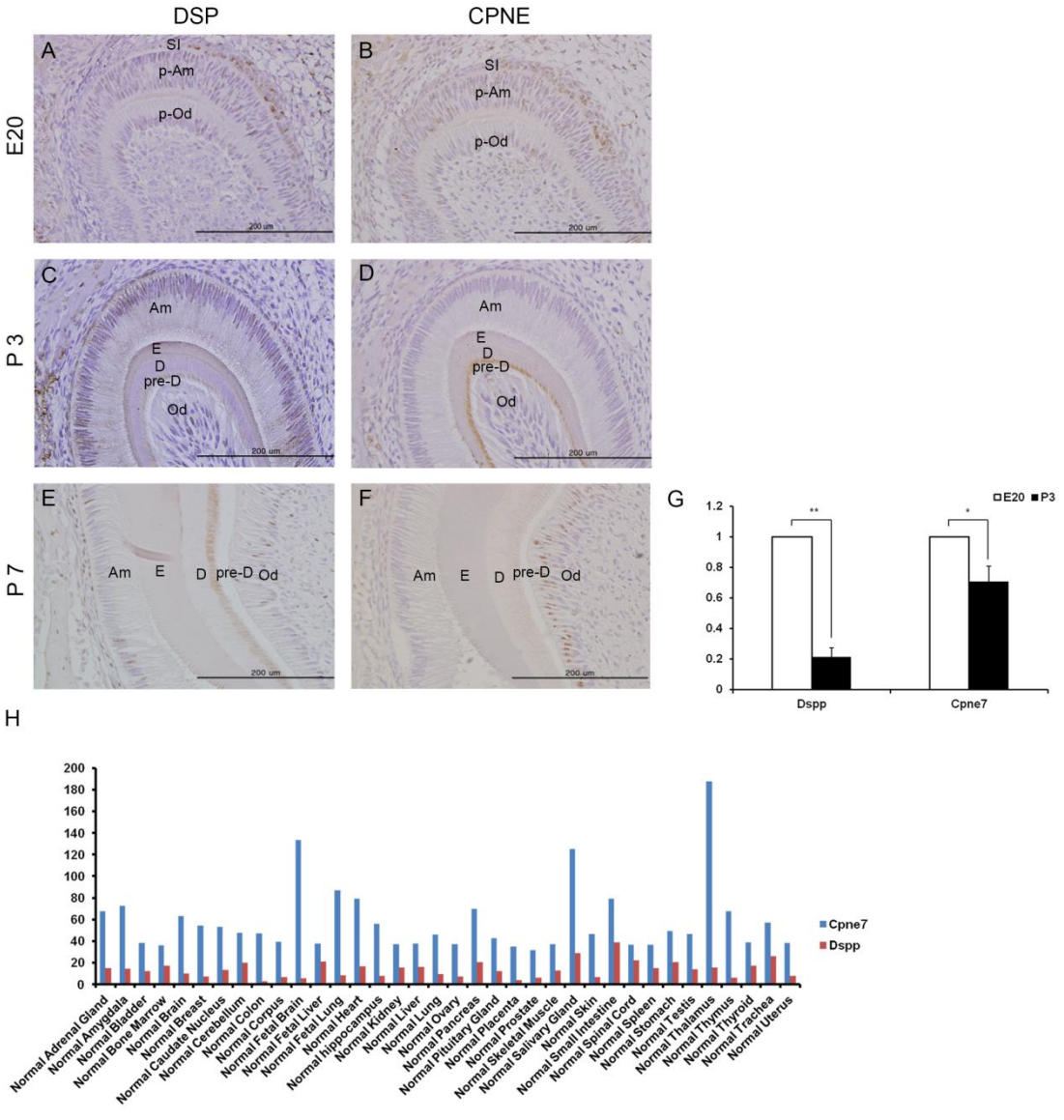
1. Spatiotemporal expression of *Dspp* and *Cpne7* (mRNA and protein) during mouse tooth development

At embryonic day 20 (E20), the bell stage of tooth development, positive staining of DSP was found in preameloblasts and the stratum intermedium of the developing mandibular first molar (Fig. 1A). At this stage, CPNE7 was also localized in preameloblasts and the stratum intermedium (Fig. 1B). At postnatal days 3 (P3), the enamel matrix was partially deposited, and the cells were entering the early secretory stage. At this point, DSP expression decreased and CPNE7 was hardly detectable in early secretory ameloblasts (Fig. 1C and D). At P7, which represented the late secretory stage, the expression of DSP decreased, and CPNE7 was no longer detected in late secretory ameloblasts but was clearly observed in odontoblasts and predentin (Fig. 1E and F). From quantitative RT-PCR results, I found that the mRNA expression level of *Dspp* and *Cpne7* was significantly decreased in tooth germ at P3 compared to at E20. These results are in accord closely with those from the IHC analysis (Fig. 1G).

2. Expression of *Dspp* is associated with *Cpne7* expression in various normal human tissues

The expression of *Dspp* is detected in various tissues including kidney, salivary gland, liver, cartilage and lung in mouse [24]. Copine (Cpne) family is also expressed ubiquitously [8]. Other investigators have shown that genes with similar functions

frequently have similar patterns of mRNA expression [29]. Thus, I analyzed microarray data from the NCBI GEO dataset to examine *Dspp* and *Cpne7* expression in various human tissues as well as in mouse dental tissues. *Dspp* was expressed in the human adrenal gland, bone marrow, colon, heart, kidney, liver, ovary, and small intestine. Similar to *Dspp*, *Cpne7* was detected in the same tissues, although the level of *Cpne7* was generally higher than that of *Dspp*. (Fig. 1H).


DSP
CPNE
A
B
C
D
E
F
G
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E20
P 3
P 7
SI
p-Am
p-Od
Am
E
D
pre-D
Od
200 um
E20
P3
Dspp
Cpne7
Normal Adrenal Gland
Normal Amygdala
Normal Bladder
Normal Bone Marrow
Normal Brain
Normal Breast
Normal Caudate Nucleus
Normal Cerebellum
Normal Colon
Normal Corpus
Normal Fetal Brain
Normal Fetal Liver
Normal Fetal Lung
Normal Heart
Normal hippocampus
Normal Kidney
Normal Liver
Normal Lung
Normal Ovary
Normal Pancreas
Normal Pituitary Gland
Normal Placenta
Normal Prostate
Normal Skeletal Muscle
Normal Salivary Gland
Normal Skin
Normal Small Intestine
Normal Spinal Cord
Normal Spleen
Normal Stomach
Normal Testis
Normal Thalamus
Normal Thymus
Normal Thyroid
Normal Trachea
Normal Uterus

Figure 1. *Dspp* and *Cpne7* (mRNA and protein) expression in developing mouse teeth and various normal human tissues.

(A–F) Immunostaining of DSP and CPNE7 in sections of mouse molar germ at embryonic day 20 (E20) and postnatal days 3 (P3) and 7 (P7). SI, stratum intermedium; p-Am, preameloblast; Am, ameloblast; p-Od, preodontoblast; Od, odontoblast; E, enamel; D, dentin; pre-D, predntin. Scale bar: 200 μm. (G) Expression of *Dspp* and *Cpne7* mRNA in tooth germ at E20 and P3 was determined by real-time RT-PCR. The data represent three independent experiments, and results are shown as the mean ± SD. ($^{*}p < 0.05$, $^{**}p <$ 0.01 compared to control). (H) *Dspp* and *Cpne7* mRNA expression in various normal human tissues. The mRNA expression of *Dspp* and *Cpne7* were compared in various normal human tissues based on gene expression data from NCBI's GEO database.

3. Expression of Dspp, Cpne7, and ameloblast-related factors during ameloblast differentiation in vitro

The expression of *Dspp* gradually increased from the first day of culture until day 5 (pre-ameloblast stage), and then decreased thereafter (Fig. 2A). The expression pattern of *Cpne7* mRNA in HAT-7 ameloblastic cells was similar to that of *Dspp* (Fig. 2B). It is axiomatic in functional genomics that genes with similar mRNA expression profiles are likely to be regulated via the same mechanisms [30]. Thus, the similarity of expression patterns between *Dspp* and *Cpne7* mRNA may suggest that two genes are interrelated. Ameloblastin (*Ambn*) and Amelogenin (*Amelx*) mRNAs increased slightly from the first day of culture until days 7 and 10, respectively, and decreased thereafter (Fig. 2C, D). ODAM, an odontogenic ameloblast-associated protein, increased during ameloblast differentiation (Fig. 2E). The expression patterns of DSP, CPNE7, AMBN, AMELX and ODAM proteins in HAT-7 ameloblastic cells were similar to those of their respective mRNAs (Fig. 2F).

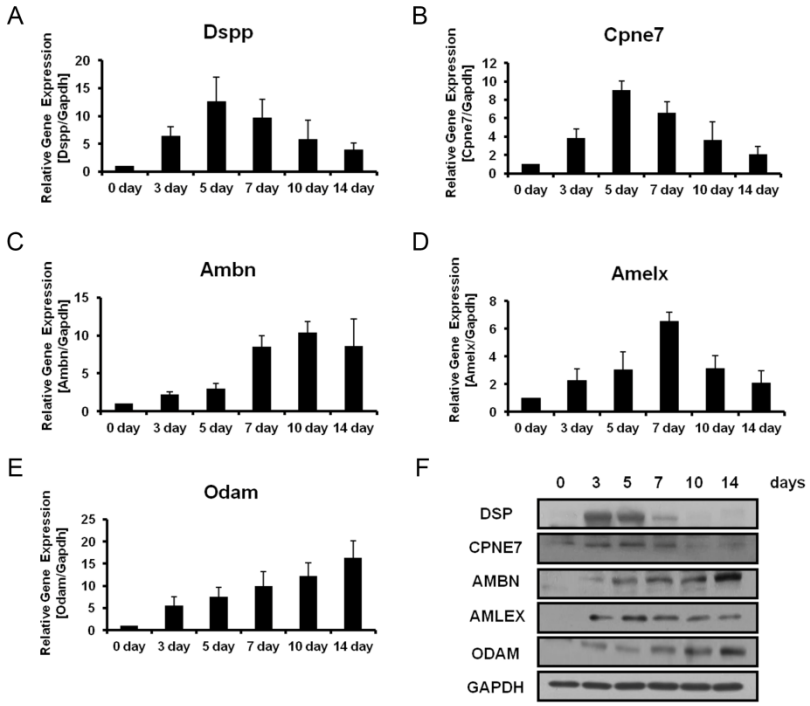

Figure 2. Quantitative real-time PCR and western analysis of *Dspp*, *Cpne7*, *Ameloblastin*, *Amelogenin* , and *Odam* during HAT-7 cell culture for 14 days.

(A–E) Quantitative real time PCR and (F) western analysis. The data represent three independent experiments, and results are shown as the mean ± SD. *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Cpne7*, Copine-7; *Ambn*, Ameloblastin; *Amelx*, Amelogenin; *Odam*, odontogenic ameloblast-associated protein.

4. Regulation of Dspp expression by Cpne7 in ameloblasts

Twenty-four hours after *Cpne7* plasmid transfection, the overexpression efficiency of *Cpne7* in HAT-7 cells was confirmed (Fig. 3A). Expression of *Cpne7* in HAT-7 cells was successfully knocked down by shRNA fragments specific for *Cpne7* (Fig. 3B). *Cpne7* overexpression upregulated *Dspp* mRNA and DSP protein, compared with controls. *Cpne7* inactivation downregulated *Dspp* mRNA and DSP protein compared with controls (Fig. 3C and D).

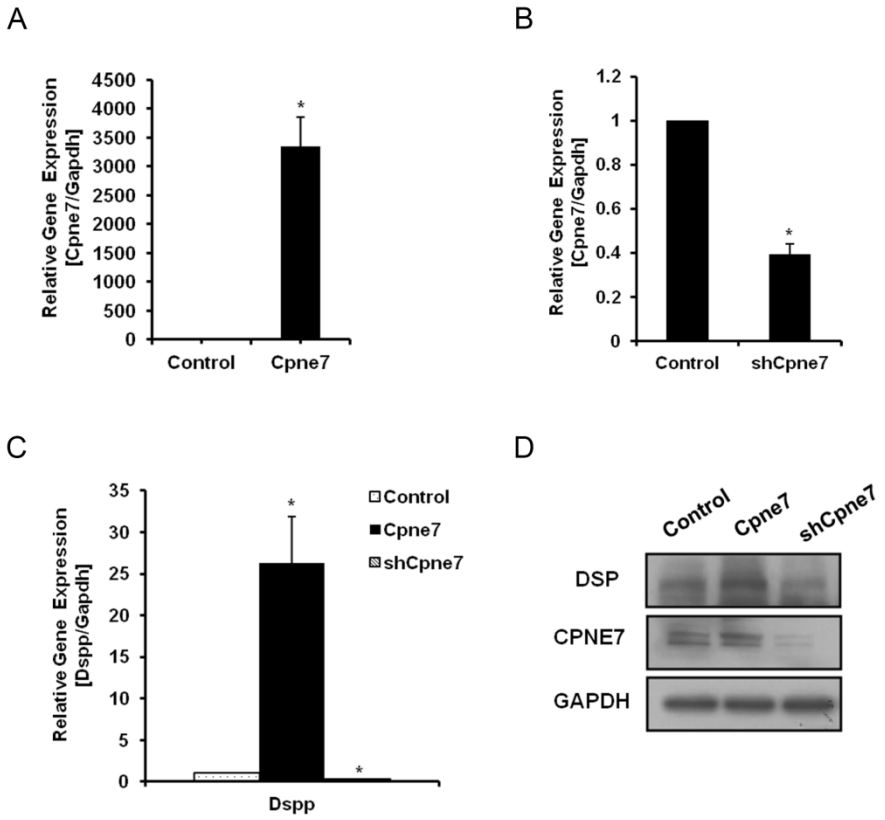

Figure 3. The effects of *Cpne7* on *Dspp* expression in ameloblastic HAT-7 cells.

HAT-7 cells were transfected with a *Cpne7* expression vector or a shRNA construct. (A–C) Expression of *Cpne7* and *Dspp* mRNA was determined by real-time RT-PCR. (D) Expression of CPNE7 and DSP proteins was analyzed by western blotting. The data represent three independent experiments, and results are shown as the mean ± SD. ($^{*}p < 0.05$ compared to control).

5. Recruitment of Cpne7 to the Dspp Promoter in ameloblasts

The transcriptional activation of the *Dspp* promoter was significantly induced by Cpne7 overexpression. These activation effects were disrupted by shRNA-mediated Cpne7 inactivation (Fig. 4A). To obtain more direct evidence for an interaction between Cpne7 and the *Dspp* promoter, I performed ChIP assay in HAT-7 cells. Results from the ChIP assay demonstrated that Cpne7 bound to the *Dspp* promoter. IgG antibody was used as a negative control with total DNA before immunoprecipitation as the input for PCR (Fig. 4B).

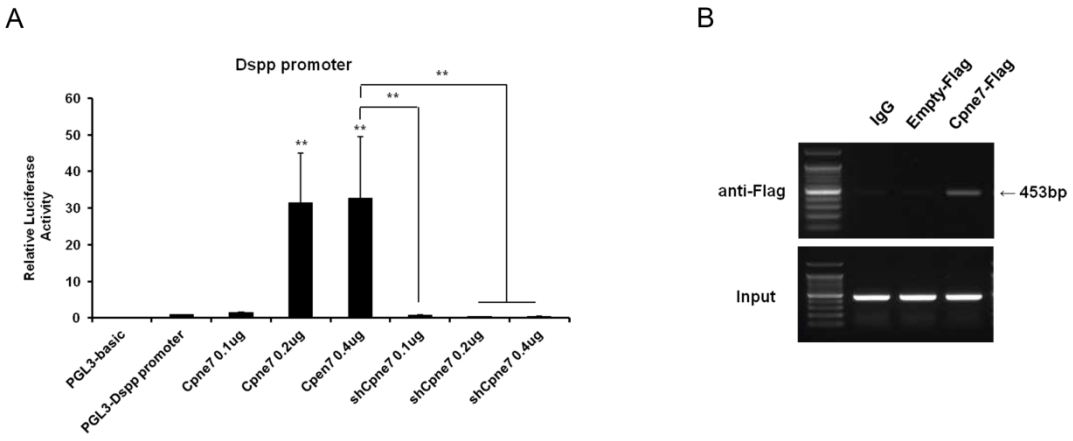

Figure 4. Transcriptional activity of *Dspp* and Cpne7 binding to the *Dspp* promoter in ameloblastic HAT-7 cells.

(A) Transcriptional activity of the *Dspp* promoter was evaluated by a luciferase assay using *Cpne7* expression vector or an shRNA construct in HAT-7 cells. (B) HAT-7 cells were transfected with a vector expressing a Flag-tagged *Cpne7* construct or an empty Flag-vector as a control. A ChIP assay was performed using anti-Flag or IgG antibodies. Chromatin samples were subjected to PCR analysis using primer pairs for the *Dspp* promoter. Input: PCR product of chromatin obtained before immunoprecipitation; IgG: preimmune serum. The data represent three independent experiments, and results are shown as the mean ± SD. ($^{*}p < 0.05$, $^{**}p < 0.01$ compared to control).

V. DISCUSSION

For over a decade, *Dspp* and its cleaved products were thought to be tooth dentin specific [9]. In human genetic studies, mutations in the *Dspp* gene cause inherited dentin defects, such as dentinogenesis imperfecta type II and dentinogenesis imperfecta type III [31, 32]. *Dspp* has been thoroughly shown to play a major role in dentinogenesis by analyses of the dentin phenotypes in *Dspp*-knockout animals [33, 34]. Although there are no major defects in the overall thickness or hardness of mature enamel in *Dspp* knockout teeth compared with their heterozygous counterparts, several studies have suggested that *Dspp* plays an important role in amelogenesis. *Dspp* has been postulated to play a role in enamel biomineralization, acting as a potential nucleator of hydroxyapatite crystal formation [15]. Recently, *Dspp* was suggested to be associated with ameloblast differentiation and physiology [26]. However, the origin of *Dspp* in early ameloblasts remains elusive.

Electron microscopic analyses have demonstrated that the basement membrane, which separates the epithelium of the dental lamina from the ectomesenchyme, is degraded during the terminal differentiation of ameloblasts [35, 36]. The disappearance of the basement membrane allows the penetration of the cytoplasmic processes of differentiating ameloblasts into the forming mantle dentin [36]. Since ameloblasts directly contact the nonmineralized matrix produced by odontoblasts, it possible that DSP found in presecretory ameloblasts obtained from the adjacent predentin via endocytosis [16, 25]. It is also possible that ameloblasts can synthesize *Dspp*. Unlike the sustained pattern of

Dspp expression observed in odontoblasts, *Dspp* was greatly reduced when predentin was detected at the epithelial-mesenchymal interface in ameloblasts. This contrast in *Dspp* expression patterns in odontoblasts and ameloblasts; suggests that *Dspp* is transcribed and translated by early secretory ameloblasts [37]. However, the exact mechanism controlling the initiation of *Dspp* expression in ameloblasts is unknown. On the other hand, previous immunohistochemical and biochemical studies have shown that preodontoblasts transiently express enamel matrix proteins such as AMELX and AMBN [38, 39]. However, the exact origins and roles of these nonspecific enamel matrix proteins are unknown.

In the present study, the level of *Cpne7* is generally higher than that of *Dspp* in diverse tissues (Fig. 1H). Copines (*Cpnes*) are evolutionally conserved and are expressed from *Paramecium* to humans [8]. Nine *Cpne* genes have been identified and most of them are expressed ubiquitously. They are thought to be associated with membrane-trafficking phenomena and protein-protein interaction because their biochemical properties and gene structure [6]. These characteristics of Cpnes propose that they may be concerned various events to control the molecular activity during biological development. Therefore, I suggest that high-level gene expression of *Cpne7* compared to that of *Dspp* in varied tissues may be involved in extensive functions of *Cpne7*.

As shown in Fig. 4B, the PCR amplifying bands with primers corresponding to the 5'-flanking region (from nucleotides (nt) -393 to +60) of rat *Dspp* gene were detected in cells transfected with *pcDNA-Cpne7-Flag* plasmids. These results indicated that *Cpne7* is

able to directly bind to *Dspp* promoter region from nt -393 to +60 *in vivo*. It is reported that Cpne7-Nucleolin complex bound AP-1 site of *Dspp* promoter and regulated the *Dspp* gene expression in odontoblastic MDPC-23 cells [5]. However, in the present study, the experiments to determine whether Cpne7 interacts with Nucleolin in ameloblastic HAT-7 cells were not carried out. There is a need to examine whether these two factors interact to bind *Dspp* promoter and control *Dspp* gene expression in ameloblast cells.

In summary, the results from this study provide confirmation for both the mRNA and protein expression of *Dspp* in differentiating ameloblasts and its expression pattern is similar to that of *Cpne7* during ameloblast differentiation. Moreover, GEO profiles indicate that there is a close correlation between *Cpne7* and *Dspp* expression in various normal human tissues. *Cpne7* overexpression promotes *Dspp* expression, whereas *Dspp* expression is downregulated by *Cpne7* inactivation in early amelogenesis. Mechansim of such regulation is confirmed by findings that Cpne7 binds to the *Dspp* promoter region and regulates its transcription. Taken together, these findings suggest that *Dspp* is synthesized in dental epithelial cells by the control of Cpne7 and its transient expression occurs in early ameloblasts.

CHAPTER III. Copine-7 binds to the cell surface receptor, nucleolin, and regulates ciliogenesis and Dspp expression during odontoblast differentiation

* This Chapter has been largely reproduced from an article published by Park SJ. and Park JC. (2017). Sci Rep., 12;7(1):11283.

I. ABSTRACT

Tooth development is a progressive process regulated by interactions between epithelial and mesenchymal tissues. Our previous studies showed that copine-7 (Cpne7), a dental epithelium-derived protein, is a signalling molecule that is secreted by preameloblasts and regulates the differentiation of preodontoblasts into odontoblasts. However, the mechanisms involved in the translocation of Cpne7 from preameloblasts to preodontoblasts and the functions of Cpne7 during odontogenesis are poorly understood. Here, I showed that the internalization of Cpne7 was mediated primarily by caveolae. This process was initiated by Cpne7 binding to the cell surface protein, nucleolin. Treatment with recombinant Cpne7 protein (rCpne7) in human dental pulp cells (hDPCs) caused an increase in the number of ciliated cells. The expression level of cilium components, Ift88 and Kif3a, and Dspp were increased by rCpne7. Treatment with Ift88 siRNA in hDPCs and MDPC-23 cells significantly down-regulated the expression of Dspp, an odontoblastic differentiation marker gene. Furthermore, the treatment with nucleolin siRNA in MDPC-23 cells decreased the expression of Dmp1, Dspp, and cilium components. These findings suggested that the binding of Cpne7 with its receptor, nucleolin, has an important function involving Cpne7 internalization into preodontoblasts and regulation of Dspp expression through ciliogenesis during odontoblast differentiation.

II. INTRODUCTION

Tooth development is a consequence of programmed, sequential, and reciprocal communications between the dental epithelium and mesenchyme, which is also mediated by specific temporal-spatial expression of a series of genes[1]. Interactions between the ectodermal tissue and underlying mesenchymal tissue form the basis of the mechanism that regulates tooth development. Epithelial and mesenchymal cells differentiate into ameloblasts and odontoblasts, respectively, during crown formation[40]. In 1887, Von Brunn suggested that odontoblasts differentiated only in the presence of the enamel epithelia[41]. This study reported that epithelial signals induced in the mesenchyme led to subsequent odontoblast differentiation and dentin formation.

Based on the concept of epithelial-mesenchymal interactions during odontogenesis, the effects of preameloblast-conditioned medium (PA-CM) on the odontogenic differentiation of human dental pulp cells (hDPCs) were investigated in previous studies. It has been showed that dental epithelium-derived factors in PA-CM induced odontogenic differentiation of hDPCs. Among those secreted dental epithelium-derived factors, copine-7 (Cpne7) was expressed in preameloblasts and secreted extracellularly during ameloblast differentiation. After secretion, the Cpne7 protein was translocated to differentiating odontoblasts and to induce the expression of Dspp, which is a major component of the non-collagenous dentin extracellular matrix and odontoblast differentiation *in vitro* and *in vivo* [42]. However, the mechanism involved in the translocation of Cpne7 from preameloblasts to prodontoblasts is poorly understood.

Cpne is a ubiquitous family of calcium-dependent phospholipid-binding proteins that is evolutionally conserved in animals, plants, and protists. Nine Cpne genes have been identified. Cpnes have conserved features consisting of two C2 domains (C2A and C2B) and the von Willebrand factor A (vWA) domain. The C2 domains of Cpnes were originally identified in conventional protein kinase C (PKC) and were involved in calcium influx[43]. The vWA domain mediates protein-protein interactions[44]. Although the function of Cpnes remains unclear, some of the biological roles of several Cpnes are known. In mammals, Cpnes are widely expressed throughout different tissues, including the brain, heart, lung, liver, and kidney[45]. Cpne1, 2, and 3 are expressed in all normal tissues. Cpnes4-7 show more limited expression. Cpne4 is found in brain, heart, and prostate glands, and Cpne6 is brain specific. Cpne7 is expressed in foetal brain, thymus, and testis[46]. All of the Cpnes exhibit calcium–dependent translocation to the plasma membrane, and Cpne 1, 2, 3, and 7 also translocate to the nucleus[47]. Cpne6 links activity-triggered calcium signals to spine structural plasticity that is necessary for learning and memory[48]. However, there have been few studies of the functions of Cpne7 besides our previous report that Cpne7, a diffusing signalling molecule, is a regulator of the differentiation of mesenchymal cells into odontoblasts.

The primary cilium has been found in almost every eukaryotic cell type as a non-motile antenna emerging from the cell and extending into the extracellular space[49]. In many tissues, primary cilia are essential for sensing mechanical, biochemical or light signals[50, 51]. Mutations in genes encoding cilium components involved in major human genetic diseases including developmental disorders, dysfunctions of the reproductive system,

airway disease, cystic disorders of the kidney, liver, and pancreas, defects in vision, smell and hearing, and oncogenesis[52]. Consequently, the primary cilium plays a fundamental role in cellular physiology and development including tooth formation, bone formation, and nerve formation[53-55]. Among the Cpne family, Cpne6 was identified in ciliary bodies[56]. However, the molecular mechanisms responsible for inducing cilianogenesis during odontogenesis remain unclear.

In this study, I showed that the internalization of Cpne7, an epithelium-derived factor, into the odontoblasts is a caveolae-dependent, receptor-mediated event and that Cpne7 regulated odontoblast differentiation through the formation of primary cilia. Three major steps highlighted in this study involved: nucleolin functioning as a cell surface receptor in Cpne7 endocytosis, Cpne7 regulation of ciliogenesis, and Cpne7 regulation of Dspp expression via Ift88, one of the ciliary components.

III. MATERIALS AND METHODS

1. Reagents, antibodies, and plasmids

All reagents used in this study [chlorpromazine, sucrose, methyl-β-cyclodextrin, nystatin, calcium chloride, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), potassium chloride, magnesium chloride and dithiothreitol (DTT)] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit and affinity-purified polyclonal anti-Cpne7, and anti-Dsp antibodies were produced as described previously[57, 58]. Commercial antibodies against Flag (Sigma-Aldrich), nucleolin (Cell Signaling; Danvers, MA, USA), Dmp1 (Abcam; Cambridge, UK), glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Santa Cruz Biotechnology; Santa Cruz, CA, USA), α-tubulin (Santa Cruz Biotechnology), and Ift-88 (Santa Cruz Biotechnology) were used for Western blot analyses. Expression vectors encoding DDK (Flag)-tagged Cpne7 (NM_153636) and recombinant Cpne7 (NP 705900) were purchased from Origene (Rockville, MD, USA). Control siRNA and nucleolin-targeting siRNA were purchased from Ambion (Carlsbad, CA, USA). Ift88 siRNA was purchased from Santa Cruz Biotechnology.

2. Cell culture

Human impacted third molars were collected at the Seoul National University Dental Hospital (Seoul, Republic of Korea), and the experimental protocol was approved by the

Institutional Review Board (S-D20140007). Informed consent was obtained from all patients. All methods were performed in accordance with the relevant guidelines and regulations. The human whole pulp cell isolation was described previously[57]. MDPC-23 cells were provided by Dr. J.E. Nor (University of Michigan, Ann Arbor, MI, USA). The hDPCs and MDPC-23 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco BRL) and antibiotic-antimycotic reagents (Gibco BRL) at 37 ℃ in an atmosphere of 5% CO_2. To induce hPDC and MDPC-23 cell differentiation, 80-90% confluent cells were cultured in DMEM supplemented with 5% FBS, ascorbic acid (50 μg/mL), and β-glycerophosphate (10 mM) for up to 1 week.

3. Real-time PCR analysis

Total RNA was extracted from cells with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total RNA (3 μg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (Invitrogen). One μL of the RT product was PCR amplified using the primer pairs. For real-time PCR, the specific primers for *Cpne7*, *nucleolin*, *Dmp1*, *Dspp*, *Ift88*, and *Kif3a* were synthesized as listed in Table 1. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA,USA) using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were 40 cycles at 95 ℃ for 1 min, 94 ℃ for 15 s, and 60 ℃

for 1 min. All reactions were performed in triplicate, and the 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.

Table 1. Oligonucleotide primer sequence used in the real-time PCR

Gene	Sequence (5'-3')
hGAPDH	F: AGG GCT GCT TTT AAC TCT GGT
	R: CCC CAC TTG ATT TTG GAG GGA
hCPNE7	F: GTC TTC ACG GTG GAC TAC TAC T
	R: ATG CGT GTC GTA CAC CTC AAA
hIFT88	F: GCA ATC CTA CGA AAC AGT GCC
	R: CAC TGA CCA CCT GCA TTA GC
hKIF3A	F: CTC GTC TTC TTC AGG ATT CC
	R: GAG ACT TTC TTT TTT CCC CTT C
mGapdh	F: AGG TCG GTG TGA ACG GAT TTG
	R: TGT AGA CCA TGT AGT TGA GGT CA
mCpne7	F: CGG GAC CCA TTG ACC AAG TC
	R: CAT ACA CCT CAA ACC GTA GCT TC
mNucleolin	F: ACA CCA GCC AAA GTC ATT CC
	R: ATC CTC ATC ACT GTC TTC CTT C
mDmp1	F: CAT TCT CCT TGT GTT CCT TTG GG
	R: TGT GGT CAC TAT TTG CCT GTG
mDspp	F: GTG AGG ACA AGG ACG AAT CTG A
	R: CAC TAC TGT CAC TGC TGT CAC T
mIft88	F: GCA GTG ACAGTG GCC AGA ACA ATA
	R: CAG CCA GGG AGC AGA GAC AAG TAG
mKif3a	F: GAA GCC CAA CAA GAG CAT CAG T
	R: CCA GTG GAC GTA GTT TTC AAT CAT

4. Western blot analysis

Whole cell lysates of cells were harvested using a lysis buffer consisting of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 1 mM PMSF supplemented with protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). Following centrifugation at 13,000 × g for 30 min, the supernatant was collected for analysis. Protein concentrations were determined using the DC^{TM} protein assay system (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (20 μg) were resolved using 8% or 10% polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The PVDF membrane was blocked with PBST (10 mM phosphate-buffered saline, pH 7.0, and 0.1% Tween-20) buffer containing 5% non-fat dry milk for 1 h at room temperature. The blots were then washed and incubated with the indicated antibodies for 24 h at 4 ℃ with gentle shaking. Blots were washed three times for 10 min each in PBST, followed by incubation with anti-rabbit or anti-mouse immunoglobulin G conjugated to horseradish peroxidase in PBST for 1 h at room temperature. After washing three times in PBST, the blots were analysed using an enhanced chemi-luminescence reagent (ECL; Santa Cruz Biotechnology) according to the manufacturer's guidelines. Protein loading was assessed by the expression of Gapdh (1:5000; Santa Cruz Biotechnology). Semi-quantitative measurements were carried out using Image J software (National Institutes of Health, USA).

5. Subcellular fractionation

MDPC-23 cells were collected and resuspended in 500 μL ice-cold fractionation buffer [250 mM sucrose, 20mM HEPES; pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1mM DTT and protease inhibitor cocktail (Roche Molecular Biochemicals)]. The cells were sheared by repeated passage through a 25-gauge needle (10 times). After 20 min, the lysates were centrifuged at 720 × g for 5 min and the pellet was washed once by adding 500 μL of fractionation buffer. The pellet (nuclear fraction) was resuspended in nuclear buffer (RIPA buffer). The supernatant was collected and centrifuged again at 10,000 × g for 60 min. The supernatant was the cytosol/membrane fraction. The pellets (membrane fraction) were lysed by adding 100 μL of RIPA buffer. The nuclear and cytosol/membrane fractions were diluted with an equal volume of 2 × sample buffer and resolved using SDS-PAGE.

6. Transient transfection

The hDPC or MDPC-23 cells were seeded into 60 mm culture plates at a density of 1.0 × 10^6 cells per well. The cells were transiently transfected with DDK (Flag)-tagged Cpne7 using the Metafectene Pro reagent (Biontex, Martinsried, Germany) or with siRNA using the Lipofectamine RNAi MAX reagent (Invitrogen) according to the manufacturer's instructions.

7. Immunoprecipitation (IP)

Cells were transfected with the indicated constructs for 48 h and harvested in IP buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 10% glycerol supplemented with protease inhibitors). The lysates were incubated with the anti-Flag antibody overnight at 4 ℃. After incubation for 4 h at 4 ℃ with A/G-agarose beads (Santa Cruz Biotechnology), the beads were washed three times with IP buffer. Immune complexes were released from the beads by boiling. Following electrophoresis using 8% or 10% SDS-PAGE, the immune-precipitates were analysed by western blot analyses or proteomic analyses.

8. In-gel digestion with trypsin and the extraction of peptides

The procedures for the in-gel digestion of protein spots excised from the Coomassie Blue-stained gels were performed as previously described[59]. In brief, the protein spots were excised from the stained gel and cut into pieces. The gel pieces were washed for 1h at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50 % (v/v) acetonitrile (ACN). Following the dehydration of gel pieces in a centrifugal vacuum concentrator for 10 min, the gel pieces were rehydrated in 50 ng of sequencing grade trypsin solution (Promega, Madison, WI, USA). After incubation in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37 ℃ overnight, the tryptic peptides were extracted with 5 uL of 0.5% formic acid containing 50% (v/v) ACN for 40 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator. Prior to

mass spectrometric analyses, the peptide solution was subjected to a desalting process using a reversed-phase column[60]. In brief, after an equilibration step with 10 μL of 5% (v/v) formic acid, the peptides solution was loaded on the column and washed with 10 μL of 5% (v/v) formic acid. The bound peptides were eluted with 5 μL of 70% ACN containing 5% (v/v) formic acid.

9. Identification of proteins by LC-MS/MS

After desalting, the eluted tryptic peptides were separated and analysed using a nano ACQUITY UPLC (Waters, Milford, MA, USA) directly coupled to a Finnigan LCQ DECA iontrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). In brief, the peptides were bound to the ACQUITY UPLC peptide BEH C18 column (1.7 μm size, 130Å pore size, 100 μm × 100 mm) with distilled water containing 0.1% (v/v) formic acid and the bound peptides were eluted with a 40 min gradient of 0 – 90% (v/v) ACN gradient with 0.1% (v/v) formic acid at a flow rate of 0.4 μL/min. For tandem mass spectrometry, the full mass scan range mode was m/z = 400 – 2000 Da. After determination of the charge states of an ion on zoom scans, the product ion spectra were acquired in the MS/MS mode with relative collision energy of 55%. The individual spectra from MS/MS were processed using SEQUEST software (Thermo Quest, San Jose, CA, USA) and the generated peak lists were used to query the NCBI database using the MASCOT program (Matrix Science., London, UK). I set the modifications of methionine and cysteine for MS analyses. The tolerance of the peptide mass was 2 Da. The MS/MS

ion mass tolerance was 1 Da, allowance of missed cleavage was 1, and the charge states (+1, +2, and +3) were taken into account for data analyses. I considered only significant hits as defined by MASCOT probability analyses.

10. Immunofluorescence staining

Cells in Laboratory-Tek chamber slides (Nunc, Rochester, NY, USA) were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized in PBS containing 0.5% Triton X-100. After washing and blocking, cells were incubated for 1 h with primary (1:200) antibodies in blocking buffer (PBS and 1% bovine serum albumin), followed by the addition of anti-FITC or Cy3-conjugated anti-mouse or rabbit IgG antibodies (1:200; Life Technologies). After washing, cells were visualized using fluorescence microscopy (AX70, Olympus, Tokyo, Japan). The chromosomal DNA in the nucleus was stained using DAPI.

11. Statistical analysis

All data were expressed as the mean ± standard deviation from at least three independent experiments. Statistical significance was analysed using one-way ANOVA by the SPSS software version 19.

12. Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

IV. RESULTS

1. Endocytosed Cpne7 is localized in the cytoplasm and nucleus

The cellular localization of endogenous Cpne7 was evaluated in odontoblastic MDPC-23 cells. Cpne7 was localized in the cytoplasm and nucleus in odontoblastic cells (Fig. 5A, B). Nuclear-localized Cpne7 was especially increased during odontoblast differentiation (Fig. 5B). To further validate the internalization process, MDPC-23 cells were treated for 2, 5, 15 and 30 min with recombinant Flag-Cpne7 protein (rCpne7). Analyses by confocal microscopy showed that Cpne7 was endocytosed and transported to the perinuclear region by 30 min (Fig. 5Ce). However, the secondary antibody treated-group, which served as the control, was not internalized (Fig. 5Ca).

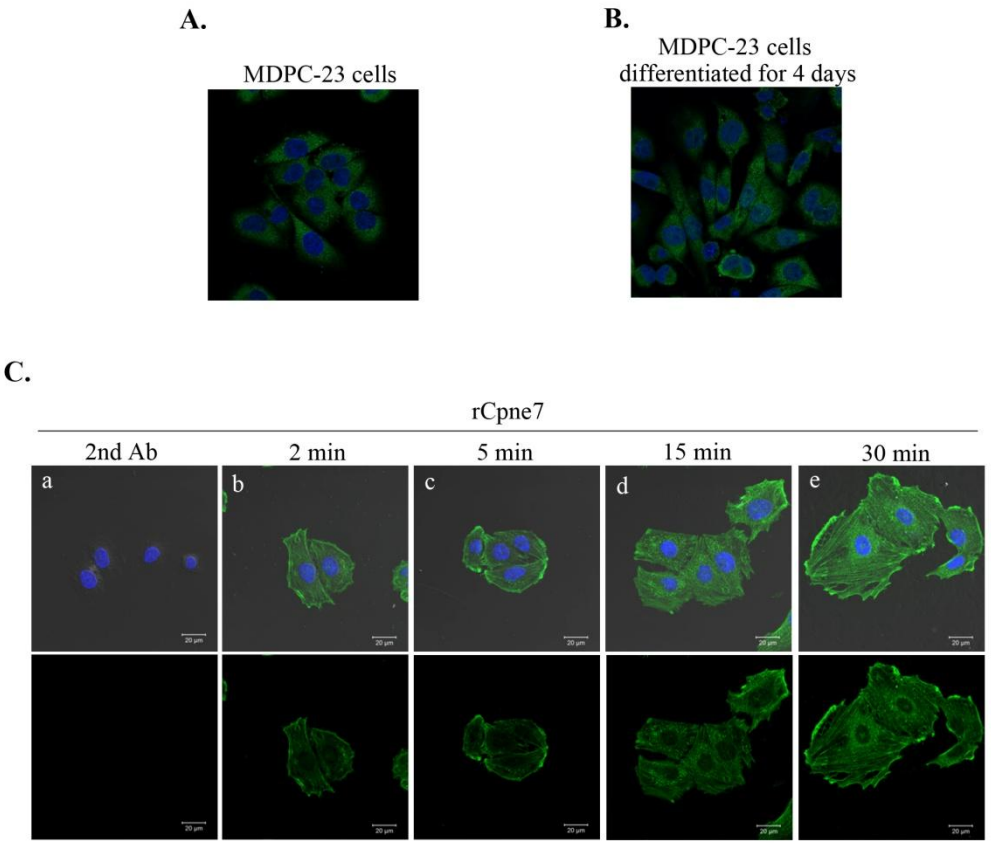

Figure 5. Intracellular distribution of Cpne7 in odontoblastic MDPC-23 cells

(A, B) Cellular localization of Cpne7 was detected by immunofluorescence before (A) or after odontoblastic differentiation (B) of MDPC-23 cells. (C) The time course confocal images of internalized Cpne7 were detected using anti-Flag antibody in MDPC-23 cells after rCpne7 treatment for 2, 5, 15, and 30 min. Each data are representative of two or three independently performed experiments. Scale bars, 20 μm

2. Cellular uptake of Cpne7 occurs through caveolae-dependent receptor-mediated endocytosis

Receptor-mediated endocytosis is a process by which cells absorb metabolites, hormones, other proteins, and, in some cases, viruses by inward budding of plasma membrane vesicles containing proteins with receptor sites specific to the molecules being absorbed. To elucidate the characteristics of the endocytic pathway of Cpne7, I tested the effect of drugs that inhibit clathrin or caveolae-mediated endocytosis. Use of chlorpromazine and 0.45M sucrose, which inhibits clathrin-mediated endocytosis, did not have any effect (Fig. 6A). Pre-treatment of the cells with inhibitors of lipid raft/caveolae-dependent endocytosis, methyl-β-cyclodextrin or nystatin, significantly inhibited cellular uptake of Cpne7 (Fig. 6B, C). Possible drug-induced cytotoxic effects were assessed by MTT cell viability assays (Fig. 6B, C). These results indicated that Cpne7 uptake into MDPC-23 cells could be mediated via the lipid raft/caveolae pathway.

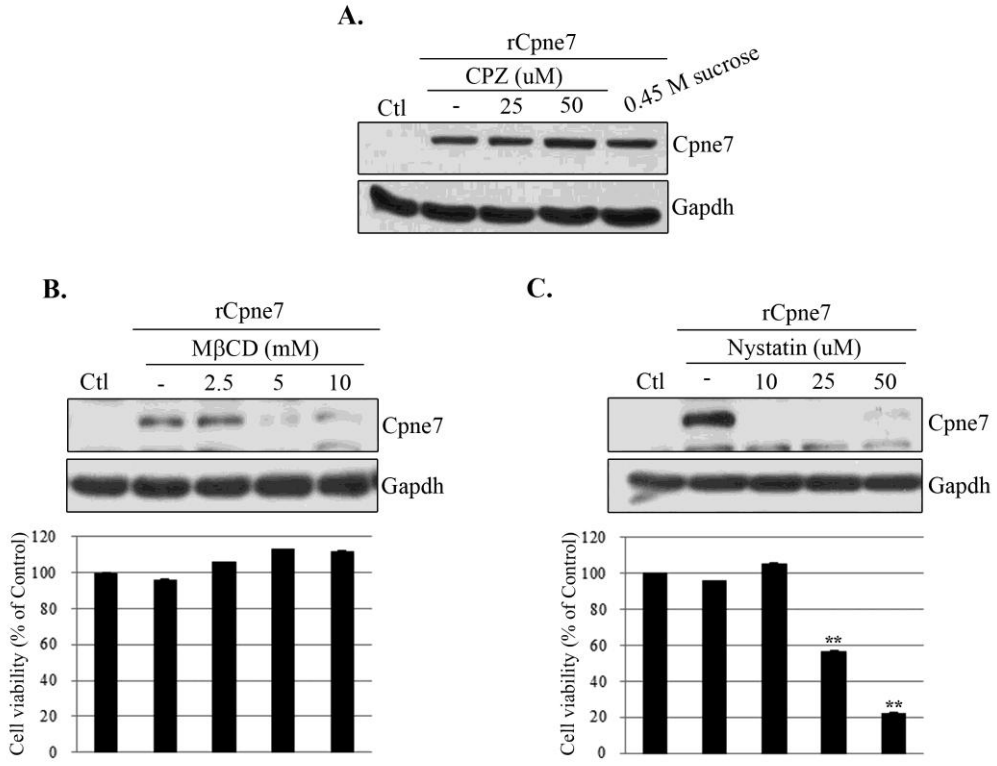

Figure 6. Effects of receptor-mediated endocytic inhibitors on Cpne7 endocytosis in odontoblastic MDPC-23 cells.

MDPC-23 cells were pre-treated with varying concentrations of chlorpromazine (CPZ ; 25 and 50 μM) (A), 0.45M sucrose (A), methyl-beta-cyclodextrin (MβCD ; 2.5, 5, and 10 mM) (B) and nystatin (10, 25, and 50 μM) (C) for 1 h before rCpne7 treatment. Internalized Cpne7 was detected by western blotting. The effects of drug treatment on cell viability were assessed using the MTT assay. All values represent the mean ± standard deviation of triplicate experiments. **P < 0.001 compared with the control.

3. Nucleolin is a cell surface receptor of Cpne7

Previous results indicated that Cpne7 underwent receptor-mediated endocytosis. To identify the receptor of Cpne7 in preodontoblasts, total lysates were immunoprecipitated with Flag antibody in Flag-Cpne7 transfected-MDPC-23 cells, and then resolved by SDS-PAGE. The individual compartments were excised from the gel, and the proteins were analysed by tandem mass spectrometry (LC-MS/MS; Fig. 7A). Most of the proteins were cytoskeletal components implicated in the maintenance and the motility of microvilli. Only two proteins (nucleolin and Slitrk1) of the Cpne7-interacting proteins were localized to the cellular membrane. Surface nucleolin, has recently attracted increasing attention as an important cell receptor for numerous ligands derived from various sources[61]. Nucleolin also mediates internalization of endostatin[62], LPS[63], DNA nanoparticles[64], and lactoferrin[65]. To confirm that Cpne7 binds specifically to nucleolin, I performed immunoprecipitation using anti-Flag antibody. Nucleolin interacted with Cpne7 in the membrane/cytosol and nuclear fractions (Fig. 7C) and was also expressed in the membrane/cytosol and nuclear fractions (Fig. 7B).

To confirm that nucleolin is a receptor that mediates endocytosis of Cpne7, I treated cells with nucleolin siRNA before rCpne7 treatment and analysed Cpne7 internalization by western blotting and immunostaining. Fig. 7D and E showed that Cpne7 internalization was abrogated with nucleolin siRNA. These results confirmed that nucleolin mediated Cpne7 endocytosis into preodontoblasts.

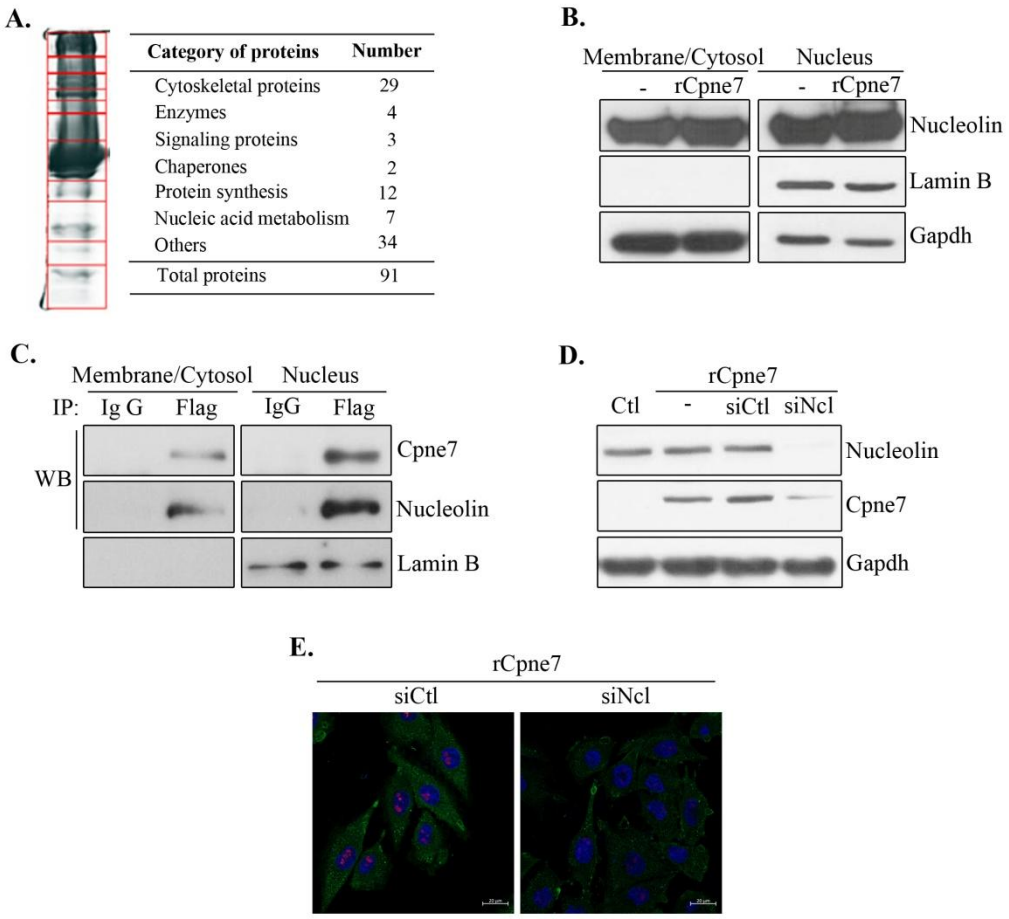

Figure 7. Identification of a receptor, nucleolin which mediates Cpne7 endocytosis.

(A) Total lysates obtained from Flag-Cpne7-overexpressed MDPC-23 cells were immunoprecipitated with anti-Flag antibody. The samples were subjected to SDS-PAGE followed by Coomassie blue staining. Eleven compartments were excised from the gel and analysed by tendem mass spectrometry. (B) The cell lysates were separated into membrane/cytoplasmic and nuclear fractions and then analysed for nucleolin proteins by

western blotting. (C) The interaction with Cpne7 and nucleolin was analysed by co-immunoprecipitation. Lamin B served as nucleus fractionation control. (D, E) Endocytosis of Cpne7 in MDPC-23 cells after treatment with nucleolin siRNA. MDPC-23 cells were pre-treated with nucleolin siRNA for 1 h before Cpne7 protein (rCpne7) treatment. Internalized Cpne7 was detected by western blotting (D) or immunostaining (E). Each data are representative of two or three independently performed experiments. siCtl, control siRNA; siNcl, nucleolin siRNA; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

4. Cpne7 endocytosis is dependent on calcium.

In many cells, endocytosis is Ca^{2+}-dependent[66]. The molecular machinery mediating Ca^{2+}-dependent endocytosis was best characterized in the context of synaptic vesicle recycling[67, 68]. The Ca^{2+} influx through Ca^{2+} channels triggering endocytosis is also well established in non-excitable cells, such as oocytes[69]. I therefore examined whether Ca^{2+} entry was responsible for Cpne7 endocytosis. Treatment with $CaCl_2$ did not alter the amount of Cpne7 endocytosis (Fig. 8A), and Ca^{2+} did not affect Cpne7 endocytosis because the MDPC-23 cell culture medium, DMEM, contained 1.8 mM $CaCl_2$. However, EGTA, Ca^{2+} chelator, prevented Cpne7 endocytosis (Fig. 8B), suggesting that Cpne7 endocytosis was dependent on Ca^{2+}.

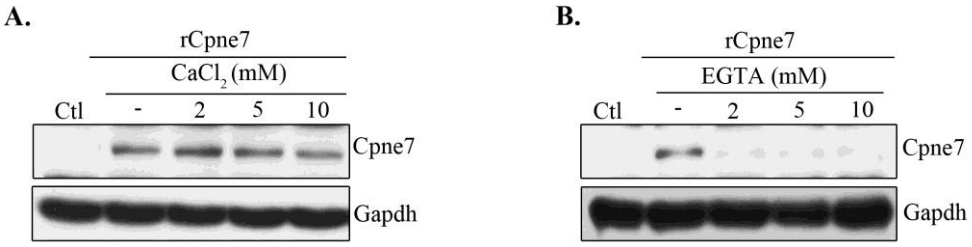

Figure 8. Calcium dependence of Cpne7 endocytosis.

Endocytosis of Cpne7 in MDPC-23 cells after treatment with $CaCl_2$ (A) or EGTA (B). MDPC-23 cells were pre-treated with varying concentrations of $CaCl_2$ or EGTA for 1 h before rCpne7 treatment. The internalized Cpne7 was detected by western blotting. Each data are representative of three independently performed experiments. Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

5. Cpne7 regulates ciliogenesis during odontoblast differentiation

Primary cilia play an essential role not only in the initiation of both osteogenic and adipogenic differentiation, but also in the maintenance of the phenotype of differentiated cells[70]. To assess whether the presence of primary cilium components had any physiological relevance in odontoblast differentiation, I examined the number of primary cilia and the expression of cilium components during odontoblast differentiation using immunofluorescence microscopy and real-time PCR. The basal body and axoneme of primary cilium can be stained with γ-tubulin and acetylated α-tubulin antibody, respectively. I detected the existence of primary cilia with anti-acetylated α-tubulin antibody. The number of ciliated cells and the cilium length gradually increased following odontoblast differentiation (Fig. 9A-C). In addition, the mRNA of Cpne7 and cilium components, Ift88 and Kif3a, respectively, increased progressively during hDPC differentiation (Fig. 9D). To examine whether Cpne7 regulated ciliogenesis during odontogenesis, I performed immunofluorescence microscopy of cilium components in hDPCs after transfection with a Cpne7 construct. Cpne7 increased the number of ciliated cells (Fig. 10A, B). To confirm this observation, I also investigated the mRNA and protein levels of cilium components after Cpne7 overexpression or shRNA treatment. As expected, the expression level of the cilium components, Ift88 and Kif3a, was increased after Cpne7 treatment and was inhibited by Cpne7 shRNA treatment in hDPCs (Fig. 10C, D) and MDPC23 cells (Fig. 11A, B). Taken together, these results suggested that Cpne7 affected ciliogenesis during odontoblast differentiation.

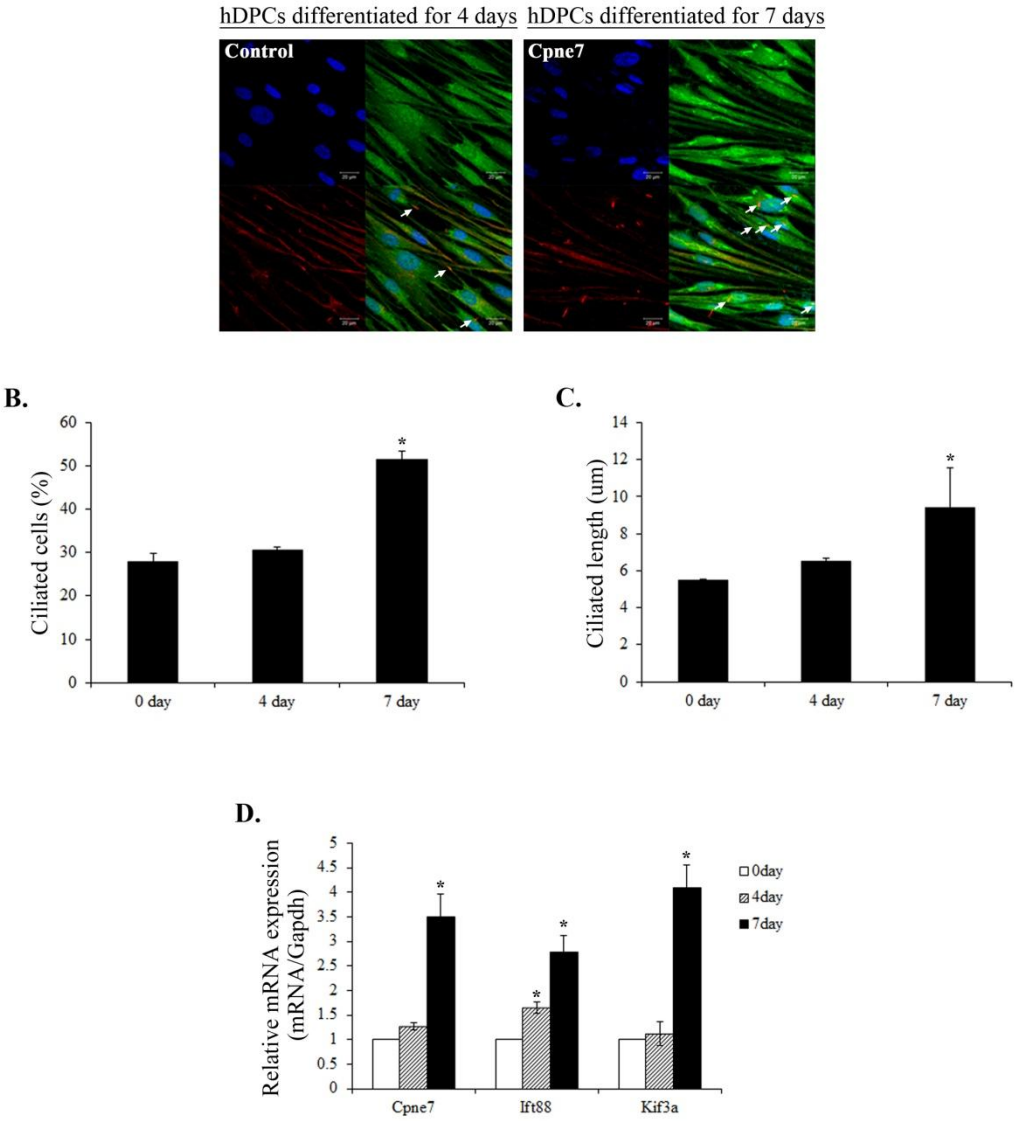
hDPCs differentiated for 4 days
hDPCs differentiated for 7 days
Control
Cpne7
B.
Ciliated cells (%)
0 day
4 day
7 day
C.
Ciliated length (um)
0 day
4 day
7 day
D.
Relative mRNA expression (mRNA/Gapdh)
0day
4day
7day
Cpne7
Ift88
Kif3a

Figure 9. Expression of Cpne7, primary cilia and cilium components during the differentiation of hDPCs.

(A) Primary cilia were detected with acetylated α-tubulin (red), Cpne7 (green) immunostaining, and counterstained with DAPI following 7 and 14 days of differentiation in defined odontogenic inductive medium. Arrows indicate α-tubulin immunostained primary cilia. (B) Counterstaining with DAPI was used to calculate the percentage of primary cilia in the hDPCs. (C) Confocal images of cilia were randomly captured and cilium length was analyzed using Olympus software. (D) Expression of Cpne7 and cilium components, Ift88 and Kif3a in hDPCs during differentiation was assessed using reverse transcription-quantitative polymerase chain reaction analysis. The data are presented as the mean ± standard deviation of three independently performed experiments. *$P < 0.05$ compared with day 0. Scale bars, 20 μm

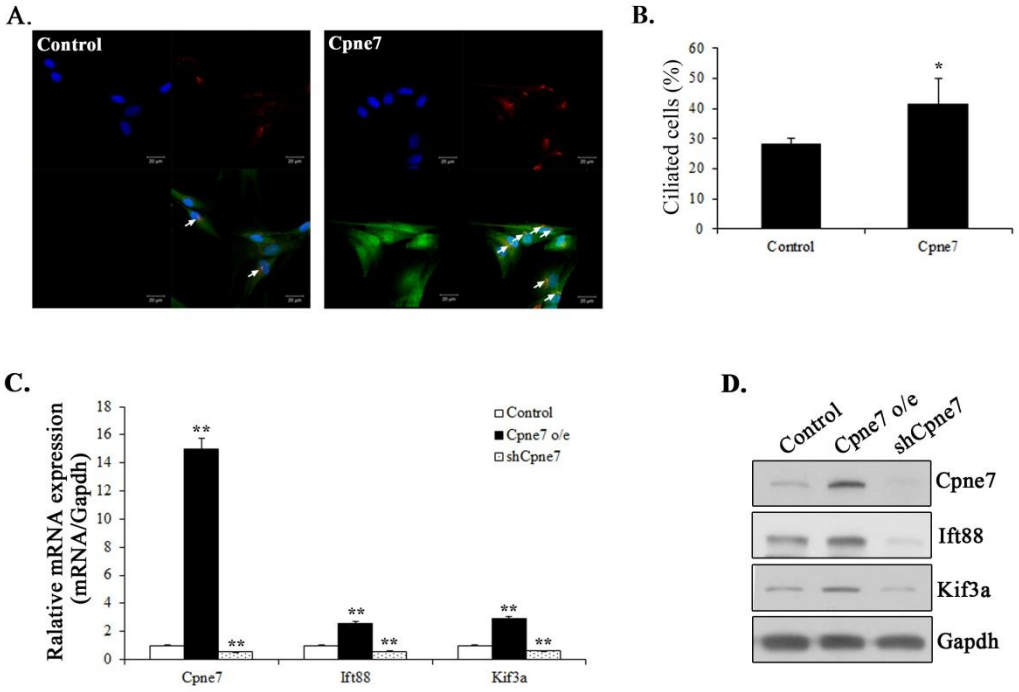

Figure 10. Effect of Cpne7 on ciliogenesis in hDPCs.

(A) Human DPCs were immunostained with acetylated α-tubulin antibody (red) in the presence or absence of Cpne7 (green) and the presence of cilia was analysed by confocal microscopy. (B) The percentage of cells with cilia was counted for the indicated group. (C, D) The expression levels of cilium components by Cpne7 were examined by quantitative real-time polymerase chain reaction (C) and western blotting (D) after Cpne7 overexpression or shRNA treatment for 2 days. All values represent the mean ± standard deviation of three independently performed experiments. **P < 0.001, *P < 0.05 compared with control. Scale bars, 20 μm

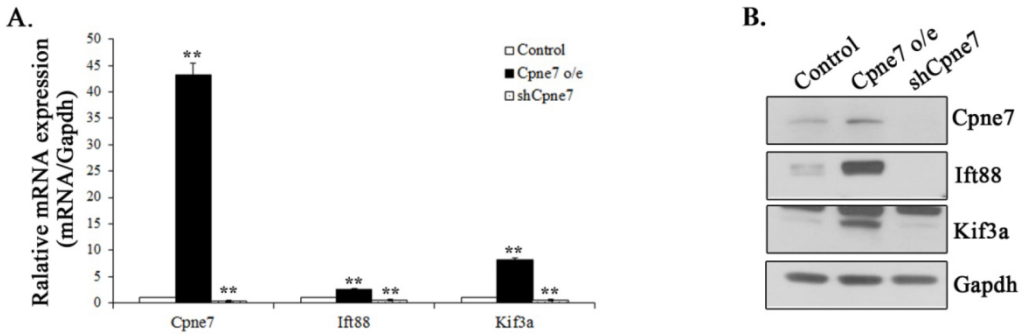

Figure 11. Regulation of the expression of cilium components by Cpne7 in MDPC-23 cells

(A, B) The expression levels of cilium components by Cpne7 were examined by quantitative real-time polymerase chain reaction (A) and western blotting (B) after Cpne7 overexpression or shRNA treatment for 2 days. All values represent the mean ± standard deviation of three independently performed experiments. **P < 0.001 compared with the control.

6. Cpne7 regulates Dspp expression via ciliogenesis in odontoblasts

In the previous study, Cpne7 controlled Dspp expression during odontoblast differentiation[42]. The results of Fig. 6 show that Cpne7 regulated ciliogenesis during odontoblast differentiation. To evaluate whether primary cilia and cilium components mediated the regulation of Dspp expression by Cpne7, I investigated the expression of Dspp after treatment of rCpne7 in the presence or absence of Ift88 siRNA in hDPCs and MDPC-23 cells using real-time PCR and western blot analyses. The presence of rCpne7 increased the transcription of Ift88 and Dspp, whereas hDPCs and MDPC-23 cells transfected with Ift88 siRNA had inhibited Dspp expression (Fig. 12A, B, Fig. 13A, B). Together, these results suggested that Cpne7 secreted from ameloblasts affected ciliogenesis and odontogenesis by controlling the expression of cilium components and Dspp.

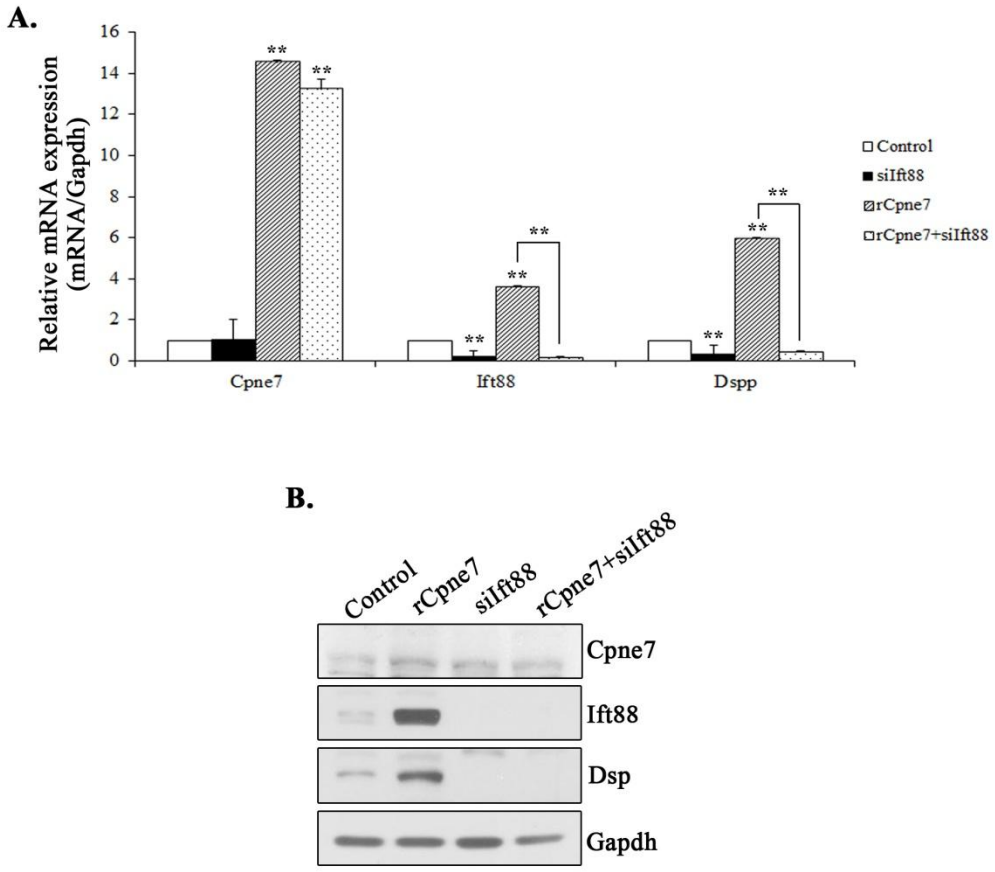

Figure 12. Effect of Ift88 knockdown on Dspp expression in hDPCs.

(A, B) Expression levels of Dspp were examined by the quantitative real-time polymerase chain reaction and western blotting after Ift88 siRNA treatment for 2 days. All values represent the mean ± standard deviation of three independently performed experiments. **P < 0.001 compared with the control.

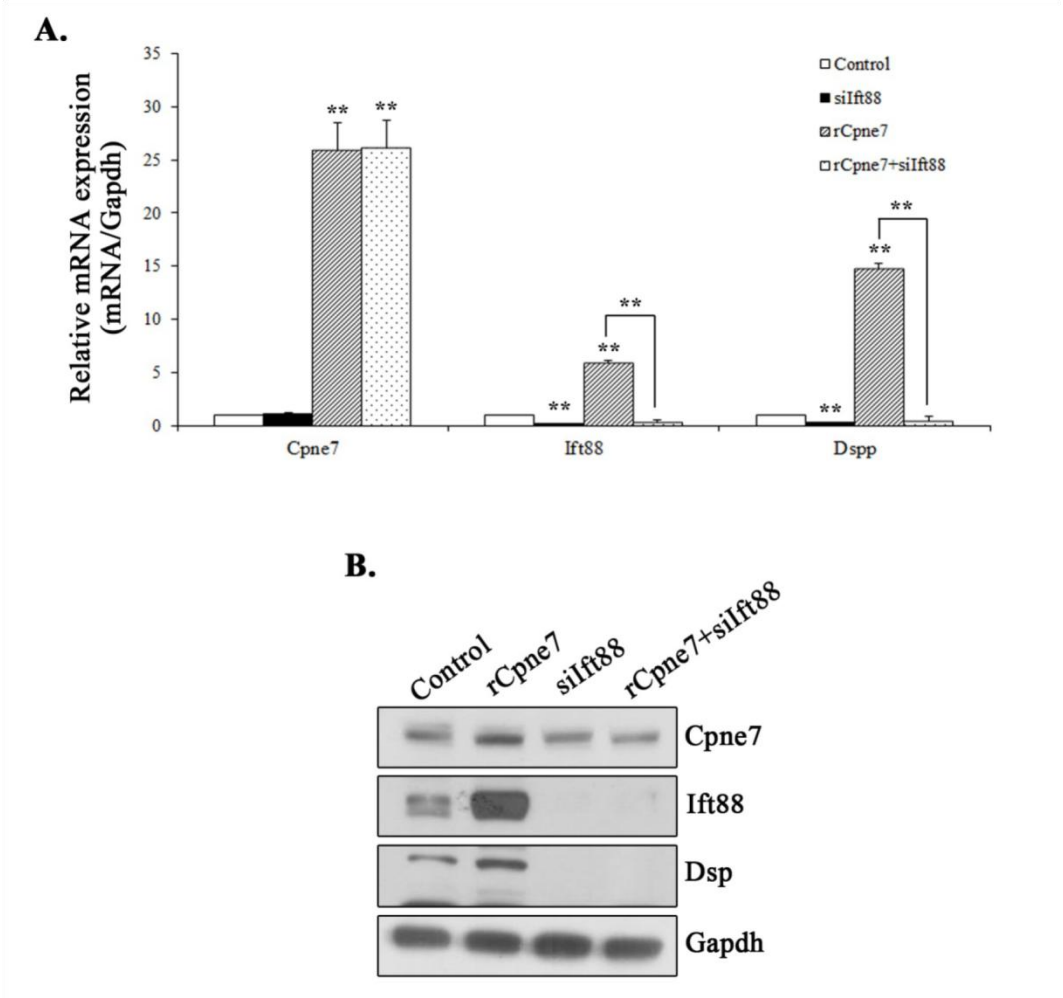

Figure 13. Effect of Ift88 knockdown on Dspp expression in MDPC-23 cells.

(A, B) Expression levels of Dspp were examined by the quantitative real-time polymerase chain reaction and western blotting after Ift88 siRNA treatment for 2 days. All values represent the mean ± standard deviation of three independently performed experiments. **P < 0.001 compared with the control.

7. Expression of ciliary components and Dspp is induced by binding of Cpne7 and its membrane receptor, nucleolin

Next, I verified whether Cpne7 influenced the expression of cilium components and Dspp through the binding of nucleolin. MDPC-23 cells were treated with rCpne7 in the presence or absence of nucleolin siRNA. Figure 14A, B and C show that the increase of Dmp1, Dspp, and cilium components, Ift88 and Kif3a, by rCpne7 was dramatically inhibited by nucleolin down-regulation by siRNA. These data indicated that the Cpne7-nucleolin complex stimulated Dspp expression through the regulation of expression of cilium components.

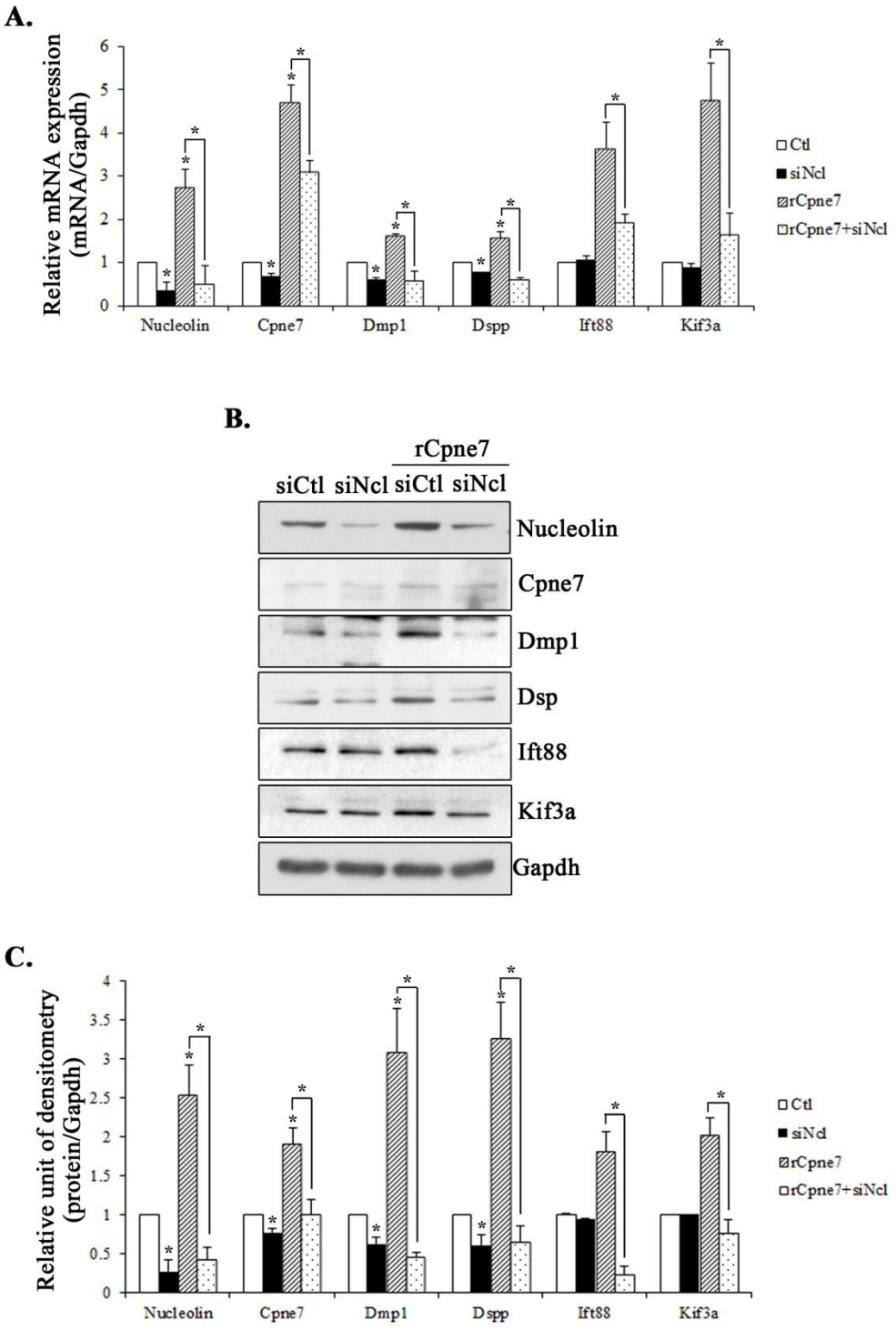
A.
Relative mRNA expression (mRNA/Gapdh)
Nucleolin
Cpne7
Dmp1
Dspp
Ift88
Kif3a
Ctl
siNcl
rCpne7
rCpne7+siNcl
B.
rCpne7
siCtl siNcl siCtl siNcl
Nucleolin
Cpne7
Dmp1
Dsp
Ift88
Kif3a
Gapdh
C.
Relative unit of densitometry (protein/Gapdh)
Nucleolin
Cpne7
Dmp1
Dspp
Ift88
Kif3a
Ctl
siNcl
rCpne7
rCpne7+siNcl

Figure 14. The expression of Cpne7-regulaing genes (Dmp1 and Dspp) and cilium components after nucleolin siRNA treatment.

The levels of nucleolin, dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (Dspp) in nucleolin siRNA-treated MDPC-23 cells were evaluated by quantitative real-time polymerase chain reaction (A) and western blotting (B) after rCpne7 treatment for 2 days. (C) Densitometry analysis of proteins on western blotting are presented according to the formula: (average densitometry assessment protein/average densitometry assessment Gapdh). All values represent the mean ± standard deviation of three independently performed experiments. *$P < 0.05$ compared with the control. siCtl, control siRNA; siNcl, nucleolin siRNA; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

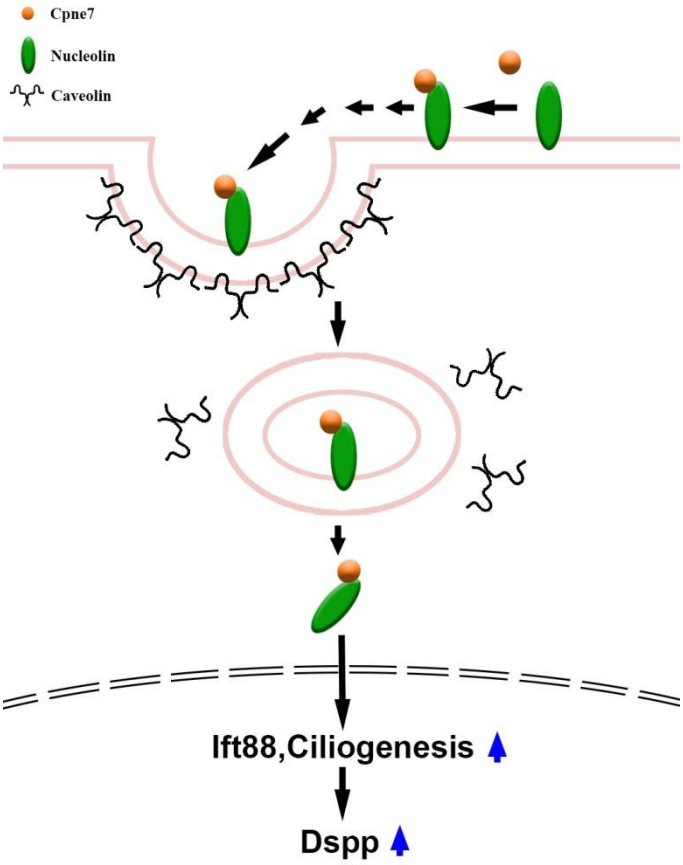

Figure 15. Proposed model for mechanism of actin of Cpne7 in odontoblast differentiation.

Cpne7 binds to cell surface nucleolin in lipid rafts, and is internalized via caveolae-mediated endocytosis. Cpne7-nucleolin complex is translocated into nucleus of a preodontoblast. Cpne7 increases the formation of primary cilia affecting the expression of Kif3a and Ift88, cilium components. Ift88 promotes up-regulation of Dspp expression.

V. DISCUSSION

Epithelial-mesenchymal interactions are typical of odontogenesis[71]. It has been suggested that Cpne7 is an epithelial factor that was secreted by preameloblasts, and regulated the differentiation of mesenchymal cells of dental or non-dental origin into odontoblasts[42]. The distribution of the Cpne7 protein in dental cells suggested an important role in epithelial-mesenchymal interactions[42]. Epithelial-mesenchymal interactions during tooth development involve the following steps, 1) interactions involving signalling molecules, 2) transmission of information to adjacent cells, 3) binding of molecules to target cell receptors, 4) activation of a cell cascade in the cytoplasm, 5) entrance into the nucleus to regulate gene expression, 6) expression of new proteins, and 7) a change in the behaviour of target cells. In the present study, I showed that Cpne7 was involved in epithelial-mesenchymal interactions during early odontoblast differentiation.

Nucleolin, with a molecular weight of 105–110 kDa, initially called C23, was originally one of 100 distinct proteins: It comprises 5–10 % of the total nucleolar protein in normal rat liver and Novikoff hepatoma ascites cells[72, 73]. Nucleolin was previously thought to be a simple RNA-binding protein involved in the organization of nucleolar chromatin, packaging of the pre-RNA, rDNA transcription and ribosome assembly by shuttling between the nucleus and cytoplasm. Recent studies have further reported that nucleolin is involved in modulating transcriptional processes, cytokinesis, nucleogenesis, signal transduction, apoptosis, induction of chromatin decondensation, and replication[74]. Although ≥ 90 % of the nucleolin is found in the nucleolus, the distribution of nucleolin

within the cell is also found in nucleus, cytoplasm, and cell-surface[75]. Cell surface nucleolin is a receptor for various ligands such as midkine, pleiotrophin, P-selectin, ErbB and hepatocyte growth factor (HGF)[61]. More importantly, nucleolin functions as a receptor for internalization of DNPs via lipid rafts[64], as a receptor for endostatin[62] and as a receptor for lactoferrin[65]. In the present study, internalization of Cpne7 by preodontoblasts occurred through endocytosis. This endocytic process was mediated by binding of Cpne7 to nucleolin on the cell surface of preodontoblasts. Nucleolin down-regulation by siRNA decreased the endocytosis of Cpne7 in MDPC-23 cells. Together, these results suggested that nucleolin functions as an important receptor for Cpne7.

Nucleolin is also involved in the movement to the nucleus of its ligand as an endocytic receptor. Cines et al reported that nucleolin, interacting with the uPA/uPAR complex, regulated the nuclear translocation of scuPA[76]. Song et al also reported that after the translocation of endostatin by nucleolin from the cell membrane to the early endosome, importin $\alpha 1\beta 1$ recognized the nuclear localization signal of nucleolin, facilitating further transports of the endostatin/nucleolin complex to the nucleus[77]. In the present study, following internalization, Cpne7 was transported to the perinuclear region. Cpne7 in differentiating MDPC-23 cells and hDPCs was present not only in the cytoplasm, but also in the nucleus. The interaction between nucleolin and internalized Cpne7 was detected both in the membrane/cytoplasm and nucleus. Thus, in addition to the internalization and cellular trafficking of Cpne7, nucleolin may also play an important role in nuclear import.

Caveolae-mediated endocytosis is essential for many important signalling pathways and

the location of many receptors such as TGFR and the hedgehog receptor[78]. Generally, cell surface caveolae have limited motility and dynamics. However, when triggered by the appropriate ligand, they can be internalized[79]. Acidic non-collagenous protein, Dmp1, was internalized by caveolae in preodontoblasts and hDPCs during tooth development[80]. Notably, Cpne6 associated with clathrin-coated vesicles in a calcium-dependent manner[47], and Cpne7 was endocytosed via a caveolae-dependent endocytic pathway when Cpne7 was bound to the cell surface.

Cpne 1, 2, 3, 6, and 7 exhibited calcium concentration-dependent translocation to the plasma membrane, and Cpne1, 2, 3, and 7 were also translocated to the nucleus[47]. Cpne7 was localized to the cytoplasm and nucleus of MDPC-23 cells and hDPCs. Moreover, expression of Cpne7 increased during differentiation, especially in the nucleus of differentiating odontoblasts. Previous study suggested that the Cpne7-nucleolin complex directly regulated transcriptional activation of the Dspp promoter and that it was responsible for Cpne7-mediated Dspp expression during odontoblast differentiation and mineralization[42]. Taken together, these findings suggested that Cpne7 serves as a transcription cofactor/factor during odontoblast differentiation.

Primary cilia, a single and immotile cilia extending from the centriole, were necessary for chemically induced differentiation of human mesenchymal stem cells[70]. During tooth development, the disruption of the function of primary cilia caused dental anomalies, as characterized by missing/supernumerary teeth and dental hypoplasia[81, 82]. Furthermore, primary cilia were required for normal tooth development by integrating

Hedgehog and Wnt signalling between the dental epithelium and mesenchyme[83]. These results suggested that primary cilia play important roles in epithelial-mesenchymal interactions. Primary cilia have specialized functions as fundamental structures for mechanical and chemical sensing by individual cells, that transfer signals to the nucleus and cell organelles to induce adequate cellular responses[84]. Odontoblasts are subjected to external stimuli involving the primary cilia as a mediator of mechano-transduction processes concomitantly with mechano-sensitive ion channels[85, 86]. Odontoblasts express cilia components such as tubulin, inversin, rootletin, Ofd1, Bbs4, Bbs6, Alsm1, Kif3a, PC1 and PC2 and cilia are aligned parallel to the dentin walls, with the top part oriented toward the pulp core[53]. Studies using human and mouse dental pulps have reported that primary cilia influenced the terminal differentiation of odontoblasts, and may be functionally connected with tooth pain transmission[53]. Jiang et al reported that inhibition of Kif3a resulted in hDPC disrupted primary cilia formation and/or function, and suggested that Kif3a was an important in the osteoblastic differentiation of hDPCs[87]. In the current study, the overexpression of Cpne7 resulted in increased formation of primary cilia in hDPCs that affected the expression of the Kif3a and Ift88 ciliary components. Furthermore, the odontogenic gene, Dspp, was significantly down-regulated following Ift88 knockdown in hDPCs and MDPC-23 cells. I therefore suggest that Cpne7 affects differentiation of odontoblast by regulating Dspp expression through ciliogenesis.

In the previous study, the expression of odontoblast differentiation markers, including Dspp, nestin, and Alp was enhanced by Cpne7 [57]. Specifically, It has been observed

that Cpne7 promoted mineralized nodule formation *in vitro*[42]. Cpne7 induced the differentiation of non-dental mesenchymal stem cells into odontoblasts and resulted in the formation of dentin-like structures including dentinal tubules *in vivo*[42]. In this study, the results strongly suggested a mechanism in which Cpne7, an epithelial factor, is internalized into preodontoblasts and transported to the nucleus, which implied possible strategies to regulate odontoblast differentiation (Fig. 15). Cpne7 acted as a ligand and bound to its receptor, nucleolin. The Cpne7-nucleolin complex was then translocated to the nucleus of preodontoblasts. Taken together, I propose that the existence of a Cpne7-nucleolin complex-primary cilia-Dspp pathway during early odontoblast differentiation.

CHAPTER VI. CONCLUDING REMARKS

In the study, 1) the roles of Cpne7 on expression of *Dspp* during early amelogenesis and 2) the mechanisms involved in the translocation of Cpne7 from preameloblasts to preodontoblasts and 3) the functions of Cpne7 in regulation of ciliogenesis and *Dspp* expression during odontogenesis were investigated.

During early amelogenesis, the expression pattern of *Dspp* was similar to that of Cpne7 *in vitro* and *in vivo*. Moreover, *Cpne7* overexpression promotes *Dspp* expression, whereas *Dspp* expression is downregulated by *Cpne7* inactivation in ameloblastic HAT-7 cells. Mechansim of such regulation is confirmed by findings that Cpne7 binds to the *Dspp* promoter region and regulates its transcription. Taken together, these findings suggest that *Dspp* is synthesized in dental epithelial cells by the control of Cpne7 and its transient expression occurs in early ameloblasts.

Next, I focused on the mechanism involved in the translocation of Cpne7 from preameloblast to preodontoblasts. The internalization of Cpne7 by preodontoblasts occurred through endocytosis. This endocytic process was mediated by binding of Cpne7 to nucleolin on the cell surface of preodontoblasts. Nucleolin down-regulation by siRNA decreased the endocytosis of Cpne7 in MDPC-23 cells. Together, these results suggested that nucleolin functions as an important receptor for Cpne7.

Primary cilia were detected in preodontoblasts and hDPCs and the number of ciliated cells and the cilium length gradually increased following odontoblast differentiation.

Furthermore, the mRNA and protein levels of cilium components were increased after Cpne7 treatment and were inhibited by Cpne7 shRNA treatment in hDPCs and MDPC23 cells. Put together, these results suggest that Cpne7 affected ciliogenesis during odontoblast differentiation.

In this work, the results strongly suggested a mechanism in which Cpne7, an epithelial factor, is internalized into preodontoblasts and transported to the nucleus, which implied possible strategies to regulate odontoblast differentiation. Cpne7 acted as a ligand and bound to its receptor, nucleolin. The Cpne7-nucleolin complex was then translocated to the nucleus of preodontoblasts. Taken together, I propose that the existence of a Cpne7-nucleolin complex-primary cilia-Dspp pathway during early odontoblast differentiation.

Collectively, these studies provide the new insights on the roles and underlying mechanisms of Cpne7 as an epithelial factor on *Dspp* expression during early amelogenesis and ciliogenesis of mesenchymal stem cells.

REFERENCES

1. Santosh, A.B. and T.J. Jones, *The epithelial-mesenchymal interactions: insights into physiological and pathological aspects of oral tissues.* Oncol Rev, 2014. **8**(1): p. 239.
2. Thesleff, I., *Epithelial-mesenchymal signalling regulating tooth morphogenesis.* J Cell Sci, 2003. **116**(Pt 9): p. 1647-8.
3. Zhang, Y.D., et al., *Making a tooth: growth factors, transcription factors, and stem cells.* Cell Res, 2005. **15**(5): p. 301-16.
4. Lee, J.H., et al., *Odontogenic differentiation of human dental pulp stem cells induced by preameloblast-derived factors.* Biomaterials, 2011. **32**(36): p. 9696-706.
5. Oh, H.J., et al., *CPNE7, a preameloblast-derived factor, regulates odontoblastic differentiation of mesenchymal stem cells.* Biomaterials, 2015. **37**: p. 208-17.
6. Nalefski, E.A. and J.J. Falke, *The C2 domain calcium-binding motif: structural and functional diversity.* Protein Sci, 1996. **5**(12): p. 2375-90.
7. Rizo, J. and T.C. Sudhof, *C2-domains, structure and function of a universal Ca2+-binding domain.* J Biol Chem, 1998. **273**(26): p. 15879-82.
8. Tomsig, J.L. and C.E. Creutz, *Copines: a ubiquitous family of Ca(2+)-dependent phospholipid-binding proteins.* Cell Mol Life Sci, 2002. **59**(9): p. 1467-77.
9. Butler, W.T., *Dentin matrix proteins and dentinogenesis.* Connect Tissue Res, 1995. **33**(1-3): p. 59-65.

10. Robinson, C., et al., *The developing enamel matrix: nature and function.* Eur J Oral Sci, 1998. **106 Suppl 1**: p. 282-91.

11. Qin, C., et al., *The expression of dentin sialophosphoprotein gene in bone.* J Dent Res, 2002. **81**(6): p. 392-4.

12. Baba, O., et al., *Detection of dentin sialoprotein in rat periodontium.* Eur J Oral Sci, 2004. **112**(2): p. 163-70.

13. Ogbureke, K.U. and L.W. Fisher, *SIBLING expression patterns in duct epithelia reflect the degree of metabolic activity.* J Histochem Cytochem, 2007. **55**(4): p. 403-9.

14. Begue-Kirn, C., et al., *Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation.* Eur J Oral Sci, 1998. **106**(5): p. 963-70.

15. MacDougall, M., et al., *Developmental regulation of dentin sialophosphoprotein during ameloblast differentiation: a potential enamel matrix nucleator.* Connect Tissue Res, 1998. **39**(1-3): p. 25-37; discussion 63-7.

16. Ritchie, H.H., et al., *Dentin sialoprotein (DSP) transcripts: developmentally-sustained expression in odontoblasts and transient expression in pre-ameloblasts.* Eur J Oral Sci, 1997. **105**(5 Pt 1): p. 405-13.

17. Satir, P., L.B. Pedersen, and S.T. Christensen, *The primary cilium at a glance.* J Cell Sci, 2010. **123**(Pt 4): p. 499-503.

18. Wheatley, D.N., A.M. Wang, and G.E. Strugnell, *Expression of primary cilia in mammalian cells.* Cell Biol Int, 1996. **20**(1): p. 73-81.

19. Goetz, S.C. and K.V. Anderson, *The primary cilium: a signalling centre during vertebrate development.* Nat Rev Genet, 2010. **11**(5): p. 331-44.

20. Veland, I.R., et al., *Primary cilia and signaling pathways in mammalian development, health and disease.* Nephron Physiol, 2009. **111**(3): p. p39-53.

21. Hisamoto, M., et al., *Developmental changes in primary cilia in the mouse tooth germ and oral cavity.* Biomed Res, 2016. **37**(3): p. 207-14.

22. Prattichizzo, C., et al., *Mutational spectrum of the oral-facial-digital type I syndrome: a study on a large collection of patients.* Hum Mutat, 2008. **29**(10): p. 1237-46.

23. Thesleff, I. and K. Hurmerinta, *Tissue interactions in tooth development.* Differentiation, 1981. **18**(2): p. 75-88.

24. Prasad, M., et al., *Expression of dentin sialophosphoprotein in non-mineralized tissues.* J Histochem Cytochem, 2011. **59**(11): p. 1009-21.

25. Bronckers, A.L., et al., *Dentin sialoprotein: biosynthesis and developmental appearance in rat tooth germs in comparison with amelogenins, osteocalcin and collagen type-I.* Cell Tissue Res, 1993. **272**(2): p. 237-47.

26. Verdelis, K., et al., *Accelerated enamel mineralization in Dspp mutant mice.* Matrix Biol, 2016. **52-54**: p. 246-59.

27. Lee, H.K., et al., *The odontogenic ameloblast-associated protein (ODAM) cooperates with RUNX2 and modulates enamel mineralization via regulation of MMP-20.* J Cell Biochem, 2010. **111**(3): p. 755-67.

28. Kawano, S., et al., *Establishment of dental epithelial cell line (HAT-7) and the*

cell differentiation dependent on Notch signaling pathway. Connect Tissue Res, 2002. **43**(2-3): p. 409-12.

29. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14863-8.
30. Schulze, A. and J. Downward, *Navigating gene expression using microarrays--a technology review.* Nat Cell Biol, 2001. **3**(8): p. E190-5.
31. Hart, P.S. and T.C. Hart, *Disorders of human dentin.* Cells Tissues Organs, 2007. **186**(1): p. 70-7.
32. Kim, J.W. and J.P. Simmer, *Hereditary dentin defects.* J Dent Res, 2007. **86**(5): p. 392-9.
33. Sreenath, T., et al., *Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III.* J Biol Chem, 2003. **278**(27): p. 24874-80.
34. Zhu, Q., et al., *Proteolytic processing of dentin sialophosphoprotein (DSPP) is essential to dentinogenesis.* J Biol Chem, 2012. **287**(36): p. 30426-35.
35. Matthiessen, M.E. and P. Romert, *Ultrastructure of the human enamel organ. II. Internal enamel epithelium, preameloblasts, and secretory ameloblasts.* Cell Tissue Res, 1980. **205**(3): p. 371-82.
36. Sawada, T., et al., *Evidence for uptake of basement membrane by differentiating ameloblasts in the rat incisor enamel organ.* J Dent Res, 1990. **69**(8): p. 1508-11.
37. D'Souza, R.N., et al., *Gene expression patterns of murine dentin matrix protein 1*

(Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. J Bone Miner Res, 1997. **12**(12): p. 2040-9.

38. Nanci, A., et al., *Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors.* J Histochem Cytochem, 1998. **46**(8): p. 911-34.

39. Nakamura, M., et al., *Translocation of enamel proteins from inner enamel epithelia to odontoblasts during mouse tooth development.* Anat Rec, 1994. **238**(3): p. 383-96.

40. Linde, A. and M. Goldberg, *Dentinogenesis.* Critical Reviews in Oral Biology & Medicine, 1993. **4**(5): p. 679-728.

41. Diekwisch, T., *The developmental biology of cementum.* International Journal of Developmental Biology, 2004. **45**(5-6): p. 695-706.

42. Oh, H.-J., et al., *CPNE7, a preameloblast-derived factor, regulates odontoblastic differentiation of mesenchymal stem cells.* Biomaterials, 2015. **37**: p. 208-217.

43. Nalefski, E.A. and J.J. Falke, *The C2 domain calcium-binding motif: structural and functional diversity.* Protein Science, 1996. **5**(12): p. 2375-2390.

44. Tomsig, J. and C. Creutz, *Copines: a ubiquitous family of Ca2+-dependent phospholipid-binding proteins.* Cellular and Molecular Life Sciences CMLS, 2002. **59**(9): p. 1467-1477.

45. Tomsig, J.L. and C.E. Creutz, *Biochemical characterization of copine: a ubiquitous Ca2+-dependent, phospholipid-binding protein.* Biochemistry, 2000. **39**(51): p. 16163-16175.

46. Cowland, J.B., et al., *Tissue expression of copines and isolation of copines I and III from the cytosol of human neutrophils.* Journal of leukocyte biology, 2003. **74**(3): p. 379-388.

47. Perestenko, P.V., et al., *Copines-1,-2,-3,-6 and-7 show different calcium-dependent intracellular membrane translocation and targeting.* FEBS journal, 2010. **277**(24): p. 5174-5189.

48. Reinhard, J.R., et al., *The calcium sensor Copine-6 regulates spine structural plasticity and learning and memory.* Nature communications, 2016. **7**.

49. Wheatley, D.N., *Primary cilia in normal and pathological tissues.* Pathobiology, 1995. **63**(4): p. 222-238.

50. Pazour, G.J. and G.B. Witman, *The vertebrate primary cilium is a sensory organelle.* Current opinion in cell biology, 2003. **15**(1): p. 105-110.

51. Praetorius, H.A. and K.R. Spring, *A physiological view of the primary cilium.* Annu. Rev. Physiol., 2005. **67**: p. 515-529.

52. Fliegauf, M., T. Benzing, and H. Omran, *When cilia go bad: cilia defects and ciliopathies.* Nature reviews Molecular cell biology, 2007. **8**(11): p. 880-893.

53. Thivichon-Prince, B., et al., *Primary cilia of odontoblasts: possible role in molar morphogenesis.* Journal of dental research, 2009. **88**(10): p. 910-915.

54. Qiu, N., et al., *Disruption of Kif3a in osteoblasts results in defective bone formation and osteopenia.* J Cell Sci, 2012. **125**(8): p. 1945-1957.

55. Lee, J.H. and J.G. Gleeson, *The role of primary cilia in neuronal function.* Neurobiology of disease, 2010. **38**(2): p. 167-172.

56. Goel, R., et al., *Characterizing the normal proteome of human ciliary body.* Clinical proteomics, 2013. **10**(1): p. 1.

57. Lee, J.-H., et al., *Odontogenic differentiation of human dental pulp stem cells induced by preameloblast-derived factors.* Biomaterials, 2011. **32**(36): p. 9696-9706.

58. Lee, H.K., et al., *The odontogenic ameloblast-associated protein (ODAM) cooperates with RUNX2 and modulates enamel mineralization via regulation of MMP-20.* Journal of cellular biochemistry, 2010. **111**(3): p. 755-767.

59. Bahk, Y.Y., et al., *Antigens secreted from Mycobacterium tuberculosis: identification by proteomics approach and test for diagnostic marker.* Proteomics, 2004. **4**(11): p. 3299-3307.

60. Gobom, J., et al., *Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry.* Journal of Mass Spectrometry, 1999. **34**(2): p. 105-116.

61. Fujiki, H., T. Watanabe, and M. Suganuma, *Cell-surface nucleolin acts as a central mediator for carcinogenic, anti-carcinogenic, and disease-related ligands.* Journal of cancer research and clinical oncology, 2014. **140**(5): p. 689-699.

62. Shi, H., et al., *Nucleolin is a receptor that mediates antiangiogenic and antitumor activity of endostatin.* Blood, 2007. **110**(8): p. 2899-2906.

63. Wang, Y., M. Mao, and J.c. Xu, *Cell-surface nucleolin is involved in lipopolysaccharide internalization and signalling in alveolar macrophages.* Cell

biology international, 2011. **35**(7): p. 677-685.

64. Chen, X., et al., *Cell surface nucleolin serves as receptor for DNA nanoparticles composed of pegylated polylysine and DNA.* Molecular Therapy, 2008. **16**(2): p. 333-342.

65. Legrand, D., et al., *Surface nucleolin participates in both the binding and endocytosis of lactoferrin in target cells.* European Journal of Biochemistry, 2004. **271**(2): p. 303-317.

66. MacDonald, P.E., L. Eliasson, and P. Rorsman, *Calcium increases endocytotic vesicle size and accelerates membrane fission in insulin-secreting INS-1 cells.* Journal of cell science, 2005. **118**(24): p. 5911-5920.

67. Jarousse, N. and R.B. Kelly, *Endocytotic mechanisms in synapses.* Current opinion in cell biology, 2001. **13**(4): p. 461-469.

68. Murthy, V.N. and P.D. Camilli, *Cell biology of the presynaptic terminal.* Annual review of neuroscience, 2003. **26**(1): p. 701-728.

69. Vogel, S.S., et al., *Calcium influx is required for endocytotic membrane retrieval.* Proceedings of the National Academy of Sciences, 1999. **96**(9): p. 5019-5024.

70. Tummala, P., E.J. Arnsdorf, and C.R. Jacobs, *The role of primary cilia in mesenchymal stem cell differentiation: a pivotal switch in guiding lineage commitment.* Cellular and molecular bioengineering, 2010. **3**(3): p. 207-212.

71. Thesleff, I., *Epithelial-mesenchymal signalling regulating tooth morphogenesis.* Journal of cell science, 2003. **116**(9): p. 1647-1648.

72. Orrick, L.R., M.O. Olson, and H. Busch, *Comparison of nucleolar proteins of*

normal rat liver and Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis. Proceedings of the National Academy of Sciences, 1973. **70**(5): p. 1316-1320.

73. BUGLER, B., et al., *Detection and localization of a class of proteins immunologically related to a 100-KDa nucleolar protein.* European Journal of Biochemistry, 1982. **128**(2-3): p. 475-480.

74. Srivastava, M. and H.B. Pollard, *Molecular dissection of nucleolin's role in growth and cell proliferation: new insights.* The FASEB Journal, 1999. **13**(14): p. 1911-1922.

75. Jordan, P., et al., *Major cell surface-located protein substrates of an ecto-protein kinase are homologs of known nuclear proteins.* Biochemistry, 1994. **33**(49): p. 14696-14706.

76. Stepanova, V., et al., *Nuclear translocation of urokinase-type plasminogen activator.* Blood, 2008. **112**(1): p. 100-110.

77. Song, N., et al., *The nuclear translocation of endostatin is mediated by its receptor nucleolin in endothelial cells.* Angiogenesis, 2012. **15**(4): p. 697-711.

78. Liu, P., M. Rudick, and R.G. Anderson, *Multiple functions of caveolin-1.* Journal of Biological Chemistry, 2002. **277**(44): p. 41295-41298.

79. Sharma, D.K., et al., *Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol.* Molecular biology of the cell, 2004. **15**(7): p. 3114-3122.

80. Ravindran, S., et al., *Endoplasmic reticulum chaperone protein GRP-78 mediates*

endocytosis of dentin matrix protein 1. Journal of Biological Chemistry, 2008. **283**(44): p. 29658-29670.

81. Nakatomi, M., et al., *Evc regulates a symmetrical response to Shh signaling in molar development.* Journal of dental research, 2013: p. 0022034512471826.

82. Blair, H.J., et al., *Evc2 is a positive modulator of Hedgehog signalling that interacts with Evc at the cilia membrane and is also found in the nucleus.* BMC biology, 2011. **9**(1): p. 1.

83. Liu, B., et al., *Primary cilia integrate hedgehog and Wnt signaling during tooth development.* Journal of dental research, 2014: p. 0022034514528211.

84. Michaud, E.J. and B.K. Yoder, *The primary cilium in cell signaling and cancer.* Cancer research, 2006. **66**(13): p. 6463-6467.

85. Allard, B., et al., *Characterization and gene expression of high conductance calcium-activated potassium channels displaying mechanosensitivity in human odontoblasts.* Journal of Biological Chemistry, 2000. **275**(33): p. 25556-25561.

86. Magloire, H., et al., *Expression and localization of TREK-1 K+ channels in human odontoblasts.* Journal of dental research, 2003. **82**(7): p. 542-545.

87. Jiang, S., et al., *Disruption of kif3a results in defective osteoblastic differentiation in dental mesenchymal stem/precursor cells via the Wnt signaling pathway.* Molecular Medicine Reports, 2016. **14**(3): p. 1891-1900.

CHAPTER VII. ABSTRACT IN KOREAN

법랑질과 상아질 형성과정에서 CPNE7의 역할

치아 발생은 치성상피와 외배엽성 간엽세포 사이의 상피-간엽 상호작용을 통해 조절되는 복잡한 발생과정이다. 이러한 개념을 바탕으로 치아상피로부터 분비된 인자들 중 중간엽 세포의 상아모세포로의 분화 유도 및 조절하는 데 중요한 유도물질로서 Cpne7이라는 새로운 분비 단백질이 발견되었다. 하지만, 법랑모세포로부터 분비된 Cpne7의 법랑질 및 상아질 형성 과정에서의 역할과 상아모세포로의 세포내이입 및 분화 조절 메커니즘에 대해서는 정확히 알려진 바가 없다.

초기 법랑질 형성 과정에서 Dspp 유전자의 일시적 발현이 보고되고 있지만 그 기원과 기능에 대해서는 명확히 밝혀져 있지 않다. 이에 본 논문에서는 초기 법랑모세포 유래 분비 물질인 Cpne7과의 연관성을 확인하기 위해 두 유전자의 치배 내 위치 변화와 분화에 따른 발현 양상을 확인하고, 초기 법랑모세포 내 Cpne7 유전자를 과발현 또는 감소시킴으로서 Dspp 유전자 발현양에 미치는 영향을 평가하였다. 또한, Dspp 전사 조절에 Cpne7이 관여하는지를 알아보기 위해 promoter assay를 진행하였다. 초기

법랑모세포에서뿐 아니라 인체 내 다양한 조직에서도 Dspp 유전자가 발현하는데 이 때 동일한 조직에서 Cpne7의 발현을 확인하였다. 이러한 결과들을 통하여 인체 내 여러 조직 및 장기에서 나타나는 Dspp의 발현이 Cpne7과 관련이 있을 것으로 추측되며, 그 중 하나로서 초기 법랑질 형성 과정에서 일시적으로 나타나는 Dspp 유전자 발현을 Cpne7이 조절한다는 사실을 확인하였다.

이전 연구에서 Cpne7이 치수줄기세포의 상아모세포로의 분화과정에서 Dspp 유전자를 조절함으로써 중요한 역할을 한다는 것이 보고된 바 있다. 하지만 초기법랑모세포로부터 상아모세포로의 이동경로와 치수줄기세포의 상아모세포로 분화 조절에 대한 상세 메커니즘에 대해서는 아직까지 명확히 밝혀진 바가 없다. 본 논문에서는 초기법랑모세포로부터 분비된 Cpne7이 상아모세포 표면에서 수용체 역할을 하는 nucleolin과 결합, Cpne7-nucleolin 복합체가 칼슘 유도에 의해 카베올리(caveolae) 피막소포 매개 방법으로 상아모세포내로 이입되는 것을 확인하였다. 또한, Cpne7-nucleolin 복합체는 중간엽세포의 분화 개시에 중요한 역할을 하는 것으로 알려진 일차섬모 관련 유전자들을 조절함으로서 Dspp 유전자 조절을 일으키는 것을 확인하였다.

종합해 볼 때, 본 연구에서는 초기 법랑질 형성시기에 일시적으로 나타나는 Dspp 유전자 발현에 Cpne7이 중요한 역할을 하며, 이러한 Cpne7이 초기 법랑모세포로부터 분비된 후 초기 상아모세포로 이동하는 경로

및 치수줄기세포의 일차섬모 형성에 관여함으로서 상아모세포로의 분화 과정에 미치는 영향 및 그 작용 메커니즘을 확인하였고, 이로서 치아 형성 관련 신호 전달 체계의 한 pathway를 제시함으로서 치아 발생 과정을 조금 더 이해할 수 있는 발판을 제공하였다.

주요어 : Cpne7 · Dspp · 상피-간엽 상호작용· 법랑질형성 · Nucleolin · 일차섬모 · 상아모세포 분화

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