Role of COUP-TFII in Odontoblast Differentiation

著者	Hur Sung-Woong
学位授与機関	Tohoku University
学位授与番号	11301甲第16936号
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Doctoral Dissertation

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Department of Dental Science

Graduate School, Chonnam National University

HUR, Sung-Woong

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Supervised by Professor Koh, Jeong-Tae

A dissertation submitted in partial fulfillment of the requirements for the Doctor of Philosophy in Dental Science.

Committee in Charge:

Kim, Sun-Hun _____

Kim, Won-Jae _____

Satoshi Fukumoto _____

Masahiro Saito _____

Koh, Jeong-Tae _____

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Abbreviations

AA	ascorbic acid
Ad	adenoviral
ALP	alkaline phosphatase
Am	ameloblast
Ambn	ameloblastin
Amel	amelogenin
AR-S	alizarin-red staining
α-MEM	α-minimal essential medium
ChIP	chromatin immunoprecipitation
COUP-TF	chicken ovalbumin upstream promoter transcription factor
DAPI	4’,6-diamidino-2-phenylindole
DE	dental epithelium
Dlx	distal-less genes
DM	dental mesenchyme
DMEM	Dulbecco’s modified Eagle’s medium
DMP-1	dentin matrix protein-1
DP	dental papilla
DSPP	dentin sialophosphoprotein
E	embryonic
EDTA	ethylenediaminetetraacetic acid
Enam	enamelin
EO	enamel organ
FBS	fetal bovine serum
GFP	green fluorescence protein
HA	hemagglutinin

HDPC	human dental pulp cells
HEK	human embryonic kidney
HRE	homeodomain response element
ICR	institute for cancer research
IEE	inner enamel epithelium
IP	immunoprecipitation
mALC	mouse ameloblast lineage cells
MDPC-23	murine dental papilla-derived cells
MM	mineralizing medium
MOI	multiplicity of infection
MSC	mesenchymal stem cells
Msx	msh homeobox
NC	negative control
NR2F2	nuclear receptor subfamily 2, group F, member 2
Od	odontoblast
OD	optical density
OCT	optimum cutting temperature
PBS	phosphate buffered saline
PN	postnatal
PPARγ	peroxisome proliferator-activated receptor-gamma
PVDF	polyvinylidenedifluoride
Runx2	runt-related transcription factor 2
si	small interfering
Sox-9	SRY-related high-mobility group box gene-9

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(Abstract)

Chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII; NR2F2, nuclear receptor subfamily 2, group f, member2), an orphan nuclear receptor belonging to the steroidthyroid hormone receptor superfamily, plays an important role in cell fate determination of various tissues. However, the specific role of COUP-TFII in tooth development has not yet been elucidated. The aim of present study is to explore the role of COUP-TFII in odontoblast differentiation. Endogenous expression of COUP-TFII in maxillary second molar germs of rats showed an increasing tendency as development of the tooth progressed. Also, COUP-TFII protein was detected in greater quantity in the odontoblastic layer of second molar germs (root formation stage) than in that of third molar germs (cap stage) of rats at post-natal day 9 (PN9). In mouse maxillary first molar tooth germ at its secretory stage (PN1), COUP-TFII protein expression was observed along with the odontoblastic layer. In primary human dental pulp cells (HDPCs) and murine dental papilla-derived cells (MDPC-23) cultured in a mineralizing medium, the expression of COUP-TFII was induced along with the increased odontoblast-specific dentin matrix protein-1 (DMP-1) and dentin sialophosphoprotein (DSPP) expression. Overexpression of

COUP-TFII using an adenoviral system up-regulated the expression of odontoblast-specific genes with increased alkaline phosphatase activity and matrix mineralization in odontoblast-lineage cells. In contrast, down-regulation of COUP-TFII using small interfering RNA decreased the expression of odontoblast-specific genes, which reduced matrix mineralization as well. Mechanistic studies revealed that COUP-TFII increased DSPP transcription by direct binding on the DSPP promoter. In addition, COUP-TFII physically interacted with the homeodomain transcription factor Msx2 and antagonistically regulated the Msx2 effect on DSPP promoter activity.

Taken together, these results suggest that COUP-TFII has a stimulatory role in DSPP expression and matrix mineralization in odontoblast-lineage cells.

INTRODUCTION

Odontoblasts differentiate from embryonic mesenchymal stem cells (MSCs) of neural crest origin in tooth-specific temporo-spatial patterns. Terminally differentiated mature odontoblasts secrete matrix proteins and induce mineral deposition to build the predentin-dentin structure (1). The process of tooth development including odontoblast maturation is tightly regulated by interactions between epithelial and mesenchymal tissues. Diverse signals in the families of bone morphogenetic protein (BMPs), fibroblast growth factor (FGFs) and Wnt, or the transcription factors Runx2, Msx-1, Msx-2 and Dlxs are involved in the process (2).

Adult dental pulp tissue has been also identified one of the possible sources of mesenchymal stem cells to form reparative dentin as a consequence against noxious stimuli. Isolated postnatal human dental pulp cells (HDPCs) possess stem-cell-like characteristics, including self-renewal capability and multi-lineage differentiation. Dental pulp cells have the ability to form a dentin/pulp-like complex in vivo, and also have the potential to differentiate into odontoblast-like cells in vitro, expressing odontoblast-specific markers including dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) (3-5). The critical roles of DSPP and DMP-1 in hard tissue development and mineralization have been examined utilizing knockout and transgenic mouse models (6,7).

Among the two proteins, DSPP is expressed predominantly in odontoblasts and at low levels in osteoblasts (8). Previous reports have shown that various mutations of the human DSPP gene cause dentinogenesis imperfecta type II (9-14), III (15) and dentin dysplasia type II (16). The distinct changes of phenotypes in relation with DSPP gene mutations are indicating that DSPP plays a critical role in odontoblast differentiation and dentinogenesis.

The orphan nuclear receptor COUP-TFII is widely distributed in the mesenchymal

compartment of developing organs. COUP-TFII is crucial in embryogenesis, angiogenesis, reproduction and metabolic homeostasis (17-20). Recent studies have demonstrated the importance of COUP-TFII in cell-fate determination, showing that COUP-TFII controls mesenchymal cell commitment and differentiation (21,22). However, the specific role of COUP-TFII in determining the fate of dental pulp cells remains unclear.

This study was undertaken to determine the functional roles of COUP-TFII in odontoblast differentiation and matrix mineralization. Here, it is suggested that COUP-TFII has the potential of stimulating DSPP expression and matrix mineralization in odontoblast lineage cells and its molecular mechanism underlying the regulation of DSPP transcription is discussed.

MATERIALS AND METHODS

Reagents and Plasmid Constructs

Commercial antibodies against COUP-TFII (Abcam, Cambridge, UK), Msx2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Cell Signaling Technology, Beverly, MA, USA) were used for Western blot analysis. Adenoviral (Ad) vector expressing COUP-TFII and HA-tagged COUP-TFII expression vector were kindly provided by Dr. Kee-Sook Lee (Chonnam National University, Gwangju, Korea). Human COUP-TFII small interfering RNA (si-COUP-TFII) was purchased from Ambion (Life Technologies, Paisley, UK). Three repeated homeodomain response element (3x HRE)-Luc reporter was kindly provided by Dr. Kwang-Ryul Lee (Chonnam National University, Gwangju, Korea).

Isolation of Rat Tooth Germ

Sprague-Dawley rat pups (Damool Science, Daejeon, Korea) at post-natal days 3, 6 and 9 were sacrificed, and maxillae including developing tooth germs were removed. The second molar tooth germs were surgically isolated using a stereomicroscope (Leica, Wetzlar, Germany), and 10 tooth germs per group were pooled for analysis of gene expression (23). All protocols were reviewed and approved by the Animal Use and Care Committee of Chonnam National University.

Immunofluorescence Staining

Immunofluorescence staining was performed with a TSA^{TM} Kit (Invitrogen, Carlsbad, CA, USA). The maxillae containing the tooth germs were fixed in 4%

paraformaldehyde and decalcified in 10% EDTA solution (pH 7.4) for 6 to 8 weeks. The tissues were treated with ethanol dehydration, embedded in paraffin, cut into 4-μm sections, and reacted with anti-COUP-TFII (Abcam) and anti-Msx2 (Santa Cruz Biotechnology) for 24 h and horseradish peroxidase-conjugated secondary antibody (1:200; Santa Cruz Biotechnology) for 1 h after the endogenous peroxidase was blocked with 1% hydrogen peroxide. The sections were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear morphology and photographed using LSM confocal microscopy (Carl Zeiss, Oberkochen, Germany). Immunological specificity was tested by substituting the primary antibody with normal serum (24).

Immunostaining

Embryo and postnatal heads of ICR (Institute for Cancer Research) mice were dissected and embedded in optimum cutting temperature compound (OCT; Sakura Fine Technical, Tokyo, Japan) for frozen sectioning. OCT blocks were cut into 8-μm sections with a 2800 Frigocut cryostat (Leica, Bensheim, Germany), held for 30 min at room temperature and then fixed in 4% paraformaldehyde for 10 min. After washing three times with phosphate buffered saline (PBS) for 5 min each, the tissue sections were incubated with 3% hydrogen peroxide in methanol for 15 min. After washing two times, the sections were further incubated with blocking solution A (Histofine DAB substrate kit, Nichirei, Tokyo, Japan) for 1 h, primary antibody for 1 h and blocking solution B (Histofine DAB substrate kit) for 10 min. After washing, mouse MAX-PO (Nichirei, Tokyo, Japan) was placed on the tissue for 10 min. The sections were washed with PBS and incubated with 3,3'-diaminobenzidine (DAB; Nichirei, Tokyo, Japan) for 10 min at room temperature (25). Then, hematoxylin and eosin stain was applied for counter-staining. For immunostaining, another commercial antibody against COUP-TFII (Perseus Proteomics, Tokyo, Japan) was used. Immunostaining images were obtained using a Biozero-8000 microscope (Keyence, Osaka, Japan).

Cell Culture and Viral Infection

Primary human dental pulp cells (HDPCs) were isolated as described previously (26). HDPCs were cultured in alpha-minimal essential medium (α-MEM; Gibco, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air containing 5% CO_2 at 37 °C. Odontoblastic differentiation was induced by adding mineralizing medium containing 10% FBS, 50 μg/ml ascorbic acid (12) and 5 mM β-glycerophosphate (β-GP), and the culture medium was replaced every other day. Murine dental papilla-derived MDPC-23 cells and mouse ameloblast lineage cells (mALCs) were similarly cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco). For viral infection, cells were treated with Ad-COUP-TFII or Ad-green fluorescence protein (GFP) as a control virus at the designated multiplicity of infection (MOI) under serum-free conditions. After 4 h, an equivalent volume of medium containing 20% FBS was added, and the cells were incubated for an additional 24 h before the mineralizing medium was changed.

Real-Time Polymerase chain reaction

Total RNA was prepared by TRI reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. Real-time PCR was performed with the ABI Step One Plus (Applied Biosystems, Foster City, CA, USA) using the Quanti Mix SYBR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The expression levels of all mRNAs were normalized to that of endogenous β-actin. Relative target gene expression was quantified using the comparative CT method (27). The primer sequences were provided in Table 1.

Table 1. The nucleotide sequences used for real-time PCR

Gene Name	*Nucleotide sequence*	*Size (bp)*
Rat β-actin	Forward 5'-GCTGACAGGATGCAGAAGGA-3' Reverse 5'-TGGACAGTGAGGCCAGGATA-3'	124
Rat COUP-TFII	Forward 5'-CAAGGCCATAGTCCTGTTCACC-3' Reverse 5'-CGTACTCTTCCAAAGCACACTGG-3'	100
Rat ALP	Forward 5'-ATCTTTGGTCTGGCTCCCATG-3' Reverse 5'-TTTCCCGTTCACCGTCCAC-3'	106
Rat DMP-1	Forward 5'-GGAGCAAGGTGACAGCGAGT-3' Reverse 5'-GAGACTGGAGGCCTTCCTGG-3'	104
Rat DSPP	Forward 5'-TGACAGCAAGGACAGCAC-3' Reverse 5'-GGGGTTCTCTGCTCTAATC-3'	145
Rat Msx2	Forward 5'-ACACAAGACCAATCGGAAGC-3' Reverse 5'-GCAGCCATTTTCAGCTTTTC-3'	222
Human β-actin	Forward 5'-ACCCACACTGTGCCCATCTAC-3' Reverse 5'-GCCATCTCCTGCTCGAAGTC-3'	206
Human COUP-TFII	Forward 5'-TGCCTGTGGTCTCTCTGATG-3' Reverse 5'-ATATCCCGGATGAGGGTTTC-3'	225
Human DMP-1	Forward 5'-GATCAGCATCCTGCTCATGTT-3' Reverse 5'-AGCCAAATGACCCTTCCATTC-3'	125
Human DSPP	Forward 5'-AGAAGGACCTGGCCAAAAAT-3' Reverse 5'-TCTCCTCGGCTACTGCTGTT-3'	280

Western Blot Analysis

Total extracts of cells and tooth germs were harvested in a lysis buffer (Cell Signaling Technology) and centrifuged at 12,000 × g for 15 min at 4 °C. Quantification of total protein was performed using the BCA protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved by 10% SDS-PAGE and transferred to a polyvinylidenedifluoride (PVDF) membrane. After blocking in 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), the membrane was incubated with the indicated antibodies diluted in 5% milk at 4 °C for overnight. After washing five times with TBS-T for 5 min each time, and finally the signals were visualized using an enhanced chemiluminescence reagent (ECL; Santa Cruz Biotechnology) in a LAS-4000 luminoimage analyzer system (Fujifilm, Tokyo, Japan).

Transient Transfection and Luciferase Reporter Assay

Transient transfections of reporter and mammal expression plasmids were carried out using Lipofectamine 2000 (Invitrogen) as described previously (28). Human embryonic kidney (HEK-293T) cells were used and a β-galactosidase reporter plasmid under the control of cytomegalovirus (CMV) promoter was co-transfected as an internal control. Luciferase activity was measured with the multiplate reader (Bio-Tek Instruments, Winooski, VT, USA) and normalized to β-galactosidase activity. All experiments were performed at least 3 times in duplicate, and the most representative results were shown.

Alkaline Phosphatase Staining and Alizarin Red Staining

For alkaline phosphatase (ALP) enzyme staining, cells were fixed with 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA), rinsed three times with deionized

water and treated with a BCIP®/NBT solution (Sigma-Aldrich) for 15 min. The stained culture plates were scanned by Epson Perfection V700 (Epson Korea, Seoul, Korea), and the degree of staining was quantitatively compared using Image J software. To evaluate mineralization, Alizarin Red stain was applied as previously described (22). Briefly, cells were fixed with 70% ethanol and then treated with a 40 mM Alizarin Red solution (pH 4.2) for 10 min. After extraction with 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate (pH 7.0) for 15 min, staining was quantified by measuring the absorbance at 540 nm using a multiplate reader (Bio-Tek Instruments).

Immunoprecipitation (IP) and Chromatin Immunoprecipitation (ChIP) Assays

MDPC-23 cells were transfected with indicated constructs for 48 h, and harvested in a lysis buffer (Cell Signaling Technology) containing protease inhibitors (Roche, Basel, Switzerland). The samples were centrifuged at 4°C for 15 min, and supernatants were pre-cleared with protein G-agarose beads (Invitrogen) prior to overnight incubation with anti-Msx2 and anti-HA antibodies (Sigma-Aldrich). Protein G-agarose beads were added to the lysate, incubated for 4 h, washed five times with lysis buffer and resuspended in SDS sample buffer. After samples were resolved by 10% SDS-PAGE, Western blots were performed with the designated antibodies and ECL reagents. For ChIP assay, MDPC-23 cells were transfected with HA-COUP-TFII or empty vector for 48 h, and fixed with 1% formaldehyde for 10 min. After washing with ice-cold PBS, the cells were harvested and sonicated in a lysis buffer (Millipore Corporation, Billerica, MA). Soluble chromatin was subjected to immunoprecipitation using monoclonal anti-HA antibody-coupled agarose beads. Then the DNA fragments were recovered by phenol/chloroform extraction, and analyzed by RT-PCR. The primer sequences for COUP-TFII binding region of the DSPP promoter were listed in Table 2.

Table 2. The nucleotide sequences of the primers used in ChIP assay.

Location	*Nucleotide sequence*	*Size (bp)*
−333/−328	(P1) Forward 5'-CCTCAGGAATGATAGGGGTCT-3' (P2) Reverse 5'-AGAGCCACTTAGACTCTGTCACC-3'	120
−184/179	(P3) Forward 5'-GACACAAAACAGTCTTCCAGGAG-3' (P4) Reverse 5'-GCTGTAATAACGCCCCACTC-3'	120

P : primer.

Statistical Analyses

All experiments were repeated at least three times. Statistical analysis was performed using the Student's t-test or analysis of variance followed by the Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. The results are expressed as the mean ± standard deviation of triplicate independent samples.

RESULTS

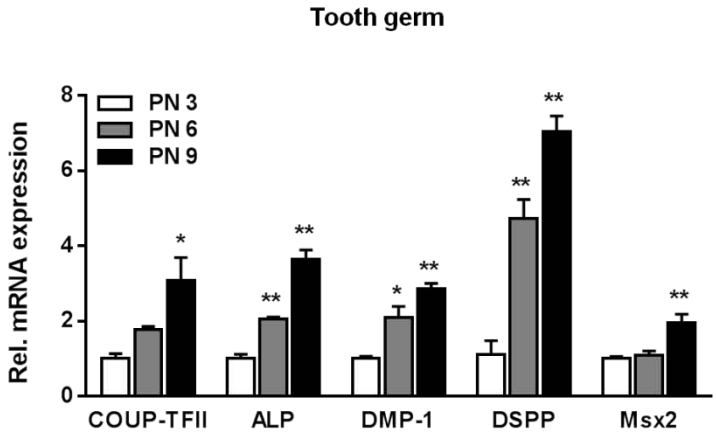
1. Endogenous Expression of COUP-TFII

Initially, to identify whether COUP-TFII is related to development of tooth germ, the level of COUP-TFII mRNA or protein was examined in the tooth germs of rat and mouse at different developmental stages.

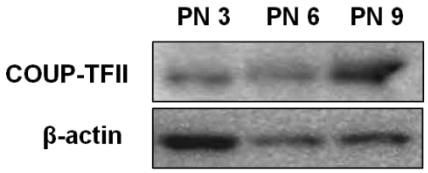
1-1. Endogenous COUP-TFII Expression in Rat Tooth Germ

In this part, the expression level of COUP-TFII mRNA was assessed in rat tooth germ. Rat tooth germs undergo different developmental stages with postnatal (PN) days; bell stage (PN 3), crown formation stage (PN 6) and root formation stage (PN 9) of maxillary second molar germs (29). As tooth germ development progressed, the expression of COUP-TFII mRNA also increased along with the expression of ALP, DMP-1, DSPP and homeoprotein Msx2 (Fig. 1A). Western blot analysis confirmed that the protein level of COUP-TFII consistently increased postnatally (Fig. 1B). Confocal microscopy analysis of PN day 9 maxilla of rat also revealed the increased positive immunoreactivity for anti-COUP-TFII and anti-Msx2 depending on germ stages; the immunoreactivity was more pronounced in the odontoblast layer of the second molar germ (root formation stage) than that of the third molar germ (cap stage) (Fig. 1C). These results suggest that the increasing expression pattern of COUP-TFII is related with the maturation of odontoblast and tooth germ development.

A


Tooth germ
Rel. mRNA expression
PN 3
PN 6
PN 9
0
2
4
6
8
COUP-TFII
ALP
DMP-1
DSPP
Msx2

B


PN 3
PN 6
PN 9
COUP-TFII
β-actin

C

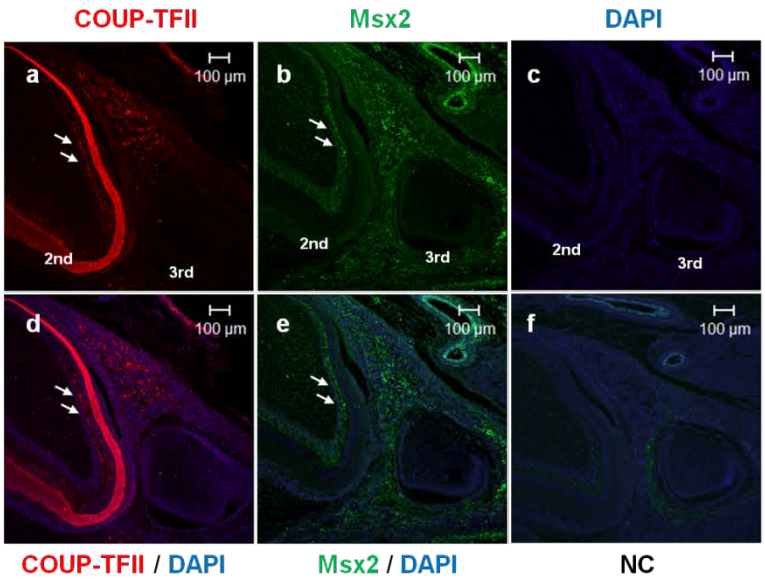

COUP-TFII
Msx2
DAPI
a
b
c
d
e
f
100 μm
2nd
3rd
COUP-TFII / DAPI
Msx2 / DAPI
NC

Figure 1. Endogenous expression of COUP-TFII in rat tooth germ. (**A**) Expression of COUP-TFII mRNA in rat tooth germ. Total RNA was isolated from tooth germ of the maxillary second molars at post-natal day 3 (bell stage), 6 (crown formation stage) and 9 (root formation stage). Real-time PCR was performed with specific primers. (**B**) COUP-TFII protein expression in rat tooth germ. Tooth germ samples were harvested at the designated time point for total protein isolation, and Western blot analysis was performed. (**C**) Immunofluorescent staining was performed with COUP-TFII or Msx2 specific antibody in serial sections of rat maxilla at post-natal day 9, and imaged by LSM confocal microscopy. Immunoreactivity to anti-COUP-TFII (red) was observed in dentin-forming odontoblasts of the second molar germ and also enamel-forming ameloblasts. Immunoreactivity to the anti-Msx2 antibody (green) was observed in the odontoblasts of the second molar germ. The nucleus was stained with DAPI (blue). The negative control stained with IgG was immunofluorescence negative (green). Notably, immunoreactivity of COUP-TFII and Msx2 in second molar germs (root formation stage) was stronger than that in third molar germs (cap stage). Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group). PN, post-natal days; 2nd, second molar; 3rd, third molar; bars, 100 μm

1-2. Endogenous COUP-TFII Expression in Mouse Tooth Germ

To compare the expressional pattern of COUP-TFII in mouse tooth germ, immunostaining experiment was carried out using anti-COUP-TFII antibody in mouse maxillary first molar germs at different developmental stages. Mouse maxillae including first molar germs were isolated at embryonic days of E13, E14, E16.5 and postnatal day of PN1; the first molar germs undergo bud, cap, bell and secretory stage, respectively. Each specimen was cryosectioned and reacted with anti-COUP-TFII antibody. As shown in Fig. 2, the signal of COUP-TFII protein was hardly detected around dental mesenchyme (DM) or dental pulp (DP) in the tooth germ of E13, E14 and E16.5. However, immunoreactivity against COUP-TFII was observed in the tooth germ of PN1 along with the odontoblastic layer (Fig. 2). Mouse maxillary first molars at PN1 are in the secretory stage when matured odontoblasts start forming predentin structure. The expression of COUP-TFII in developing tooth germs of mouse started to be detected from PN1. These findings provide supportive evidence that COUP-TFII might be related on the process of odontoblast differentiation or dentin matrix mineralization.

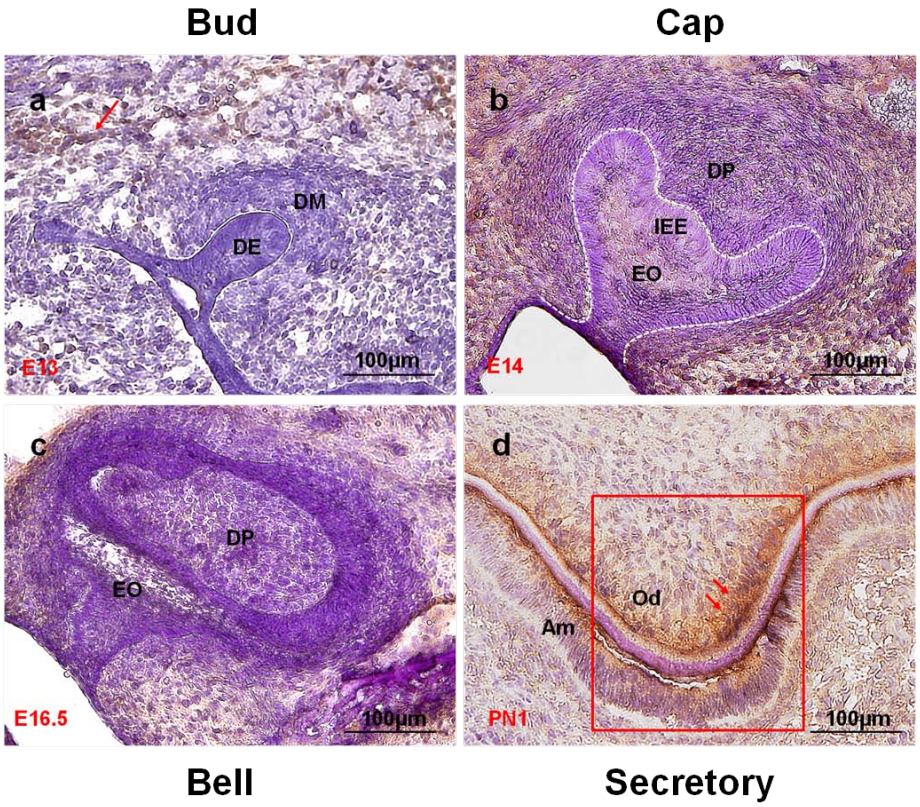


Figure 2. Endogenous expression of COUP-TFII in mouse tooth germ. Immunohistochemistry. Mouse maxillae including first molar germs were isolated at E13, E14, E16.5 and PN1 (a-d); the first molar germs undergo bud, cap, bell and secretory stage, respectively. Each specimen was cryosectioned and reacted with anti-COUP-TFII antibody. The signal of COUP-TFII protein was hardly detected around dental mesenchyme (DM) or dental pulp (DP) in the tooth germ of E13, E14 and E16.5. However, immunoreactivity against COUP-TFII was observed in PN1 along the odontoblastic layer and ameloblastic layer as well. Red arrows indicate expression of COUP-TFII. E, embryonic; DE, dental epithelium; DM, dental mesenchyme; EO, enamel organ; IEE, inner enamel epithelium; DP, dental papilla; Od, odontoblast; Am, ameloblast; bars , 100 μm

2. Endogenous COUP-TFII Expression during Odontoblast Differentiation

To determine if there is a change in endogenous COUP-TFII expression profile during induced odontoblast differentiation, the level of COUP-TFII mRNA expression was evaluated in cultured odontoblast lineage cells, primary human dental pulp cells (HDPCs) and murine dental papilla-derived cells (MDPC-23). Odontoblast differentiation of HDPCs and MDPC-23 cells were induced by incubation with mineralizing medium containing 10% fetal bovine serum (FBS), 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate (β–GP), and the culture medium was replaced every other day.

As shown in Fig. 3, the expression of odontoblast-specific DMP-1 and DSPP expression was continuously increased along with the culture period up to day 7 in HDPCs (Fig. 3A) and day 4 in MDPC-23 cells (Fig. 3B), respectively. These results imply the cells were undergoing odontoblast differentiation by mineralizing medium. Under these conditions, COUP-TFII mRNA expression was also gradually induced as the expression of odontobalst differentiation marker genes was increased. These results suggest COUP-TFII might play some roles during the differentiation of odontoblast lineage cells.

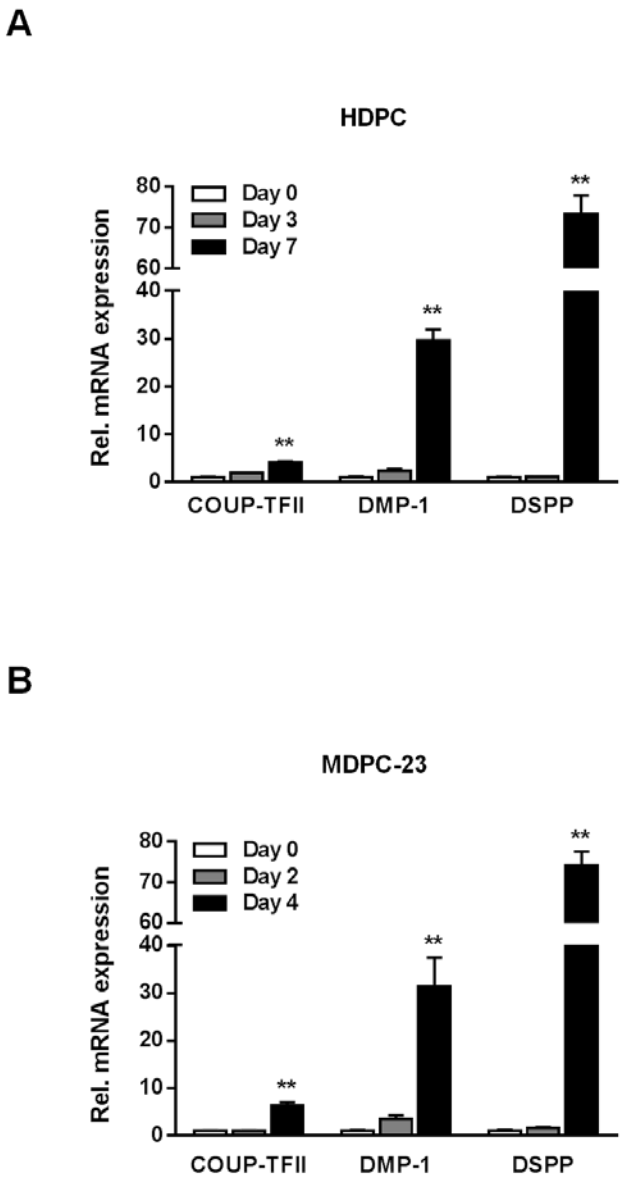


Figure 3. COUP-TFII expression profile during odontoblast differentiation. (**A**) HDPCs were cultured with mineralizing medium and harvested at designated time points of 0, 3 and 7 days for total RNA isolation. Real-time PCR was performed with specific primers for COUP-TFII, DMP-1 and DSPP. β-actin was used as an internal control. (**B**) MDPC-23 cells were harvested at 0, 2 and 4 days and real-time PCR was performed with the specific primers for COUP-TFII, DMP-1 and DSPP. Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group).

3. The Effects of COUP-TFII on Odontoblast Differentiation and Matrix Mineralization

In previous part, the results showed that COUP-TFII mRNA expression was increased during the differentiation of odontoblast lineage cells. Based on those results, the effects of COUP-TFII on odontoblasts were clarified through gain-or-loss of function experiments.

3-1. The Effects of COUP-TFII Overexpression in Odontoblast

To clarify the potential roles of COUP-TFII in odontoblast differentiation, adenovirus encoding COUP-TFII (Ad-COUP-TFII) or control adenovirus vector, Ad-green fluorescence protein (Ad-GFP) was transduced in HDPCs. The cells were treated with Ad-COUP-TFII or Ad-GFP at the designated multiplicity of infection (MOI) under serum-free conditions. After 4 h, an equivalent volume of medium containing 20% FBS was added, and the cells were incubated for an additional 24 h before the medium was changed. Then, after cultured for 5 days in mineralizing medium, the expressions of COUP-TFII, DMP-1 and DSPP were examined by real-time PCR analysis. When COUP-TFII was overexpressed in HDPCs using Ad-COUP-TFII, the levels of DMP-1 and DSPP mRNA expression were increased in a dose dependent manner (Fig. 4A). Notably, the expression of DSPP mRNA in the highest dose of Ad-COUP-TFII infection was approximately 12 fold compared with the control group.

To determine whether COUP-TFII can affect matrix mineralization, alkaline phosphatase (ALP) and Alizarin Red staining (AR-S) were performed in the culture of HDPCs or MDPC-23 cells. ALP staining for detecting ALP enzyme activity was analyzed after 7 days in HDPCs, and AR-S for detecting calcium nodules formation was analyzed after 24 days in HDPCs and 6 days in MDPC-23 cells. The cells were cultured with or without GFP-tagged Ad-COUP-TFII in the presence or absence of AA and β-GP for the designated culture days. Prior to ALP staining, infectivity of Ad-COUP-TFII for COUP-TFII overexpression was confirmed by fluorescent microscopic analysis. As shown in Fig. 4B

(upper panel), adenovirus infection was successfully carried out by dose dependent manner. As a result of ALP staining, COUP-TFII overexpression significantly enhanced the ALP activity in HDPCs. Quantitative changes of ALP activity compared with the control group, were measured by using Image J software (Fig. 4B, lower panel).

Next, AR-S for detecting calcium nodules formation was conducted in HDPCs and MDPC-23 cells. The results of AR-S showed similar pattern as ALP staining in both HDPCs and MDPC-23 cells, demonstrating that mineralized nodule formation was significantly increased depending on doses of Ad-COUP-TFII (Fig. 4C-D, upper panels). For quantitative analysis, the stain was extracted with 10% cetylpyridinium chloride (CPC) and then concentration of AR-S was measured by spectrophotometry in 562nm of optical density (Fig. 4C-D, lower panels).

The gain-of-function study indicates that overexpression of COUP-TFII enhances the expression of odontoblast differentiation marker genes, DMP-1 and DSPP, also stimulates mineralization in odontoblast lineage cells. Taken together, the results provide some evidences that COUP-TFII has a stimulatory role in odontoblast differentiation and matrix mineralization.

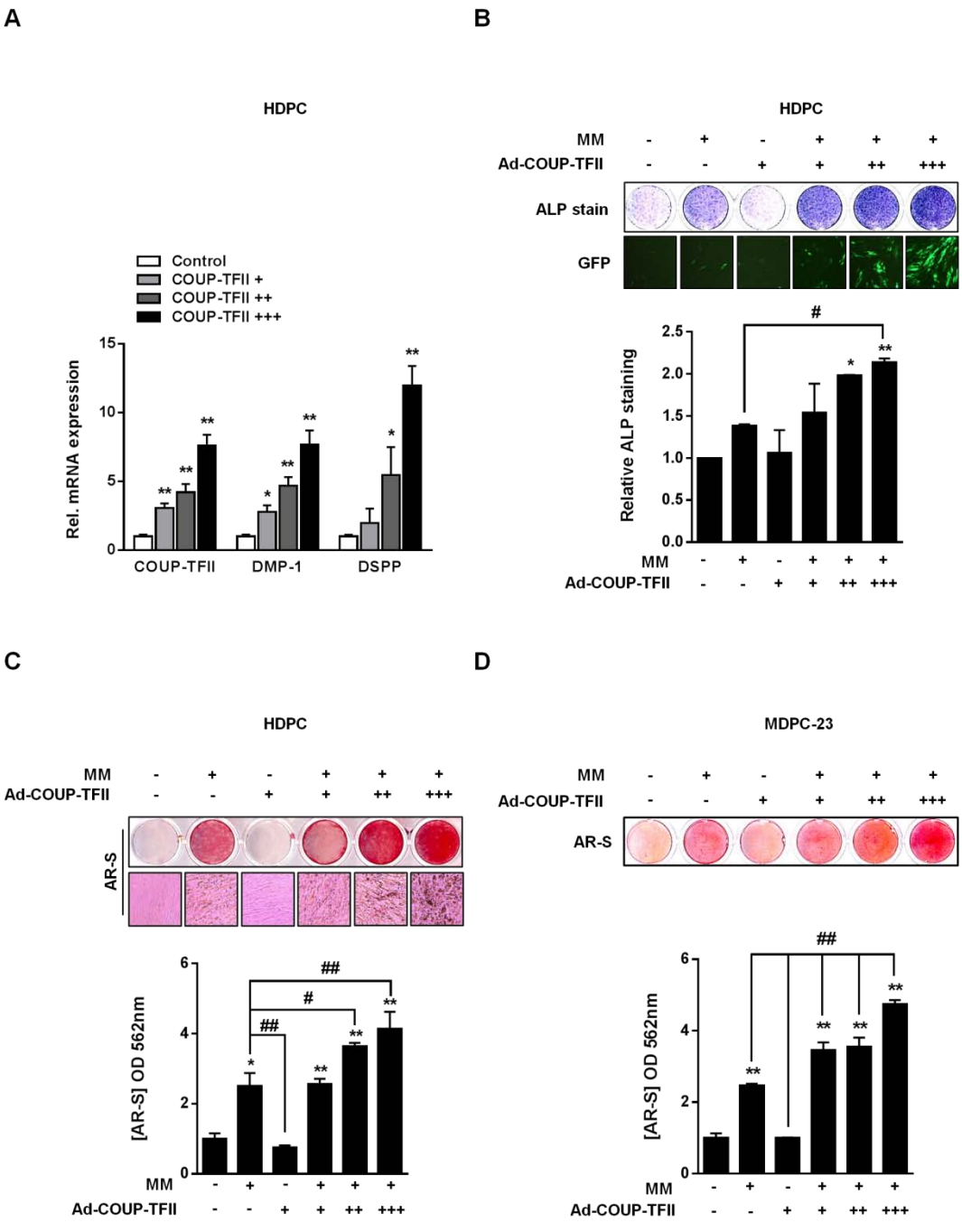

A
HDPC
Control
COUP-TFII +
COUP-TFII ++
COUP-TFII +++
Rel. mRNA expression
COUP-TFII
DMP-1
DSPP
B
HDPC
MM
Ad-COUP-TFII
ALP stain
GFP
Relative ALP staining
C
HDPC
AR-S
[AR-S] OD 562nm
D
MDPC-23

Figure 4. Overexperssion of COUP-TFII stimulates DSPP expression and matrix mineralization in HDPCs and MDPC-23 cells. (**A**) Real-time PCR analysis. HDPCs were infected with Ad-COUP-TFII or Ad-GFP and cultured for 5 days in mineralizing medium. (**B**) Alkaline phosphatase staining. HDPCs were cultured with green fluorescence protein (GFP)-tagged Ad-COUP-TFII for 7 days. The infectivity of Ad-COUP-TFII for COUP-TFII overexpression was confirmed by fluorescence microphotography. Alkaline phosphatase staining was performed with a BCIP®/NBT solution, scanned, and quantitatively compared using Image J software (lower panel). (**C, D**) Alizarin Red staining (AR-S). HDPCs and MDPC-23 cells were cultured for 24 days or 6 days under the condition of overexpression of COUP-TFII, and stained with Alizarin Red solution. Magnified images represent mineralized nodule formation of the relevant wells. For quantitative analysis, the stain was extracted with 10% cetylpyridinium chloride and then concentration of AR-S was measured by spectrophotometry (lower panels). Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group. #, $p < 0.05$; ##, $p < 0.01$ *versus* the indicated group). Ad-COUP-TFII (+, 50 MOI; ++, 100 MOI; +++, 200 MOI), Ad-GFP (Control, 50 MOI). MM, mineralizing medium

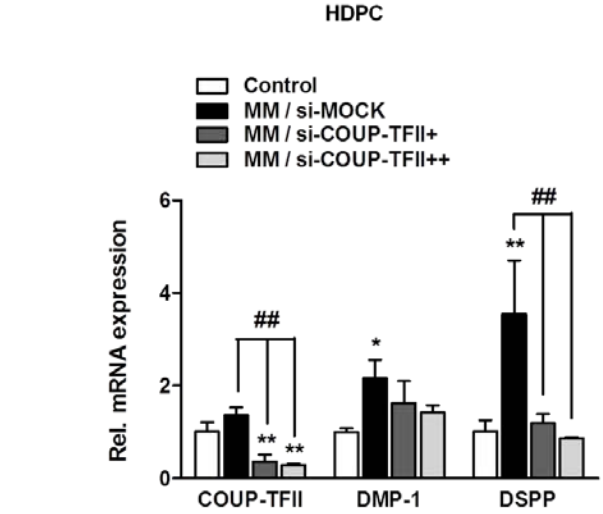
3-2. The Effects of COUP-TFII Down-regulation in Odontoblast

To confirm further understanding of the roles of COUP-TFII in odontoblast differentiation and matrix mineralization, loss-of-function study was carried out by using si-RNA mediated down-regulation of COUP-TFII. To silence the endogenous expression of COUP-TFII, HDPCs were transfected with human COUP-TFII small interfering RNA (si-COUP-TFII) or control siRNA and cultured for 5 days with or without mineralizing medium. When HDPCs were transfected with si-COUP-TFII, the expression of COUP-TFII mRNA was significantly decreased depending on the transfected doses of si-COUP-TFII, accompanied by decrease of DMP-1 and DSPP expression, analyzed by real-time PCR. In particular, the reduction of DSPP expression was more remarkable compared with that of DMP-1 (Fig. 5A).

AR staining was carried out in the similar way with the gain-of-function study. HDPCs were cultured for 24 days under the condition of silencing of COUP-TFII, and stained with Alizarin Red solution. As shown in Fig. 5B, si-COUP-TFII transfected groups demonstrated an inhibition of mineralized nodule formation, indicating that the down-regulation of COUP-TFII have caused an inhibitory effect on the mineralization in HDPCs. Magnified images represent mineralized nodule formation of the relevant wells. Quantitative analysis was performed; the stain was extracted with 10% cetylpyridinium chloride and then concentration of AR-S was measured by spectrophotometry (Fig. 5B, lower panel).

These results were consistent with the previous gain-of-function study, suggesting that COUP-TFII may play a positive role in odontoblast lineage cells and their function of inducing dentinal matrix mineralization.

A


HDPC
Control
MM / si-MOCK
MM / si-COUP-TFII+
MM / si-COUP-TFII++
Rel. mRNA expression
6
4
2
0
##
**
**
*
**
##
COUP-TFII
DMP-1
DSPP

B

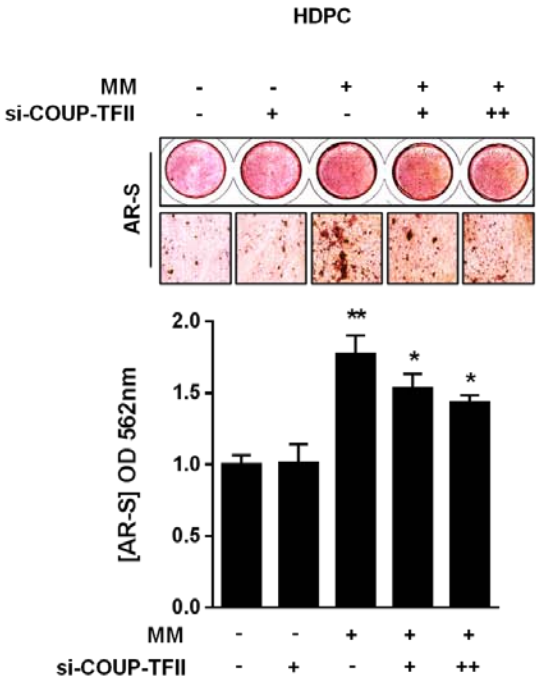

HDPC
MM - - + + +
si-COUP-TFII - + - + ++
AR-S
[AR-S] OD 562nm
2.0
1.5
1.0
0.5
0.0
**
*
*
MM - - + + +
si-COUP-TFII - + - + ++

Figure 5. Down-regulation of COUP-TFII inhibits DSPP expression and matrix mineralization in HDPCs. (**A**) Real-time PCR analysis. HDPCs were transfected with si-COUP-TFII or control siRNA and cultured for 5 days with or without mineralizing medium. (**B**) Alizarin Red staining (AR-S). HDPCs were cultured for 24 days under the condition of silencing of COUP-TFII, and stained with Alizarin Red solution. Magnified images represent mineralized nodule formation of the relevant wells. For quantitative analysis, the stain was extracted with 10% cetylpyridinium chloride and then concentration of AR-S was measured by spectrophotometry (lower panels). Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group. #, $p < 0.05$; ##, $p < 0.01$ *versus* the indicated group). si-COUP-TFII (+, 100 ng/well; ++, 200 ng/well; +++, 300 ng/well); MM, mineralizing medium

4. The Molecular Mechanism of COUP-TFII Action

The previous results suggest that COUP-TFII has the positive effects on odontoblast differentiation and matrix mineralization. In this part, molecular mechanism of COUP-TFII action was explored focused on the transcriptional regulation of DSPP and interaction with Msx2, another major transcription factor of odontoblast differentiation.

4-1. COUP-TFII Activation of Promoter Gene

As shown in the previous part, expressions of DMP-1 and DSPP, the specific odontoblast differentiation marker genes, were regulated by expression of COUP-TFII (Fig. 4A and 5A). The expressional change was much greater in DSPP rather than DMP-1, although both of them are the matrix proteins secreted by matured odontoblast. Increased DSPP expression is an indicator of odontoblastic differentiation and is also necessary for tooth development and matrix mineralization (30). Therefore, to understand the mechanism by which COUP-TFII regulates odontoblastic gene expression and matrix mineralization, the effects of COUP-TFII on DSPP promoter activity were examined.

Previous studies have reported that there exist specific binding elements (AGGTCA) for COUP-TFII binding (31). Hence, DSPP promoter region has been searched to see if there exist some putative COUP-TFII binding motifs (AGGTCA), and there two candidate elements were found at -333/-328 and -184/-179 bp from the transcription start site in the DSPP promoter gene.

To assess if COUP-TFII could have actual effects on the regulation of DSPP transcription, serial deletion promoters of DSPP gene were constructed; 2.6-, 1.5-, 0.7-kb and 50-bp DSPP-Luc reporter with or without the putative COUP-TFII binding motifs (Fig. 6A). Then, luciferase reporter assay was performed with those serial deletion promoters of DSPP gene. For the luciferase reporter assay, HEK-293T cells were used and a β-galactosidase reporter plasmid under the control of cytomegalovirus promoter was co-transfected as an internal control. Luciferase activity was measured with the multiplate

reader. When COUP-TFII expression vector was co-transfected with the 2.6-, 1.5- or 0.7-kb DSPP-Luc reporter, luciferase activity of each reporter was increased in proportion to the dose of COUP-TFII vector. On the other hand, no response was seen with 50-bp DSPP-Luc reporter, which did not contain putative COUP-TFII binding element (Fig. 6B). These results indicate that COUP-TFII positively regulates the transcriptional activity of DSPP.

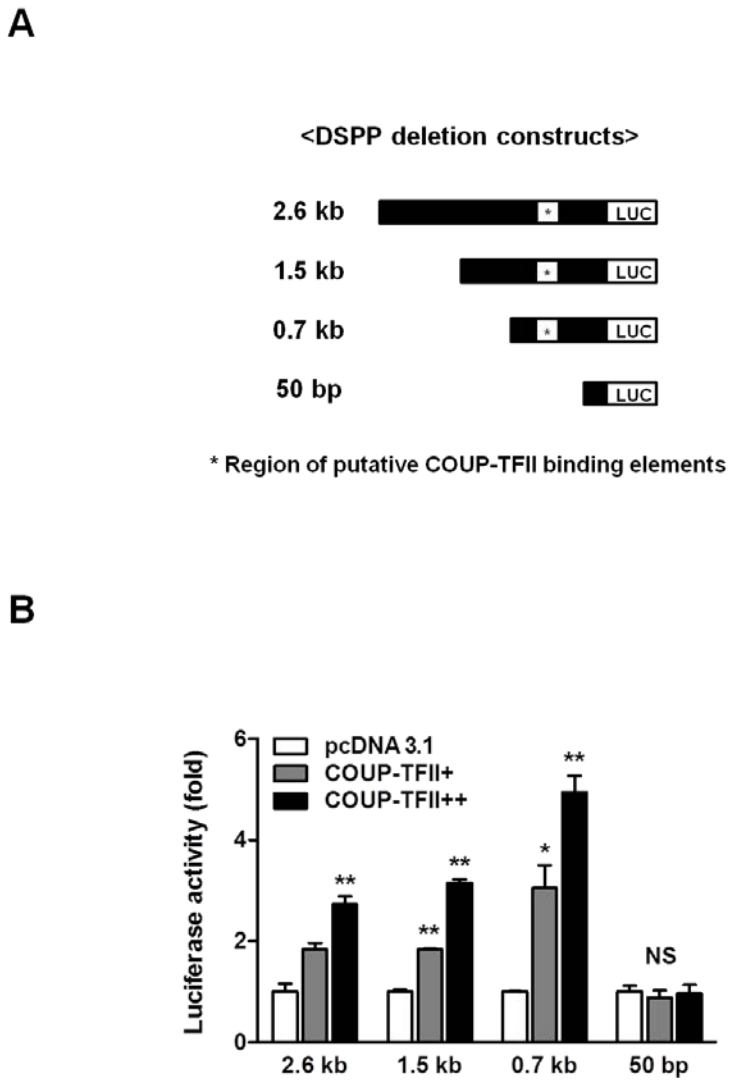


Figure 6. COUP-TFII enhances the activity of DSPP promoter. (**A**) Schematic representation of serial deletion constructs of DSPP-Luc promoter. (**B**) Luciferase reporter assay. HEK-293T cells were co-transfected with 100 ng of the indicated luciferase reporter constructs (2.6-, 1.5-, 0.7-kb and 50-bp DSPP-Luc) and COUP-TFII expressional vector (+, 100 ng/well; ++, 200 ng/well). Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group). NS, not significant

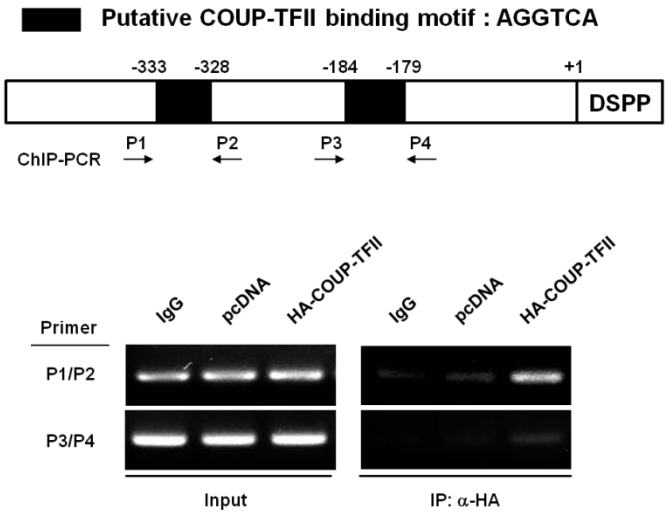
4-2. COUP-TFII Protein Binding on DSPP Promoter Gene

To determine if COUP-TFII protein directly binds on DSPP promoter region, the COUP-TFII expression vector was co-transfected with the 0.7-kb DSPP promoter in MDPC-23 cells and ChIP assay was performed. MDPC-23 cells were transfected with or without HA-COUP-TFII. After 48 h, the ChIP assay was carried out using the anti-HA antibody. Immunoprecipitated products were amplified by PCR with primers specific for the putative COUP-TFII binding sites on DSPP promoter (-333/-328bp or -184/-179bp). Two loci of the DSPP gene were interacted with the COUP-TFII protein, analyzed by the ChIP assay, although the -333/-328 element was more dominant than the -184/-179 element (Fig. 7A). This result suggests that the COUP-TFII protein directly binds to the DSPP promoter region.

Moreover, it also implies that the -333/-328 element is more responsible for the transcriptional regulation of DSPP by COUP-TFII than the other. Based on the observation, a mutant form of DSPP-Luc reporter targeting the -333/-328 element was produced (Fig 7B, upper diagram).

To confirm the COUP-TFII binding to DSPP gene, COUP-TFII expression vector was transfected with the wild type (WT) or the mutant (Mut, -333/-328) of 0.7-kb DSPP-Luc promoter. Compared to the activity of wild-type DSPP-Luc reporter activity, COUP-TFII failed to activate the luciferase activity of mutated form of DSPP-Luc reporter, in which six nucleotides of the COUP-TFII binding site (-333/-328) was substituted (Fig. 7B, lower panel). These findings suggest that COUP-TFII might directly control DSPP transcription through the specific COUP-TFII binding elements.

A


Putative COUP-TFII binding motif : AGGTCA
-333
-328
-184
-179
+1
DSPP
ChIP-PCR
P1
P2
P3
P4
IgG
pcDNA
HA-COUP-TFII
IgG
pcDNA
HA-COUP-TFII
Primer
P1/P2
P3/P4
Input
IP: α-HA

B

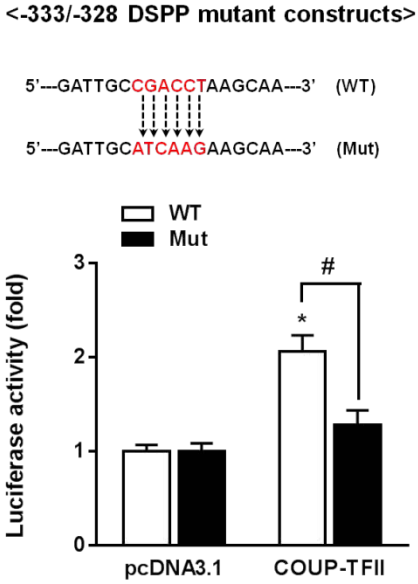

<-333/-328 DSPP mutant constructs>
5'---GATTGCCGACCTAAGCAA---3' (WT)
5'---GATTGCATCAAGAAGCAA---3' (Mut)
WT
Mut
Luciferase activity (fold)
0
1
2
3
*
#
pcDNA3.1
COUP-TFII

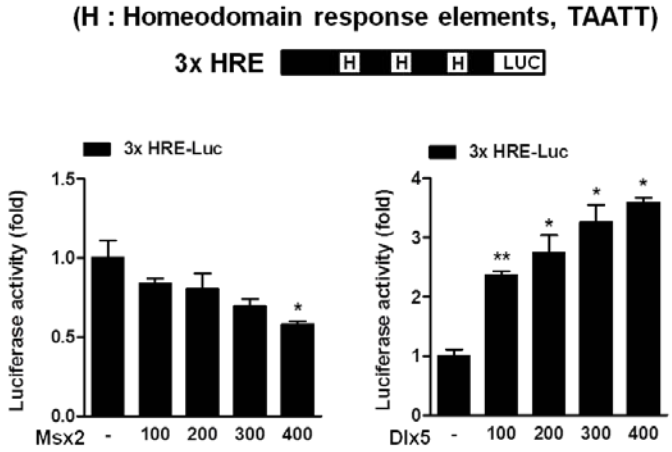
Figure 7. COUP-TFII directly binds to DSPP promoter. (**A**) MDPC-23 cells were transfected with or without HA-COUP-TFII. After 48 h, the ChIP assay was carried out using the anti-HA antibody. Immunoprecipitated products were amplified by PCR with primers specific for the putative COUP-TFII binding sites on DSPP promoter (-333/-328bp or -184/-179bp). (**B**) COUP-TFII was transfected on the wild type (WT) or the mutant (Mut, -333/-328) of 0.7-kb *DSPP*-Luc promoter, and then luciferase reporter assay was carried out. Note that only the activity of wild type DSPP promoter was increased. Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$ *versus* control group. #, $p < 0.05$ *versus* the indicated group).

4-3. Antagonistic Effect of COUP-TFII and Msx2 on the Activity of DSPP Transcription

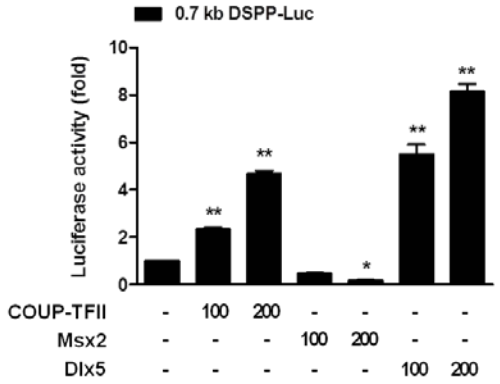
Previously, COUP-TFII demonstrated a positive regulation on DSPP transcription. Another important transcription factors in tooth development, Msx2 and Dlx5, so called as homeoproteins, control DSPP transcription in opposite directions by competing with homeodomain response element (HRE, TAATT) (32,33). To confirm the action of Msx2 and Dlx5 on DSPP transcription, 3x HRE-Luc and 0.7-kb DSPP-Luc reporter were prepared and luciferase reporter assay was carried out. When Msx2 expression vector was co-transfected with 3x HRE-Luc or 0.7-kb DSPP-Luc reporter, the luciferase activity was dose-dependently decreased, as reported previously (33). In contrast, transfection of Dlx5 expression vector increased luciferase activity (Figs. 8A, B).

To explore the interaction in between COUP-TFII protein and homeoproteins, HEK-293T cells were co-transfected with 0.7-kb DSPP-Luc reporter together with Msx2 and COUP-TFII expression vectors at the indicated doses. COUP-TFII dose-dependently increased the activity of 0.7-kb DSPP-Luc reporter even in the presence of Msx2, and on the contrary Msx2 dose-dependently inhibited COUP-TFII-induced luciferase activity (Fig. 8C). Western blot analysis confirmed the induction of COUP-TFII and Msx2 protein by the vectors (Fig. 8C, lower panel). The results suggest that Msx2 could be a potential counter-partner for COUP-TFII to regulate the DSPP transcription.

A


(H : Homeodomain response elements, TAATT)
3x HRE
H
H
H
LUC
3x HRE-Luc
Luciferase activity (fold)
Msx2 - 100 200 300 400
3x HRE-Luc
Luciferase activity (fold)
Dlx5 - 100 200 300 400

B


0.7 kb DSPP-Luc
Luciferase activity (fold)
COUP-TFII - 100 200 - - - -
Msx2 - - - 100 200 - -
Dlx5 - - - - - 100 200

C

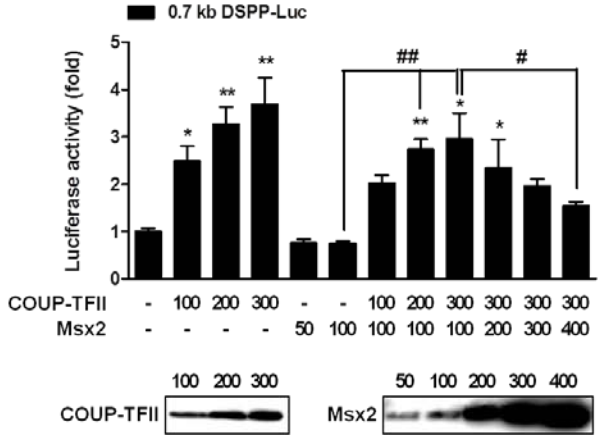

0.7 kb DSPP-Luc
Luciferase activity (fold)
##
#
COUP-TFII - 100 200 300 - - 100 200 300 300 300 300
Msx2 - - - - 50 100 100 100 100 200 300 400
100 200 300
COUP-TFII
50 100 200 300 400
Msx2

Figure 8. COUP-TFII regulates the activity of DSPP promoter through associating with homeoprotein Msx2. (**A-C**) Luciferase reporter assay. (**A-B**) Effects of homeodomain transcription factor Msx2 and Dlx5 on the activation of 3x HRE-Luc reporter or 0.7-kb DSPP-Luc. HEK-293T cells were co-transfected with 200 ng of 3x HRE-Luc or 0.7-kb DSPP-Luc together with Msx2 or Dlx5 expression vectors at the indicated doses (ng/well). (**C**) Cells were co-transfected with 0.7-kb DSPP-Luc reporter together with Msx2 and COUP-TFII expression vectors at the indicated doses (ng/well), and then luciferase reporter assay was done. Western blot analysis confirmed the induction of COUP-TFII and Msx2 protein by the vectors (lower panel). COUP-TFII and Msx2 showed opposite regulation on 0.7-kb *DSPP* promoter. Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group. #, $p < 0.05$; ##, $p < 0.01$ *versus* the indicated group); H, homeodomain response element (HRE)

4-4. Physical Interaction of COUP-TFII and Msx2

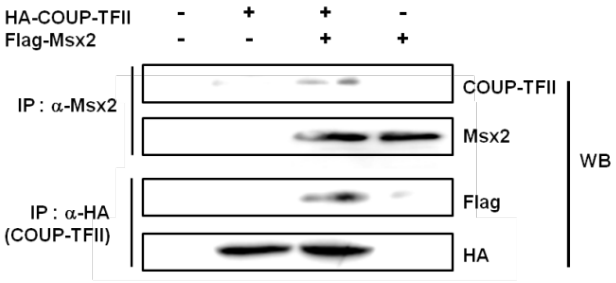
To better understand the mechanism by which COUP-TFII overcame the negative effect of Msx2 on DSPP promoter activity, the protein interaction between COUP-TFII and Msx2 was examined using an immunoprecipitation (IP) assay.

MDPC-23 cells were transfected with HA-tagged COUP-TFII and/or Flag-tagged Msx2 constructs. Immunoprecipitation was performed with anti-Msx2 or anti-HA antibodies, and Western blot analysis was performed with the indicated antibodies. Only HA-COUP-TFII and Flag-Msx2 transfected group showed the positive reactivity, suggesting that COUP-TFII protein can physically interact with Msx2 protein (Fig. 9A).

To examine whether the two proteins are co-localized in the cell level, subsequent immunofluorescence assay was done with anti-COUP-TFII and anti-Msx2 antibodies. MDPC-23 cells were fixed with ethanol, and then reacted with anti-COUP-TFII and anti-Msx2 antibodies and horseradish peroxidase-conjugated secondary antibody. Then, the immunoreactivity for COUP-TFII (red) and Msx2 (green) was photographed with LSM confocal microscopy. DAPI staining was performed for nuclear morphology (Fig. 9B). The signals of COUP-TFII and Msx2 were co-localized in the nucleus (white arrows). The co-localization of COUP-TFII and Msx2 also supports the physical interaction between them.

Taken together, these results indicate that COUP-TFII stimulates DSPP transcription through physically interacting with Msx2.

A


HA-COUP-TFII - + + -
Flag-Msx2 - - + +
IP : α-Msx2
IP : α-HA
(COUP-TFII)
COUP-TFII
Msx2
Flag
HA
WB

B

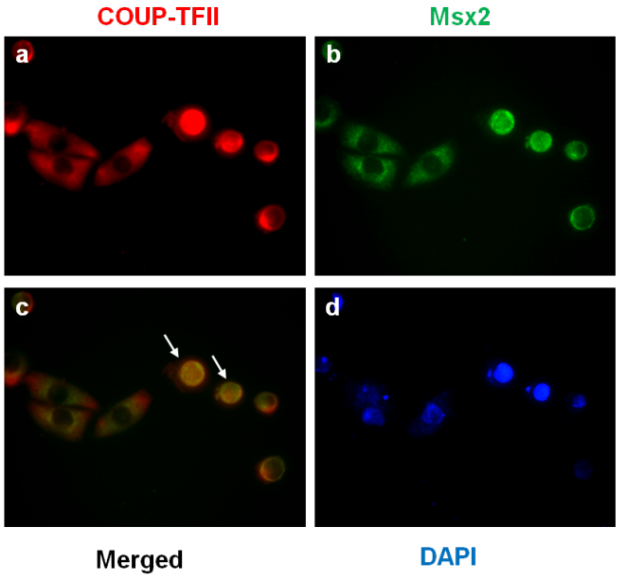

COUP-TFII
Msx2
a
b
c
d
Merged
DAPI

Figure 9. COUP-TFII physically interacts with Msx2. (**A**) Immunoprecipitation assay. MDPC-23 cells were transfected with HA-tagged COUP-TFII and/or Flag-tagged Msx2 constructs. Immunoprecipitation was performed with anti-Msx2 or anti-HA antibodies, and Western blot analysis was performed with the indicated antibodies. (**B**) Fluorescent microphotographs. MDPC-23 cells were fixed with ethanol, and then reacted with anti-COUP-TFII and anti-Msx2 antibodies and horseradish peroxidase-conjugated secondary antibody. Immunoreactivity for COUP-TFII (red) and Msx2 (green) was photographed with LSM confocal microscopy. DAPI staining was performed for nuclear morphology. Note that the signals of COUP-TFII and Msx2 were co-localized in the nucleus (white arrows).

5. Endogenous COUP-TFII Expression during Amloblast Differentiation

Until here, the novel effects of COUP-TF-II on the expression of odontoblastic specific genes (e.g. DMP-1 and DSPP), and the function of dental pulp cells to induce matrix mineralization were discussed.

Previous results (Fig.2) showed that COUP-TFII also expressed in the enamel-forming ameloblast layer. Additional experiment was undertaken to make it clear if COUP-TFII expression is also related to ameloblast differentiation. Mouse ameloblast lineage cells (mALCs) were cultured for 12 days with mineralizing medium under the similar condition with HDPCs or MDPC-23 cells. The mRNA expressions of ALP, COUP-TFII, ameloblast-specific genes ameloblastin, amelogenin and enamelin were analyzed by real-time PCR. Interestingly, the expressions of ALP and the ameloblast-specific genes ameloblastin, amelogenin and enamelin were increased over time, but COUP-TFII expression was not altered, indicating that the action of COUP-TFII could be excluded in ameloblast differentiation (Fig. 10).

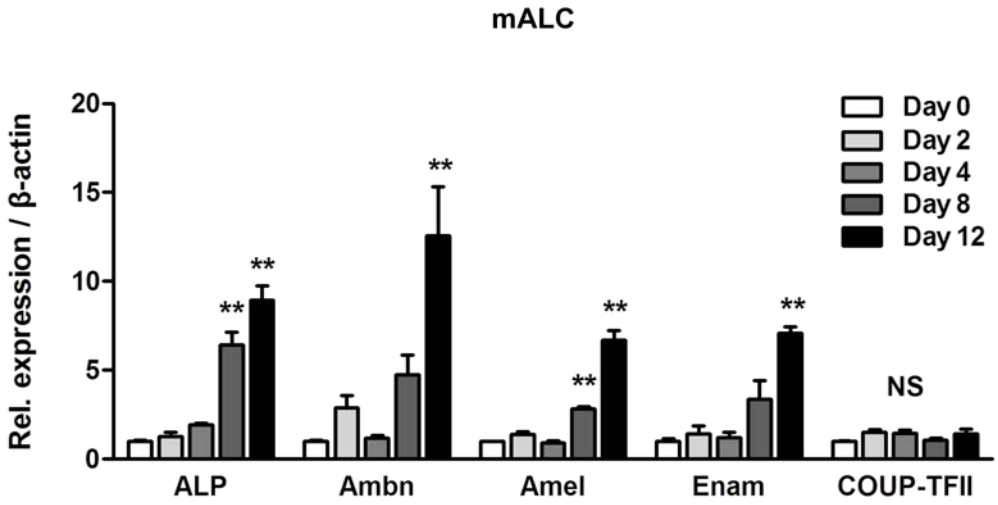


Figure 10. COUP-TFII expression profile during ameloblast differentiation. Real-time PCR analysis. Mouse ameloblast lineage cells (mALCs) were cultured for 12 days in mineralization medium containing 10% FBS, 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate. The culture medium was replaced every other day. Data are expressed as the mean ± S.D. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$ *versus* control group). Ambn, ameloblastin; Amel, amelogenin; Enam, enamelin; NS, not significant

DISCUSSION

In the present study, it was showed that the orphan nuclear receptor COUP-TFII might be another important regulator of DSPP expression and mineralization in odontoblast lineage cells. COUP-TFII expression was increased during the differentiation of isolated HDPCs and development of rat tooth germ. Overexpression of COUP-TFII increased the expression of DMP-1 and DSPP, and mineralized nodule formation. In contrast, knockdown of COUP-TFII expression decreased them. Besides, COUP-TFII stimulated DSPP transcription by directly binding to the DSPP promoter and physically interacting with Msx2 protein.

COUP-TFII is a key regulator that decides the commitment and differentiation of mesenchymal stem cells (MSCs) into multiple cell lineages. For example, COUP-TFII stimulates the differentiation of precursors into adipocyte and chondrocyte lineages with the increase of peroxisome proliferator-activated receptor-gamma (PPARγ) and Sox-9 expression, while it inhibits Wnt signaling and Runx2 activity to impede MSC access to osteogenic and myogenic lineages (21). Because odontoblasts are also differentiated from MSCs, I aimed to explore the role of COUP-TFII in odontoblastic differentiation of HDPCs. The gain-or-loss of function studies showed that COUP-TFII may be a stimulatory regulator for matrix mineralization or expression of odontoblast-specific gene in HDPCs. To clarify the stimulatory role of COUP-TFII in the body, more extensive animal studies including knockout or transgenic mice are needed.

DSPP and DMP-1 are highly phosphorylated proteins in the dentin matrix, and they are essential for the proper development of teeth and bones (34-37). According to the previous reports, homeodomain transcriptional factors including Hox group, Dlxs and Msxs are involved in the hard tissue development along with changes in DSPP and DMP-1 expression (38-40). Among them, Msx2 and Dlx5 have an opposite effect on expression of

mineralization-related genes, such as Runx2 (41,42), Osteocalcin (43) and DSPP (33).

In this study, altered expression of COUP-TFII could induce or inhibit DSPP and DMP-1 expression in odontoblast lineage cells (Figs. 4A and 5A). The results prompted an exploration of a possible molecular mechanism by which COUP-TFII regulates DSPP expression with promoter study and immunoprecipitation assays. COUP-TFII could stimulate DSPP transcription by directly binding into specific regions of DSPP promoter gene. In addition, COUP-TFII activated DSPP transcription, overcoming the negative effect of Msx2 on DSPP promoter and the two proteins physically interacted. Based on these results, I assume that COUP-TFII can also stimulate dentin matrix mineralization with transcriptional activation of matrix protein genes. However, there was no evidence that COUP-TFII activates DMP-1 transcription, although it is also another important matrix protein of dentin and possesses the potential to enhance DSPP expression (35,36). Further studies will be needed to determine whether COUP-TFII directly regulates DMP-1 transcription.

In the immunostaining experiment, COUP-TFII expression in pulp tissues increased as tooth germ development progressed (Fig. 1C and Fig. 2), supporting the hypothesis that COUP-TFII might stimulate dentinal matrix mineralization. However, the immunoreactivity of COUP-TFII at PN day 1 tooth germ of mouse was observed in the odontoblast layer as well as the ameloblast layer (Fig. 2). To figure out whether COUP-TFII expression is related to ameloblast differentiation, additional real-time PCR analysis was carried out in mouse ameloblast lineage cells cultured with mineralizing medium. Expression of the ameloblast-specific genes ameloblastin, amelogenin and enamelin increased over time, but COUP-TFII expression was not altered (Fig. 10), indicating that COUP-TFII may not be involved in amelogenesis. More supportive experiments will be needed about this issue.

Runx2 is also a key transcription factor that regulates bone and tooth formation. Osteoblast differentiation occurs along with Runx2 activation. However, at the onset of odontoblast differentiation, Runx2 expression is markedly down-regulated (44,45).

Prolonged Runx2 expression inhibits the terminal differentiation into odontoblasts along with a decrease in DSPP expression. The severe reduction of the dentin structure results in trans-differentiation into osteoblasts (46,47). In addition, our previous study showed that COUP-TFII negatively regulates osteoblast differentiation by inhibiting Runx2 activity (22). It is still possible that COUP-TFII might regulate DSPP expression and matrix mineralization through another pathway modulating Runx2 activity in odontoblasts.

Overall, this study provides evidence that COUP-TFII has a stimulatory role in DSPP transcription and matrix mineralization in odontoblast lineage cells. The findings also provide new insights that COUP-TFII has potential as a novel therapeutic target for dentin regeneration.

REFERENCES

1. Ruch, J. V., Lesot, H., and Begue-Kirn, C. (1995) Odontoblast differentiation. *Int J Dev Biol* **39**, 51-68
2. Thesleff, I. (2003) Epithelial-mesenchymal signalling regulating tooth morphogenesis. *J Cell Sci* **116**, 1647-1648
3. Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* **97**, 13625-13630
4. Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., DenBesten, P., Robey, P. G., and Shi, S. (2002) Stem cell properties of human dental pulp stem cells. *J Dent Res* **81**, 531-535
5. Gopinathan, G., Kolokythas, A., Luan, X., and Diekwisch, T. G. (2013) Epigenetic marks define the lineage and differentiation potential of two distinct neural crest-derived intermediate odontogenic progenitor populations. *Stem Cells Dev* **22**, 1763-1778
6. Sreenath, T., Thyagarajan, T., Hall, B., Longenecker, G., D'Souza, R., Hong, S., Wright, J. T., MacDougall, M., Sauk, J., and Kulkarni, A. B. (2003) Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. *J Biol Chem* **278**, 24874-24880
7. Balic, A., and Mina, M. (2011) Identification of secretory odontoblasts using DMP1-GFP transgenic mice. *Bone* **48**, 927-937
8. Qin, C., Brunn, J. C., Cadena, E., Ridall, A., Tsujigiwa, H., Nagatsuka, H., Nagai, N., and Butler, W. T. (2002) The expression of dentin sialophosphoprotein gene in bone. *J Dent Res* **81**, 392-394

9. Kim, J. W., Hu, J. C., Lee, J. I., Moon, S. K., Kim, Y. J., Jang, K. T., Lee, S. H., Kim, C. C., Hahn, S. H., and Simmer, J. P. (2005) Mutational hot spot in the DSPP gene causing dentinogenesis imperfecta type II. *Hum Genet* **116**, 186-191

10. Lee, S. K., Lee, K. E., Jeon, D., Lee, G., Lee, H., Shin, C. U., Jung, Y. J., Lee, S. H., Hahn, S. H., and Kim, J. W. (2009) A novel mutation in the DSPP gene associated with dentinogenesis imperfecta type II. *J Dent Res* **88**, 51-55

11. Zhang, X., Zhao, J., Li, C., Gao, S., Qiu, C., Liu, P., Wu, G., Qiang, B., Lo, W. H., and Shen, Y. (2001) DSPP mutation in dentinogenesis imperfecta Shields type II. *Nat Genet* **27**, 151-152

12. Holappa, H., Nieminen, P., Tolva, L., Lukinmaa, P. L., and Alaluusua, S. (2006) Splicing site mutations in dentin sialophosphoprotein causing dentinogenesis imperfecta type II. *Eur J Oral Sci* **114**, 381-384

13. McKnight, D. A., Suzanne Hart, P., Hart, T. C., Hartsfield, J. K., Wilson, A., Wright, J. T., and Fisher, L. W. (2008) A comprehensive analysis of normal variation and disease-causing mutations in the human DSPP gene. *Hum Mutat* **29**, 1392-1404

14. Song, Y., Wang, C., Peng, B., Ye, X., Zhao, G., Fan, M., Fu, Q., and Bian, Z. (2006) Phenotypes and genotypes in 2 DGI families with different DSPP mutations. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **102**, 360-374

15. Dong, J., Gu, T., Jeffords, L., and MacDougall, M. (2005) Dentin phosphoprotein compound mutation in dentin sialophosphoprotein causes dentinogenesis imperfecta type III. *Am J Med Genet A* **132A**, 305-309

16. Rajpar, M. H., Koch, M. J., Davies, R. M., Mellody, K. T., Kielty, C. M., and Dixon, M. J. (2002) Mutation of the signal peptide region of the bicistronic gene DSPP affects translocation to the endoplasmic reticulum and results in defective dentine biomineralization. *Hum Mol Genet* **11**, 2559-2565

17. Pereira, F. A., Tsai, M. J., and Tsai, S. Y. (2000) COUP-TF orphan nuclear receptors in development and differentiation. *Cell Mol Life Sci* **57**, 1388-1398

18. Pereira, F. A., Qiu, Y., Tsai, M. J., and Tsai, S. Y. (1995) Chicken ovalbumin upstream promoter transcription factor (COUP-TF): expression during mouse embryogenesis. *J Steroid Biochem Mol Biol* **53**, 503-508

19. Qin, J., Chen, X., Xie, X., Tsai, M. J., and Tsai, S. Y. (2010) COUP-TFII regulates tumor growth and metastasis by modulating tumor angiogenesis. *Proc Natl Acad Sci U S A* **107**, 3687-3692

20. Lin, F. J., Qin, J., Tang, K., Tsai, S. Y., and Tsai, M. J. (2011) Coup d'Etat: an orphan takes control. *Endocr Rev* **32**, 404-421

21. Xie, X., Qin, J., Lin, S. H., Tsai, S. Y., and Tsai, M. J. (2011) Nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) modulates mesenchymal cell commitment and differentiation. *Proc Natl Acad Sci U S A* **108**, 14843-14848

22. Lee, K. N., Jang, W. G., Kim, E. J., Oh, S. H., Son, H. J., Kim, S. H., Franceschi, R., Zhang, X. K., Lee, S. E., and Koh, J. T. (2012) Orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) protein negatively regulates bone morphogenetic protein 2-induced osteoblast differentiation through suppressing runt-related gene 2 (Runx2) activity. *J Biol Chem* **287**, 18888-18899

23. Kim, J. W., Choi, H., Jeong, B. C., Oh, S. H., Hur, S. W., Lee, B. N., Kim, S. H., Nor, J. E., Koh, J. T., and Hwang, Y. C. (2014) Transcriptional factor ATF6 is involved in odontoblastic differentiation. *J Dent Res* **93**, 483-489

24. Oh, S. H., Hwang, Y. C., Yang, H., Kang, J. H., Hur, S. W., Jung, N. R., Jang, W. G., Lee, K. N., Oh, W. M., Park, J. C., Kim, S. H., and Koh, J. T. (2012) SHP is involved in BMP2-induced odontoblast differentiation. *J Dent Res* **91**, 1124-1129

25. Yamanobe, T., Okada, F., Iuchi, Y., Onuma, K., Tomita, Y., and Fujii, J. (2007) Deterioration of ischemia/reperfusion-induced acute renal failure in SOD1-deficient mice. *Free Radic Res* **41**, 200-207

26. Hwang, Y. C., Hwang, I. N., Oh, W. M., Park, J. C., Lee, D. S., and Son, H. H. (2008) Influence of TGF-beta1 on the expression of BSP, DSP, TGF-beta1 receptor I and Smad proteins during reparative dentinogenesis. *J Mol Histol* **39**, 153-160

27. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408

28. Lee, Y. S., Kim, D. K., Kim, Y. D., Park, K. C., Shong, M., Seong, H. A., Ha, H. J., and Choi, H. S. (2008) Orphan nuclear receptor SHP interacts with and represses hepatocyte nuclear factor-6 (HNF-6) transactivation. *Biochem J* **413**, 559-569

29. Kim, H. J., Jeon, S. K., Kang, J. H., Kim, M. S., Ko, H. M., Jung, J. Y., Koh, J. T., Kim, W. J., Lee, E. J., Lim, H. P., and Kim, S. H. (2009) Expression of DCC in differentiating ameloblasts from developing tooth germs in rats. *Arch Oral Biol* **54**, 563-569

30. Chen, S., Gluhak-Heinrich, J., Martinez, M., Li, T., Wu, Y., Chuang, H. H., Chen, L., Dong, J., Gay, I., and MacDougall, M. (2008) Bone morphogenetic protein 2 mediates dentin sialophosphoprotein expression and odontoblast differentiation via NF-Y signaling. *J Biol Chem* **283**, 19359-19370

31. Fang, B., Mane-Padros, D., Bolotin, E., Jiang, T., and Sladek, F. M. (2012) Identification of a binding motif specific to HNF4 by comparative analysis of multiple nuclear receptors. *Nucleic Acids Res* **40**, 5343-5356

32. Zhang, H., Hu, G., Wang, H., Sciavolino, P., Iler, N., Shen, M. M., and Abate-Shen, C. (1997) Heterodimerization of Msx and Dlx homeoproteins results in functional antagonism. *Mol Cell Biol* **17**, 2920-2932

33. Cho, Y. D., Yoon, W. J., Woo, K. M., Baek, J. H., Park, J. C., and Ryoo, H. M. (2010) The canonical BMP signaling pathway plays a crucial part in stimulation of dentin sialophosphoprotein expression by BMP-2. *J Biol Chem* **285**, 36369-36376

34. D'Souza, R. N., Cavender, A., Sunavala, G., Alvarez, J., Ohshima, T., Kulkarni, A. B., and MacDougall, M. (1997) Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. *J Bone Miner Res* **12**, 2040-2049

35. Narayanan, K., Gajjeraman, S., Ramachandran, A., Hao, J., and George, A. (2006) Dentin matrix protein 1 regulates dentin sialophosphoprotein gene transcription during early odontoblast differentiation. *J Biol Chem* **281**, 19064-19071

36. Gibson, M. P., Zhu, Q., Wang, S., Liu, Q., Liu, Y., Wang, X., Yuan, B., Ruest, L. B., Feng, J. Q., D'Souza, R. N., Qin, C., and Lu, Y. (2013) The rescue of dentin matrix protein 1 (DMP1)-deficient tooth defects by the transgenic expression of dentin sialophosphoprotein (DSPP) indicates that DSPP is a downstream effector molecule of DMP1 in dentinogenesis. *J Biol Chem* **288**, 7204-7214

37. Suzuki, S., Haruyama, N., Nishimura, F., and Kulkarni, A. B. (2012) Dentin sialophosphoprotein and dentin matrix protein-1: Two highly phosphorylated proteins in mineralized tissues. *Arch Oral Biol* **57**, 1165-1175

38. Thomas, B. L., Tucker, A. S., Ferguson, C., Qiu, M., Rubenstein, J. L., and Sharpe, P. T. (1998) Molecular control of odontogenic patterning: positional dependent initiation and morphogenesis. *Eur J Oral Sci* **106 Suppl 1**, 44-47

39. Alappat, S., Zhang, Z. Y., and Chen, Y. P. (2003) Msx homeobox gene family and craniofacial development. *Cell Res* **13**, 429-442

40. Premkumar, S. (2011) Textbook of Craniofacial Growth; In Genetics and caniofacial growth. pp 328-329

41. Lee, M. H., Kim, Y. J., Yoon, W. J., Kim, J. I., Kim, B. G., Hwang, Y. S., Wozney, J. M., Chi, X. Z., Bae, S. C., Choi, K. Y., Cho, J. Y., Choi, J. Y., and Ryoo, H. M. (2005) Dlx5 specifically regulates Runx2 type II expression by binding to homeodomain-response elements in the Runx2 distal promoter. *J Biol Chem* **280**, 35579-35587

42. Shirakabe, K., Terasawa, K., Miyama, K., Shibuya, H., and Nishida, E. (2001) Regulation of the activity of the transcription factor Runx2 by two homeobox proteins, Msx2 and Dlx5. *Genes Cells* **6**, 851-856

43. Newberry, E. P., Latifi, T., and Towler, D. A. (1998) Reciprocal regulation of osteocalcin transcription by the homeodomain proteins Msx2 and Dlx5. *Biochemistry* **37**, 16360-16368

44. D'Souza, R. N., Aberg, T., Gaikwad, J., Cavender, A., Owen, M., Karsenty, G., and Thesleff, I. (1999) Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. *Development* **126**, 2911-2920

45. Komori, T. (2010) Regulation of bone development and extracellular matrix protein genes by RUNX2. *Cell Tissue Res* **339**, 189-195

46. Miyazaki, T., Kanatani, N., Rokutanda, S., Yoshida, C., Toyosawa, S., Nakamura, R., Takada, S., and Komori, T. (2008) Inhibition of the terminal differentiation of odontoblasts and their transdifferentiation into osteoblasts in Runx2 transgenic mice. *Arch Histol Cytol* **71**, 131-146

47. Li, S., Kong, H., Yao, N., Yu, Q., Wang, P., Lin, Y., Wang, J., Kuang, R., Zhao, X., Xu, J., Zhu, Q., and Ni, L. (2011) The role of runt-related transcription factor 2 (Runx2) in the late stage of odontoblast differentiation and dentin formation. *Biochem Biophys Res Commun* **410**, 698-704

상아질모세포 분화에서의 COUP-TFII 의 역할

허 성 웅

전남대학교 대학원 치의학과

(지도교수 : 고정태)

(국문초록)

스테로이드-갑상선 호르몬 수용에 속하는 고아 핵 수용체인 Chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII; NR2F2, nuclear receptor subfamily 2, group f, member2)는 다양한 조직들에서 세포분화의 운명을 결정하는 중요한 역할을 담당한다. 그러나 치아발생에 있어서 COUP-TFII 의 구체적인 역할은 아직 밝혀지지 않고 있다. 이에 본 연구에서는 COUP-TFII 가 상아질 모세포 분화에 미치는 영향에 대해 규명하고자 하였다.

흰쥐 상악 제2대구치에서 내인성 COUP-TFII 의 발현은 치아발달이 진행됨에 따라 증가하는 경향을 보였다. 또한, 생후 9일째 흰쥐의 제2대구치(치근형성기)의 상아질모세포층에서는 제3대구치(모상기)에서의 그것보다 더 많은 양의 COUP-TFII 단백질이 탐지되었다. 생후 1일에 기질단백 분비기에 있는 생쥐 상악 제1대구치 치배에서도 COUP-TFII 단백질이 상아질모세포층을 따라 발현하였다. 일차배양한 인간 치수세포와 생쥐 치수유래세포를 석회화 배지에서 배양

하였을 때, COUP-TFII 의 발현이 상아질모세포 특이 단백질 DMP-1 과 DSPP 의 증가와 동반하여 함께 증가되었다. 상아질모세포에서 아데노바이러스 시스템을 이용한 COUP-TFII 의 과발현은 상아질모세포 특이 유전자들의 발현을 상향 조절함과 동시에 알칼리성 인산가수분해효소(alkaline phosphatase)의 활성과 기질 석회화 정도를 모두 증가시켰다. 반대로, siRNA을 이용한 COUP-TFII 의 하향 조절은 상아질 모세포 특이 유전자들의 발현과 기질 석회화를 모두 감소시켰다. 기전적인 연구에서는 COUP-TFII 가 DSPP 프로모터에 직접 결합함으로써 DSPP 의 전사를 증가시킴을 보여주었다. 뿐만 아니라, COUP-TFII 는 호메오도메인 (homeodomain) 전사인자인 Msx2 와 물리적인 상호작용을 하여 DSPP 프로모터 활성에 대한 Msx2의 작용을 길항적으로 조절하였다.

이상의 결과들은 COUP-TFII 가 상아질모세포에서 DSPP 의 발현을 증가시킴과 더불어 기질의 석회화를 촉진시키는 역할을 한다는 것을 시사한다.