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Nuclear Factor I-C (NFIC) Regulates Dentin Sialophosphoprotein (DSPP) and E-cadherin via Control of Krüppel-like Factor 4 (KLF4) During Dentinogenesis*

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Hye-Kyung Lee[‡], Dong-Seol Lee[‡], Su-Jin Park[‡], Kwang-Hee Cho[§], Hyun-Sook Bae[§], and Joo-Cheol Park[‡1]

From the [‡]Department of Oral Histology-Developmental Biology and Dental Research Institute, School of Dentistry, Seoul National University, 101 Daehagro, Chongro-gu, Seoul 110-749, Korea and the [§]Department of Dental Hygiene, Namseoul University, Cheon-An, Chung-Nam 331-707, Korea

Background: The role of NFIC and KLF4 and their interrelationship during dentinogenesis remain unclear.
Results: Our study establishes the *Nfic-Klf4* dentin matrix protein 1 (*Dmp1*)-*Dspp* pathway in odontoblasts.
Conclusion: NFIC regulates KLF4 during dentinogenesis.
Significance: These data demonstrate the regulation of DSPP via NFIC-KLF4 axis.

Odontoblasts are a type of terminally differentiated matrix-secreting cells. A number of molecular mechanisms are involved in the differentiation of odontoblasts. Several studies demonstrated that Krüppel-like factor 4 (KLF4) promotes odontoblast differentiation via control of dentin sialophosphoprotein (DSPP). Because nuclear factor I-C (NFIC) is also known to control DSPP, we investigated the relationship between NFIC and KLF4 during odontoblast differentiation. *Klf4* mRNA expression was significantly decreased in *Nfic*$^{-/-}$ pulp cells compared with wild type cells. In immunohistochemistry assays, dentin matrix protein 1 (Dmp1), and DSP protein expression was barely observed in *Nfic*$^{-/-}$ odontoblasts and dentin matrix. *Nfic* bound directly to the *Klf4* promoter and stimulated *Klf4* transcriptional activity, thereby regulating Dmp1 and DSPP expression during odontoblast differentiation. *Nfic* or *Klf4* overexpression promoted mineralized nodule formation in MDPC-23 cells. In addition, *Nfic* overexpression also decreased *Slug* luciferase activity but augmented E-cadherin promoter activity via up-regulation of *Klf4* in odontoblasts. Our study reveals important signaling pathways during dentinogenesis: the *Nfic-Klf4-Dmp1-Dspp* and the *Nfic-Klf4*-E-cadherin pathways in odontoblasts. Our results indicate the important role of NFIC in regulating KLF4 during dentinogenesis.

Odontoblasts are a type of neural crest-derived matrix-secreting cells that are vital for dentin formation and mineralization (1). During odontoblast differentiation, regulation of dentin sialophosphoprotein (*DSPP*)[2] expression is critical because *DSPP* is a representative odontoblast marker gene (2).

The nuclear factor I (NFI) family of site-specific transcription factors, encoded by four genes in vertebrates (termed *Nfia*, *Nfib*, *Nfic*, and *Nfix*), plays essential developmental roles in the transcriptional modulation of various cell types (3). NFI proteins contain a highly conserved NH_2-terminal domain that mediates DNA binding and a dimerization domain is gene-specific (4). Disruption of the *Nfic* gene in mice leads to abnormal tooth roots that are predominantly caused by abnormal odontoblast differentiation and dentin formation during root formation (5–7). Previously, we suggested that *Nfic* transcriptionally activates *DSPP* expression (8).

The roles of Krüppel-like factor 4 (KLF4) have been extensively studied in many different physiological processes, including development, cytodifferentiation, and maintenance of normal tissue homeostasis (9). KLF4 is specifically expressed in the polarizing and elongating odontoblasts of the mouse incisor at embryonic day 16.5, and in the molar from embryonic day 18.5 to postnatal day 3 (10). Recently, *KLF4* overexpression in human dental pulp cells led to down-regulate the cell proliferation rate and up-regulate odontoblast-related genes, such as dentin matrix protein 1 (*DMP1*), *DSPP*, and alkaline phosphatase (*ALP*) (11, 12). Therefore, NFIC and KLF4 are important regulators of odontoblast-related genes, but the interrelationship between NFIC and KLF4 during odontoblast differentiation remains unclear. We investigated the roles of NFIC and KLF4 and their relationship during odontoblast differentiation and dentin formation.

EXPERIMENTAL PROCEDURES

DNA Microarray—Total RNA from pulp cells of wild type (*Nfic*$^{+/+}$) and *Nfic*$^{-/-}$ mice were sent to Origen Labs for DNA microarray analysis on the GeneChip Sample Cleanup Module (Qiagen, Valencia, CA). Expression data were analyzed using Affymetrix MicroArray Suite (version 5.0). Signal intensities of all probe sets were scaled to the target value of 500.

Primary Cell Culture and Cell Lines—The mandibles were removed from 17-day-old (P17) wild type and *Nfic*$^{-/-}$ mice, and primary pulp cell culture was conducted as described previously (6, 13). Briefly, after the incisors were dissected out, they were cracked longitudinally using a 27-gauge

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[1] To whom correspondence should be addressed: Dept. of Oral Histology-Developmental Biology, School of Dentistry, Seoul National University, 101 Daehagro, Chongro-gu, Seoul 110-749, Korea. Tel.: 82-2-740-8668; Fax: 82-2-763-3613; E-mail: jcapark@snu.ac.kr.
[2] The abbreviations used are: DSPP, dentin sialophosphoprotein; NFIC, nuclear factor I-C; KLF4, Krüppel-like factor 4; DMP1, dentin matrix protein 1; ALC, ameloblast lineage cells.

needle on a 1-ml syringe. Pulp tissues were then minced to explants and placed in 60-mm culture dishes (Nunc, Rochester, NY). Explants were weighed down with a sterile cover glass and cultured in DMEM Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics (100 units/ml, penicillin-G, 100 mg/ml streptomycin, and 2.5 mg/ml fungizone, Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, and cells at passage 2 were used in the experiments. All experiments using mice were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-111013-2).

MDPC-23 (a generous gift from Dr. J. E. Nör, School of Dental Medicine, University of Michigan, Ann Arbor, MI) and HEK293 cells (ATCC, Rockville, MD) were grown and maintained in DMEM supplemented with 10% FBS and antibiotics in a 5% CO_2 atmosphere at 37 °C. Ameloblast lineage cells (ALC; a generous gift from Dr. T. Sugiyama, Akita University School of Medicine, Akita, Japan) were cultured in minimum essential medium (Invitrogen) supplemented with 5% FBS, 10 ng/ml recombinant human epithelial growth factor (EGF; Sigma-Aldrich), and antibiotics. To induce differentiation of MDPC-23 and ALC cells, 80–90% confluent cells were cultured in DMEM or minimum essential medium supplemented with 5% FBS, ascorbic acid (50 mg/ml), and β-glycerophosphate (10 mM) for up to 1 or 2 weeks.

Plasmid Constructs—The pCH-*Nfic* expression plasmid was provided by Dr. R. M. Gronostajski (State University of New York at Buffalo, Buffalo, NY) (14). siRNAs were synthesized (Integrated DNA Technologies, San Diego, CA) based on the chosen 19 nucleotides of *Nfic* (5′-CCG GTG AAG AAG ACA GAG A-3′), and these siRNA plasmids were prepared using the pSUPER-retro-neo-GFP retro virus siRNA expression vector (OligoEngine, Seattle, WA) according to the manufacturer's instructions. *Klf4* cDNAs were amplified by PCR and subcloned into FLAG-tagged pcDNA3 vector (Invitrogen). The pcDNA3-FLAG-*Slug*, pGL2-*Klf4*, -*Slug*, and -E-cadherin plasmids were purchased from Origene Company (Rockville, MD). Deletion mutants of the *Klf*4 promoter regions were generated by PCR amplification and subcloned into pGL3 (Promega, Madison, WI).

Real-time PCR Analyses—Total RNA was extracted from MDPC-23 cells as well as pulp tissue using TRIzol® reagent according to the manufacturer's instructions (Invitrogen). Total RNA (2 μg) was reverse transcribed for 1 h at 50 °C with 0.5 mg oligo(dT) and 1 μl (50 international units) Superscript III enzyme (Invitrogen) in a 20-μl reaction. One microliter of the reverse transcription product was PCR-amplified using the primer pairs. For real-time PCR, the specific primers for *Nfic*, *Snail*, *Slug*, *Klf4*, vimentin, E-cadherin, N-cadherin, *Dmp1*, and *Dspp* were synthesized as listed in Table 1. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA) using SYBR GREEN PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were 95 °C for 1 min, 94 °C for 15 s, and 60 °C for 34 s for 40 cycles. All reactions were run in triplicate and were normalized to the housekeeping gene GAPDH. Relative differences in PCR results were calculated using the comparative cycle threshold (C_T) method.

TABLE 1
Real-time PCR primer sequences

Gene name	Primer
mNfic	
Forward	5′-GAC CTG TAC CTG GCC TAC TTT G-3′
Reverse	5′-CAC ACC TGA CGT GAC AAA GCT C-3′
mKlf4	
Forward	5′-CTG AAC AGC AGG GAC TGT-3′
Reverse	5′-GTG TGG GTG GCT GTT CTT TT-3′
mSnail	
Forward	5′-TCT GAA GAT GCA CAT CCG AAG C-3′
Reverse	5′-TTG CAG TGG GAG CAG GAG AAT-3′
mSlug	
Forward	5′-GGC TGC TTC AAG GAC ACA TTA GAA C-3′
Reverse	5′-GGT CTG CAG ATG TGC CCT CA-3′
mE-cadherin	
Forward	5′-CGT CCT GCC AAT CCT GAT GA-3′
Reverse	5′-ACC ACT GCC CTC GTA ATC GAA C-3′
mVimentin	
Forward	5′-AAA GCG TGG CTG CCA AGA AC-3′
Reverse	5′-GTG ACT GCA CCT GTC TCC GGT A-3′
mN-cadherin	
Forward	5′-CGC CAA TCA ACT TGC CAG AA-3′
Reverse	5′-TGG CCC AGT GAC GCT GTA TC-3′
mDmp1	
Forward	5′-AGT GAAG TCA TCA GAA GAA AGT CA-3′
Reverse	5′-TAC TGG CCT CTG TCG TAG CC-3′
mDspp	
Forward	5′-ATT CCG GTT CCC CAG TTA GTA-3′
Reverse	5′-CTG TTG CTA GTG GTG CTG TT-3′
mGapdh	
Forward	5′-AGG TCG GTG TGA ACG GAT TTG-3′
Reverse	5′-TGT AGA CCA TGT AGT TGA GGT CA-3′

Western Blot Analyses—To prepare whole cell extracts, cells were washed three times with PBS, scraped into 1.5-ml tubes, and pelleted by centrifugation at 12,000 rpm for 2 min at 4 °C. After removal of the supernatant, pellets were suspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 2 mM EDTA, pH 7.4) and incubated for 15 min on ice. Cell debris was removed by centrifugation. Proteins (30 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). 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 °C with the primary antibody diluted in PBS-T buffer (1:1000). Rabbit polyclonal anti-Nfic and anti-DSP antibodies were produced as described previously (6). The mouse monoclonal anti-HA (MMS-101P) antibody was purchased from Covance (Emeryville, CA). Other antibodies, including E-cadherin (sc-7870), N-cadherin (sc-7939), TGFβ-RI (sc-398), Slug (sc-1539), and GAPDH (sc-25778), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-E-cadherin (3195) antibody was purchased from Cell Signaling Technology (Danvers, MA). Antibodies against N-cadherin (ab12221) and Dmp1 (ab103203) were purchased from Abcam (Cambridge, MA). After washing, membranes were incubated for 1 h with anti-mouse (sc-2031), anti-rabbit (sc-2004), or anti-goat (sc-2768) IgG secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Labeled protein bands were detected using an enhanced chemiluminescence system (Dogen, Cambridge, MA). The quantification analyses were performed using ImageJ (http://imagej.nih.gov/ij/).

TABLE 2
The list for ChIP assay primer

Promoter name	Primer
mSlug pro	
Forward	5′-GAA GCC AAG TCG CCG TAG-3′
Reverse	5′-GGC GCC TCT GAA GTC ACC-3′
mKlf4 pro	
Forward	5′-CAT TGC AGC GCT CCT CTT C-3′
Reverse	5′-GGC TCG AAA GTC CTG CCA-3′
mE-cad pro	
Forward	5′-AAA AGG GAG CCG GTC AGG T-3′
Reverse	5′-AGG AGT CTA GCA GAA GTT CTT-3′

Transient Transfection and Luciferase Assays—HEK293 and MDPC-23 cells were seeded in 12-well culture plates at a density of 1.5×10^5 cells per well. Cells were transiently transfected with reporter constructs using Metafectene PRO reagent (Biontex, Planegg, Martinsried, Germany). pGL2-*Klf4*, -*Slug*, -E-cadherin, or pGL3-*DSPP* were transfected into cells with *Nfia*, *Nfib*, *Nfic*, *Nfix*, *Klf4*, or *Slug* vectors, or the *Nfic* siRNA vector. Also, cells were transiently transfected with *Klf4* siRNA (Santa Cruz Biotechnology) using Lipofectamine RNAiMAX reagent (Invitrogen). Following the addition of luciferin (50 μl) to the cell lysate (50 μl), luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions (Promega, Madison, WI).

Cells were transiently transfected with microRNA (miR) control and miR145 (Invitrogen) using Lipofectamine RNAiMAX reagent (Invitrogen). Following the addition of lysis buffer (100 μl) to the cell lysate, Western blot assay was performed.

Chromatin Immunoprecipitation (ChIP) Assays—After transfection with the indicated plasmid DNA using the metafectene Pro reagent (Biontex), MDPC-23 cells were treated with formaldehyde (1% final concentration) for 10 min at 37 °C, rinsed twice with cold PBS, and swollen on ice in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1)) for 10 min. Nuclei were collected and sonicated on ice. Supernatants were obtained by centrifugation for 10 min and were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl). The fragmented chromatin mixture was incubated for 4 h with anti-Nfic, anti-Klf4, or anti-FLAG antibodies (F3165, Sigma-Aldrich, 1:250) on a rotator at 4 °C. Protein A/G PLUS-agarose (30 μl; sc-2003, Santa Cruz Biotechnology) was added and incubated at 4 °C for 1 h with rotation to collect the antibody/chromatin complex. The precipitated chromatin complexes were recovered and reversed according to the manufacturer's protocol (Upstate Biotechnology). The final DNA pellets were recovered and analyzed by PCR using the specific primers for *Slug* (300 bp), *Klf4* (650 bp), or the E-cadherin promoter region (750 bp) listed in Table 2. The following PCR conditions were used: 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 1 min for 35 cycles. PCR products were electrophoresed in 1% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

DNA Affinity Precipitation Assays—Transfected MDPC-23 cells were washed with ice-cold PBS, collected by centrifugation, and resuspended in RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF) supplemented with protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). Lysates were rotated on a rotating platform for 30 min at 4 °C and purified by centrifugation at 13,000 rpm for 5 min at 4 °C. Binding assays were performed by mixing nuclear extract proteins (600 μg) and biotinylated specific wild type or mutated Nfic binding site oligonucleotides (6 μg) (Cosmogenetech, Seoul, Korea) of Klf4 promoter in binding buffer (12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 4 mM Tris-Cl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT). Mutated positions in the sequence are *underlined*. Lysates were incubated at room temperature for 30 min. Next, 60 μl of streptavidin-agarose beads (Thermo Scientific, Rockford, IL) were added. The mixture was incubated for 2 h at 4 °C with rotating. Beads were pelleted and washed three times with PBS. Nfic (wild type or mutant forms) bound to the oligonucleotides was detected by SDS-PAGE and immunoblotting using the mouse monoclonal anti-Nfic antibody.

Tissue Preparation and Immunohistochemistry—All experiments involving animals were performed according to the Dental Research Institute guidelines of Seoul National University. Mice tooth were decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and processed for immunohistochemistry. Sections were incubated overnight at 4 °C with rabbit polyclonal Nfic, Klf4, E-cadherin, N-cadherin, Dmp1, and DSP as the primary antibodies (dilutions of 1:100–1:200). Secondary anti-rabbit or -mouse IgG antibodies were added to the sections for 30 min at room temperature and then reacted with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Signals were converted using a diaminobenzidine kit (Vector Laboratories). Nuclei were stained with hematoxylin.

Alizarin Red S Staining—Cells were fixed for 20 min with 4% paraformaldehyde in PBS. Cell were stained with 1% alizarin red S (Sigma-Aldrich) solution in 0.1% NH_4OH (pH 4.2) for 20 min at room temperature. Stained cells were collected by centrifugation at 13,000 rpm for 10 min at 4 °C. Cell lysates were solubilized by vortexing or pipetting with 0.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature. Solubilized stain (0.1 ml) was transferred to the wells of a 96-well plate, and absorbance was measured at 405 nm.

Fluorescence Microscopy—Cells in Laboratory-Tek chamber slides (Nunc) were washed with PBS, fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, and permeabilized for 5 min in PBS containing 0.5% Triton X-100. After washing and blocking, cells were incubated for 1 h with anti-Nfic (1:200) and Alexa Fluor® 488 Phalloidin (A12379, Invitrogen) antibodies in blocking buffer (PBS and 1% bovine serum albumin), followed by the addition of a fluorescein isothiocyanate (FITC) or Cy3-conjugated anti-rabbit IgG antibody (1:200, Amersham Biosciences). After washing, cells were visualized using fluorescence microscopy (AX70; Olympus Optical Co., Tokyo, Japan). Chromosomal DNA in the nucleus was stained using DAPI.

Statistical Analyses—Statistical analyses were performed using Student's t tests. Statistical significance is denoted (*, $p <$ 0.01). All statistical analyses were performed using SPSS software (version 19.0; IBM Corp., Armonk, NY).

RESULTS

Differential Gene Expression Profiling in Nfic$^{-/-}$ Pulp Cells—*Nfic*$^{-/-}$ mice exhibited a lack of dentin formation by abnormal odontoblasts, which failed to express *Dspp* (6). To determine which genes cause abnormal differentiation of odontoblasts in *Nfic*$^{-/-}$ mice, we performed microarray analyses with pulp cells isolated from *Nfic*$^{-/-}$ and wild type mice. Among 190,230 probes on the BeadChip, 2,338 genes differed by factors of greater than 2-fold in pulp cells of *Nfic*$^{-/-}$ mice compared with wild type. Of these, 1,343 genes were up-regulated, whereas 995 genes were down-regulated. Among these genes, some are related to morphologic changes of the cells and are reported to impact odontoblast differentiation, including *Snail*, *Fox*, *KLF*, and TGF-β family genes (Table 3).

TABLE 3
Summary of microarray analyses between WT and *Nfic*$^{-/-}$ primary pulp cells

Gene symbol	GenBank™ accession no.	Gene function	Fold change
Snail1	NM_011427	Snail homolog 1	0.649
Snail2 (*Slug*)	NM_011415	Snail homolog 2	2.414
Klf3	NM_008453	Kruppel-like factor 3	0.557
Klf5	NM_009769	Kruppel-like factor 5	0.371
Klf8	NM_173780	Kruppel-like factor 8	1.582
Klf12	AK012473	Kruppel-like factor 12	4.845
Tgfβ RI	NM_009370	Transforming growth factor, β receptor I	2.807
Dmp1	NM_016779	Dentin matrix protein 1	0.115

Klf4 Is Down-regulated in Nfic$^{-/-}$ *Odontoblasts*—KLF5 belongs to the KLF family, which has several members that regulate epithelial-mesenchymal transition, including KLF4 (15), KLF8 (16), and KLF17 (17). In the microarray results, *Klf5* showed a greater than 2-fold decrease in expression in *Nfic*$^{-/-}$ pulp cells compared with wild type. However, *Klf4* was not identified (Table 3). Nevertheless, KLF5 and KLF4 have similarities and differences in regulating dental cell differentiation (10). In addition, KLF4 function during tooth development has been reported, whereas KLF5 has not (12). Therefore, we examined *Klf4* mRNA expression in pulp cells from *Nfic*$^{-/-}$ and wild type mice using real-time PCR. Notably, *Klf4* mRNA expression was significantly decreased in *Nfic*$^{-/-}$ pulp cells compared with wild type (Fig. 1*A*).

To investigate the functional consequences of *Nfic*-induced *Klf4* expression, we determined the effect of *Nfic* on *Klf4*-mediated transcriptional activation. *Nfic* overexpression accelerated Klf4 mRNA and protein levels, whereas siRNA-mediated silencing of *Nfic* decreased Klf4 expression in odontoblastic MDPC-23 cells (Fig. 1, *B* and *C*). In addition, increasing concentrations of *Nfic* significantly enhanced expression of luciferase reporter genes under the control of the mouse *Klf4* promoter. In contrast, depletion of *Nfic* using a specific siRNA suppressed promoter activity of the *Klf4* reporter construct in 293 cells (Fig. 1*D*).

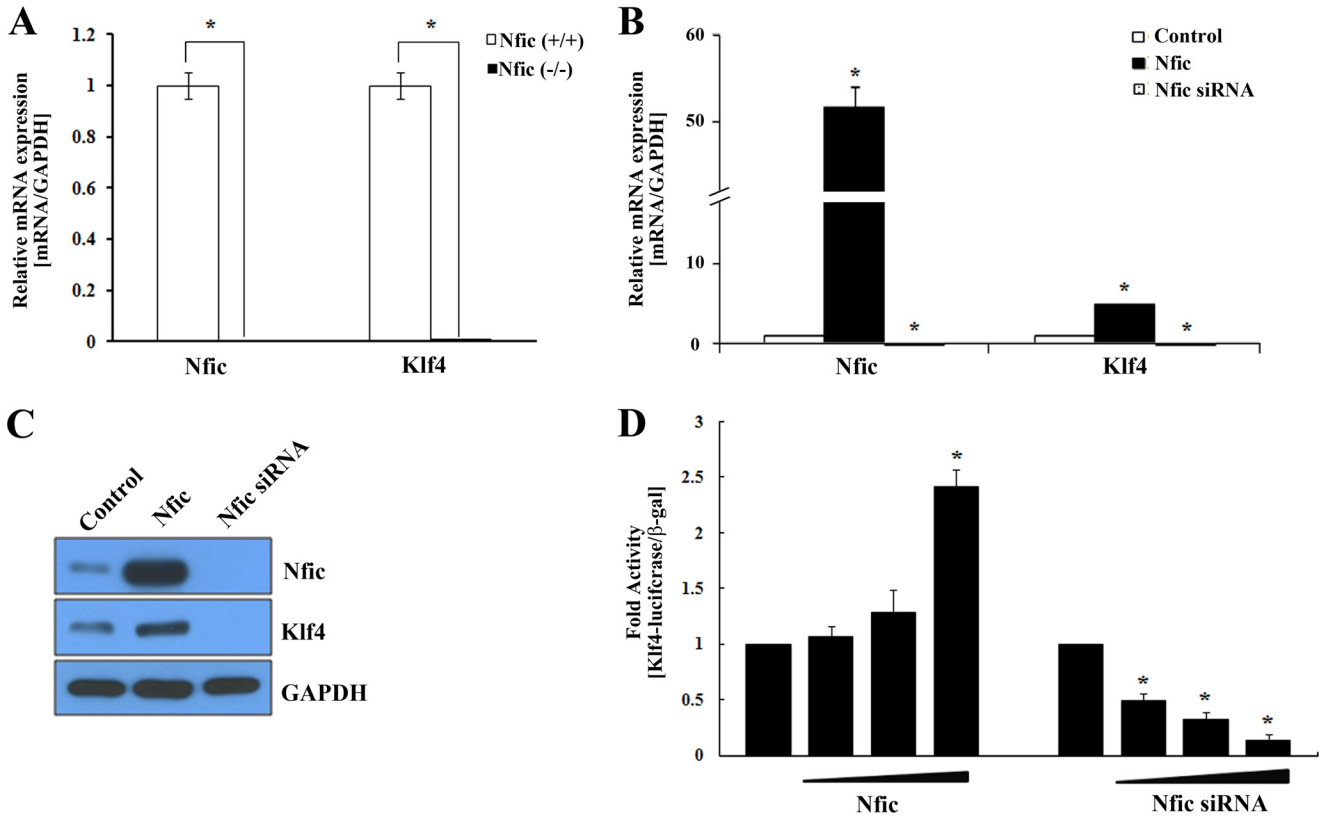


FIGURE 1. **The effect of Nfic on Klf4 expression in human dental pulp and MDPC-23 odontoblastic cells.** *A*, Nfic and Klf4 expression was analyzed using real-time PCR in *Nfic*$^{+/+}$ (wild type) and *Nfic*$^{-/-}$ primary pulp cells. An *asterisk* denotes values significantly different from the wild type ($p < 0.01$). *B* and *C*, expression of Nfic and Klf4 mRNA, and protein was analyzed by real-time PCR (*B*) and Western blotting (*C*) after transfection with *Nfic*-expressing or *Nfic* siRNA constructs in MDPC-23 cells. GAPDH was used as a whole cell loading control. *D*, *Klf4* promoter activity was evaluated by luciferase assays in HEK293 cells transfected with *Nfic*-expressing or *Nfic* siRNA constructs. Data are presented as the mean ± S.D. of triplicate experiments. An *asterisk* denotes values significantly different from the control ($p < 0.01$).

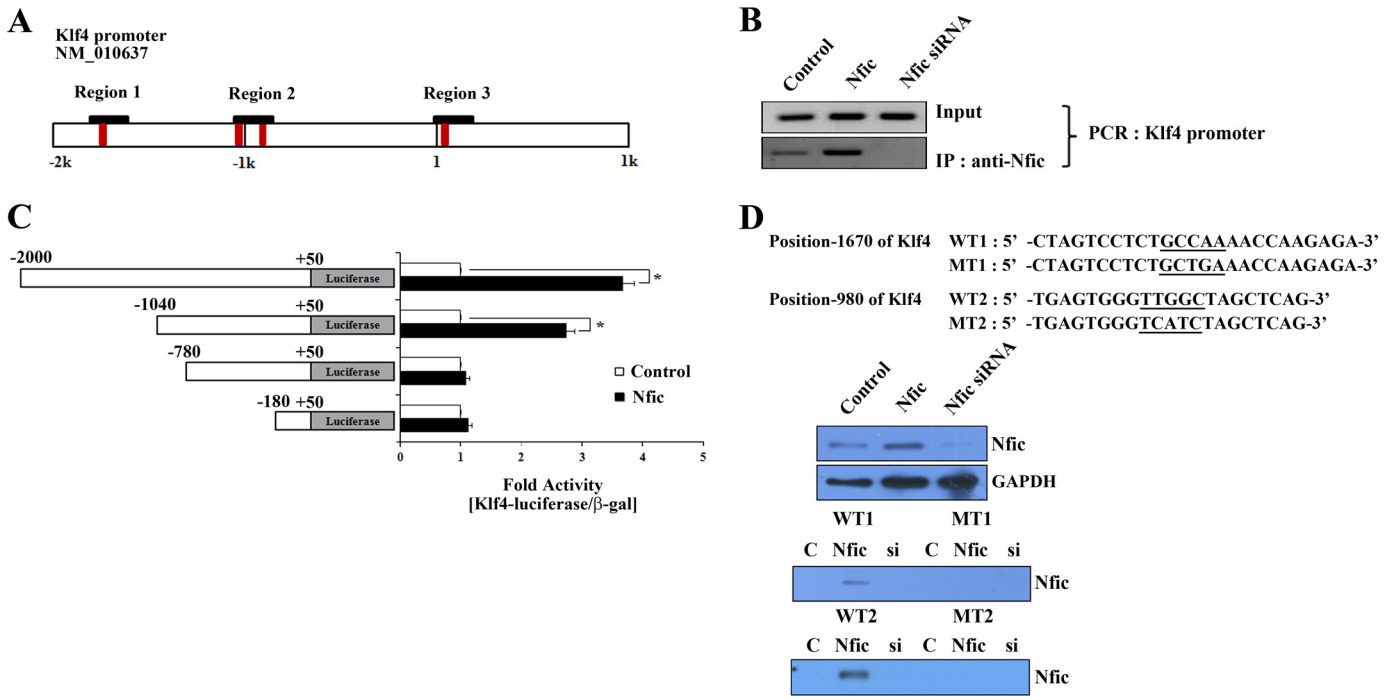


FIGURE 2. **Nfic binding to the *Klf4* promoter and transcriptional activity of *Klf4* in odontoblasts.** *A*, a schematic illustration of the Nfic putative binding elements in the 2-kb mouse *Klf4* promoter. *B*, Nfic binding to the *Klf4* promoter was investigated using ChIP assays after transfection with *Nfic*-expressing or *Nfic* siRNA constructs in MDPC-23 cells. *C*, an Nfic-binding motif in the *Klf4* promoter was identified by luciferase reporter assays in MDPC-23 cells co-transfected with *Nfic*-expressing constructs and truncated *Klf4* promoter reporter constructs. Luciferase activity was normalized to β-gal. Data are presented as the mean ± S.D. of triplicate experiments. An *asterisk* denotes values significantly different from the control ($p < 0.01$). *D*, the Nfic binding motif in the *Klf4* promoter was confirmed by DNA affinity precipitation (*DNAP*) assays using extracts from MDPC-23 cells that had been transfected with *Nfic*-expressing or *Nfic* siRNA constructs. Biotinylated oligonucleotides corresponding to the *Klf4* promoter (WT or mutated) or the −1670/−980 region of the *Klf4* promoter were used as probes. A schematic illustration shows the sequence of the proximal mouse *Klf4* promoter at the nucleotide −1670 and −980 regions. *C*, control; *MT*, mutant type.

Nfic Is Recruited to the Klf4 Promoter in Odontoblasts—A computer search using the TESS program revealed four *Nfic*-binding sites, TTGGC*XXXXX*GCCAA regions, in the *Klf4* promoter (Fig. 2*A*). Results were similar to those predicted by other programs. To investigate whether *Nfic*-mediated activation of the *Klf4* promoter occurs through recruitment of Nfic to the endogenous *Klf4* promoter, we performed ChIP assays with the primer containing region 2 of Klf4 promoter. The *Klf4* promoter could be precipitated using an Nfic-specific antibody in *Nfic*-expressing MDPC-23 cells but not in *Nfic*-silenced MDPC-23 cells (Fig. 2*B*). These results suggest that *Nfic* has an important role in activating *Klf4* transcriptional activity and expression in odontoblasts.

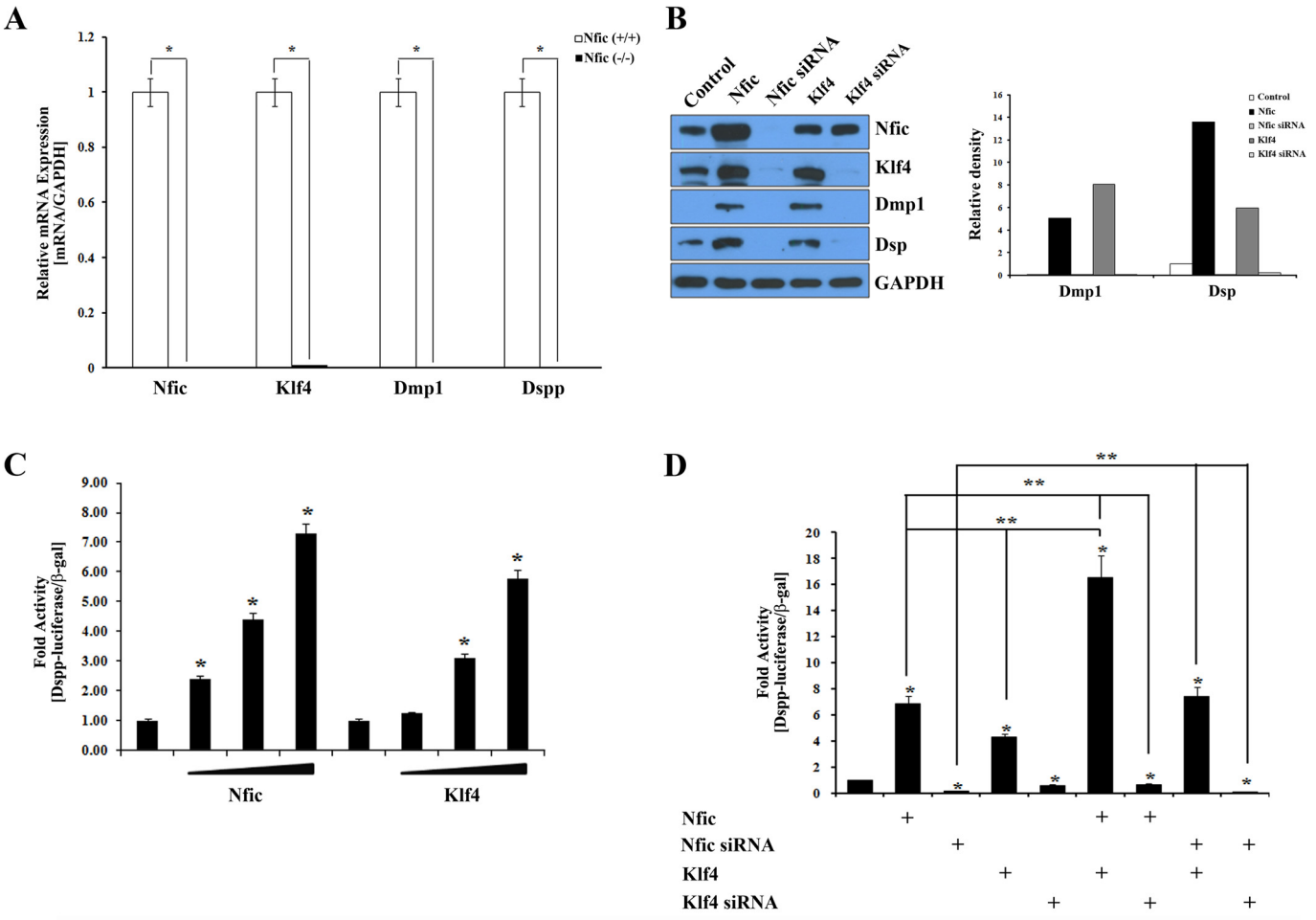
To identify the responsive *Nfic* sites among the four putative sites, we isolated the *Klf4* promoter, which is a region spanning to −2,000, using genomic DNA PCR. We created a series of deletion constructs in pGL3 and transiently transfected them into MDPC-23 cells in the presence or absence of *Nfic*-expressing constructs. Subsequent luciferase assays indicated that *Nfic* stimulated *Klf4* promoter activity. Luciferase assays of the truncation mutants revealed that the region near −1,670 and −980 of *Klf4* promoter were required for promoter activation. Therefore, the region near −1,670 and −980 appears to be involved in *Nfic*-mediated activation of the *Klf4* promoter. However, the −980 region of *Klf4* promoter was more important than the −1,670 region because the reporter construct without the −980 region exhibited severely diminished transcriptional activity compared with the reporter construct without the −1,670 region (Fig. 2*C*).

To confirm Nfic recruitment to the *Klf4* promoter *in vitro*, we performed DNA affinity precipitation experiments using wild type- or mutant-biotinylated oligonucleotides corresponding to the −1,670/−980 region of the *Klf4* promoter containing the Nfic-binding sites (Fig. 2*D*) and extracts from MDPC-23 cells that had been transfected with *Nfic*-expressing or *Nfic* siRNA constructs. In agreement with the luciferase assay data, the wild type −1,670/−980 region of the *Klf4* promoter bound Nfic protein, but not Nfic proteins with mutations in these regions. In addition, the −980 region of *Klf4* promoter recruited Nfic more strongly than the −1,670 region of the *Klf4* promoter (Fig. 2*D*). These results suggest that these regions are important for Nfic recruitment to the *Klf4* promoter in odontoblasts.

Nfic Cooperates with Klf4 to Regulate Dmp1 and Dspp in Odontoblasts—Mesenchymal cells express *Dmp1* and *Dspp* in mouse incisors, which are important for odontoblast differentiation (18). We observed regulation of *Klf4* mRNA and protein by *Nfic* in odontoblasts (Fig. 1). Therefore, we next examined *Dmp1* and *Dspp* expression using real-time PCR in primary pulp cells from wild type and $Nfic^{-/-}$ mice. The expression levels of *Klf4*, *Dmp1*, and *Dspp* mRNAs were significantly decreased in the $Nfic^{-/-}$ primary pulp cells compared with wild type pulp cells (Fig. 3*A*). To confirm these findings, we analyzed the expression of their respective proteins using Western blot-

ting in MDPC-23 cells that were transfected with *Nfic*-expressing, *Nfic* siRNA, or *Klf4*-expressing constructs or *Klf4* siRNA. The data showed that Dmp1 and Dsp proteins were significantly increased in *Nfic*- or *Klf4*-expressing cells compared with control. However, *Nfic* or *Klf4* inactivation by siRNA decreased Klf4, Dmp1, and Dsp protein expression (Fig. 3*B*).

To investigate whether *Nfic* and *Klf4* influence odontoblast differentiation by up-regulating *Dspp*, we measured *Dspp* pro-



moter activity after transfection with *Nfic*- and *Klf4*-expressing constructs in MDPC-23 cells. Interestingly, *Nfic* and *Klf4* increased *Dspp* promoter activity approximately ~6–7-fold compared with control in a dose-dependent manner (Fig. 3*C*). Next, we investigated whether *Nfic*, acting as an upstream regulator of *Klf4*, is required for *Klf4*-mediated *Dmp1-Dspp* transcriptional regulation using a mouse *Dspp*-luciferase construct in MDPC-23 cells, which express quantifiable levels of *Klf4*. As expected, overexpression of *Nfic* or *Klf4* significantly induced *Dspp* transcriptional activity in MDPC-23 cells (~5–7-fold; *lanes 2* and *4*). Overexpression of *Nfic* and *Klf4* showed a synergistic effect on *Dspp* transcriptional activity (16.5-fold; *lanes 2* and *4* compared with *lane 6*). In contrast, when endogenous *Nfic* or *Klf4* was suppressed by *Nfic* siRNA or *Klf4* siRNA, activity of the *Dspp* promoter was disrupted (2–9-fold; *lanes 3* and *5*). Moreover, *Klf4* facilitated *Dspp* promoter activity in *Nfic*-silenced cells (7.4-fold; *lane 3* compared with *lane 8*), but not in Klf4-silenced cells (0.5-fold; *lane 8* compared with *lane 9*, Fig. 3*D*). However, *Nfic* did not enhance *Dspp* promoter activity in *Klf4*-silenced cells (0.4-fold; *lane 2* compared with *lane 7*), but in Klf4-overexpressed cells (16-fold; *lane 2* compared with *lane 6*, Fig. 3*D*). These results suggest the *Nfic-Klf4-Dmp1-Dspp* cascade has an important role during odontoblast differentiation.

To confirm regulation of the *Klf4-Dmp1-Dspp* axis by *Nfic in vivo*, immunohistochemical staining was performed on tooth sections of 14-day-old $Nfic^{-/-}$ and wild type mice using polyclonal anti-Klf4, -Dmp1, and -Dsp antibodies. In postnatal day 14 molars, Klf4 protein was noticeably reduced in $Nfic^{-/-}$ odontoblasts and predentin compared with wild type. In wild type mice, Dmp1 and Dsp protein expression was observed predominantly in the dentin matrix and pulp cells, whereas their expression was barely detectable in $Nfic^{-/-}$ odontoblasts and the dentin matrix (Fig. 3*E*).

To gain insight into the expression pattern of Nfic and Dsp during odontoblast differentiation *in vitro*, MDPC-23 cells were grown in differentiation medium for 14 days. Nfic protein was expressed at the beginning of the culture, decreased from days 3 to 5, and increased from days 7 to 14. Dsp expression was detected on day 5 and continued to increase through day 14 during odontoblast differentiation (Fig. 4*A*). We also determined the effect of altered Klf4 expression on dentin mineralization. Alizarin red S staining was performed to evaluate mineralization nodules by culturing MDPC-23 cells in odontoblastic induction medium for 7 days. Overexpression of *Nfic* or *Klf4* induced mineralized nodule formation on day 7 in MDPC-23 cells. Remarkable nodules were noted in the negative control group, whereas scarce nodules were observed in the *Nfic* siRNA transfected group (Fig. 4*B*). These results demonstrate that enforced expression of Nfic or Klf4 significantly promotes odontoblast differentiation and mineralization.

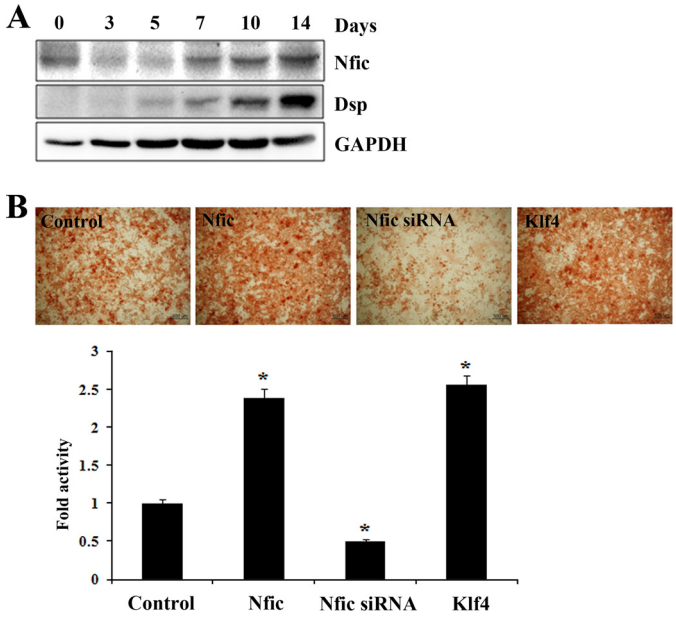


FIGURE 4. **The effect of Nfic and Klf4 on odontoblast differentiation and dentin mineralization *in vitro*.** *A*, Nfic and Dsp protein expression after MDPC-23 odontoblastic cell culture for 14 days in differentiation medium analyzed by Western blotting. *B*, the effect of *Nfic* and *Klf4* on mineralized nodules formation in MDPC-23 cells 7 days after transfection of *Nfic*-expressing, *Nfic* siRNA, or *Klf4*-expressing constructs analyzed by alizarin red S staining. Quantification of mineralization was measured by colorimetric spectrophotometry. Data are presented as the mean ± S.D. of triplicate experiments. An *asterisk* denotes values significantly different from the control ($p < 0.01$). *Scale bars*, 200 μm.

Nfic Is a Target for miR-145 in Odontoblasts—miR-145 decreased *Dmp1* and *Dspp* expression by binding to the 3′-UTRs of *Klf4* genes in mouse primary dental pulp cells and controlling odontoblast differentiation and dentin formation (19). We examined the effect of miR-145 on *Nfic* regulation. *In silico* assays revealed binding sites for miR-145 within the 3′-UTR of *Nfic* (Fig. 5*A*). In Western blot analyses, Nfic, Klf4, and DSP expression was decreased when miR-145 was enforced (Fig. 5*B*). These data demonstrate that miR-145 may bind to the 3′-UTR of the *Nfic* gene and decrease both Klf4 and Dsp expression. This suggests that miR-145 regulates cell differentiation via *Nfic-Klf4* pathways.

Nfic Maintains Epithelial Differentiation Status in Odontoblasts—The previous reports have shown the expression change of E-cadherin and N-cadherin in ameloblasts and odontoblasts during tooth development. In addition, these cells undergo actin rearrangement and cell shape change during amelogenesis and odontogenesis (20, 21). KLF4 regulated epithelial cell marker genes such as E-cadherin as a transcription factor, and then it induced epithelial cellular characters (22). To investigate the expression patterns of E-cadherin, N-cadherin, TGFβ-RI, TGF-β, and Nfic during ameloblast and odontoblast

FIGURE 3. **Enhanced Dmp1 and Dspp expression by the *Nfic-Klf4* axis in human dental pulp cells and odontoblasts.** *A*, Nfic, Klf4, Dmp1, and Dspp expression was analyzed by real-time PCR in $Nfic^{+/+}$ (wild type) and $Nfic^{-/-}$ primary pulp cells. *B*, Nfic, Klf4, Dmp1, and DSP protein expression was analyzed by Western blotting (*left panel*) and its quantification (*right panel*) in MDPC-23 cells transfected with *Nfic*-expressing, *Nfic* siRNA, or *Klf4*-expressing constructs or *Klf4* siRNA. GAPDH was used as a whole cell loading control. *C* and *D*, transcriptional activity of the *Dspp* promoter was evaluated by luciferase assays using *Nfic*-expressing, *Nfic* siRNA, or *Klf4*-expressing constructs, or *Klf4* siRNA in MDPC-23 cells. Control samples were transfected with only Dspp promoter constructs. *Nfic*-expressing and *Klf4*-expressing constructs were transfected in dose-dependent manner (*B*; 0.1, 0.5, or 1 ug). Data are presented as the mean ± S.D. of triplicate experiments. An *asterisk* denotes values significantly different from the control ($p < 0.01$); *double asterisks* denote values significantly different among the groups ($p < 0.01$). *E*, Klf4, Dmp1, and DSP protein expression (*arrows*) was detected by immunohistochemistry of $Nfic^{+/+}$ (wild type) and $Nfic^{-/-}$ mice on postnatal day 14. *Scale bars*, 100 μm.

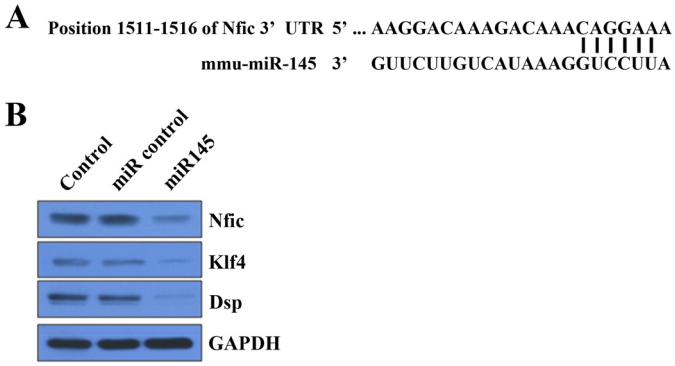


FIGURE 5. **The regulation *Nfic-Klf4* axis by miR-145 in odontoblasts.** *A*, *in silico* assays reveal binding sites for miR-145 within the 3′-UTR of *Nfic*. *B*, NFIC, Klf4, and DSP protein expression was analyzed by Western blotting in MDPC-23 cells transfected with miR control or miR-145. *mmu, M. musculus*.

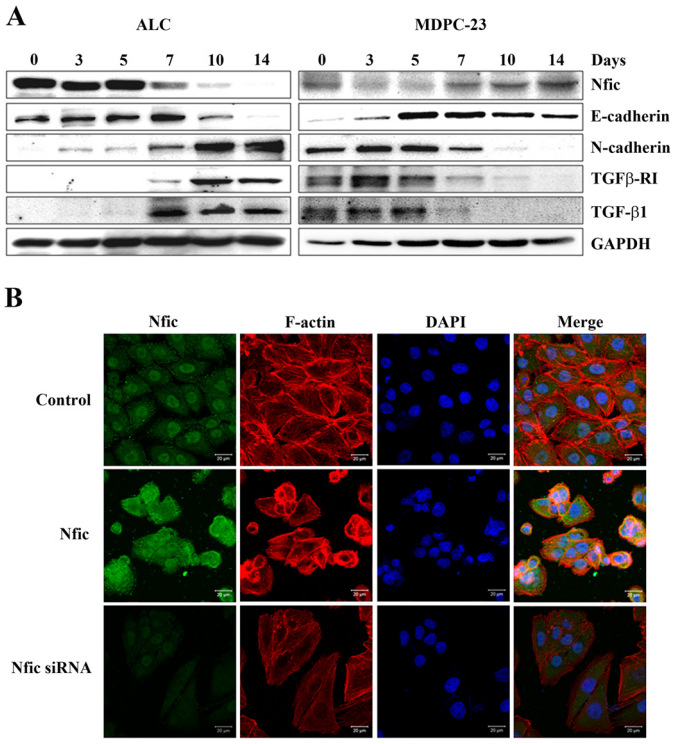


FIGURE 6. **Expression pattern of *Nfic*, cadherins, and TGF-β signaling factors during ameloblast and odontoblast differentiation *in vitro*.** *A*, protein levels of Nfic, E-cadherin, N-cadherin, TGFβ-RI, and TGF-β were determined by Western blotting in ALC (*left panel*) or odontoblastic MDPC-23 cell (*right panel*) cultures after 14 days in differentiation medium. GAPDH was used as a loading control. *B*, Nfic expression (*green*) and phalloidin staining (F-actin, *red*) with nuclei counterstained (*blue*) was detected by fluorescence microscopy in MDPC-23 cells transfected with *Nfic*-expressing or *Nfic* siRNA constructs. *Scale bars*, 50 μm.

differentiation *in vitro*, we cultured ameloblastic ALC and odontoblastic MDPC-23 cells for 14 days and evaluated protein expression using Western blot analyses. Ameloblastic ALC and odontoblastic MDPC-23 cells showed opposite patterns in the expression of E-cadherin, N-cadherin, TGFβ-RI, TGF-β, and Nfic during their differentiation. During odontoblast differentiation, Nfic and E-cadherin expression gradually increased over time, whereas the expression of N-cadherin and TGF-β gradually decreased (Fig. 6*A*). We also investigated the correlation between cellular morphology and actin arrangement in odontoblasts in the presence or absence of Nfic. Parental MDPC-23 cells grow as dispersed cells and rarely formed clusters. In contrast, *Nfic*-expressing MDPC-23 cells formed tightly packed patches of epithelial sheets, were smaller than control cells, and had a nearly complete disappearance of central actin stress fibers with a transition to circumferential actin cables. The altered morphology and cytoskeletal modifications were reversible by siRNA-mediated inactivation of *Nfic* in MDPC-23 cells (Fig. 6*B*).

The Snail family members, Snail 1 and Slug, which encode zinc finger-type transcription factors, have been shown to be involved in neural crest formation and subsequent epithelial status (23, 24). Consistent with microarray data, *Slug* expression was significantly increased in $Nfic^{-/-}$ pulp cells compared with wild type, whereas E-cadherin expression was decreased (Fig. 7*A*).

A distribution of vimentin accompanies odontoblast polarization (25). *Nfic* overexpression decreased expression of Slug and vimentin, which play important roles in the polarization of odontoblasts, but enhanced E-cadherin expression, which plays an important role in cellular adhesion. However, E-cadherin expression was decreased after *Nfic* inactivation by siRNA (Fig. 7, *B* and *C*). Additionally, *Nfic* overexpression decreased *Slug* luciferase activity but augmented E-cadherin promoter activity, whereas siRNA-mediated *Nfic* inactivation increased *Slug* promoter activity but decreased *E-cadherin* promoter activity (Fig. 7, *D* and *E*).

Klf4 Is Recruited to the E-cadherin Promoter—A computer search using the TESS program revealed two *Nfic*-binding sites in the *Slug* promoter and four *Nfic*-binding sites in the E-cadherin promoter (Fig. 8*A*). To investigate whether Nfic could bind to the *Slug* or E-cadherin promoter, we performed ChIP assays. As shown in Fig. 8*B*, Nfic did not associate with the *Slug* or E-cadherin promoters. However, ChIP assays using a primer set for E-cadherin indicated that Klf4 proteins were recruited to the E-cadherin promoter after expression of either *Klf4* or *Nfic* (Fig. 8*C*). Klf4 activated E-cadherin expression by binding the GC-rich/E-box region of the E-cadherin promoter, resulting in alterations in epithelial cell morphology and migration (26). Thus, Nfic is critical for recruitment to the *Klf4* promoter, but not to the *Slug* and E-cadherin promoters, *in vivo*.

Nfic and Nfix, but Not Nfia and Nfib, Influence Klf4 Transcription—To investigate the function of the four NFI factors on the regulation of KLF4 and E-cadherin, we examined the effects of NFI family member overexpression on *Klf4* and E-cadherin promoter activity. Expression of each of the NFI isoforms was confirmed by Western blotting with the anti-HA antibody after transfection. *Nfic* and *Nfix* overexpression significantly induced expression of *Klf4* and E-cadherin in odontoblasts (Fig. 9*A*).

Next, expression vectors for *Nfia*, *Nfib*, *Nfib*, or *Nfix* were co-transfected with *Klf4* or E-cadherin reporter constructs into MDPC-23 cells. Transfection of *Nfic* or *Nfix* constructs significantly enhanced the *Klf4* and E-cadherin promoter activity, suggesting that the two isoforms function as transcriptional activators for the expression of *Klf4* and E-cadherin. In contrast, transfection with *Nfia* or *Nfib*-expressing constructs did not noticeably activate *Klf4* or E-cadherin (Fig. 9, *B* and *C*).

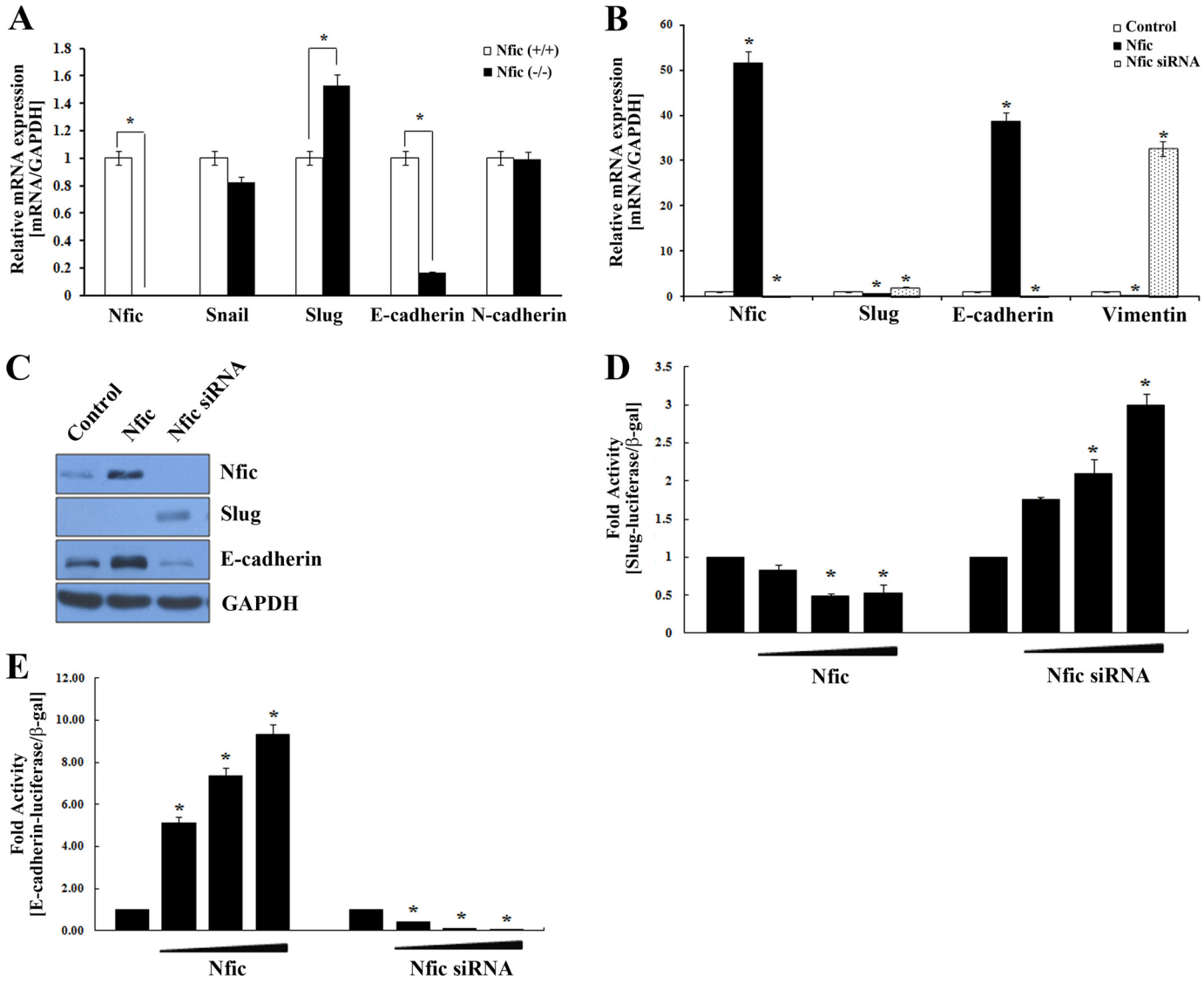


FIGURE 7. **Effects of Nfic on Snail, Slug, E-cadherin, N-cadherin, vimentin, and F-actin in human dental pulp cells and odontoblastic MDPC-23 cells.** *A*, *Nfic*, *Snail*, *Slug*, E-cadherin, and N-cadherin mRNA expression in $Nfic^{+/+}$ (wild type) and $Nfic^{-/-}$ primary pulp cells analyzed by real-time PCR. *B* and *C*, expression of *Nfic*, *Slug*, E-cadherin, and vimentin mRNA (*B*) and protein (*C*) in MDPC-23 cells transfected with *Nfic*-expressing or *Nfic* siRNA constructs analyzed by real-time PCR and Western blotting, respectively. *D*, the transcriptional activity of *Slug* promoter was evaluated by luciferase assay in HEK293 cells transfected with *Nfic*-expressing or *Nfic* siRNA constructs. *E*, the transcriptional activity of E-cadherin promoter was evaluated by luciferase assay in HEK293 cells transfected with *Nfic*-expressing or *Nfic* siRNA constructs. Data are presented as the mean ± S.D. of triplicate experiments. An *asterisk* denotes values significantly different from the control ($p < 0.01$).

DISCUSSION

Tooth development involves a complex sequence of reciprocal epithelial-mesenchymal interactions. Dental epithelial cells from the dental organ differentiate into ameloblasts, whereas ectomesenchymal cells from the dental papilla differentiate into odontoblasts (27). Differentiating odontoblasts elongate, polarize, and produce dentin by synthesizing and secreting Dmp1 and DSPP, important markers of odontoblast differentiation (2, 18). *Klf4* promotes odontoblast differentiation via up-regulation of *Dmp1* by binding its promoter and *Dmp1* subsequently induces *Dspp* expression during odontoblast differentiation (12, 19, 28, 29). These studies imply that *Klf4-Dmp1-Dspp* signaling controls odontoblast differentiation and dentin formation. In our study, we report that Nfic directly binds to the *Klf4* promoter and transactivates its expression. Therefore, we suggest that the *Nfic-Klf4-Dmp1-Dspp* cascade controls odontoblast differentiation and dentin formation. This view is also supported by two other findings. First, Klf4, Dmp1, and Dsp protein expression was significantly decreased in $Nfic^{-/-}$ odontoblasts compared with wild type odontoblasts. In addition, *Nfic* or *Klf4* overexpression promoted mineralized nodule formation in odontoblastic MDPC-23 cells. Dmp1 protein was expressed in the abnormal dentin matrix of $Nfic^{-/-}$ mice but was not expressed in the normal dentin matrix of wild type mice. This result is in agreement with our previous investigations that abnormal dentin is formed by aberrant odontoblasts in $Nfic^{-/-}$ mice that exhibit a bone-like phenotype, as observed the expression of Dmp1 in surrounding alveolar bone (6).

In our previous studies, $Nfic^{-/-}$ mice developed normal molar crowns but short molar roots that contain aberrant

odontoblasts and abnormal dentin formation. Gross observations of the teeth of *Nfic*$^{-/-}$ mice revealed normal molar crowns, short molar roots, and thin incisors (14). However, in microscopic observations, the pulp side of a root forming molar of an *Nfic*$^{-/-}$ mouse was covered with polygonal, disorganized, and abnormal odontoblasts because ectomesenchymal pulp cells failed to differentiate into normal odontoblasts (5). An incisor from *Nfic*$^{-/-}$ mice also contained abnormal odontoblasts in the pulp, and some of the cells were trapped in osteodentin-like mineralized tissues (6). In this study, we have used pulp cells of *Nfic*$^{-/-}$ mice incisors for the experiments because we can easily obtain the more pulp cells form incisors than molars.

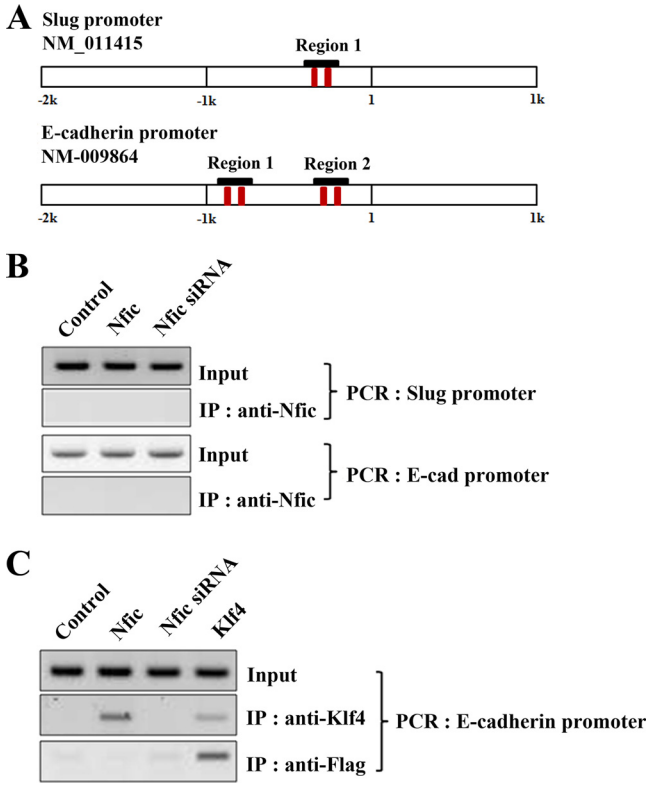


FIGURE 8. **Klf4 binds the E-cadherin promoter.** *A*, a schematic illustration of the putative *Nfic* binding elements in the 2-kb mouse *Slug* and E-cadherin promoters. *B*, Nfic binding to the *Slug* and E-cadherin promoter was investigated using ChIP analyses after transfection with *Nfic*-expressing or *Nfic* siRNA constructs in MDPC-23 cells. *C*, Klf4 binding to the E-cadherin promoter was investigated using ChIP analyses after transfection with the *Nfic*-expressing or *Nfic* siRNA, or *Klf4*-expressing constructs, in MDPC-23 cells.

It was also reported that miR-143 and miR-145 control odontoblast differentiation via regulation of *Klf4* and *Osx* transcription factors. miR-143 and miR-145 expression decreases during odontoblast differentiation *in vitro* and thus releases the expression of *Klf4* and *Osx* regulating *Dspp* (19). Consistent with these results, in the present study, miR-145 decreased Nfic, Klf4, and Dsp expression in odontoblasts. It implies that miR-143 and miR-145 regulated *Klf4* and *Dsp* via the control of Nfic, an upstream effector molecule of *Klf4*. Furthermore, we have shown that *Nfic* directly regulates *Osx* expression but acts downstream of the BMP2-Runx2 pathway in osteoblasts (30). These findings suggest that miR-143 and miR-145 also regulates odontoblast differentiation via the miR-143/145-*Nfic*-*Klf4*/*Osx*-*Dspp* pathway.

E-cadherin and N-cadherin, which are expressed during odontogenesis, are involved in the regulation of various biological processes such as cell recognition, intercellular communication, cell fate, cell polarity, boundary formation, and morphogenesis (31). Although not observed *in vivo*, E-cadherin is expressed in cultured pulp cells (32). In this study, odontoblastic MDPC-23 cells showed opposite patterns in the expression of Nfic/E-cadherin and TGF-β/N-cadherin. This result is in agreement with our previous investigations that demonstrate

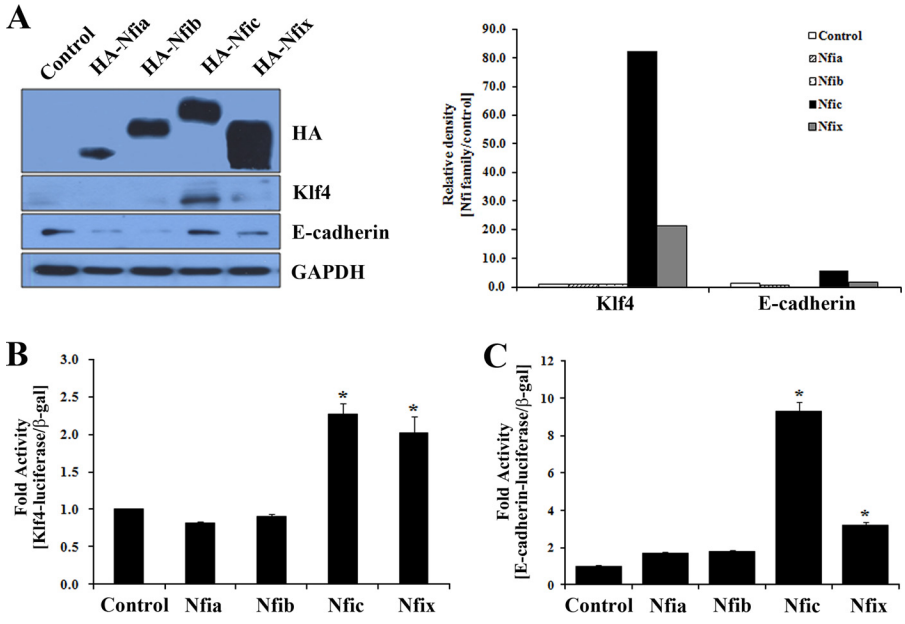


FIGURE 9. **Effects of NFI family members on the expression of Klf4 and E-cadherin.** *A*, expression of HA, Klf4, and E-cadherin protein was analyzed by Western blotting (*left panel*) and its quantification (*right panel*) in MDPC-23 cells transfected with HA-tagged *Nfia*-, *Nfib*-, *Nfic*-, or *Nfix*-expressing constructs. *B* and *C*, the transcriptional activity of the *Klf4* or E-cadherin promoters was evaluated by luciferase assay in HEK293 cells transfected with *Nfia*-, *Nfib*-, *Nfic*-, or *Nfix*-expressing constructs. The data are presented as the mean ± S.D. of triplicate experiments. An *asterisk* denotes values significantly different from the control ($p < 0.01$).

cross-talk between Nfic and TGF-β1 signaling regulates cell differentiation in odontoblasts (33).

During the cytodifferentiation stage, odontoblasts become highly complex, polarized secretory cells based on the appearance of a complex cytoskeleton (34). Differentiating odontoblasts establish palisade-like layers due to the development of junctional complexes containing tight junctions and adherens junctions (35, 36). In our previous study, Nfic expression strikingly alters the morphology and intercellular adhesion of odontoblasts (37). siRNA-mediated inactivation of *Nfic* also caused altered morphology and cytoskeletal modifications in MDPC-23 cells. These results suggest that Nfic induces the transition of cellular morphology by regulating the expression of Klf4, Slug, and E-cadherin in odontoblasts.

Nfi genes (*Nfia*, *Nfib*, *Nfic*, and *Nfix*) exhibit promoter-specific differences in their maximal transcriptional activation potentials due to differences in their carboxyl-terminal regions (38). NFI proteins exhibit cell type- and promoter-specific differences in their repression properties, with NFIC and NFIX, but not NFIA and NFIB, repressing the mouse mammary tumor virus promoter in HeLa cells. NFIC-mediated repression occurs by interference with coactivator (*e.g.* p300/CBP or SRC-1A) function at the mouse mammary tumor virus promoter (39). Although it is widely accepted that NFI proteins differ in their repression and activation potentials, their ability to regulate transcription is poorly understood. In our study, *Nfic* and *Nfix* influenced *Klf4* transcription but *Nfia* and *Nfib* did not. *Nfic* and *Nfix* are thought to regulate *Klf4* because the DNA-binding affinities of NFIC and NFIX were higher than those of NFIA and NFIB; however, no direct evidence yet supports this notion.

To our knowledge, this work is the first to investigate the *Nfic-Klf4-Dmp1-Dspp* and the *Nfic-Klf4*-E-cadherin signaling pathways in odontoblasts, as well as their functional implications during dentin formation. This information will lead to a comprehensive understanding of the role of NFIC in dentinogenesis.

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