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Zinc Balance is Critical for NFI-C Mediated Regulation of Odontoblast Differentiation

Hyun-Jung Oh,[1] Hye-Kyung Lee,[1] Su-Jin Park,[1] Young-Sik Cho,[2] Hyun-Sook Bae,[2] Moon-Il Cho,[3] and Joo-Cheol Park[1*]

[1] *Department of Oral Histology-Developmental Biology, School of Dentistry and Dental Research Institute, BK 21, Seoul National University, Seoul 110-749, Korea*

[2] *Department of Dental Hygiene, Namseoul University, Cheon-An 331-707, Korea*

[3] *Department of Oral Biology, School of Dental Medicine, State University of New York at Buffalo, New York 14214-3092*

ABSTRACT

Zinc is trace element essential for diverse metabolic and cellular signaling pathways for the growth, development, and maintenance. Zinc deficiency is involved in bone malformations and oral disease. Mice deficient in zinc transporter Zip13 show connective tissue and skeletal disorders, abnormal incisor teeth, and reduced root dentin formation in the molar teeth and share a morphologically similar phenotype to nuclear factor I-C (NFI-C)-deficient mice. However, the precise function of zinc in NFI-C signaling-mediated odontoblast differentiation and dentin formation remains unclear. Here, we show that zinc stimulated the expression of metal transcription factor-1, but decreased NFI-C expression in odontoblastic MDPC-23 cells. Zinc also enhanced the phosphorylation of Smad2/3 (p-Smad2/3) and increased the binding efficiency of NFI-C and p-Smad2/3 in the cytoplasm. In contrast, zinc deficiency resulted in the accumulation of NFI-C into nucleus. Consequently, NFI-C had the biologic properties of a transcription factor, including DNA binding affinity for metallothionein-1 and the dentin sialophosphoprotein (DSPP) promoter, and transcriptional activation of the DSPP gene. Furthermore, zinc deficiency condition promoted DSPP expression in odontoblasts and dentin mineralization, while zinc sufficiency condition decreased DSPP expression and slightly delayed dentin mineralization. These data suggest that zinc equilibrium is required for odontoblast differentiation and dentin formation during dentinogenesis through the nuclear accumulation and modulation of NFI-C. J. Cell. Biochem. 113: 877–887, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: ZINC; NFI-C; DENTIN; ODONTOBLAST; DIFFERENTIATION

The trace element zinc is essential for growth, development, and health maintenance, and is involved in cellular metabolism, replication, growth, and tissue repair [Prasad, 1995]. Zinc modulates transcription and other biological functions through more than 300 zinc containing enzymes and 2,000 transcription factors that contain a DNA-binding Zn-finger motif, a Ring-finger, and an LIM domain [Vallee and Falchuk, 1993]. Consequently, intracellular zinc concentrations are extremely important for the maintenance of regulatory systems. Moreover, zinc deficiency results in malformation and retardation of bone growth [Yamaguchi, 2010] as well as the impairment of immune responses and brain functions [Hambidge, 2000; Truong-Tran et al., 2001]. Conversely, excess zinc can induce apoptosis and neuronal death [Koh et al., 1996]. Therefore, it is important not only the element of zinc but also the equilibrium in intracellular zinc concentrations for mediating proper intracellular signaling events.

Zinc homeostasis is tightly controlled by various proteins, which include zinc transporters, zinc binding molecules, such as metallothioneins (MTs), and zinc sensing molecules, such as metal transcription factor-1 (MTF-1). MTF-1 is a six-zinc finger domain-containing transcription factor that induces the expression of MTs in response to heavy metal ions, such as zinc. MTs play important roles in zinc homeostasis [Vallee, 1995; Saydam et al., 2002; Eide, 2004].

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*Correspondence to: Joo-Cheol Park, Department of Oral Histology-Developmental Biology, School of Dentistry, Seoul National University, 28, Yeongun-Dong, Chongro-Gu, Seoul 110-749, Korea. E-mail: jcapark@snu.ac.kr

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The nuclear factor I (NFI) family of transcription factors consists of four members (Nfia, Nfib, Nfic, and Nfix) in mammals. NFI proteins contain an N-terminal DNA-binding/dimerization domain and a C-terminal NFI transactivation and repression domain [Gronostajski, 2000]. Interestingly, *Nfic*-deficient mice exhibit thin and brittle mature mandibular incisors, dimorphic maxillary incisors, and the absence of a molar root. Thus, NFI-C is important for odontoblast differentiation, which is essential for the production of the thick dentin layer that forms the bulk of the tooth [Park et al., 2007]. The zinc transporter Slc39a13/Zip13 influences intracellular zinc levels by transporting zinc from the Golgi to the cytosol. Mice that are deficient for the zinc transporter Zip13 show connective tissue and skeletal disorders, abnormal incisor teeth, and reduced root dentin formation in the molar teeth [Fukada et al., 2008]. Interestingly, these mice share a morphologically similar phenotype to *Nfic*-deficient mice [Lee et al., 2009]. However, the precise mechanism by which zinc regulates the NFI-C gene for odontoblast differentiation and dentin formation remains unknown.

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that regulates cellular processes, such as mediating odontoblast differentiation and dentin formation [Smith et al., 1998]. During TGF-β signaling, the activated TGFβ-R1 phosphorylates Smad2 and Smad3, which form a complex with a common partner, Smad4, which then translocates into the nucleus to control the transcription of target genes [Miyazono et al., 2001].

There are many studies showing that zinc is associated with osteogenic regulatory genes of bone formation and clinical dental studies. Indeed, zinc can promote osteoblastic bone formation and inhibit osteoclastic bone resorption [Yamaguchi et al., 1992, 2008]. In addition, zinc insufficiency has been related to defective collagen synthesis and delayed skeletal maturation [Prasad and Bose, 1975; Agren, 1991]. Furthermore, zinc deficiency has been linked to oral disease, such as human taste impairments, parakeratosis in the black plaque buccal epithelium [Chen et al., 1975], and susceptibility to dental caries [Hambidge et al., 1972; Fang et al., 1980]. However, the role of zinc in the odontoblast differentiation and dentin mineralization has not yet been clearly defined.

The present study was conducted to assess how NFI-C signaling and zinc concentration changes affect odontoblast differentiation and mineralization in a mouse odontoblast cell line (MDPC-23). We also determined whether zinc equilibrium is required for dentin formation through the nuclear accumulation and modulation of NFI-C.

MATERIALS AND METHODS

CELL CULTURE

MDPC-23 cells were provided by Dr. J.E. Nör (University of Michigan, MI). 293T (human embryonic kidney) and MC3T3-E1 (mouse osteoblast-like) cells were obtained from ATCC (Rockville, MD, USA). MDPC-23 and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM), and MC3T3-E1 cells were cultured in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum and antibiotic–antimycotic (Invitrogen, Carlsbad, CA) in a 5% CO_2 atmosphere at 37°C. To induce cell differentiation and mineralized nodule formation, confluent MDPC-23 cells were treated with 50 μg/ml ascorbic acid and 10 mM ß-glycerophosphate for up to 2 weeks.

PLASMIDS

Flag-tagged Smad3 cDNA was placed on a pcDNA3 vector (Invitrogen). HA-tagged NFI-C was constructed with a pcDNA3 vector (Invitrogen) from Dr. R. M. Gronostajski (State university of New York at Buffalo, Buffalo, NY). The green fluorescent protein (GFP)-tagged NFI-C gene was placed into pEGFP-C3 (BD Biosciences, San Jose, CA). The dentin sialophosphoprotein (DSPP) promoter (pGL3LUC-791 to +54) plasmid was a kind gift from Dr. W.X. He (Qin Du Stomatological, Xian, China). A 1.5-kb genomic fragment was amplified from mouse genomic DNA to obtain the NFI-C promoter (−1,543 to +88). One microliter of the mouse genomic DNA was subjected to PCR using the following cycling conditions: 94°C for 1 min; 60°C for 1 min; and 72°C for 1 min for a total of 35 cycles. The forward and reverse primers were as follows: 5′-CTC GAG GGA CTG TAA CTG CTG AGC TGT-3′ and 5′-AAG CTT CAG AGC GGG GAG GAA TAC AT-3′. The amplified 1.5-kb fragment was subcloned into PCR® 2.1 T vector and ligated into the XhoI and Hind III sites of pGL3 luciferase (LUC) basic expression vector (Promega, Madison, WI). The construct was confirmed by sequencing and was designated pGL3-mNFI-C.

COLORIMETRIC MTT ASSAY FOR CELL PROLIFERATION

MDPC-23 cells were seeded at a density of 5×10^3 cells per well in 48-well culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing various concentrations of $ZnCl_2$ (Sigma–Aldrich, St. Louis, MO) and cell membrane-permeable zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, Sigma–Aldrich) for 24 h. The mitochondria activity of proliferating cells was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The plates were measured at 540 nm using a plate reader. Triplicate samples were analyzed from three independent experiments.

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was extracted using TRIzol® Reagent (Invitrogen), and 3 μg of RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (New England Biolabs, Beverly, MA). One microliter of the RT product was amplified by PCR using the following primer pairs: MT-1 (399 bp), forward, 5′-gtggtccggaacctcaaagt-3′ and reverse, 5′-ccggagcaggatagcaaagt-3′; MTF-1 (289 bp), forward, 5′- 5′-cctgcagtccagcctagtca-3′ and reverse, 5′-tggggaagaacatccctttc-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 452 bp), forward, 5′-accacagtccatgccatcac-3′ and reverse, 5′-tccaccaccctgttgctgt-3′. The following PCR conditions were used: 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min for a total of 30 cycles. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

PREPARATION OF CYTOPLASMIC AND NUCLEAR PROTIEN EXTRACTS

Zinc or TPEN was administered to MDPC-23 cells dose-dependently for 4 h. At the end of each experiment, the cells were collected by centrifugation at 3,000 rpm for 5 min at 4°C. Cell lysis was performed in ice-cold hypotonic lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1% NP-40) supplemented with protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) for 15 min. Nuclear and cytoplasmic fractions were separated by centrifugation at 3,000 rpm for 5 min at 4°C. The resulting supernatant (the cytoplasmic fraction) was stored at 4°C until further analysis. The membrane pellet was resuspended in ice-cold hypertonic lysis buffer (10 mM HEPES [pH 7.9], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol) supplemented with protease inhibitors and incubated for 15 min at 4°C. The soluble fraction was isolated by centrifugation at 3,000 rpm for 5 min at 4°C. The resulting supernatant (the nuclear fraction) was stored at 4°C until further analysis.

WESTERN BLOT ANALYSIS

To prepare whole cell extracts, the cell were washed three times with PBS, scraped into 1.5-ml tubes, and pelleted by centrifugation at 12,000 rpm for 5 min at 4°C. After removal of the supernatant, the pellet was resuspended in lysis buffer (50 mM Tris–Cl [pH 7.4], 150 mM NaCl, 1% NP-40, 2 mM EDTA [pH 7.4]) and incubated for 15 min on ice. Cell debris was removed by centrifugation at 12,000 rpm for 15 min at 4°C. The proteins were resuspended in lysis buffer as described above. Thirty microgram of samples were separated on denaturing 10–12% Tris–HCl polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% nonfat dry milk in phosphate buffered saline containing 0.1% Tween 20 (PBS-T), washed with the PBS-T, and incubated overnight with primary antibody diluted in PBS-T buffer (1:1,000) at 4°C. Antiserum against NFI-C was produced by immunization in rabbit with the synthetic peptides NH2-RPTRPLQTVPLWD-COOH (amino acid residues 427-439 of NFI-C) and NH2-GNKSIITKESGKLSGS-COOH (amino acid residues 372–387 of DSP) [Lee et al., 2009]. The mouse monoclonal anti-HA (E10176EF) and anti-Flag (F-3165) antibodies were purchased from Sigma–Aldrich. The antibodies against p-Smad2/3 (sc-11769), MTF-1 (sc-48775), Smad 1/5/8 (sc-6031) p-Smad 1 (sc-101800), Lamin B (sc-6216), CDK2 (sc-6248), osteocalcin (sc-30044), Runx2 (sc-10758), and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-Smad3 antibody (9523) was purchased from Cell Signaling Technology (Beverly, MA). After washing, the membranes were incubated with anti-mouse (sc-2031), -rabbit (sc-2004), or-goat (sc-2768) IgG secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h. The labeled protein bands were detected using an enhanced chemiluminescence system (Dogen, Cambridge, MA), and the bands were measured by densitometric analysis of the autoradiograph films.

FLUORESCENCE MICROSCOPY

MDPC-23 cells in Laboratory-Tek chambered cover glasses (Nunc, Wiesbaden, Germany) were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized for 4 min in PBS containing 0.5% Triton X-100. After washing, the cells were incubated with anti-NFI-C antibody (1:200 dilution) in blocking buffer (PBS and 1% bovine serum albumin) for 1 h followed by the addition of a fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (1:200 dilution; Amersham Pharmacia Biotech, Orsay, France). After washing, the cells were visualized using a fluorescence microscope (AX70; Olympus Optical Co., Tokyo, Japan). DAPI (4′, 6-diamidino-2-phenylindole) was used to stain chromosomal DNA in the nucleus.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

293T cells were seeded in 12-well culture plates at a density of 1.5×10^5 cells per well. Cells were transiently transfected with the reporter constructs described above. Depending on the experimental conditions, pGL3-NFI-C and pGL3-DSPP was transfected into the cell, which were treated with zinc or TPEN (dose-dependent) 2 days later. Following the addition of 50 μl Luciferin to 50 μl of the cell lysate, the luciferase activity was determined using an Analytical Luminescence Luminometer according to the manufacturer's instructions (Promega).

Co-IMMUNOPRECIPITATION (CO-IP) ASSAY

After transfection with the indicated plasmid DNA using Metafectene pro reagent (Biontex, Munich, Germany), 293T cells were washed in PBS, and the cell lysates were prepared by adding 1 ml of Co-IP buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediaminetetraacetic acid [EDTA]) supplemented with protease inhibitors (Roche Molecular Biochemicals). The lysates were incubated at 4°C for 2 h with a 1:200 dilution of mouse monoclonal anti-HA antibodies. Thirty microliter of protein A/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology) was added and incubated at 4°C for 1 h with rotation. The immune complexes were then released from the beads by boiling in sample buffer for 5 min. Following electrophoresis on 10% SDS–polyacrylamide gels, the immunoprecipitates were analyzed by Western blotting with either anti-HA or anti-Flag antibodies.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

MDPC-23 cells were treated with zinc or TPEN. The cells were treated with the cross-linking reagent formaldehyde (1% final concentration) for 10 min at 37°C, rinsed twice with cold PBS, and swollen on ice in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl [pH 8.1]) for 10 min. The nuclei were collected and sonicated on ice. The 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 with anti-MTF-1 (1 ml) and anti-NFI-C antibodies (30 μl) on a rotator at 4°C for 4 h. Thirty microliter of protein A/G PLUS-agarose (Santa Cruz Biotechnology) was added and incubated at 4°C for 1 h with rotation to collect the antibody/chromatin complex. Cross-linked, precipitated chromatin complexes were recovered and reversed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). The final DNA pellets were recovered and analyzed by PCR using primers that encompass the MT-1 promoter region (183 bp);

mouse MT-1 promoter −238 region: forward, 5′-cactatagggacat-gatgttc-3′ and mouse MT-1 promoter −55 region: reverse, 5′-cagcaggcggttgctcca-3′, and the DSPP promoter region (390 bp), mouse DSPP-400 region: forward 5′-gggtcttaaatagccagtcg-3′ and mouse DSPP-10 region: reverse, 5′-ctgagagtggcacactgt-3′. The following PCR conditions were used: 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min for a total of 35 cycles. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

ALIZARIN RED S STAINING

The cells were fixed with 70% ethanol for 20 min and stained with 1% alizarin red S (Sigma–Aldrich) in 0.1% NH_4OH at pH 4.2–4.4. The mineralization assays were performed by staining MDPC-23 cells treated with either zinc or TPEN with alizarin red S solution. The cells were evaluated at 0, 4, 7, 10, and 14 days. To quantify the intensity of mineralization, we measured density of stained nodules by colorimetric spectrophotometry. The stained cells were collected by centrifugation at 13,000 rpm for 10 min at 4°C. Cell lysis was solubilized with 0.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature (vortexing or pipetting). Solubilized stain (0.1 ml) was transferred to wells of a 96-well plate, and absorbance was measured at 405 nm.

STATISTICAL ANALYSIS

Statistical analyses were carried out using a Student's *t*-test. Statistical significance is displayed as; $^{*}P < 0.05$; $^{**}P < 0.01$. All statistical analyses were performed using SPSS software ver. 19.0.

RESULTS

EFFECT OF ZINC ON NFI-C GENE EXPRESION IN ODONOTOBLASTS

Physiological concentrations of zinc is 1.5–100 μM in Hep-2 cells [Rudolf et al., 2003]. To select suitable does of physiological concentration, we analyzed the cell viability by MTT assay. We treated various concentrations of zinc and zinc chelator, TPEN, in mouse odontoblast cells (MDPC-23). In the present study, odontoblast cell viability was increased in additive zinc concentration (10–30 μM) and decreased in a dose dependent manner of high zinc level and TPEN (supplemental Fig. S1). Therefore, we had chosen 10, 30, 50, or 80 μM concentration of zinc and 1, 5, or 10 nM concentration of TPEN for experiments.

Cellular zinc homeostasis and zinc cellular signaling processes are controlled by MTs [Vallee, 1995]. Also, intracellular zinc concentrations and MT gene expression are dependent on the supplement of zinc or zinc chelator in THP-1 cells [Cao et al., 2001]. When compared with levels in control cells, MT-1 mRNA increased in MDPC-23 cells following the addition of zinc to the culture medium. However, MT-1 mRNA levels decreased after the addition of TPEN (Fig. 1A). Similar to previous reports, these data showed that changes in MT-1 mRNA expression are linked to zinc or TPEN-mediated changes in intracellular zinc concentrations in MDPC-23 cells.

We further investigated whether zinc is involved in the regulation of the MTF-1, NFI-C, Smad 2/3, and Smad 1/5/8 genes in odontoblasts. Zinc treatment enhanced the expression of MTF-1 mRNA (Fig. 1A) and protein (Fig. 1B), but decreased NFI-C protein expression. However, TPEN treatment hardly influenced NFI-C expression. In addition, zinc also enhanced phosphorylation of the Smad2/3 protein (p-Smad2/3) and Smad 1 protein (p-Smad 1) (Fig. 1B). To confirm the zinc-mediated expression change in NFI-C, we performed an NFI-C promoter assay. We discovered that NFI-C promoter activity was decreased by zinc in a concentration-dependent manner. However, TPEN barely influenced NFI-C promoter activity in both MDPC-23 and 293T cells (Fig. 1C). These results support the hypothesis that intracellular zinc concentrations change NFI-C expression in odontoblasts.

EFFECT OF ZINC ON THE SUBCELLULAR LOCALIZATION OF NFI-C

A number of stress-responsive transcription factors are primarily localized to the cytoplasm, but they can translocate to the nucleus when required. Like these, MTF-1 has a dual nuclear and cytoplasmic localization. MTF-1 is normally found in the cytoplasm; however, it translocates into the nucleus and induces the expression of MTs in response to zinc in the human kidney [Saydam et al., 2001]. NFI-C2 is also translocated into the nucleus upon prolactin stimulation in mammary glands cells [Johansson et al., 2005]. To examine the effect of zinc on the intracellular localization of MTF-1, NFI-C, and p-Smad2/3, we isolated nuclear and cytoplasmic protein fractions and investigated their cellular localization by Western blot analysis. Zinc promoted the rapid nuclear translocation of MTF-1 in MDPC-23 cells (Fig. 2A). In control cells, endogenous NFI-C was located in the cytoplasm and the nucleus. In cells that received 50 μM of zinc for 4 h, we did not observe a change in localization patter for NFI-C compared to the control. However, in zinc deficiency, endogenous NFI-C was localized and accumulated in the nucleus (Fig. 2A and supplemental Fig. S2). Similar with endogenous NFI-C, exogenous GFP-tagged NFI-C also accumulated in the nucleus of MDPC-23 cells (supplemental Fig. S2). Moreover, Smad2/3, which is phosphorylated by zinc, was observed in cytoplasm in a dose-dependent manner. However, the activity and nuclear translocation of p-Smad2/3 were not altered by TPEN (Fig. 2A).

TGF-β regulates proliferation, cellular differentiation, and other functions in most cells [Ignotz and Massague, 1987]. During dentinogenesis, Smads and NFI-C signaling works via negative feedback. Indeed, TGF-β receptor 1 and Smad3 are over-expressed in *Nfic*-deficient mice [Lee et al., 2009]. TGF-β induced the interaction between p-Smad3 and NFI-C, leading to the degradation of NFI-C (unpublished data). We examine that zinc has the capacity to induce the interaction between p-Smad2/3 and NFI-C. 293T cells were transfected with an HA-tagged NFI-C expression construct and a Flag-tagged Smad3 expression construct for IP assay. The IP assay with an anti-HA antibody and a subsequent Western blot with an anti-Flag antibody demonstrated the zinc-dependent interaction between p-Smad2/3 and NFI-C. Furthermore, zinc deficiency in MDPC-23 cells rendered p-Smad2/3 unable to bind to NFI-C (Fig. 2B). These results suggest that zinc changes the binding efficiency between NFI-C and p-Smad2/3. Altogether, our data suggests that zinc is acceptable for regulation of expression and localization.

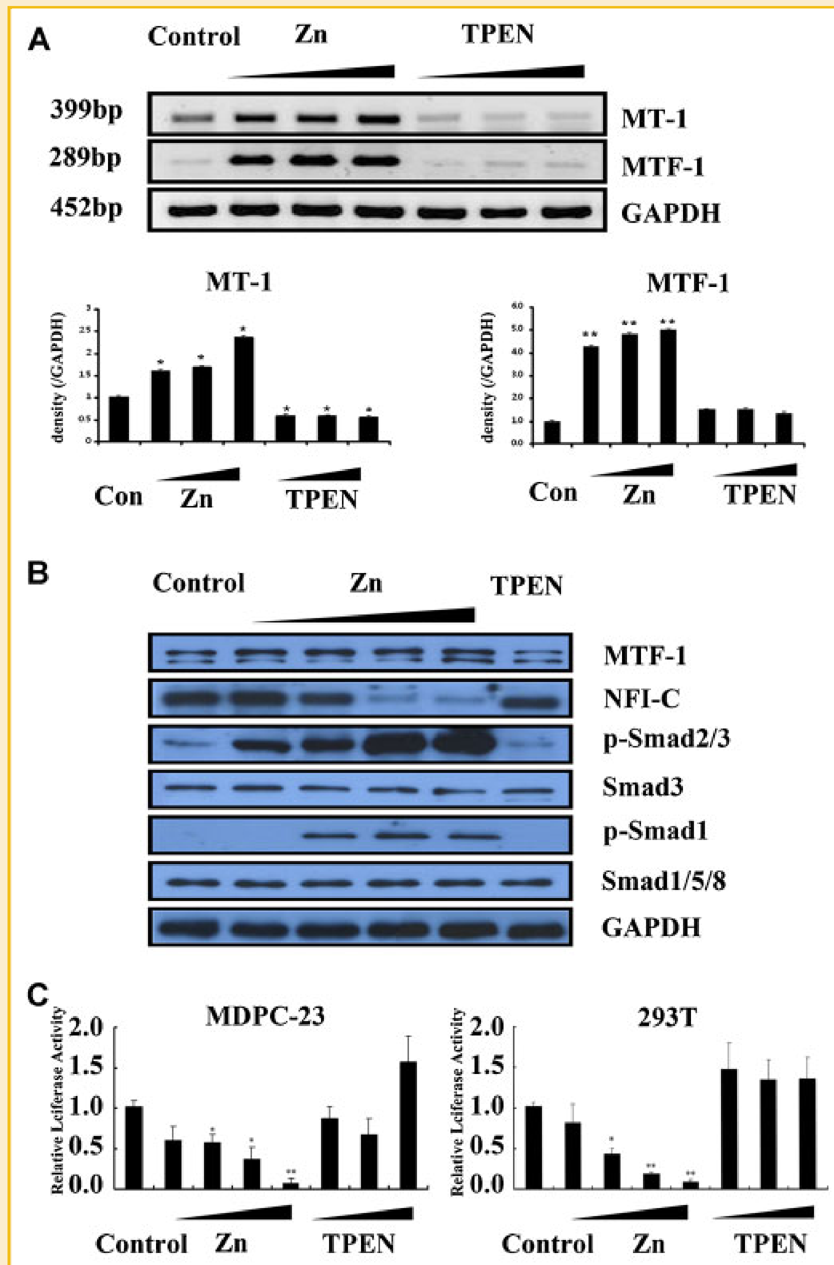

Fig. 1. MDPC-23 cellular expression of MT-1, MTF-1, NFI-C, Smad 2/3, and Smad1/5/8 mRNA and protein in the presence or absence of zinc. A: MDPC-23 cells were treated with 10, 50, or 80 μM of zinc or 1, 5, or 10 nM of TPEN. MT-1 and MTF-1 expression were monitored by RT-PCR. Densitometry analysis of the MT-1 and MTF-1 gel images (lower panel). Con; control. Zinc induced MT-1 and MTF-1 mRNA expression. The data are presented as the mean ± SD of triplicate experiments. Asterisks denote values significantly different from the control ($^{*}P < 0.05$; $^{**}P < 0.01$). B: MDPC-23 cells were treated with 10, 30, 50, or 80 μM of zinc or 10 nM of TPEN. The cell lysates were analyzed by Western blot using antibodies against MTF-1, NFI-C, p-Smad 2/3, Smad3, p-Smad 1, Smad 1/5/8, and GAPDH. Zinc increased MTF-1 protein levels and induced the phosphorylation of Smad2/3 and Smad 1, but decreased NFI-C expression. C: MDPC-23 and 293T cells were transfected with the NFI-C reporter gene. Cells were treated with increasing concentrations of zinc (10, 30, 50, and 80 μM) or TPEN (1, 5, and 10 nM) for 4 h, and the cell lysates were subjected to luciferase assays. The activity of the NFI-C promoter was decreased after zinc administration. Promoter activity was determined as luciferase light units/protein and expressed as fold activation compared to control. The data are presented as the mean ± SD of triplicate experiments ($^{*}P < 0.05$; $^{**}P < 0.01$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

RECRUITMENT OF NFI-C TO THE MT-1 PROMOTER IN RESPONSE TO ZINC

In a zinc-dependent manner, MTF-1 binds to DNA sequence motifs, such as metal response elements (MREs), with a core consensus of TGCRCNC in the promoter region (−200 to −30) of MT genes. Also, NFI is known as a transcription factor that binds overlapping MRE element sites in the mouse MT-1 promoter region and mediates MT-1 gene synergistically with MTF-1 [LaRochelle et al., 2008]. To clarify the role of NFI-C and MTF-1, which could affect zinc-responsive transcriptional activity by binding to MT genes in

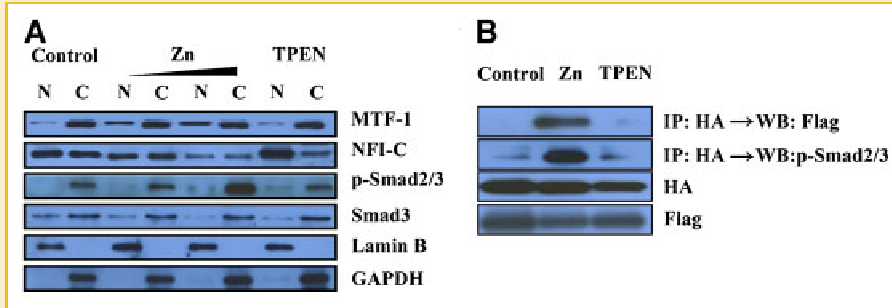

Fig. 2. Subcellular localization of NFI-C in the presence or absence of zinc. A: MDPC-23 cells were treated with 50 or 80 μM of zinc or 10 nM of TPEN. The cell lysates were then analyzed by Western blot using antibodies against NFI-C. Endogenous NFI-C was located in nucleus and cytoplasm, but NFI-C proteins were translocated by TPEN. N: nucleus, C: cytosol B: 293T cells were transfected with both HA-tagged NFI-C and Flag-tagged Smad3 and treated with 80 μM zinc or 10 nM TPEN. The cell lysates were subjected to IP followed by Western blot with anti-HA and anti-Flag antibodies, or vice versa. In the presence of zinc, NFI-C interacted with p-Smad2/3. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

odontoblasts, we performed a ChIP assay for transcription factor binding properties. Chromatin DNA fragments were precipitated with the indicated antibodies and amplified using selective primers for binding sites in the MT-1 promoter region. The MT-1 promoter could be precipitated using an MTF-1 and NFI-C-specific antibodies. However, negative control (pre-immune serum) could not precipitate MT-1 promoter (Fig. 3A). In the zinc sufficiency condition, MTF-1 translocated into the nucleus, directly interacted with the MRE site on the MT-1 promoter (Fig. 3B), and eventually upregulated the MT-1 gene (Fig. 1A). In contrast, in the zinc deficiency condition, the recruitment of NFI-C to the MT-1 promoter was further increased (Fig. 3B), and the MT-1 gene was decreased (Fig. 1A). Consequently, MTF-1 and NFI-C can bind to the MRE site on the MT-1 promoter individually and can regulate MT-1 expression in response to zinc.

ZINC INFLUENCES PROLIFERATION AND DIFFERENTIATION

In tooth development, odontoblasts differentiate in response to epithelial–mesenchymal interactions, which regulate proliferation, differentiation, and polarization [Thesleff and Sharpe, 1997]. Odontoblasts produce collagenous and non-collagenous proteins to form the dentin matrix [Butler, 1998]. Additionally, zinc is a structural element for DNA synthesis enzymes and is involved in regulating cell proliferation by second messenger mitogenic signaling [Grummt et al., 1986]. MC3T3-E1 cells were increased in cell viability by additional zinc treatment of 15 μM for 1, 5, and 15 days [Kwun et al., 2010]. However, the zinc-mediated cellular mechanisms in odontoblast cells have not been fully clarified.

To evaluate the effect of zinc or TPEN on the proliferation and differentiation of odontoblasts, we treated MDPC-23 cells with zinc (10 μM) or TPEN (1 nM) constantly in differentiated media and examined the protein levels of NFI-C, CDK2, osteocalcin, and DSP by Western blot analysis. The results showed that the NFI-C protein, a key transcription factor in odontoblasts, was expressed at the beginning of culture, was decreased at day 4, and increased again by day 10 in control cells. In the TPEN-treated group, NFI-C was strongly expressed throughout the entire culture period. In contrast,

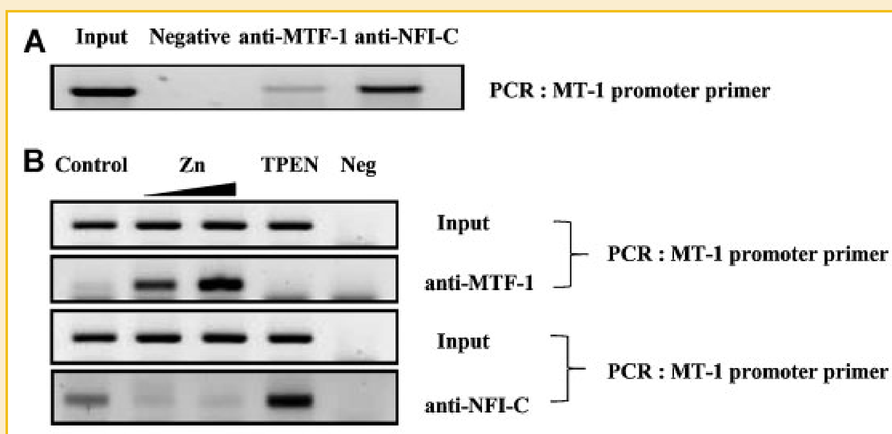

Fig. 3. Recruitment of MTF-1 and NFI-C to chromatin. A: Cross-linked chromatin was prepared and immunoprecipitated with pre-immune serum or MTF-1, NFI-C specific antibody. B: Cross-linked sheared chromatin was prepared from MDPC-23 cells treated with zinc (50 and 80 μM) or TPEN (10 nM) for 4 h. Chromatin samples were subjected to PCR analysis using primer pairs spanning the nearest MRE element site on MT-1 promoter. Zinc recruited MTF-1 and TPEN recruited NFI-C to the MT-1 promoter. The control represents the PCR product of chromatin obtained before IP with the MTF-1 and NFI-C antibodies. Neg: negative control.

this protein decreased from the beginning of the culture to day 14 in the zinc-treated group. The level of CDK2, a cellular proliferation marker, was decreased from the beginning of the culture to day 10 in control, TPEN-, and zinc-treated groups. However, in zinc-treated group, although CDK2 protein was slightly decreased from the beginning of the culture to day 10, it was still clearly expressed by day 10 (Fig. 4). Moreover, we measured cell proliferation during early time points of differentiation by MTT assay. In this result, additional zinc-treated group showed a slight increase in cell proliferation of early stage but TPEN-treated group did not show (supplemental Fig. S3). There were also notable differences in the expression levels of osteocalcin and DSP protein between the zinc- and TPEN-treated groups. The expression of osteocalcin and DSP protein, a marker of differentiated odontoblasts, was significantly increased by TPEN treatment, but slightly decreased by zinc treatment (Fig. 4). These results suggest that zinc may maintain odontoblast proliferation properties during early differentiation stage, while zinc deficiency likely promotes odontoblast differentiation.

NUCLEAR ACCUMLATION OF NFI-C REGULATES DSPP TRANSCRIPTIONAL ACTIVATION

DSPP is secreted by odontoblasts and is involved in the dentin biomineralization process [Butler, 1998]. To elucidate whether the nuclear accumulation of NFI-C leads to transcription factor functionality, we next assessed DNA binding affinity and transcriptional activation. We performed a ChIP assay for the binding of NFI-C to the TTGGC (N5) GCCAA/CCAAT motif site on the DSPP promoter. In response to zinc, NFI-C was dissociated from the DSPP promoter. In contrast, zinc deficiency mediated by TPEN caused the recruitment of the NFI-C protein to the DSPP promoter (Fig. 5A). A second approach was taken to confirm the functional consequences of nuclear-accumulated NFI-C on DSPP promoter activity. Increasing concentrations of zinc significantly repressed the transcriptional activity of the DSPP promoter. However, TPEN enhanced its transcriptional activity in both MDPC-23 and 293T cells (Fig. 5B). Therefore, it was suggested that DSPP might be upregulated through NFI-C binding, which is likely caused by zinc deprivation. Based on these results, nuclear NFI-C is biologically active for binding affinity and transcriptional activity in odontoblasts.

EFFECTS OF ZINC ON THE MINERALIZATION OF DENTIN IN VITRO

Finally, we determined the effects of altered zinc concentrations on dentin mineralization. MDPC-23 cells were cultured for 14 days in differentiation media with continuous zinc or TPEN, and the mineralized nodules were evaluated by Alizarin red S staining. In normal MDPC-23 cells, mineralized nodules appeared after 10 days of culture. The zinc-treated group showed mineralized nodules after 10 days of culture in the same pattern as the control group. However, the number of mineralized nodules was slightly lower in the zinc-treated group compared to the control. Interestingly, the TPEN-treated group showed mineralized nodules after only 4 days, and the mineralized nodules increased with time during the culture period (Fig. 6A,B). These results suggest that zinc sufficiency can slightly delay dentin mineralization in vitro, while zinc deficiency initiates mineralization earlier and accelerates the formation of mineralized nodules.

DISCUSSION

TGF-β1 overexpressing transgenic mice develop distinct dentin defects similar to those seen in *Nfic*-deficient mice [Thyagarajan et al., 2001]. Therefore, NFI-C and TGF-β1 are essential factors for both normal odontoblast differentiation and dentin formation.

Zinc plays important roles in TGF-β signaling. Preconditioning with zinc stimulates the phosphorylation of a downstream factor, protein kinase C [Aras et al., 2009], and phosphorylated protein kinase C phosphorylates the MH1 domain of Smad3 and directly abrogates promoter DNA binding of Smad3 [Yakymovych et al., 2001]. However, the functional relationship between zinc and NFI-C during odontoblast differentiation remains uncharacterized. In the present study, zinc stimulated the phosphorylation of Smad2/3 in cytoplasm and also induced the binding of p-Smad2/3 to the NFI-C protein. In contrast, TPEN treatment leads to NFI-C

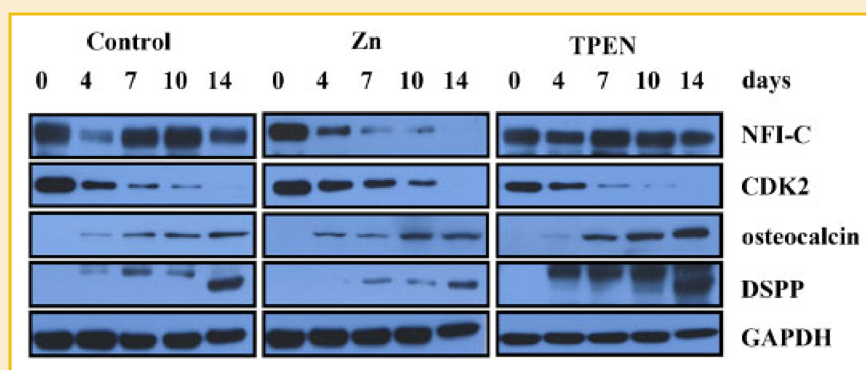

Fig. 4. NFI-C, CDK2, osteocalcin, and DSP protein expression during odontoblast differentiation in vitro. Western blot analysis of NFI-C, CDK2, osteocalcin, and DSP expression during MDPC-23 cell differentiation in the presence of zinc (10 μM) or TPEN (1 nM). The cells were evaluated at 0, 4, 7, 10, and 14 days. NFI-C was strongly expressed in TPEN-treated cultures. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

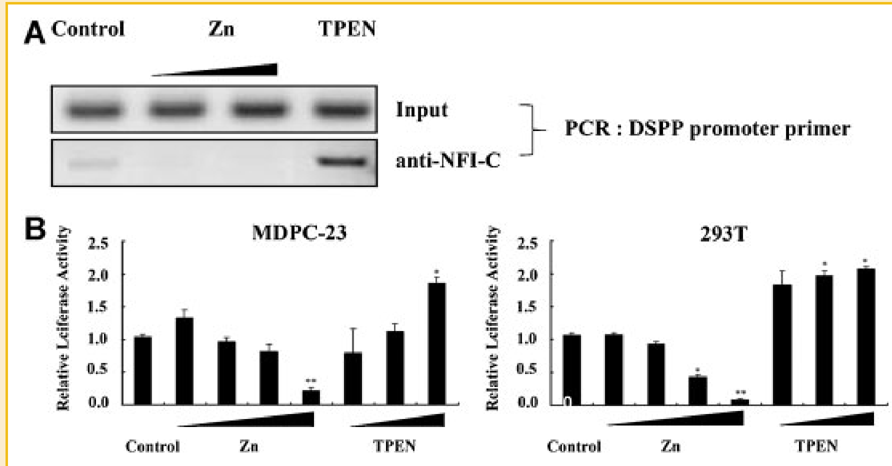

Fig. 5. NFI-C functions as a transcription factor. A: Cross-linked sheared chromatin was prepared from MDPC-23 cells treated with zinc (50 and 80 μM) or TPEN (10 nM) for 4 h. Chromatin samples were subjected to PCR analysis using primer pairs spanning the nearest TTGGC (N5) GCCAA/CCAAT motif site on the DSPP promoter. NFI-C was recruited by TPEN to the DSPP promoter. The control represents the PCR product of chromatin obtained before IP with the NFI-C antibody. B: MDPC-23 and 293T cells were transfected with pGL3-DSPP. The cells were treated with increasing concentrations of zinc (10, 30, 50, and 80 μM) or of TPEN (1, 5, and 10 nM) for 4 h, and the lysates were then subjected to luciferase assays. The transcriptional activity of the DSPP promoter was inhibited by zinc in a dose-dependent manner, but was activated by TPEN. The data are presented as the mean ± SD of triplicate experiments. Asterisks denote values significantly different from the control ($^{*}P < 0.05$; $^{**}P < 0.01$).

translocation to the nucleus. These results suggest that zinc may play a role in holding NFI-C in the cytoplasm by inducing the tight interaction between NFI-C and p-Smad2/3. Therefore, zinc could be a key element for mediating interactions between NFI-C and p-Smad2/3.

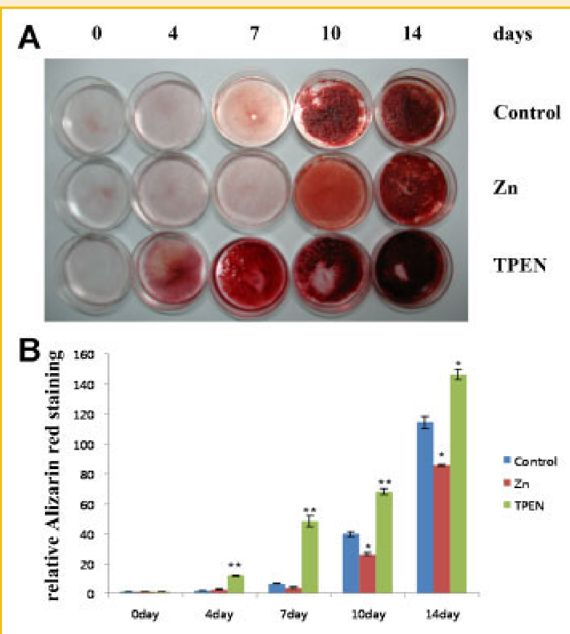

Fig. 6. Mineralization in MDPC-23 cells in the presence or absence of zinc. A: MDPC-23 cells were treated with 10 μM zinc or 1 nM TPEN for 14 days, and the mineralization was evaluated by Alizarin red S staining. B: Quantification of mineralization by colorimetric spectrophotometry. Odontoblast mineralization was altered by zinc administration. The data are presented as the mean ± SD of triplicate experiments. Asterisks denote values significantly different from the control ($^{*}P < 0.05$; $^{**}P < 0.01$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Differently spliced isoforms of NFI proteins (NFI-A, NFI-B, NFI-C, and NFI-X) play different roles in various tissues or with different promoters. The overexpression of NFI-C downregulates MT-1 promoter activity in HepG2 cells, whereas NFI-A and -B activate it [Majumder et al., 2001]. In the present study, NFI-C interacted with the MT-1 promoter, and MT-1 was decreased in the zinc deficiency condition. We suggest that MT-1 gene expression in MDPC-23 cells is regulated by a transcriptional activator of MTF-1 and a transcriptional repressor of NFI-C, and this regulation is likely associated with different isoforms of NFI proteins.

It was recently discovered that zinc and MT-1 induce DNA synthesis and mitogenic gene induction through transitioning the S-phase of cell cycle [Beyersmann and Haase, 2001]. Also, MTF-1 is involved in tumor development, and the loss of MTF-1 results in the augmentation of ECM deposition [Haroon et al., 2004]. In the present study, zinc sufficiency condition increased the expression of MT-1 and MTF-1, decreased NFI-C expression, and maintained the proliferation of MDPC-23 cells through the CDK2. In contrast, zinc deficiency condition reduced MT-1 and MTF-1 expression, and induced the differentiation of MDPC-23 cells through the upregulation of DSPP. These results suggest that the balance of zinc concentrations regulates odontoblast proliferation and differentiation.

Mutations in the DSPP gene cause dentinogenesis imperfecta and dentin dysplasia [Zhang et al., 2001; Rajpar et al., 2002]. In addition, the DSPP gene promoter contains TATA and CAATT box sequences [Feng et al., 1998]. Transcription factors that recognize the CCAAT motif have been identified, such as CCAAT/enhancer-binding protein (C/EBP), CAAT-box transcription factor (CTF/NF-I), and CP1 (NF-Y/CBF) [Dorn et al., 1987]. Furthermore, C/EBPβ and NF-Y mediate DSPP expression and odontoblast differentiation [Naraya-

nan et al., 2004; Chen et al., 2008]. Thus far, there few reports indicate a direct interrelationship between NFI-C and DSPP promoters. However, in the present study, NFI-C interacted with the DSPP promoter, enhanced DSPP promoter activity, and, consequently, promoted the differentiation of odontoblasts and dentin mineralization. These observations suggest that NFI-C could mediate DSPP gene expression through recognized TTGGC (N5) GCCAA/CCAAT motifs.

Zinc stimulates bone formation by inducing the expression of osteoblast differentiation marker genes, such as Runx2 and osteoprotegerin, and by inducing ALP activity and Ca deposition [Yamaguchi et al., 2008]. However, in the present study, zinc inhibited or delayed dentin formation. Also, zinc increased NFI-C in osteoblast-like MC3T3-E1 cells (supplemental Fig. S4), but decreased NFI-C in odontoblastic MDPC-23 cells. Zinc induces contrasting effects of NFI-C expression in osteoblasts and odontoblasts and regulates the transcriptional activity of the DSPP gene. It was reported that zinc-deficient diet mice decreased fetal long bone growth [Kim et al., 2009]. However, we could not find any tooth defects in these mice, including dentin (data not shown). The nutritional influence of zinc may not be sufficient to affect in dentin mineralization. Therefore, the relationship between zinc and NFI-C may act by different mechanisms between bone and dentin. Similar to our results, zinc plays a reciprocal role for calcification in bone (hard tissue) and blood vessels (soft tissue). In physiological process of bone formation, zinc enhances skeletal growth; however, in pathological process, zinc deficiency increases the calcification of smooth muscle cells, which is associated with plaque formation [Vattikuti and Towler, 2004; Shen et al., 2008; Yamaguchi et al., 2008].

Zip13-KO mice showed reduced dentin formation of molar teeth in addition to reduced osteogenesis and abnormal cartilage development [Fukada et al., 2008]. However, in the present study, zinc deficiency induced by TPEN promoted dentin mineralization in vitro. Based on these findings, we speculated that TPEN treatment resulted in the overall deficiency of zinc in the cytoplasm of odontoblasts, while Zip13-KO affects the distribution of zinc within the cytoplasm, causing a local zinc deficiency in the cytoplasm, especially near the Golgi complex. Recently, it was reported that dietary zinc-deficient mice up-regulate Zip13 mRNA expression [Guo et al., 2011]. This report has also provided evidence supporting our data.

As summarized in Figure 7, we arrived at several conclusions. The addition of zinc or TPEN to the culture media in MDPC-23 cells is associated with the induction of NFI-C expression, subcellular localization, and biological activity as a transcription factor. Thus, these data indicate that NFI-C which was affected by zinc sufficiency condition may inhibit or delay the differentiation and mineralization of odontoblasts; however, the nuclear accumulation of NFI-C following zinc deficiency could initiate earlier and accelerated differentiation and mineralization of odontoblasts. Finally, zinc cellular equilibrium is extremely important for dentinogenesis. Further investigations will provide greater insight into the NFI-C signaling and translocation mechanisms in dentin.

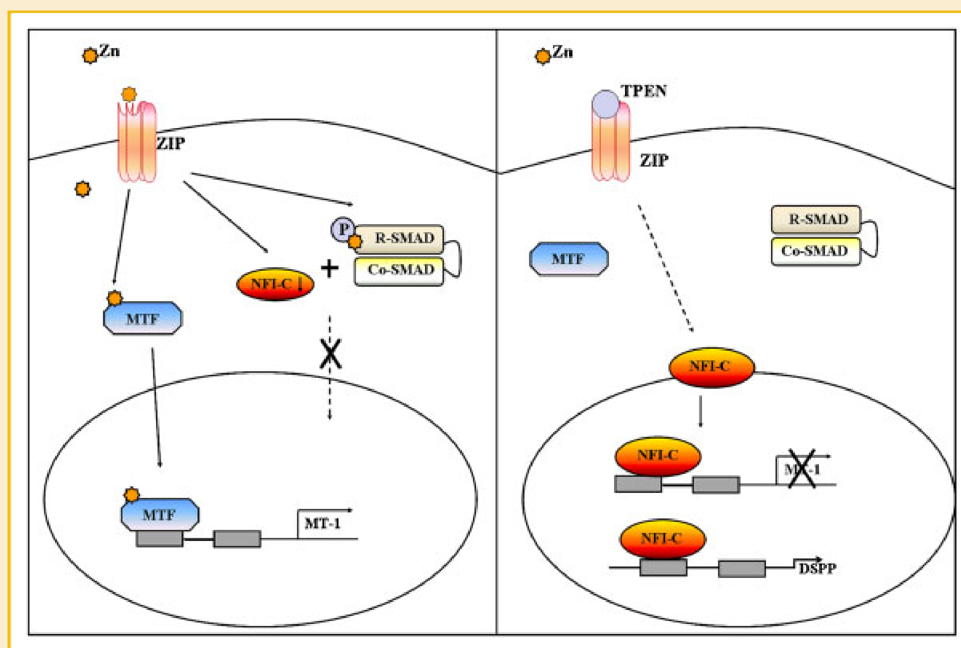

Fig. 7. A crucial role for zinc in odontoblast biology (a mechanistic summary). Zinc promotes the binding of NFI-C and Smads in the cytoplasm, while the lack of zinc causes the NFI-C protein to function as a transcription factor in the nucleus. The balance of zinc is important for odontoblast differentiation and mineralization. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

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