



The role of nuclear factor I-C in tooth and bone development

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Nuclear factor I-C (NFI-C) plays a pivotal role in various cellular processes such as odontoblast and osteoblast differentiation. *Nfic*-deficient mice showed abnormal tooth and bone formation. The transplantation of *Nfic*-expressing mouse bone marrow stromal cells rescued the impaired bone formation in *Nfic*^{-/-} mice. Studies suggest that NFI-C regulate osteogenesis and dentinogenesis in concert with several factors including transforming growth factor- β 1, Krüppel-like factor 4, and β -catenin. This review will focus on the function of NFI-C during tooth and bone formation and on the relevant pathways that involve NFI-C.

Key words: Nuclear factor I-C, Dentinogenesis, Osteogenesis, Osteoporosis

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I. Introduction

Dentin makes up the bulk of the teeth along with other tissues such as enamel, cementum, and pulp. Dental papilla cells differentiate to form dentin-pulp complex; various types of cells are found in the dental pulp including odontoblasts, fibroblasts, and immune cells such as macrophages, granulocytes, and mast cells¹. The dental follicle can differentiate into cementoblasts, osteoblasts, and fibroblasts, which later produce cementum, alveolar bone, and periodontal ligament, respectively². Strong nuclear factor I-C (*Nfic*) expression was detected in the dental papilla and dental follicle during molar development in rats from postnatal day 5 to day 16³. *Nfic*^{-/-} mice exhibited unique phenotypes, such as defective tooth root and incisors and decreased bone density and volume⁴ caused by

aberrant odontoblast formation⁵. The importance of NFI-C in tooth and bone formation was confirmed; therefore, many studies have been conducted in order to elucidate the function of NFI-C and to determine how NFI-C plays a role in tooth and bone formation. This review will introduce several studies of signaling pathways and key factors correlated with the function of NFI-C in tooth and bone formation.

II. General Description of the NFI Family of Transcription Factors

The nuclear factor I (NFI) family of transcription factors plays pivotal roles in the development of the brain, lungs, muscles, and many other organs⁶. NFI protein was first isolated from HeLa cell nuclear extract and was identified as a stimulator of adenovirus DNA replication initiation⁷ and a transcription factor for many viral and cellular genes⁸.

There are two motifs in a consensus sequence of NFI binding site TTGGCNNNN(N)GCCAA. Five base pair spacer region in the middle regulates the NFI binding affinity⁹ and NFI proteins can bind to the TTGGC and/or GCCAA regions as a dimer or individually, with reduced binding affinity¹⁰. By measuring the binding specificity of individual sites, NFI was found to be identical to the TGGCA-binding protein, which binds to chicken lysozyme's enhancer region¹¹, and

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the CAAT box transcription factor, which binds to the CAAT box of the promoter region in multiple genes¹². Subsequent studies found that NFI can also bind to the gene enhancer, promoter, and silence regions.

In vertebrates, the NFI family of transcription factors consists of four members, NFI-A, NFI-B, NFI-C, and NFI-X^{8,13}. Some of the features, such as high mortality rate and delayed glial and neuronal differentiation are commonly observed in mice deficient in NFI-A, NFI-B, and NFI-X¹⁴⁻¹⁶. NFI-B deficiency also causes defects in lung maturation¹⁷, and NFI-X deficiency leads to skeletal defects¹⁸. Unlike other NFI family members, NFI-C is known to play a critical role in tooth and bone formation. NFI-C knockout mice showed reduced bone density and abnormal tooth formation, but no life-threatening defects were observed⁴.

III. Function of NFIC in Dentinogenesis

Odontoblasts are differentiated from the dental papilla cells that were derived from neural crest origin ectomesenchymal cells¹⁹. Epithelial-mesenchymal interaction induces the dental papilla cells to differentiate into odontoblasts during crown formation²⁰ while Hertwig's epithelial root sheath (HERS) cells drive the differentiation of the dental papilla cells into odontoblasts during root formation²¹.

When *Nfic*^{-/-} mice were generated by removing the second exon of the *Nfic* gene, which contains the binding and dimerization domain of the *Nfic* gene, intriguing morphologies such as the formation of brittle and short mandibular incisors and abnormal maxillary incisors along with molars with no roots were observed⁴. Markedly different morphology of the *Nfic*^{-/-} mice from that of the wild type mice in the root area suggested that the failure of HERS formation may cause abnormal odontoblast differentiation in the *Nfic*^{-/-} mice. However, during the tooth root formation, both groups of mice formed normal HERS by the time crown formation was completed. Aberrant, polygonal odontoblasts with no polarity were observed around the short and abnormal roots and labial crown analog of *Nfic*^{-/-} mice. Since crown formation occurs during the embryonic stage and root formation takes place postnatally, it was hypothesized that NFI-C plays a crucial role in odontoblast differentiation during postnatal tooth development and dentin formation during molar development, but not in HERS⁵.

Odontoblasts and dentin were observed in a more precise manner with electron microscopy to analyze the cause of defects in molars and incisors of *Nfic*^{-/-} mice. Compared to

those of wild type mice, many of the aberrant odontoblasts of *Nfic*-deficient mice were disorganized and round in shape. The cells were also trapped in an osteodentin-like mineralized tissue and showed no intracellular junctional complexes. These odontoblasts showed characteristics of apoptotic cells²² which can be defined as aggregated chromatin, formation of apoptotic bodies, and swollen mitochondria²³. Localization and expression levels of several structural proteins, such as zonula occludens-1 (ZO-1), occludin, E-cadherin (E-Cad), and connexin 43 (CX 43), which comprise the junctional complexes, were observed *in vivo* and *in vitro*. Expression of ZO-1, occludin, and E-Cad appeared to be decreased or absent, whereas that of CX 43 was observed consistently in both, wild type and *Nfic*^{-/-} mice²². ZO-1 is a tight junction protein that can be found on the surface of cell membranes; it acts as a scaffold protein for other tight junction strand proteins^{24,25}. CX 43, also called gap junction alpha-1 protein, composes gap junctions (as the name suggests), allowing gap junction intercellular communication that regulates various cellular processes^{26,27}. Occludin is an integral cell-membrane protein that composes tight junctions as well. When E-Cad (a cell-adhesion glycoprotein) is downregulated, cellular adhesion strength decreases and cellular motility increases, which then may help in the invasion of adjacent tissues; this may result in cancer progression and metastasis^{28,29}. This data suggested that NFI-C is involved in the formation of intercellular junctions, which occurs during the differentiation of odontoblasts from preodontoblasts³⁰. Similar appearance of aberrant odontoblasts was observed in osteodentin, which is formed during dentin repair. When dentin is severely damaged and the odontoblasts are killed, the pulp cells differentiate into odontoblast-like cells to form reparative dentin^{22,31}.

Since *Nfic*^{-/-} mice and transforming growth factor β 1 (TGF- β 1) transgenic mice showed similar tooth morphologies, further investigation was conducted to identify the relationship between NFI-C and TGF- β during odontoblast differentiation. The TGF- β family consists of TGF- β , activins, inhibins, and bone morphogenic proteins (BMPs)^{32,33}. Members of this family of growth factors contribute to various cellular processes such as proliferation, differentiation, embryonic development, and epithelial to mesenchymal interactions³⁴. One of the TGF- β family members, TGF- β 1, is known to promote cell growth arrest through the Smad signaling pathway by activating the cyclin-dependent kinase inhibitor p21^{35,36}. In HeLa cells, NFI contributes to the inhibition of p21³⁷. After TGF- β treatment on the odontoblast-like cell line MDPC23, NFI-C degradation increased in

both, the cytosol and the nucleus. Treatment with MG132, a proteasome inhibitor, prevented the degradation of NFI-C by TGF- β 1 treatment. The increased mRNA level and decreased protein level of NFI-C after the treatment with TGF- β 1 suggested that TGF- β regulates NFI-C by degradation via 26S proteasome. In the primary pulp cells of *Nfic*^{-/-} mice, the expression levels of TGF- β , TGF- β receptor type I (TGF- β RI), p-SMAD2/3, and p21 appeared to be increased. Overexpression of *Smad3* induced degradation of NFI-C, and silencing of *Smad3* inhibited the degradation of NFI-C by TGF- β 1. When *Nfic* was overexpressed, p-SMAD3 was dephosphorylated and lost its ability to form an NFI-C degradation complex with smad4, inhibiting NFI-C degradation. Therefore, TGF- β 1 signaling is suggested to be negatively regulated by NFI-C during odontoblast differentiation. Smad ubiquitination regulatory factor (Smurf) is an E3 ubiquitin-protein ligase that contributes to the degradation of Smad protein, thereby negatively regulating TGF- β /BMP signaling³⁸⁻⁴⁰. Amongst several major MAPK pathways, the Jun N-terminal kinase (JNK) pathway is thought to be more likely involved in the interaction between NFI-C and Smurf 1/2⁴¹ since JNK inhibitor decreased the interaction, whereas p38, another major MAPK pathway, inhibitor did not affect the interaction between those two⁴²⁻⁴⁵. In all, NFI-C is thought to be degraded by TGF- β 1 through the Smad pathway with the aid of Smurf 1/2 during early odontoblast differentiation. In the late stage of odontoblast differentiation and mineralization, NFI-C dephosphorylates p-SMAD2/3 in both the cytoplasm and the nucleus, and is translocated into the nucleus, binding its target gene's promoter and regulating the gene expression⁴².

Zinc deficiency also results in a similar tooth phenotype that is observable in *Nfic*^{-/-} mice. Zinc transporter *Slc39a13/Zip13* transports zinc from the Golgi apparatus to the cytosol, and therefore regulates intracellular zinc level. *Slc39a13/Zip13*-deficient mice were morphologically similar to *Nfic*-deficient mice, in terms of skeletal disorders, reduced root dentin formation in the molars, and abnormal incisors⁴⁶. Zinc is a trace element and regulates various processes in living organisms including cellular metabolism, growth, replication, and tissue repair⁴⁷. Maintaining the appropriate zinc level is crucial since zinc deficiency causes impairment of brain functions and immune responses, while an excessive amount of zinc can cause apoptosis and neuronal death^{48,49}. Adequate zinc level is also required for odontoblast differentiation and dentin formation. After zinc treatment of the MDPC23 cell line, the nuclear translocation of p-SMAD2/3, metallothioneins (MT-1), and metal transcription factor-1 (MTF-1)

were increased, whereas no marked change was observed in NFI-C localization. When TPEN, a zinc chelator, was used, translocation of NFI-C into the nucleus increased. MTF-1 is a transcription factor that contains six zinc fingers in the DNA binding domain. When zinc is present, MTF-1 binds to DNA motifs such as metal response elements (MREs) in the promoter region of MT genes and increases the transcription of MT⁵⁰⁻⁵². NFI-C is known to bind to overlapping regions of MRE sites⁵³. During the early odontoblast differentiation period, adequate amount of zinc promotes nuclear translocation of MTF-1 and phosphorylation of SMAD2/3 in the cytoplasm in a dose dependent manner. MTF-1, a transcriptional activator of MT-1, increased MT-1 transcription and induced NFI-C degradation simultaneously through the TGF- β pathway. During the late stage of odontoblast differentiation, decreased zinc levels lead to the translocation of NFI-C into the nucleus. NFI-C acts as a transcriptional repressor of MT-1 and enhancer of dentin sialophosphoprotein (DSPP) promoter, accelerating differentiation and mineralization of odontoblasts⁵⁴.

Krüppel-like factor 4 (KLF4) is a member of specificity protein/KLF (SP/KLF) transcription factor family⁵⁵. This family of transcription factors is characterized by three zinc finger motifs in the carboxyl terminal sequences⁵⁶. KLF4 participates in various cellular processes such as cell proliferation, differentiation, cell growth, and maintenance of normal tissue homeostasis⁵⁷. Several members, including KLF17 and KLF4, are known to control epithelial-mesenchymal interactions⁵⁸. KLF4 is also known to regulate odontoblast cell proliferation and differentiation via control of dentin matrix protein 1 (*Dmp1*), *Dspp*, and alkaline phosphatase (*Alp*) expression^{59,60}. NFI-C is also known to control *Dspp* expression in odontoblasts; *Klf4* expression and protein level decreased significantly in the *Nfic*^{-/-} mice compared to the wild type mice. Therefore, the relationship between NFI-C and KLF4 during odontoblast differentiation was investigated. The binding of NFI-C to -980 region of *Klf4* promoter was confirmed, which lead to increased expression of *Klf4*, *Dmp1*, and *Dspp*. When *Nfic* was silenced, the expression of those genes decreased significantly. Overexpressing or silencing *Klf4* affected only the expression levels of DMP1 and DSPP, but those of NFI-C was not affected. KLF4 binds to *Dmp1* promoter, and DMP1 binds to *Dspp* promoter to increase its transcription⁶¹; therefore, the *Nfic-Klf4-Dmp1-Dspp* pathway was suggested⁶². Although odontoblasts are cells of mesenchymal origin, they exhibit epithelial cell-like characteristics as they differentiate. When MDPC-23 cells were transfected

with *Nfic* expressing vectors and cultured in osteogenic medium, they became tightly packed like patches of epithelial cells, and actin fibers rearranged to the outer surfaces of the cells. Expression of E-Cad, a mesenchymal-epithelial transition (MET) marker, and N-cadherin, an epithelial-mesenchymal transition (EMT) marker, were observed during the differentiation. Expression of E-Cad and NFI-C increased, and N-Cad, TGF- β 1, TGF- β RI decreased as differentiation progressed, which corresponded to the result of the study that investigated the relationship between NFI-C and TGF- β . Combining all the studies, it was confirmed that NFI-C controls odontoblast differentiation and mineralization through the *Nfic-Klf4-Dmp1-Dspp* pathway, and also contributes to MET induction via the *Nfic-Klf4-E-Cad* cascade⁶².

IV. Function of NFIC in Osteogenesis

Osteoblasts and adipocytes differentiate from the same progenitor, bone marrow stromal cells (BMSCs). Many proteins and signaling pathways are involved in the differentiation and cell fate determination of BMSCs. Proteins such as TGF- β , BMP, hedgehog, RUNX2, osterix (OSX), and β -catenin are reported to contribute to osteogenesis. Adipogenesis is regulated by many factors such as peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT/enhancer-binding protein (C/EBP) α , and C/EBP β . NFI-C serves as one of the critical factors for postnatal bone formation and osteogenic cell differentiation. The expression pattern of *Nfic* in mice was observed from the embryonic stage until postnatal week 60. Significant increase in *Nfic* mRNA level was observed from postnatal day 16 (P16) to 6 weeks, and then the mRNA level gradually decreased from 6 weeks until 60 weeks. *In vitro* results from mice BMSCs and MC3T3-E1, a mouse osteoblast precursor cell line, also showed a similar pattern—an increased level of *Nfic* mRNA during the early stages of osteoblast differentiation (day 4 to day 10) and decreased *Nfic* mRNA level during the late stages of osteoblast differentiation and mineralization (day 10 to day 21). Along with the delayed tooth root development, *Nfic*^{-/-} mice showed reduced bone volume and thickness for both cortical and trabecular bones, and decreased bone mineral density compared with those of wild type mice. Since prenatal bone development was not affected by *Nfic* deficiency, it was suggested that NFI-C controls bone formation during the postnatal stages, and not in the prenatal stages. Osteoblast activity was determined with femora of 6-week-old mice. *Nfic*^{-/-} mice showed decreased number of osteoblasts and proliferation rate, which

possibly impaired osteoblast function during bone formation. *In vitro* studies suggested that the decrease was not due to the osteoclast activity, since *in vivo* results showed no difference in the number of osteoclast in *Nfic*^{-/-} and wild type mice. In fact, the expression of receptor activator of nuclear factor kappa-B ligand (Rankl) was decreased in *Nfic*^{-/-} mice. When Rankl binds to its receptor, Rank, osteoclast activation and differentiation is induced. Osteoprotegerin (Opg) is secreted by osteoblasts, and binds to Rank, inhibiting Rankl from binding to its receptor and therefore inhibiting osteoclast formation. *Nfic*^{-/-} mice showed normal Opg but decreased Rankl expression, suggesting that decrease in the number of osteoblasts and bone formation is not caused by osteoclast activation. On the other hand, *in vitro* results with *Nfic*^{-/-} BMSCs showed an increase in the number of oil red-O positive cells and increased expression of adipocyte differentiation marker, *Ppar γ* . This coincides with the observation from bone sections of osteoporotic patients, which showed decreased *NFIC* expression and increased number of adipocytes. *Nfic* overexpression in *Nfic*^{-/-} BMSCs showed increased ALP expression and mineralized nodule formation when cultured in odontogenic differentiation media. In adipogenic differentiation media, cells showed significantly decreased number of oil red-O positive cells along with the expression of *Ppar γ* . To investigate whether NFI-C could rescue impaired bone formation, *Nfic*-overexpressing BMSCs were transplanted into femur cavities of *Nfic*^{-/-} mice. Increased trabecular bone volume and number and decreased bone marrow adiposity were observed, along with the osteoblast differentiation from the transplanted BMSCs. When BMSCs overexpressing *Osx* were transplanted in the same manner, partial rescue of bone formation was observed. OSX is one of key factors that contributes to osteogenesis. BMP-2 is known to regulate osteogenic transcription factors such as runt-related transcription factor 2 (RUNX2), DLX5, MSX2, and OSX, therefore modulating osteogenesis⁶³⁻⁶⁵. Expression of *Nfic* was increased after the BMP-2 treatment. Silencing *Runx2* decreased *Nfic* expression even with the BMP-2 treatment. The results confirmed that *Nfic* promoter had a binding motif for RUNX2, and RUNX2 directly bound to that site *in vitro*. An *in vivo* study showed that *Nfic* expression decreased through BMP-2 pathway in *Runx2*^{-/-} mice⁶⁶. Results from *in vivo* and *in vitro* studies demonstrated that *Runx2* acts as an upstream regulator of *Nfic*. Age-related osteoporosis patients exhibit increased bone adiposity and decreased bone formation due to impairment in osteoblast activity and differentiation. *In vitro* results using *Nfic*^{-/-} mice BMSCs also showed decreased

osteoblast differentiation and increased formation of oil red-O positive cells. Since the osteoblasts and adipocytes differentiate from the same progenitor cell, BMSCs, the formation of osteoblasts and adipocytes could be inversely proportional. Finding a key factor or pathway that is capable of shifting BMSC differentiation toward the formation of osteoblasts rather than adipocytes could be a novel candidate for treatment of age-related osteoporosis⁶⁶.

V. Other Prominent Factors in Odontogenesis and Osteogenesis

Canonical Wnt signaling pathway regulates mesenchymal cell fate⁶⁷. During adipocyte differentiation, *C/EBPβ* and *C/EBPδ* are upregulated, stimulating the expression of *C/EBPα* and *PPARγ* that in turn promote the expression of adipocyte specific genes⁶⁸. *C/EBPβ* binds to the promoter region of *Wnt10b*, one of the Wnt ligands, inhibiting its expression. As a result, Wnt signaling is inhibited while adipogenesis is activated⁶⁹. During osteogenesis, *Wnt10b* suppresses the expression of *C/EBPα* and *PPARγ* to shift the mesenchymal cell fate towards osteoblasts⁷⁰. Therefore, Wnt signaling needs to be suppressed during adipogenesis, and activated during osteogenesis^{71,72}. β -Catenin is a protein with a dual function; it is a cell adhesion molecule⁷³ and a transducer within the canonical Wnt pathway^{74,75}. When Wnt ligands bind to the receptor complex of frizzled and low-density lipoprotein receptor-related protein 5/6, β -catenin gets accumulated in the cytosol and translocated into the nucleus. In the nucleus, β -catenin controls its target gene expression and cellular differentiation. Tissue specific inactivation of β -catenin in mice leads to the tooth phenotype that is very similar to that of *Nfic*^{-/-} mice⁷⁶. *In vitro* studies also demonstrated that β -catenin deficiency causes the shift of preosteoblast cell fate from osteoblast to adipocyte⁶⁷. In *Nfic*^{-/-} mice, the level of β -catenin expression decreased significantly, and the silencing of β -catenin using siRNA resulted in the decreased expression of *Nfic* *in vitro* (unpublished data).

A recent study suggested that NFI-C regulates adipocyte and osteocyte differentiation through the *Nfic-Wnt-Runx2-Osx* pathway⁷⁷. However, further investigations should be conducted to explain a previous study that reported that changes in *Nfic* expression have no effect on the *Runx2* expression level⁶⁶.

The C/EBP family of transcription factors and NFI-C have the same binding motif, CCAAT^{9,78}. *C/EBPα*, β , and δ are known to play important roles in adipocyte differentiation,

and NFI-C inhibits adipogenesis; therefore, it is also possible that competitive binding between C/EBP family members and NFI-C might determine the mesenchymal cell differentiation, thereby promoting or inhibiting osteoblast and odontoblast differentiation. Further studies should be conducted on this issue.

VI. Conclusion

Several signaling pathways and proteins have been reported to regulate mineralized tissue formation. Even though NFI-C is one of the key transcription factors that induces odontoblast and osteoblast differentiation, its expression has to be precisely up- or down-regulated throughout the differentiation period in order to generate natural bone and tooth. The mechanism of maintenance of *Nfic* expression at an appropriate level throughout the differentiation period is still unknown. Further understanding of NFI-C and other factors of odontogenesis and osteogenesis will lead to the advancement of oral and maxillofacial regenerative therapies.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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