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Essential role of Osterix for tooth root but not crown dentin formation

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Abstract

Tooth is made of crown and root. It is widely believed that dentin formation in crown and root uses the same regulatory mechanism. However, identification of NFIC's unique function in determining root but not crown dentin formation challenges the old thought. In searching for the target molecules downstream of NFIC, we unexpectedly found a sharp reduction of OSX (osterix), the key transcription factor in skeleton formation, in the *Nfic* knockout (KO) tooth root. We then demonstrated a dose-dependent increase of *Osx* in the odontoblast cell line due to a transient transfection of *Nfic* expression plasmid. Studies of global and conditional *Osx* KO mice revealed no apparent changes in the crown dentin tubules and dentin matrix. However, the OSX conditional KO mice (crossed to the 2.3 kb Col 1-Cre) displayed an increase in cell proliferation but great decreases in expressions of root dentin matrix proteins (DMP1 and DSPP), leading to an inhibition in odontoblast differentiation, and short thin root dentin with few dentin tubules. Compared to the *Nfic* KO tooth, which contains essentially no dentin tubules and remains in a "root-less" status at adult stages, the Osx cKO root phenotype had partially improved at the late stage, indicating that other factors can compensate for OSX function. Thus, we conclude that OSX, one of the key downstream molecules of NFIC, plays a critical role in root, but not crown, formation.

Keywords

Osterix; tooth root; Nfic; odontoblast; dentin

INTRODUCTION

Tooth is composed of crown and root. There has been substantial progress in understanding the formation and regeneration of the enamel-covered crown dentin since the molecular biology era began (1,2). However, the root formation mechanisms remain unclear, partly

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due to the lack of appropriate animal models, plus the inherent difficulties associated with handling mineralized root dentin within the bone socket. For many years the knowledge obtained from the crown studies has been “logically applied” in root dentin formation. The discovery of NFIC (nuclear factor 1 C), a transcription factor essential for root but not for tooth germ and crown dentin, points to a mechanism in postnatal root formation that is potential different from that in the tooth germ/crown formation (3–5). Furthermore, recent studies suggested that the TGFβ/SMAD4-SHH βsignaling from HERS (Hertwig's epithelial root sheath, the cell that induces the differentiation of root progenital cells into odontoblasts) directly activates NFIC (6). It consequently regulates the proliferation and differentiation of odontogenic cells, although the key target molecules downstream of NFIC signal in postnatal root formation is unknown. Interestingly, a recent study reported alveolar bone defects in the *Nfic*-KO mice, in which NFIC controls OSX in osteoblast cells (7).

OSX, a zinc-finger-containing transcription factor, has long been known to act downstream of Runx2, a master gene for bone formation (8). The conventional *Osx*-KO pups died at birth and displayed a complete cartilaginous skeleton, demonstrating that Osx is essential for embryonic osteoblast differentiation and bone formation (8). The late conditional KO (cKO) work confirmed that OSX is vital for both osteoblast and osteocyte maturation during postnatal development (9). Recent studies also showed the critical role of Osx in cementogenesis (10), and the Osx expression in pulp and odontoblasts (11), in which Osx directly regulates DSPP expression *in vitro* (12). Further, Osx was activated in pulp cells during tooth pulp trauma (13). However, the *in vivo* role of Osx in odontogenesis is largely unknown.

In this study, we attempted to study roles of the *Nfic*-*Osx* pathway in tooth root formation in *Nfic* KO and both *Osx*-KO and -cKO mice using multiple techniques combining x-ray, uCT, backscattered and acid-etched casted SEM, FITC-Confocal microscope, mineral double labeling, in situ hybridization, immunohistochemistry and Toluidine blue (Supplemental Material and Methods). Our data demonstrated that OSX is the key downstream molecule of NFIC, and plays an essential role for root, but not for crown, dentin formation.

RESULTS

OSX, a downstream molecule of NFIC during molar root formation, is not required for crown dentin formation

In the search for the key downstream molecule of *Nfic* KO rootless molars (Fig. 1a), we initially examined OSX expression levels in the first mandibular molars, which were greatly reduced in the 2-week-old *Nfic* KO odontoblasts compared to the age-matched control (Fig. 1b). We also isolated and cultured the rootless pulp cells from the *Nfic* KO mandibular molars and found an approximately 40% reduction in *Osx* expression (Supplemental Fig. S1a), suggesting that OSX is likely a downstream molecule of *Nfic*. We then overexpressed CMV-*Nfic* in the odontoblast cell A4 line (14), and demonstrated a dose-dependent increase in *Osx* mRNA levels by real-time RT-PCR (Supplemental Fig. S1b), which correlated with a small but significant reduction in cell proliferation and a significant upregulation of *Col1*, *Dmp1* and *Dspp* mRNAs (Supplemental Fig. S1c), plus an accelerated mineral nodule

formation (data not shown). Together, both the *in vivo* and *in vitro* results support the notion that Osx is a downstream molecule of NFIC in the odontoblasts.

It was previously shown that there was no apparent defect in the Osx-KO tooth germ at early embryonic development (8). We then asked whether Osx is required for crown dentin formation at E18.5 and newborn by analyzing the conventional Osx KO jaw (Supplemental Fig. S2). As anticipated, the radiograph and von Kossa stain images revealed a lack of mineralized tissue in the KO jawbone, and the H&E stain image displayed a fiber-like tissue in the KO calvaria compared to the control. Unexpectedly, there were no apparent changes in the Osx KO molar crowns and incisor crown-analog compared to the age-matched control, suggesting that OSX is not required for crown dentin formation.

Osx is critical for both molar root and incisor root-analog (dentin at the lingual side) during postnatal development

Because *Osx* KO mice die at neonatal stage due to a lack of bone (8), we investigated the effect of *Osx* cKO on tooth root formation by crossing mice harboring a floxed allele of *Osx* (Osx^{flox}) (15) to the 2.3 *Col 1*-Cre mice that is active in pulp and odontoblast cells (16). These cKO mice presented no gross abnormalities in the molar crown in radiographs at the ages of 1, 2 and 4 weeks (Supplemental Fig. S3a), and by μCT images at ages of 2- and 4-week-old (Fig. 1c), although the molar root and incisor dentin at the lingual side were short and thin accompanied by poorly formed alveolar bone (Fig. 1c) and the cKO osteocytes were larger with few dendrites (indicating a defect in maturation) by backscattered SEM (Fig. 1d). The detailed analyses of dentin tubules by FITC (fluorescein isothiocyanates) confocal images (Fig. 1e) revealed no apparent changes in the crown dentin tubular structure at the 4-week-old stage (Fig. 1c). In contrast, *Osx cKO* mice displayed a sharp reduction in tooth roots by radiographs, uCT and H&E stain as shown in the above figures. The backscattered SEM (Fig. 1f) and acid-etched SEM results (Fig. 1g) divulged a lack of dentin tubules in the region adjacent to the PDL in the cKO in contrast to the control dentin tubules, which were long in length, well defined in structure, and spread over in the entire dentin area. Interestingly, there was essentially no sign of molar roots before the age of 2 weeks but the phenotype had improved by age of 4 weeks. A similar defect occurred in the Osx cKO incisor root-analog dentin which was observed to be thin with malformed dentin tubules in uCT, H&E and SEM images (Supplemental Fig. S3b–d).

To address whether OSX plays a role in the mineralization in root dentinogenesis, we did a fluorochrome labeling assay, in which the *Osx* cKO mineral deposition rate was greatly reduced in the root dentin compared to the age-matched control (Fig. 2a, arrows). The quantitative analysis showed the distance between the two lines was much narrower in the cKO group (at ~4 μm/day) than that in the WT group (~10 μm/day), which is statistically significant ($p < 0.01$; Fig. 2a).

Together, the above data support the notion that OSX is required for establishing the normal architecture and organization of the dentin tubules in root, and is especially critical in the first two weeks postnatally. This time-dependent function points to unknown molecules that compensates for the OSX role in late root formation, as the 2.3 *Col 1*-Cre activity at the age of 4 weeks remained high (Supplementary Fig. 5).

OSX is responsible for root-polarized odontoblast formation partly through Dmp1 and DSPP

In search for the cellular and molecular mechanisms by which OSX controls root dentin formation, we initially studied cell proliferation in the 2-week-old *Osx* cKO pulp, which displayed significantly more BrdU positive-stained cells compared to the age-matched control, indicating an inhibitory role of OSX in controlling cell proliferation (Fig. 2b). We showed a flat or cuboid-like odontoblast layer in the Osx cKO molar in contrast to the polarized and well defined odontoblast layer in the age-matched control, suggesting a critical role of OSX in cell differentiation (Fig. 2c). To further address changes in the molecules critical for odontoblast differentiation, we examined mRNA expression levels of *Col 1* (type one collagen, the major component of odontoblasts, Fig. 2d), *Dmp1* [dentin matrix protein 1, a matrix protein critical for odontoblast differentiation and dentin formation (17), Fig. 2e] and *Dspp* [dentin sialophosphoprotein, a critical gene essential for postnatal dentin formation (18), Fig. 2f] by in situ hybridization. Although the levels of *Col 1* and *Dmp1* are reduced in the cKO mice, the *Dspp* expression pattern is more striking, and there is a sharp reduction in the root but not in the crown. This distinctive spacial change pattern is in agreement with the change of tubulin, a cytoskeletal marker (19) (Fig. 2g). To further define the specificity of cellular and molecular regulation, we transiently transfected PEX3-Osx plasmids into the odontoblast cell line A4 (14). The *in vitro* data analyses presented a moderate but significantly inhibition in cell proliferation (data not shown), a great acceleration of mineralization by alizarin red stain assay, and a significant increase in *Dspp* expressions according to the Real-time PCR (Fig. 2h).

DISCUSSION

In this study, we demonstrated that OSX is one of the key molecules downstream of NFIC based on the following evidence: 1) Both *Nfic* KO (Supplemental Fig. S4) and *Osx* cKO (Supplemental Fig. S3) mice display modest changes in the tooth crown but severe defects in both molar root and incisor root-analog; 2) There is a sharp reduction in OSX expression in *Nfic* KO; and 3) The *in vitro* overexpression of *Nfic* leads to a dosage-dependent increase in Osx expression that is not linked to the change in cell numbers. The recent finding that NFIC directly regulates Osx expression in osteoblasts (7) is in agreement with our discoveries in tooth studies. However, Osx is only one of the downstream molecules of NFIC, as the *Nfic* KO “root remnant” does not contain any dentin or dentin tubules but the *Osx* cKO root has malformed dentin tubules. Second, the *Osx* cKO root phenotype is partially improved at the age of 4 weeks when the 2.3 Col I-Cre is still highly active (Supplemental Fig. S5), whereas the *Nfic* root-less phenotype remains at the age of 6-weeks (Supplemental Fig. S4), indicating that there must be other molecules downstream of *Nfic*, which compensates for Osx function (Fig. 2i).

Although we do not understand the complexity of the unique role of OSX in the same pulp and odontoblast cells but different anatomical areas, the poorly formed bone environment as shown in Fig 1d, as a secondary factor, may affect the root formation. Further, we recently found that deletion of *Osx* in cartilage (crossing Osx-loxP to Aggrecan-Cre and induced by tamoxifen) leads to a virtual cessation of endochondral bone formation in the condyle but a

relatively modest change in the articular and growth plate cartilage at the same tested age (20). These novel findings will trigger future studies to understand how and why different local environments change or modify the gene function during different developmental stages.

In summary, this study demonstrates that OSX, one of the key downstream molecules of NFIC, plays an essential role in postnatal tooth root formation, but not in early tooth germ (8) and crown formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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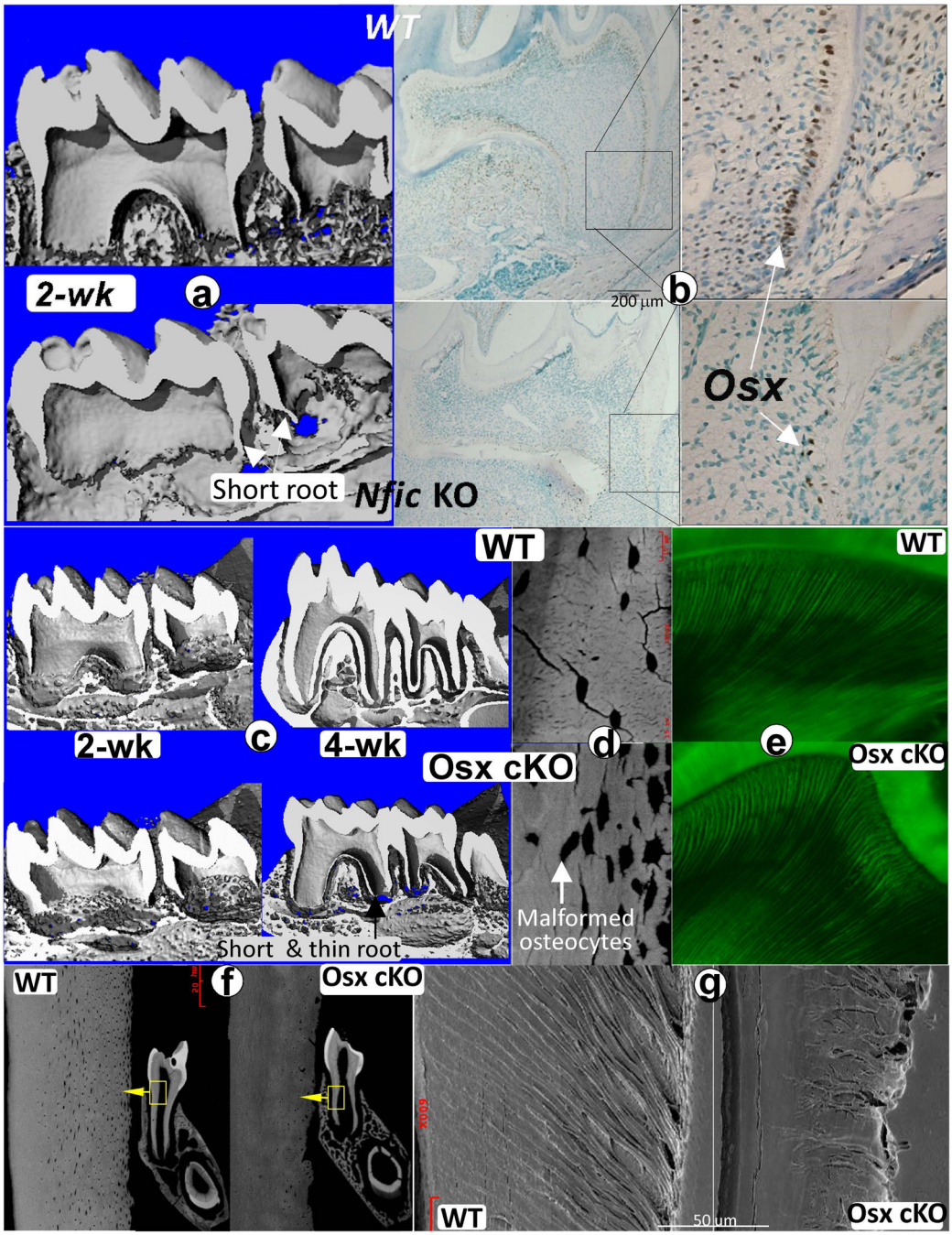

Fig 1.

Osx, downstream of *Nfic*, is not required for crown formation but is needed for root dentin formation during postnatal development. (a) The 2-week-old *Nfic* KO displayed short molar roots as viewed by uCT (*lower panel*); (b) Immunohistochemistry stains showed few positive OSX signals in the KO odontoblasts (*lower panel*); (c) Osx cKO molar roots (*lower panel*) were short and thin according to uCT findings; (d) Backscattered SEM images revealed large and immature osteocytes in the Osx cKO alveolar bone (*lower panel*); (e) FITC-confocal images displayed no apparent differences in the cKO (*lower panel*) and

control crown dentin tubules; (f–g) backscattered SEM (f) and acid-etched SEM (g) showed that the cKO dentin tubules were poorly formed in structure and sharply reduced in number with ~1/3 region without dentin tubules (*right panels*).

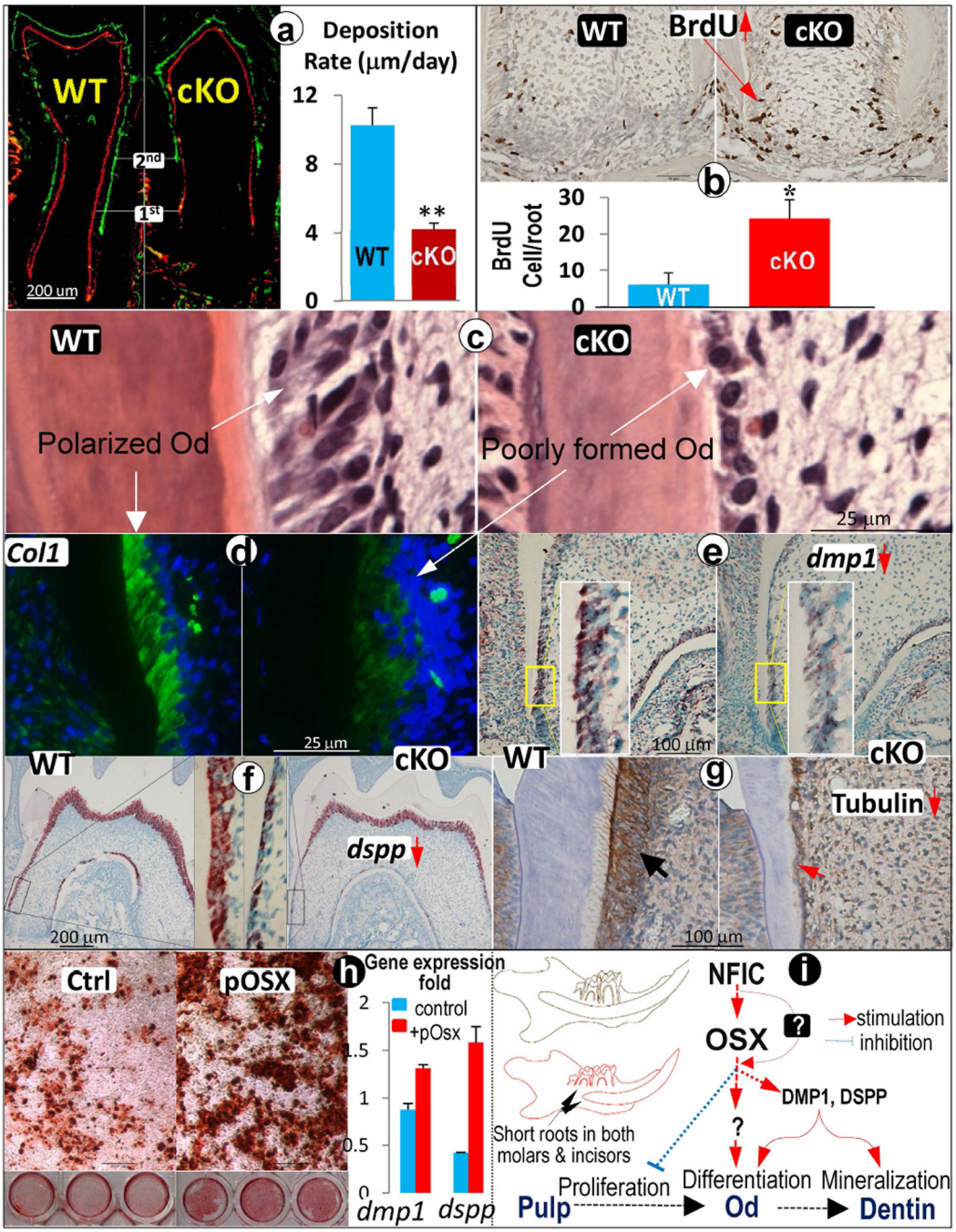

Fig 2.

Defects in dentin mineralization in *Osx* cKO mice were caused by an increase in cell proliferation and a decrease in odontoblast differentiation (right panels). (a) The double-labeling images unveiled a great reduction in the Osx cKO root at the age of 4 weeks, which is significantly different from the age-matched control (n = 4, **, P < 0.01); (b) The BrdU assay showed a sharp increase in the cKO odontoblast cell proliferation with a statistical significance from the control (n=4, * P<0.01); (c) The H&E stain documented an immature odontoblast cell layer with no sign of polarization morphology in the cKO root; (d–f) The in

situ hybridization assays displayed a great decrease in type 1 collagen (d), *Dmp1* (e), and *Dspp* (f) in the cKO root; (g) The immunostain images confirmed a great reduction in tubulin expressions in the immature flat odontoblast in cKO; (h) overexpression of PEX3-Osx plasmids in the odontoblast cell line led to an increase in mineral nodules and expressions of *Dmp1* and *Dspp*; and (i) The working hypothesis: OSX is the key downstream molecule of NFIC, which controls root but not crown dentin via its inhibitory role in cell proliferation and stimulatory function in the cell differentiation/dentin tubule formation (*right panel*). Deletion of Osx will lead to root but not crown defects (*left panel*). In addition, there must be unknown factors, which partly compensate for OSX function during late root formation.