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Effect of high phosphate diet on the formation of dentin in *Fam20c*-deficient mice

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Abstract

FAM20C (family with sequence similarity 20-member C), a kinase that phosphorylates secretory proteins, plays essential roles in various biological processes. In humans, mutations in *FAM20C* gene cause Raine syndrome, an autosomal recessive hereditary disease manifesting a broad spectrum of developmental defects including skeletal and craniofacial deformities. Our previous studies revealed that inactivation of *Fam20c* in mice led to hypophosphatemic rickets and that high phosphate (hPi) diet significantly improved the development of the skeleton in *Fam20c*-deficient mice. In this study, we evaluated the effects of hPi diet on the formation of dentin in *Fam20c*-deficient mice, using plain x-ray radiography, micro–computed tomography (μCT), histology, and immunohistochemistry. Plain x-ray radiography and μCT analyses showed that the hPi diet improved the dentin volume fraction and dentin mineral density of the *Fam20c*-deficient mice. Histology analyses further demonstrated that the hPi diet dramatically improved the integrity of the mandibular first molars and prevented pulp infection and dental abscesses in *Fam20c*-deficient mice. Our results support that the hPi diet significantly increased the formation and mineralization of dentin in *Fam20c*-deficient mice, implying that hypophosphatemia is a significant contributor to the dentin defects in *Fam20c*-deficient subjects.

Keywords

FAM20C; hypophosphatemia; high phosphate diet; dentin; mouse

Introduction

Dentin is formed by odontoblasts, which are derived from neural crest cells (1). During dentinogenesis, the odontoblasts synthesize collagen and non-collagenous proteins, and secrete them to form the organic component of the extracellular matrix, in which hydroxyapatite crystals are deposited to make mineralized dentin (2). Some of the non-

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Author Contributions

Conceptualization: Hua Zhang, Yongbo Lu, Chunlin Qin; **Formal analysis**: Hua Zhang, Yongbo Lu; Chunlin Qin; **Investigation:** Hua Zhang, Qian Xu, Yongbo Lu, Chunlin Qin. **Writing- original draft preparation and Writing-review and editing:** Hua Zhang, Qian Xu, Yongbo Lu, Chunlin Qin; **Supervision and Project administration:** Hua Zhang, Chunlin Qin.

Conflicts of interests

The authors state no conflicts of interests.

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collagenous proteins, such as dentin sialophosphoprotein and dentin matrix protein 1 are phosphoproteins. Protein kinases are required to catalyze the attachment of phosphates to the amino acid residues in proteins to synthesize phosphoproteins. Family with sequence similarity 20 – member C (FAM20C) is a protein kinase that catalyzes the attachment of phosphates to serine residues within the Ser-x-Glu/pSer motifs of secretory proteins (3–6). FAM20C, which is highly expressed in odontoblasts (7, 8), is believed to be the enzyme responsible for phosphorylating dentin non-collagenous proteins including dentin sialophosphoprotein and dentin matrix protein 1 (3, 5, 9). Our previous studies showed that global inactivation of *Fam20c* led to hypophosphatemic rickets and dentin defects in mice (10). While a deficiency in the phosphorylation of dentin non-collagenous proteins associated with the loss of FAM20C function may lead to dentin defects, a lower level of serum phosphorus (hypophosphatemia) may also contribute to dentin abnormalities observed in the *Fam20c*-deficient mice. It is unclear to what extent the dentin defects of the *Fam20c*-deficient mice may be attributed to hypophosphatemia.

In our previous study, we observed that high phosphate (hPi) diet significantly improved the skeletal development of the *Fam20c*-deficient mice (11). In this study, we evaluated the effects of the hPi diet on the formation and mineralization of dentin in the *Fam20c*-deficient mice.

Material and Methods

Experimental mice and administration of hPi diet

The mice used in this study were the same as those described in our previous study (11). Briefly by mating male *Sox2-Cre;Fam20c*$^{fl/+}$ mice with female *Fam20c*-floxed mice (*Fam20c*$^{f/f}$), we created *Sox2-Cre;Fam20c*$^{fl/fl}$ mice (designated as “cKO mice”). The breeding pairs of *Sox2-Cre;Fam20c*$^{fl/+}$ male and *Fam20c*$^{fl/fl}$ female mice were divided into two groups: “A” and “B”. The pregnant *Fam20c*$^{fl/fl}$ mice in Group A were fed a standard rodent chow (0.7% phosphorus, T.2918; Harlan Teklad), while those in Group B were given a high phosphate rodent chow (1.5% phosphorus, TD. 3625; Harlan Teklad, Table S1) at approximately 14.5 days (a time when bone mineralization begins) after the appearance of a vaginal plug. *Fam20c*$^{fl/+}$ or *Fam20c*$^{fl/fl}$ mice from Group A (receiving standard diet) or Group B (receiving hPi diet) were used as the normal controls (referred to as “Norm”); *Fam20c*$^{fl/+}$ or *Fam20c*$^{fl/fl}$ mice have been shown to have normal skeleton and tooth development (10, 12). After weaning at postnatal 3 weeks, cKO and Norm offspring mice in Group A were continuously fed a standard diet while those in Group B continued to receive hPi diet. The cKO and Norm mice were euthanized at 7 weeks after birth and their mandibles were dissected for analyses. The experiments with mice were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the animal protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University College of Dentistry (Dallas, TX, USA). All mice had free access to food and water.

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Plain x-ray radiography and micro–computed tomography (μCT)

We dissected the mandibles from the 7-week-old cKO and Norm mice and first used a plain X-ray radiography system (Faxitron MX-20 DC12; Faxitron Bioptics Tucson, Arizona, USA) to scan the mandibles with a focus on assessing the radiodensity of the molars. The μCT (Scanco μCT35 imaging system; Scanco Medical) analysis was done with a low-resolution scan (12 μm slice increment) for an overall morphological assessment of the mandibular jaw, followed by a high-resolution scan in 6 μm slice increments of the first molars as previously reported (13). The data acquired from the high-resolution scans of four samples per group ($n = 4$) were used for the quantitative analyses. Dentin was analyzed together with cementum as these two types of tissues were indistinguishable by density in μCT imaging (for additional detail see: Supplemental Material and Methods in the Supporting Information section of this article). The quantitative data were analyzed using IBM SPSS Statistics 25 software and were expressed as mean ± SD (standard deviation). One-way ANOVA was used for multiple-group comparison. If significant differences were found with the one-way ANOVA, LSD post-hoc test was used to determine the differences between two individual groups. $P < 0.05$ was considered as the statistically significant difference.

Histology and immunohistochemistry

For histologic analysis, the mandibles were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (pH 7.4) at 4°C overnight and then processed as previously reported (13). Serial sections were cut at a thickness of 5 μm and used for Hematoxylin and Eosin (H&E) staining and immunohistochemistry using the polyclonal antibodies against dentin sialoprotein (DSP, N-terminal fragment of dentin sialophosphoprotein) and dentin matrix protein 1 as previously reported (13). More details on immunohistochemistry can be found in Supplemental Material and Methods in the Supporting Information section of this article.

Results

The serum biochemistry data associated with this high phosphate diet have been published in our previous report (9). Briefly, the hPi diet did not have significant effects on serum calcium and phosphorus levels in the normal mice. However, it fully recovered the serum calcium levels of cKO mice and increased the serum phosphorus level of cKO mice by 38.7% at the age of 4 weeks. This report focused on the effects of the hPi diet on dentin formation in the *Fam20c*-deficient cKO mice.

Our x-ray analyses showed that there were no obvious differences between the molars of Norm mice receiving hPi diet (designated "Norm^{+Pi} mice") versus those of Norm mice given a standard rodent chow (Figure S1). In this report, we used Norm^{+Pi} mice as the normal controls for comparative analyses versus cKO mice receiving a standard rodent chow (designated "cKO") and cKO mice receiving hPi diet (designated "cKO^{+Pi}" mice). Our analyses primarily focused on the mandibular first molars from these three groups of mice.

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Plain x-ray radiography analyses showed that the molars of the cKO mice had enlarged pulp chamber and thinner pulp chamber walls (Figure 1B, E), compared to those of the Norm^{+Pi} mice (Figure 1A, D). Evidently, the pulp chamber walls of cKO^{+Pi} mice were much thicker (Figure 1C, F) than those of the cKO mice (Figure 1B, E). The short mandibular incisors in cKO mice were not rescued by high phosphate diet (Figure 1B, C). The reconstructed high-resolution scan μCT images confirmed that the thinner pulp chamber wall and enlarged pulp chamber in the mandibular first molars of the cKO mice were markedly improved by the hPi diet (Figure 2A–C). Quantitative analyses of the high-resolution μCT scanning data showed that the hPi diet increased the dentin volume fraction (expressed as the ratio of dentin volume to the total volume, DV/TV) of cKO mice by 87.5% (Figure 2D) and restored the dentin apparent density by 43.3% (Figure 2E) and material density by 26.5% (Figure 2F). Nevertheless, the apparent density and material density of the cKO^{+Pi} mice were still significantly lower than the Norm^{+Pi} mice.

H&E staining showed that the mandibular first molars of the 7-week-old cKO mice showed remarkably decreased dentin thickness (Figure 3B, E), compared to those of the Norm^{+Pi} mice (Figure 3A, D), and the high phosphate diet markedly increased the dentin thickness in the cKO^{+Pi} mice (Figure 3C, F). In addition, numerous small rounded areas appeared to be “empty spaces” that were present in the dentin matrices of both cKO and cKO^{+Pi} mice (Figure 3E, F). Moreover, the first molars in the cKO mice showed pulp exposure, severe pulp infection and necrosis, and lack of odontoblasts in the dental pulp. In contrast, the first molars in the cKO^{+Pi} mice had no obvious signs of direct pulp exposure or infection, and odontoblasts were still evident at the border of the dentin and pulp. All four cKO mice fed with standard diet had dental abscesses by the age of 7-weeks, but all Norm^{+Pi} mice and cKO^{+Pi} mice examined in this study did not form dental abscesses. Dentin sialophosphoprotein, the most abundant non-collagenous protein in the dentin matrix, which exists primarily in the forms of processed N-terminal fragment known as dentin sialoprotein and C-terminal fragment referred to as dentin phosphoprotein, is critical for normal formation and mineralization of tooth dentin (14). Anti- dentin sialoprotein immunohistochemistry (Figure 3G–I) revealed that the dentin sialoprotein immunostaining intensity was similar in the crown dentin among Norm^{+Pi}, cKO and cKO^{+Pi} groups. Nevertheless, many small rounded areas, which showed weak or no dentin sialoprotein immunostaining signals, were found in the dentin matrixes of both cKO and cKO^{+Pi} mice; these small rounded areas corresponded to those observed in the H&E stained histological sections.

Discussion

The genes whose mutations cause hypophosphatemia include phosphate-regulating gene with homologies to endopeptidases on the X chromosome (*PHEX*) (15–17), fibroblast growth factor 23 (*FGF23*) (18–20), dentin matrix protein 1 (*DMP1*) (21, 22), ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) (23–25) and *FAM20C* (10).

Several studies have examined the pathogenic role of hypophosphatemia in the skeletal defects in subjects with hypophosphatemic rickets/osteomalacia, but its roles in dental abnormalities are less well studied. A combination of oral phosphate supplement and

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calcitriol has been shown to restore phosphate homeostasis and to improve bone mineralization when taken by subjects with hereditary hypophosphatemic rickets/ osteomalacia, yet the findings are inconsistent concerning its effects in improving dental development (17, 26–28). The high phosphate diet (hPi) alone also improved the skeletal development of the *Hyp* mice with *Phex* mutations; however, information is very limited in terms of whether or not the hPi diet improved the dental development of the *Phex*-deficient subjects (17, 29–31). Long-time (more than 40 days) but not short-time (less than 30 days) high-calcium/-phosphate diet improved some of the histopathological features of the incisor dentin of *Hyp* mice (30, 31). Moreover, application of the hPi diet to *Dmp1*-null mice, which also manifested hypophosphatemic rickets/osteomalacia, showed that hPi improved the skeletal development of the *Dmp1*-null mice (21); yet this report did not describe the effects of the dietary phosphorus supplement on tooth development. Similarly, we previously only reported that the hPi diet improved the skeletal development of the *Fam20c*-defieicent mice (11). In this study, we described the rescue effects of the hPi diet on the dental development in the *Fam20c*-defieicent mice.

The mandibular first molar of cKO mice receiving standard diet had very thin dentin in the crown and root whose mineral density was dramatically lower than in the normal control mice. The phosphorus supplementation significantly increased the dentin volume and density of the cKO mice. At 7 weeks after birth, the first molar pulp chamber of the cKO mice fed with standard diet was exposed and the dental pulp had severe infection along with remarkable pulp necrosis. The pulp chamber wall of the cKO mice with phosphate supplementation (cKO^{+Pi} mice) was intact, the dental pulp had no apparent infection, and the odontoblasts were evident at the boundary of the dental pulp. While the dentin volume and mineral density of cKO^{+Pi} mice were remarkably better than those of the cKO mice fed with standard diet, these dentin parameters in the cKO^{+Pi} mice were still below the levels in the Norm^{+Pi} mice. These observations indicate the dietary phosphorus supplement significantly improved but not completely rescued the dentin defects in the cKO mice.

The partial rescue of the dentin defects may be attributed to loss of phosphorylation of certain dentin matrix proteins. Dentin sialophosphoprotein and dentin matrix protein 1 are two highly phosphorylated non-collagenous matrix proteins (32–34). They are essential for dentin formation (14, 35). It has been demonstrated that dentin matrix protein 1 phosphorylation is compromised in the *Fam20c*-deficient mice (36). *In vitro* studies also suggest that FAM20C alone is responsible for phosphorylating majority of secreted phosphoproteins (6). Therefore, in addition to hypophosphatemia, the loss of phosphorylation in the matrix proteins may also contribute to the dentin defects of the *Fam20c* cKO mice.

In addition to dentin, the hPi diet improved the formation of alveolar bone in the cKO mice. Plain x-ray radiography (Figure 1A–C) and reconstructed low-resolution μCT images (Figure S2A–C) demonstrated that the cKO mice receiving standard diet had a much porous mandible and alveolar bone, compared to the Norm^{+Pi} mice, and that the hPi diet dramatically improved the overall structure of the mandible and alveolar bone of the cKO mice. The high-resolution μCT scan analyses demonstrated that the hPi diet improved the formation and mineralization of the alveolar bones in the cKO mice (Figure S2D–F).

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Quantitative analyses showed that the bone volume fraction (expressed as BV/TV ratio), bone apparent density and material density in the cKO^{+Pi} mice were significantly higher than those in the cKO mice (Figure S2G–I). H&E staining and anti-DMP1 immunohistochemistry also revealed a dramatic loss of periodontal tissues (including alveolar bone, cellular cementum and periodontal ligament) in the cKO mice, compared to those in the Norm^{+Pi} mice, and high phosphate diet partially restored these tissues in the cKO^{+Pi} mice (Figure S3).

In summary, these findings suggest that hypophosphatemia is a significant contributor to the dentin defects in *Fam20c*-deficient subjects and that the partial rescue of the dentin defects may be due to loss of phosphorylation in certain dentin matrix proteins that cannot be restored by high phosphate diet. Moreover, this study pinpoints the pathogenic role of hypophosphatemia in the alveolar bone defects in the *Fam20c*-deficient subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

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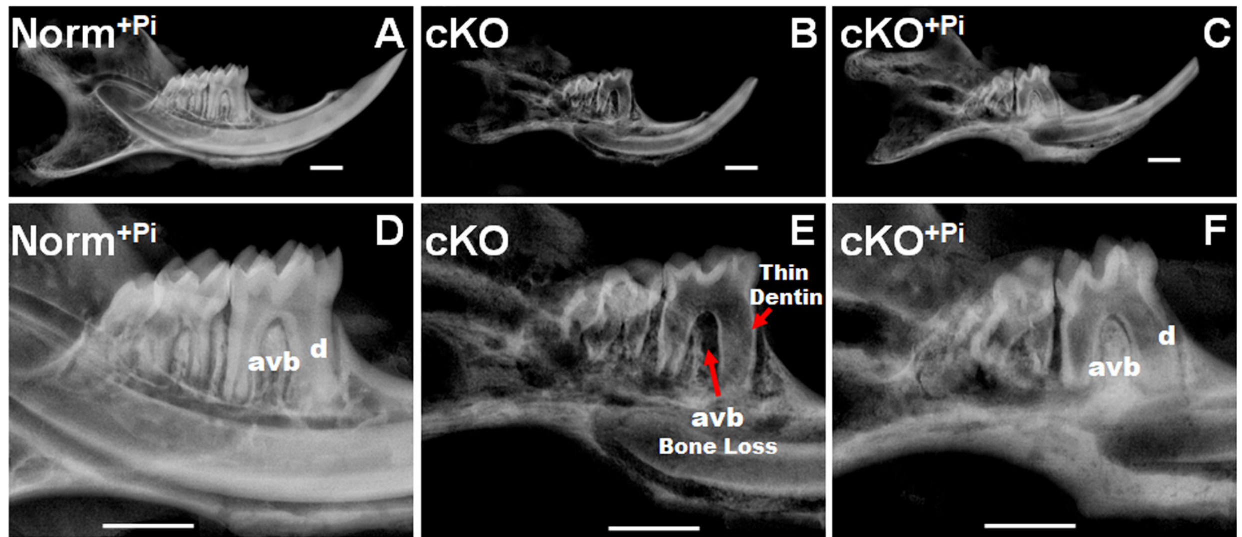

Figure 1: Plain X-ray radiography of the mandibles from 7-week-old mice.

Shown are representative plain X-ray radiography of the mandibles from 7-week-old Norm^{+Pi} (A and D), cKO (B and E) and cKO^{+Pi} (C and F) mice. D, E and F are enlarged views of the molar regions in A, B and C, respectively. The pulp chamber wall of the first molar in the cKO mice was thinner than that in the Norm^{+Pi} mice. High phosphate diet remarkably increased the thickness of the pulp chamber wall in the cKO^{+Pi} mice. High phosphate diet also markedly improved the alveolar bone in the cKO^{+Pi} mice. d: dentin; avb: alveolar bone. Bars in A to F: 1 mm.

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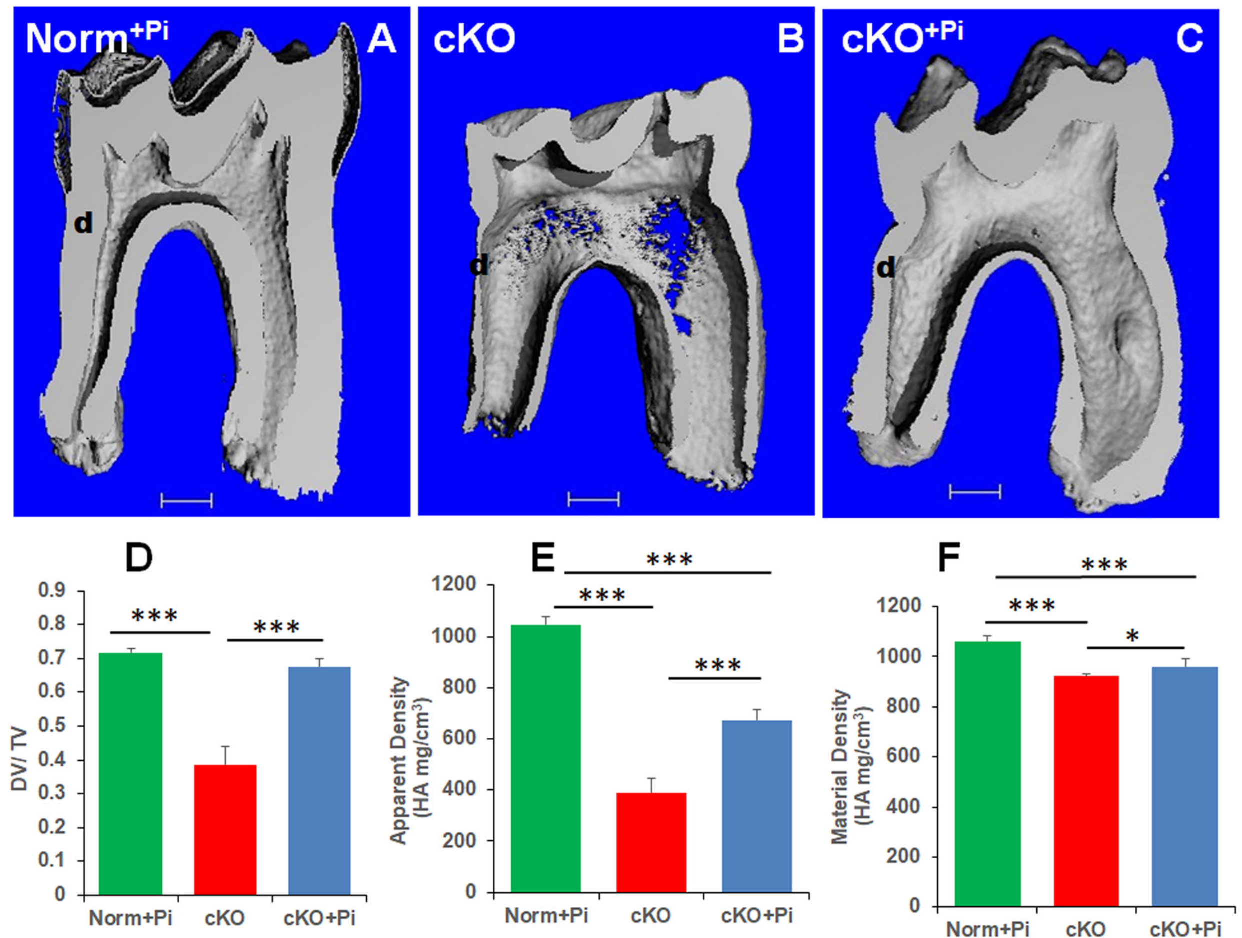

Figure 2: High-resolution μCT scan and quantitative μCT analyses of the mandibular first molars from 7-week-old mice.

A-C, Reconstructed high-resolution μCT images of the mandibular first molars from 7-week-old Norm^{+Pi} (A), cKO (B) and cKO^{+Pi} (C) mice. The dentin of the first molar in the cKO mice was remarkably thinner than in the Norm^{+Pi} mice. High phosphate diet dramatically increased the dentin thickness in the cKO^{+Pi} mice. Note the porous appearance of the pulp chamber wall in the cKO mice but not in the Norm^{+Pi} mice. d: dentin. Bars in A, B, C: 200 μm. D-F, Quantitative μCT analyses of the mandibular first molars from 7-week-old Norm^{+Pi}, cKO and cKO^{+Pi} mice. Shown are the quantitative μCT analysis results of the dentin volume fraction (expressed as the ratio of dentin volume to the total volume, DV/TV) (D), dentin apparent density (E) and material density (F). *: $P< 0.05$. ***: $P< 0.001$.

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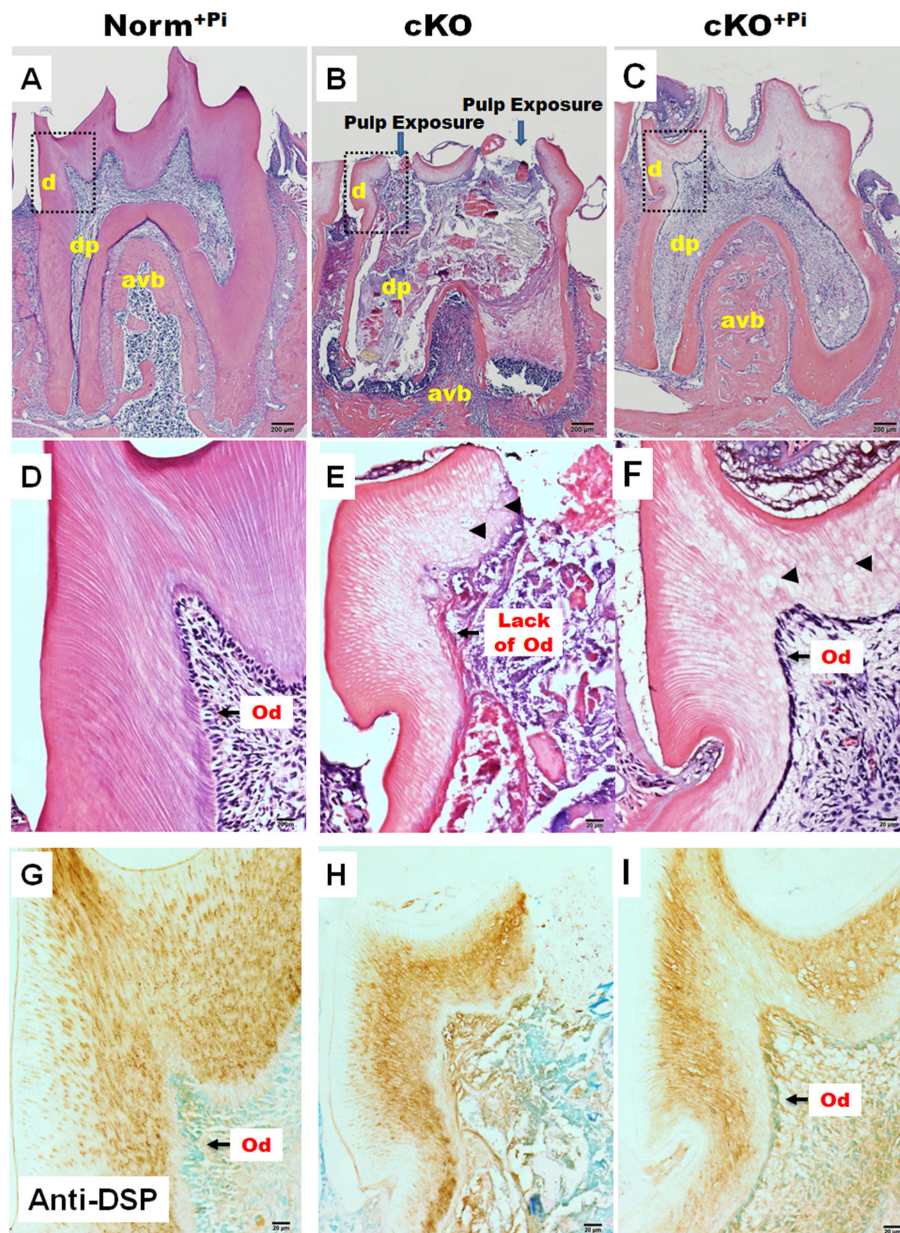

Figure 3: H&E and anti-dentin sialoprotein immunohistochemistry staining of the mandibular first molars from 7-week-old mice.

A-F, H&E staining of the mandibular first molars from 7-week-old Norm^{+Pi} (A and D), cKO (B and E) and cKO^{+Pi} (C and F) mice. D, E and F are higher magnifications of the upper right boxed areas in A, B and C, respectively. The dental pulp of the first molars in the cKO mice had severe infection with necrosis, and were devoid of odontoblasts (B and E), compared to the pulp of the Norm^{+Pi} mice (A and D). The dental pulp of the cKO^{+Pi} mice had no obvious signs of infection, and odontoblasts were evident at the border of dentin and pulp (C and F). Numerus small round areas (arrowheads) were present in the dentin matrixes of both cKO and Norm^{+Pi} mice (E and F). G-I, Anti- **dentin sialoprotein** immunohistochemistry staining of the mandibular first molars from 7-week-old Norm^{+Pi} (G), cKO (H) and cKO^{+Pi} (I) mice. The immunostaining signal intensity for **dentin**

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sialoprotein in the dentin matrixes was similar among Norm^{+Pi} (G), cKO (H) and cKO^{+Pi} (I) mice. Numerous small round areas (arrowheads), which showed weak, or no **dentin sialoprotein** immunostaining signals, were evident in both cKO (H) and cKO^{+Pi} (I) mice, but not in Norm^{+Pi} (G) mice. d: dentin; dp: dental pulp; Od: odontoblasts; avb: alveolar bone. Bars in A, B, C: 200 μm. Bars in D-I: 20 μm.