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The Specific Role of FAM20C in Amelogenesis

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ABSTRACT

Previously, we showed that *Sox2-Cre;Fam20C*$^{fl/fl}$ mice in which *Fam20C* was ubiquitously inactivated had severe defects in dentin, enamel, and bone, along with hypophosphatemia. It remains to be determined if the enamel defects in the mice with universal inactivation of Family with sequence similarity 20-C (FAM20C) were associated with the dentin defects and whether hypophosphatemia in the knockout mice contributed to the enamel defects. In this study, we crossed *Fam20C*$^{fl/fl}$ mice with keratin 14-Cre (*K14-Cre*) transgenic mice to specifically inactivate *Fam20C* in the epithelial cells, including the dental epithelial cells that are responsible for forming tooth enamel. X-ray, backscattered scanning electron microscopic, and histological analyses showed that the *K14-Cre;Fam20C*$^{fl/fl}$ mice had severe enamel and ameloblast defects, while their dentin and alveolar bone were not significantly affected. Accordingly, serum biochemistry of the *K14-Cre;Fam20C*$^{fl/fl}$ mice showed normal phosphate and FGF23 levels in the circulation. Analysis of these data indicates that, while FAM20C is a molecule essential to amelogenesis, its inactivation in the dental epithelium does not significantly affect dentinogenesis. Hypophosphatemia makes no significant contribution to the enamel defects in the mice with the ubiquitous deletion of *Fam20C*.

KEY WORDS: FGF23, biomineralization, hypophosphatemia, FAM20C, amelogenesis, kinase.

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INTRODUCTION

Family with sequence similarity 20-C (FAM20C) is an evolutionarily conserved molecule that is highly expressed in the mineralized tissues (Nalbant *et al*., 2005; Hao *et al*., 2007; Wang *et al*., 2010). In humans, loss-of-function mutations are associated with lethal osteosclerotic bone dysplasia (Raine Syndrome) (Simpson *et al*., 2007), while ubiquitous or mineralized tissue-specific inactivation of FAM20C in mice leads to hypophosphatemic rickets (Wang *et al*., 2012a) and severe tooth defects (Wang *et al*., 2012b). These genetic findings highlight the critical role of FAM20C in the development of mineralized tissues. Recently, *in vitro* biochemistry studies identified FAM20C as a Golgi-enriched kinase responsible for the phosphorylation of the secretory calcium-binding phospho-*protein* (*SCPP*) family, which includes "small-integrin-binding ligand, N-linked glycoproteins" (SIBLINGs) and several enamel-matrix proteins (Ishikawa *et al*., 2012; Tagliabracci *et al*., 2012). A recent study reported that FAM20C functions intracellularly within both ameloblasts and odontoblasts (Wang *et al*., 2013). These findings indicate that the bone and tooth defects in the *Fam20C*-deficient mice may be associated with a failure in the phosphorylation of SCPP proteins. In addition, we observed significant transcriptional alterations of several SCPP members in the bone or tooth (Wang *et al*., 2012a,b), suggesting that FAM20C may also modulate the transcription of these molecules through an unidentified mechanism.

Previously, we showed that FAM20C was expressed in the tooth germ as early as the cap stage and is highly expressed in differentiated odontoblasts and ameloblasts (Wang *et al*., 2010). *Sox2-Cre;Fam20C*$^{fl/fl}$ mice, in which FAM20C was ubiquitously inactivated, exhibited remarkable defects in the dentin, enamel, and bone, along with hypophosphatemia (Wang *et al*., 2012a,b). It remains to be determined whether the enamel/ameloblast defects in the mice with global inactivation of FAM20C are associated with the dentin/odontoblast abnormalities. Moreover, it is unclear to what extent the significantly reduced serum phosphate level in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice contributed to the enamel defects.

In this study, we sought to address the above-mentioned questions by defining the biological role of FAM20C in enamel development through the specific inactivation of FAM20C in the epithelial cells using a Cre-loxP system mediated by the *K14-Cre* transgene (Vasioukhin *et al*., 1999; Dassule *et al*., 2000).

MATERIALS & METHODS

Protocol Approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M-Baylor College of Dentistry (Dallas, TX,

USA) and performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Generation of *K14-Cre;Fam20C$^{fl/fl}$* Mice and *Sox2-Cre;Fam20C$^{fl/fl}$* Mice

Fam20C$^{fl/fl}$ mice were crossbred with *K14-Cre* transgenic mice. The resulting *K14-Cre;Fam20C$^{fl/+}$* mice were inbred to generate *K14-Cre;Fam20C$^{fl/fl}$* mice, *i.e., K14*-Cre-mediated conditional knockout (KO) mice. *Sox2-Cre;Fam20C$^{fl/fl}$* mice were generated as previously described (Wang *et al*., 2012a). Tail biopsies were analyzed by PCR genotyping with primers specific for *Cre* transgene and *Fam20C* floxed allele, as previously described (Wang *et al*., 2012a).

Plain X-ray and Backscattered Scanning Electron Microscopy (SEM)

The mandibles and hind legs dissected from three-week-old *K14-Cre;Fam20C$^{fl/fl}$* mice and their WT littermates were analyzed with plain x-ray radiography (Faxitron Bioptics, Tucson, AZ, USA). For SEM analyses, the mandibles were fixed in 4% paraformaldehyde overnight and then dehydrated through a graded series of ethanol concentrations (70%–100%) and embedded in methylmethacrylate (MMA) without prior decalcification. The frontal section at the first lower molar level was mounted, carbon-coated, and examined by field emission scanning electron microscopy (Philips XL30, FEI Company, Hillsboro, OR, USA).

Preparation of Decalcified Sections and H&E Staining

The mandibles were fixed overnight at 4°C with 4% paraformaldehyde in 0.1% diethyl pyrocarbonate (DEPC)-treated PBS solution and then decalcified in 0.1% DEPC-treated 15% EDTA (pH 7.4) at 4°C for 1 to 7 days. The samples were processed for paraffin embedding, and 5-μm serial sections were prepared for hematoxylin and eosin (H&E) staining and *in situ* hybridization staining.

In situ Hybridization (ISH)

The RNA probes for dentin matrix protein 1 (*DMP1*) and dentin sialophosphoprotein (*DSPP*) were prepared as previously described (Wang *et al*., 2012b). The RNA probes for ameloblastin (*AMBN*) and amelotin (*AMTN*) were obtained by PCR with mouse incisor cDNA as a template and were synthesized as previously described (Wang *et al*., 2012b). DIG-labeled RNA probes were detected by an enzyme-linked immunoassay with a specific anti-DIG-AP antibody conjugate (Roche, Indianapolis, IN, USA) and an improved substrate (Vector Laboratories, Burlingame, CA, USA), with red indicating positive signals. Methyl green was used for counterstaining.

Quantitative Real-time PCR

The tooth germs of lower incisors containing the entire enamel organs were dissected from newborn mice under a stereomicroscope (Olympus), and the first lower molars of three-week-old mice were extracted from jaws as previously described (Lu *et al*., 2007). The total RNAs were isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and converted into cDNAs with a Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. The cDNAs derived from the incisor tooth germ containing the entire enamel organ in new born mice were used for the real-time PCR of ameloblast markers (*AMBN, AMTN*, and *AMEL*), while the cDNAs obtained from the first lower molars of three-week-old mice were used for the real-time PCR of odontoblast markers (*DSPP* and *DMP1*). Quantitative real-time PCR was performed on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) with SYBR Green Master Mix (Stratagene, La Jolla, CA, USA), as previously described (Wang *et al*., 2012a). The Ct values were normalized to the reference gene 18s rRNA (SABiosciences, Frederick, MD, USA), and expressed as fold-changes over the experimental controls. The primers for mouse 18s rRNA, mouse *AMBN, AMTN, AMEL, DMP1*, and *DSPP* were purchased from SABiosciences.

Serum Biochemistry and Statistics

The sera of three-week-old *Sox2-Cre;Fam20C$^{fl/fl}$* mice, *K14-Cre;Fam20C$^{fl/fl}$* mice and their WT littermates were collected as previously described (Wang *et al*., 2012a). Serum phosphate was measured by the phosphomolybdate-ascorbic acid method. Serum calcium was measured with a colorimetric calcium kit (Stanbio Laboratory, Boerne, TX, USA), and the serum FGF23 was measured with a full-length FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan), as we previously described (Wang *et al*., 2012a).

For the statistical analyses, data expressed as the mean ± SD of 6 individual determinations were tested by analyses of variance (ANOVA) followed by a *post hoc* test.

RESULTS

Inactivation of FAM20C in Dental Epithelium Affected Enamel Formation But Not Dentin and Alveolar Bone

The teeth of *K14-Cre*-mediated *Fam20C*-KO mice appeared chalky white compared with those in the WT mice, indicating hypoplastic or hypocalcified enamel (Appendix Fig. 1), while the other tissues derived from epithelial cells had no significant gross defects. The *K14-Cre;Fam20C$^{fl/+}$* mice (heterozygotes) showed no difference from their WT littermates.

Plain x-rays showed that the *K14-Cre;Fam20C$^{fl/fl}$* mice had normal jaw bones and dentin, while the crown portion where the enamel is located had significantly less mineralization than normal (Fig. 1A); the long bones of the *K14-Cre;Fam20C$^{fl/fl}$* mice were normal compared with those of their WT littermates (Fig. 1B).

Backscattered SEM analyses showed very thin, poorly formed enamel matrices in the *K14-Cre;Fam20C$^{fl/fl}$* mice in contrast with the normal enamel containing well-organized rods in the WT mice (Figs. 1C-1F), while the dentin and alveolar bone in the *K14-Cre;Fam20C$^{fl/fl}$* mice were not significantly affected (Figs. 1C-1F).

Histological analysis of the incisors from E16.5 and E18.5 embryos showed that ameloblasts became significantly defective from E18.5 in the *K14-Cre;Fam20C$^{fl/fl}$* mice (Fig. 2 and

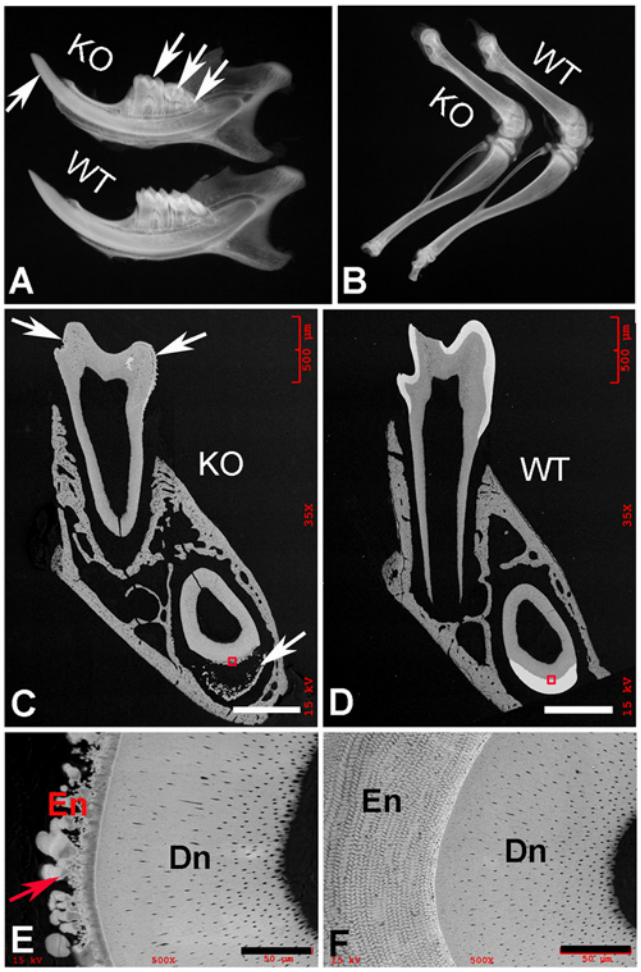

Figure 1. Plain x-ray and backscattered SEM analyses of jaws and long bones. **(A)** Plain x-ray of the mandibles from three-week-old mice. The *K14-Cre;Fam20C*$^{fl/fl}$ (KO) mice (upper) had normal jawbones and dentin, while the enamel showed significant hypomineralization (arrows) compared with that of the WT littermates (lower). **(B)** Plain x-ray of the long bones from three-week-old mice. The *K14-Cre;Fam20C*$^{fl/fl}$ mice (left) showed normal long bones compared with those in their WT littermates (right). **(C, D)** Backscattered SEM analyses of a frontal section at the first lower molar level in the mandibles of five-week-old mice. The *K14-Cre;Fam20C*$^{fl/fl}$ mice in (C) had severe defects in enamel (arrows) compared with their WT littermates in (D), while their dentin and alveolar bone were not significantly affected. **(E, F)** Higher magnification views of the red boxed area in (C) and (D). The very thin enamel (En, arrow) of the *K14-Cre;Fam20C*$^{fl/fl}$ mice in (E) showed disorganization and hypomineralization compared with the well-organized enamel rods in the WT mice in (F), while the dentin (Dn) of the KO mice did not show significant differences from that in the WT mice. Scale bars: 500 μm in C and D, 50 μm in E and F.

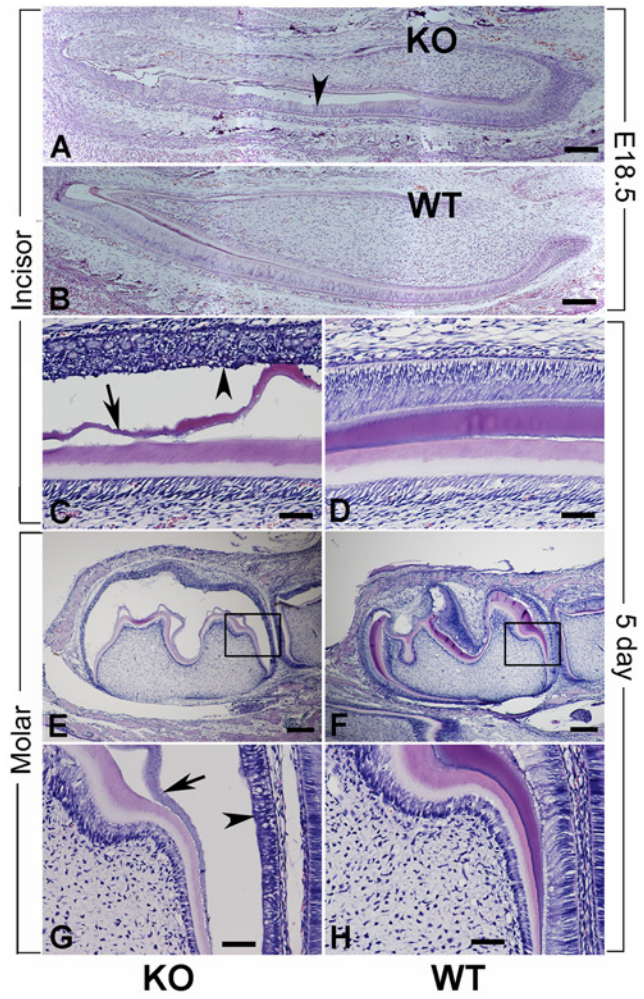

Figure 2. H&E staining of the teeth. **(A, B)** Sagittal section of the lower incisor from *K14-Cre;Fam20C*$^{fl/fl}$ mouse embryos at E18.5 showed that the malformed ameloblasts (arrowhead) peeled off from the dentin matrix, in comparison with the normal structure in the WT mice. **(C, D)** Sagittal section of the lower incisor from five-day-old *K14-Cre;Fam20C*$^{fl/fl}$ mice in (C) showed that the disorganized ameloblasts (arrowhead) dissociated from the very thin enamel (arrow), and the enamel detached from the dentin surface, while the odontoblasts and dentin were not significantly affected compared with those in their WT littermates in (D). **(E, F)** Sagittal section of the first lower molar from five-day-old *K14-Cre;Fam20C*$^{fl/fl}$ mice and their WT littermates. **(G, H)** Higher magnification views of the boxed areas in (E) and (F). The malformed ameloblasts of the *K14-Cre;Fam20C*$^{fl/fl}$ mice (arrowhead) in (G) dissociated from the very thin enamel matrices (arrow), and the enamel matrices detached from the dentin surfaces, while the odontoblasts and dentin of the KO mice were normal compared with those in their WT littermates in (H). Scale bars: 100 μm in A and B; 50 μm in C, D, G, and H; 200 μm in E and F.

Appendix Fig. 2); the ameloblasts, which differentiated into secretory and maturation stages, peeled off from the disorganized enamel matrices and showed morphological defects (Figs. 2A, 2B), while the dentin and odontoblasts in the *K14-Cre;Fam20C*$^{fl/fl}$ mice were not significantly affected, in comparison with their WT littermates.

At 5 post-natal days, the *K14-Cre;Fam20C*$^{fl/fl}$ mice formed very thin enamel matrices that peeled off from the dentin surfaces and were separated from the malformed ameloblasts, while the odontoblasts and dentin matrices in the *K14-Cre;Fam20C*$^{fl/fl}$ mice were not significantly affected compared with those in their WT littermates (Figs. 2C-2H).

ISH and Real-time PCR analyses showed a significant down-regulation of *AMBN* and *AMTN* in the ameloblasts of the *K14-Cre;Fam20C*$^{fl/fl}$ mice (Figs. 3A-3D), while *DMP1* and *DSPP* were not significantly affected in the odontoblasts of the *K14-Cre;Fam20C*$^{fl/fl}$ mice (Figs. 3E-3H, Appendix Table).

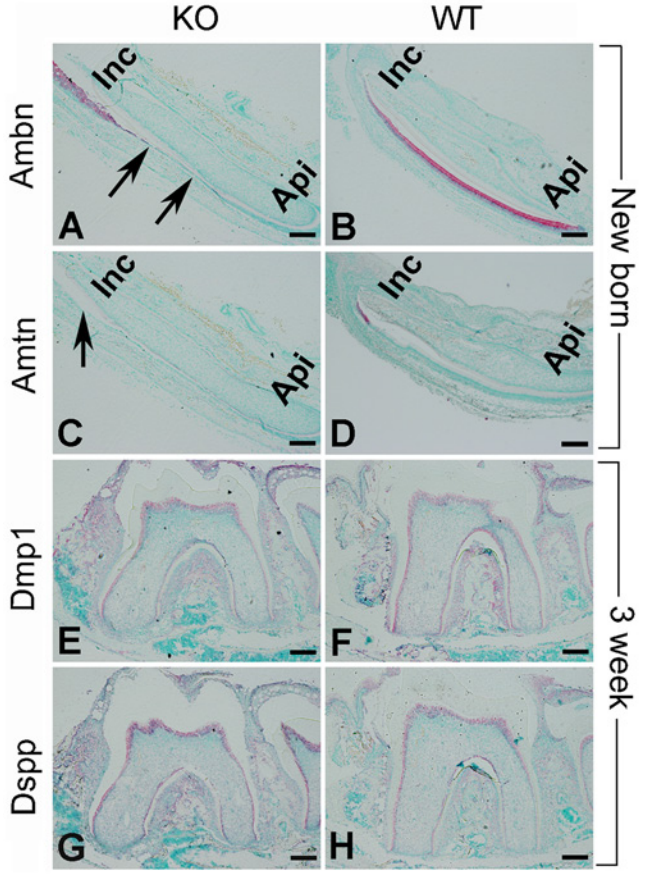

Figure 3. *AMBN* and *AMTN* were down-regulated in *Fam20C*-deficient ameloblasts. **(A, B)** ISH on the sagittal section of the lower incisor from newborn *K14-Cre;Fam20C*$^{fl/fl}$ mice in (A) displayed significant down-regulation of *AMBN* in the ameloblasts (arrows) compared with that in the WT littermates in (B). **(C, D)** ISH on the sister sections of (A) and (B) had less *AMTN* in the ameloblasts (arrow) compared with that in the WT littermates in (D). **(E, F)** ISH on the sagittal section of the first lower molar from 3-week-old *K14-Cre;Fam20C*$^{fl/fl}$ mice in (E) showed no significant difference of *DMP1* expression in the odontoblasts compared with that in the WT littermates in (F). **(G, H)** ISH on the sister sections of (E) and (F). There was no significant difference of *DSPP* expression between *K14-Cre;Fam20C*$^{fl/fl}$ and WT mice. Inc, incisal side. Api, apical side. Scale bars are equal to 200 μm.

Table. Serum biochemistry of Three-week-old WT, *K14-Cre;Fam20C*$^{fl/fl}$, and *Sox2-Cre;Fam20C*$^{fl/fl}$ Mice

Serum Biochemistry (mean ± SD)	WT Mice (n = 6)	*K14-Cre; Fam20C*$^{fl/fl}$ (n = 6)	*Sox2-Cre; Fam20C*$^{fl/fl}$ (n = 6)
Pi (mg/dL)	12.86 ± 2.32	13.24 ± 2.61	5.70 ± 0.59**
Fgf23 (pg/mL)	171.8 ± 58.5	204.7 ± 66.8	14,030.2 ± 2,982.8**
Ca (mg/dL)	15.44 ± 1.89	15.33 ± 2.21	13.54 ± 1.63*

*$p < .05$; **$p < .01$. A p value of < .05 was seen as a statistically significant difference from that of the WT mice. Note that the serum biochemistry results of *K14-Cre;Fam20C*$^{fl/fl}$ mice had no statistical difference from those of WT mice.

Inactivation of FAM20C in the Epithelial Cells Did Not Affect Phosphate Homeostasis

The *K14-Cre;Fam20C*$^{fl/fl}$ mice had normal long bones compared with those of their WT littermates. Accordingly, no significant alteration in serum biochemistry was detected in the *K14-Cre;Fam20C*$^{fl/fl}$ mice, in comparison with their WT littermates (Table).

DISCUSSION

FAM20C is essential to the formation and mineralization of all mineralized tissues, including bone, dentin, enamel, and cementum. Previously, we reported severe defects of enamel and dentin in the teeth of *Sox2-Cre;Fam20C*$^{fl/fl}$ mice (Wang *et al*., 2012b); these defects may arise from the phosphorylation failure of the SCPP proteins (Ishikawa *et al*., 2012; Tagliabracci *et al*., 2012) and/or the significant down-regulation of these matrix molecules in the odontoblasts and ameloblasts (Wang *et al*., 2012b). While the bone defects in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice likely correlated with the hypophosphatemia as well as the local effects of Fam20C inactivation (Feng *et al*., 2006; Liu *et al*., 2006), it was unclear if the lower serum phosphate level significantly affected enamel formation in these mice (Wang *et al*., 2012a). In addition, FAM20C is expressed in both ameloblasts and odontoblasts (Wang *et al*., 2010), and the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice had both enamel and dentin defects (Wang *et al*., 2012b). It was unclear whether the enamel defects of the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice were related to or independent of the dentin defects.

To address these questions, we utilized the *K14*-Cre-mediated Cre-loxp system to specifically inactivate FAM20C in the epithelial cells, including the dental epithelial cells forming the enamel (Vasioukhin *et al*., 1999; Dassule *et al*., 2000). Enamel formation is a strictly controlled step-wise process in which ameloblasts continuously secrete organic extracellular matrix while slowly moving in the opposite direction until the desired thickness of the matrix is achieved. The amelogenesis process is divided into the pre-secretory stage, secretory stage, and maturation stage, with marker genes expressed at each phase. The earliest ameloblast defects were detected in E18.5 *K14-Cre;Fam20C*$^{fl/fl}$ mice; the incisor ameloblasts that differentiated into the secretory stage and stages thereafter showed morphological defects and peeled off from the disorganized enamel matrices, indicating that FAM20C is essential for the proper organization of enamel matrices and the terminal differentiation of ameloblasts.

Inactivation of FAM20C in the epithelial cells did not affect FGF23 and phosphate homeostasis, while the ameloblasts of the *K14-Cre;Fam20C*$^{fl/fl}$ mice formed poorly organized, hypomineralized, very thin enamel matrices; the enamel defects in the *K14-Cre;Fam20C*$^{fl/fl}$ mice are very similar to those in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice. These results suggest that hypophosphatemia did not significantly contribute to the enamel defects in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice in which *Fam20C* was globally inactivated.

While the enamel defects of the *K14-Cre;Fam20C*$^{fl/fl}$ mice had severity similar to that in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice, the

K14-Cre;Fam20C$^{fl/fl}$ mice showed normal dentin formation and odontoblast differentiation, indicating that the enamel/ameloblast defects in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice were independent of the dentin/odontoblast defects. In addition, the *K14-Cre;Fam20C*$^{fl/fl}$ mice, in which *Fam20C* was inactivated in all epithelium-derived tissues including the tooth cervical loop, did not show a shorter length in the dental roots (Figs. 1A, 3E-3H), suggesting that the short-root phenotype in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice (Wang *et al.*, 2012b) was more likely associated with the dentin defects and/or phosphate homeostasis. In this respect, future study with the *Wnt1-Cre* transgene to specifically inactivate FAM20C in the odontoblasts/dentin may help clarify this postulation.

Our previous study showed that the enamel defects in the *Sox2-Cre;Fam20C*$^{fl/fl}$ mice might be a combined effect of *AMBN* and *AMTN* down-regulation (Wang *et al*., 2012b), as well as the assumed phosphorylation failure of several enamel proteins pertaining to the SCPP family (Fukumoto *et al*., 2004; Moffatt *et al*., 2006; Tagliabracci *et al*., 2012). Although the detailed mechanisms by which the lower transcription and phosphorylation of the SCPP molecules impair the enamel formation were not elucidated in this study, we confirmed that the significant down-regulation of *AMBN* and *AMTN* occurred in the ameloblasts of the *K14-Cre;Fam20C*$^{fl/fl}$ mice as well. Future studies are warranted to examine the phosphorylation status of the SCPP proteins in the enamel of the *Fam20C*-deficient mice.

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