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Overexpression of Dmp1 Fails to Rescue the Bone and Dentin Defects in *Fam20C* Knockout Mice

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Abstract

FAM20C is a kinase phosphorylating the small-integrin-binding ligand, N-linked glycoproteins (SIBLINGs), a group of extracellular matrix proteins that are essential for bone and dentin formation. Previously, we showed that *Sox2-Cre;Fam20C$^{fl/fl}$* mice had bone and dentin defects, along with hypophosphatemia and significant downregulation of dentin matrix protein 1 (DMP1). While the assumed phosphorylation failure of the SIBLINGs is likely associated with the defects in the *Fam20C*-deficient mice, it remains unclear if the downregulation of *Dmp1* contributes to these phenotypes. In this study, we crossed 3.6 kb *Col1-Dmp1* transgenic mice with 3.6 kb *Col1-Cre;Fam20C$^{fl/fl}$* mice to overexpress *Dmp1* in the mineralized tissues of *Fam20C* conditional knockout (cKO) mice. X-ray, micro-computed tomography, serum biochemistry and histology analyses showed that expressing the *Dmp1* transgene failed to rescue the bone and dentin defects, as well as the serum levels of FGF23 and phosphate in the *Fam20C*-cKO mice. These results indicated that the downregulation of *Dmp1* may not directly associate with, or significantly contribute to the bone and dentin defects in the *Fam20C*-cKO mice.

Keywords

FAM20C; DMP1; FGF23; bone; dentin; hypophosphatemia

INTRODUCTION

Bone and dentin closely resemble each other in composition and mechanism of formation/ mineralization. Among the essential components needed to form bone and tooth is a type of non-collagenous proteins (NCPs), termed the “small-integrin-binding ligand, N-linked glycoproteins” (SIBLINGs).

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DECLARATION OF INTEREST

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Family with sequence similarity 20-C (FAM20C) is highly expressed in the mineralized tissues [1]. Previously, we showed that ubiquitous (mediated by *Sox2-Cre*) or mineralized tissue-specific (mediated by 3.6 kb *Col1-Cre*) inactivation of FAM20C led to hypophosphatemic rickets and severe dentin defects in mice [2,3]. These defects are very similar to those in mice with inactivated *Dmp1* [4], a SIBLING protein known to be critical at controlling the initiation, rate and extent of biomineralization. Recent biochemical studies identified FAM20C as a Golgi-enriched kinase phosphorylating SIBLING protein [5,6], suggesting that the skeletal and dental defects in the *Fam20C*-deficient mice might be associated with the phosphorylation failure of one or several of the SIBLINGs. In addition to these assumed mechanisms, we also observed a significant downregulation of *Dmp1* in *Fam20C* conditional knockout (cKO) mice [2,3], which, however, remains elusive as to its role in the development of the defects in bones and teeth. To address this question, we crossed transgenic mice expressing the full-length DMP1 with *Fam20C*-cKO mice to overexpress *Dmp1* in the bone and dentin of *Fam20C*-cKO mice.

MATERIALS & METHODS

Animals and Genotyping

To generate *Dmp1*-Tg;*Fam20C*-cKO mice, we crossed 3.6 kb *Col1-Dmp1* transgenic mice [7] with 3.6 kb *Col1-Cre;Fam20C*$^{fl/+}$ heterozygous mice. The resulting *Dmp1Tg;Col1-Cre;Fam20C*$^{fl/+}$ offspring were inbred to generate *Fam20C*-cKO mice with or without the *Dmp1* transgene. Tail biopsies were analyzed by PCR genotyping with primers specific for the *Dmp1* and *Cre* transgenes and the *Fam20C* floxed allele, as previously described [2,7]. 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 NIH *Guide for the Care and Use of Laboratory Animals*.

X-ray and Micro-computed Tomography

The mandibles and hind legs dissected from 4-week-old mice were examined by plain X-ray radiography (Faxitron Bioptics) and micro-computed tomography (μ-CT35, Scanco Medical) at a medium resolution (7.0 μm slice increment). The images were reconstructed with EVS Beam software using a global threshold of 200 Hounsfield units. The middle shaft of tibias was analyzed by high-resolution μ-CT (3.5 μm slice increment) using the Scanco software for quantitative analysis of the ratio of bone volume to total volume (BV/TV), apparent density and material density. The mandibles and hind legs dissected from 3-month and 6-month old mice were examined by plain X-ray.

Immunohistochemistry (IHC)

The mandibles and tibias fixed with 4% paraformaldehyde were decalcified in 8% EDTA (pH 7.4) at 4°C for 12 days and then processed for paraffin embedding; 5-μm serial sections were prepared for IHC analyses. The IHC experiments were carried out using ABC and DAB kits (Vector Laboratories) according to the manufacturer’s instructions. A monoclonal anti-DMP1 C-terminus antibody (8G10.3) was used at a concentration of 3 μg/ml. Methyl green was used for counterstaining.

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Quantitative Realtime PCR

The total RNAs were isolated from long bones of 4-week old mice using an RNeasy Mini Kit (Qiagen) and converted into cDNAs using a Reverse Transcription Kit (Qiagen), as previously described [2]. Quantitative real-time PCR was performed on a Bio-Rad CFX96 system (Bio-Rad) using SYBR Green Master Mix (Stratagene), following the manufacturer's instruction. The Ct values were normalized to the reference gene 18s rRNA (SABiosciences), and expressed as fold of changes over the experimental controls. The primers for mouse 18s rRNA and *DMP1* were purchased from SABiosciences.

Goldner's Masson Trichrome Staining and Von Kossa Staining

The tibias and mandibles dissected from the mice were fixed overnight in 4% paraformaldehyde, dehydrated using a graded series of ethanol (70–100%), and then embedded in methylmethacrylate. Sagittal sections (5 μm) were prepared and used for Goldner-Masson trichrome staining and Von Kossa staining, as previously described [1,2].

Serum Biochemistry and Statistics

The serum of 4-week-old *Dmp1*-Tg;*Fam20C*-cKO mice, *Fam20C*-cKO mice and their WT littermates was collected, and the serum levels of FGF23 and phosphate were measured as previously described [2,8].

For statistical analyses, the data were expressed as mean ± SD of 6 individual determinations unless otherwise indicated. ANOVA was used to test the difference among 3 groups (WT, *Fam20C*-KO and *Dmp1*-Tg;*Fam20C*-KO). Post hoc (Fisher's LSD) analysis was employed to test the differences between two individual groups.

RESULTS

Overexpression of *Dmp1* in the *Fam20C*-cKO mice

IHC analysis on the first lower molars showed an elevated expression of DMP1 in the *Dmp1*-Tg;*Fam20C*-cKO mice (Figure 1A1) and less DMP1 in the *Fam20C*-cKO mice without the *Dmp1*-Tg (Figure 1A2), in comparison with their WT littermates (Figure 1A3). Realtime PCR using mRNA extracted from long bones revealed that the expression level of *Dmp1* in the *Dmp1*-Tg;*Fam20C*-cKO mice was ~3-fold higher than their WT littermates, and that the level of *Dmp1* in the *Fam20C*-cKO mice was ~1/4 of the normal level (Figure 2).

Transgenic Expression of Full-length DMP1 Failed to Rescue the Bone and Dentin Defects in the *Fam20C*-cKO Mice

Plain X-ray (Figures 1B1 and C1) and μ-CT (Figures 1B2, C2 and C3) analyses of the 4-week-old *Dmp1*-Tg;*Fam20C*-cKO mice showed a thinner than normal bone cortex and dentin and generalized hypomineralization, which were mineralized-tissue defects similar to those in the *Fam20C*-cKO mice. In contrast to the results for the WT mice, Goldner staining (Figure 1B3) and Von Kossa staining (Figure 1C4) disclosed more osteoids in the cortical bone and less mineralization in the dentin and alveolar bone in the *Dmp1*-Tg;*Fam20C*-cKO mice, which was similar to the situation for the *Fam20C*-cKO mice.

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Quantitative μ-CT on the middle shaft of the cortical bones of the *Dmp1*-Tg;*Fam20C*-cKO mice and the *Fam20C*-cKO mice showed similar bone volume and density, while the amounts for these parameters were significantly lower than the WT mice (Table 1).

Plain X-ray of 3-month and 6-month old mice did not show any improvement of the defects in the bone and teeth of *Dmp1*-Tg;*Fam20C*-cKO mice (data not shown).

As a whole, these data indicated that the full-length *Dmp1* transgene failed to rescue the bone and dentin defects in the *Fam20C*-cKO mice.

Overexpression of DMP1 Failed to Rescue the Serum FGF23 and Phosphate Levels in the *Fam20C*-cKO Mice

Serum biochemistry detected similar levels of FGF23 and phosphate in the *Dmp1*-Tg;*Fam20C*-cKO mice and the *Fam20C*-cKO mice (Table 2), indicating that the overexpression of DMP1 did not rescue the aberrant phosphate homeostasis in the *Fam20C*-cKO mice.

DISCUSSION

Fam20C-cKO mice share many similarities with *Dmp1* knockout mice, which implies that there might be a potential association of *Dmp1* dysfunction with the defects in the *Fam20C*-deficient subjects. In previous studies, we reported significant downregulation of *Dmp1* in the bone and tooth of the *Fam20C*-cKO mice [2,3]. To examine if the downregulation of *Dmp1* significantly contributed to the mineralized-tissue defects in these mice, we introduced the full-length *Dmp1* transgene (which had successfully rescued the defects in the *Dmp1* knockout mice [7]) into the *Fam20C*-cKO mice. Multipronged approaches showed that the *Dmp1* transgene failed to rescue the bone and dentin defects, as well as the serum levels of FGF23 and phosphate in the *Fam20C*-cKO mice, indicating that the downregulation of *Dmp1* may not directly associate with, or significantly contribute to the mineralized-tissue defects in the *Fam20C*-cKO mice.

FGF23 is a phosphaturic hormone produced by osteoblasts and osteocytes. A number of studies have shown that loss-of-function of DMP1 increased the plasma level of FGF23 which targets the Klotho/FGF receptor (FGFR) complexes in the kidney and reduces the expression of the renal sodium-phosphate cotransporters NaPi-2a/2c in the proximal tubules, thereby accelerating phosphate excretion into the urine and led to hypophosphatemia.

DMP1 is proteolytically processed into N-terminal and C-terminal fragments. The C-terminal fragment is much more highly phosphorylated than the N-terminal fragment. A number of *in vitro* studies showed that the phosphorylation status of DMP1 was significantly associated with the apatite crystal size and direction, indicating that phosphorylation is essential for DMP1 function [9]. Recent finding that FAM20C is a kinase phosphorylating the serine residue in the S-x-E motif of SIBLING proteins suggested that the mineralization defects in the *Fam20C*-KO mice may be associated with an assumed phosphorylation failure in one or several SIBLINGs (especially DMP1) [5,6]. Thus, an assumed phosphorylation failure of DMP1 in the *Fam20C* knockout mice may lead to loss of function of DMP1,

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thereby result in hypophosphatemia through FGF23 via the bone-kidney axes. With this in mind, it was not a surprise that complementing the downregulated *Dmp1* with *Dmp1*-transgene could not regain the function of DMP1 in the *Fam20C*-cKO mice, as the overexpressed DMP1 may still be unphosphorylated.

A recent study showed that FAM20C phosphorylates Ser^{180} and inhibits O-glycosylation on Thr^{178} in FGF23, thereby facilitated proteolysis (inactivation) of this protein, suggesting that the phosphorylation failure of FGF23 may contribute to the hypophosphatemic rickets in the *Fam20C*-KO mice [10]. However, our recent studies have clarified that hypophosphatemia did not significantly contribute to the dental defects in the *Fam20C*-KO mice ([8] and unpublished data). To clarify if the assumed phosphorylation failure of DMP1 contributed to the hypophosphatemic rickets and dental defects, future studies are warranted to examine the phosphorylation status of DMP1/overexpressed DMP1 in *Fam20C*-KO mice.

CONCLUSIONS

The downregulation of *Dmp1* may not directly associate with, or significantly contribute to the defects in the mineralized-tissues of the *Fam20C* knockout mice.

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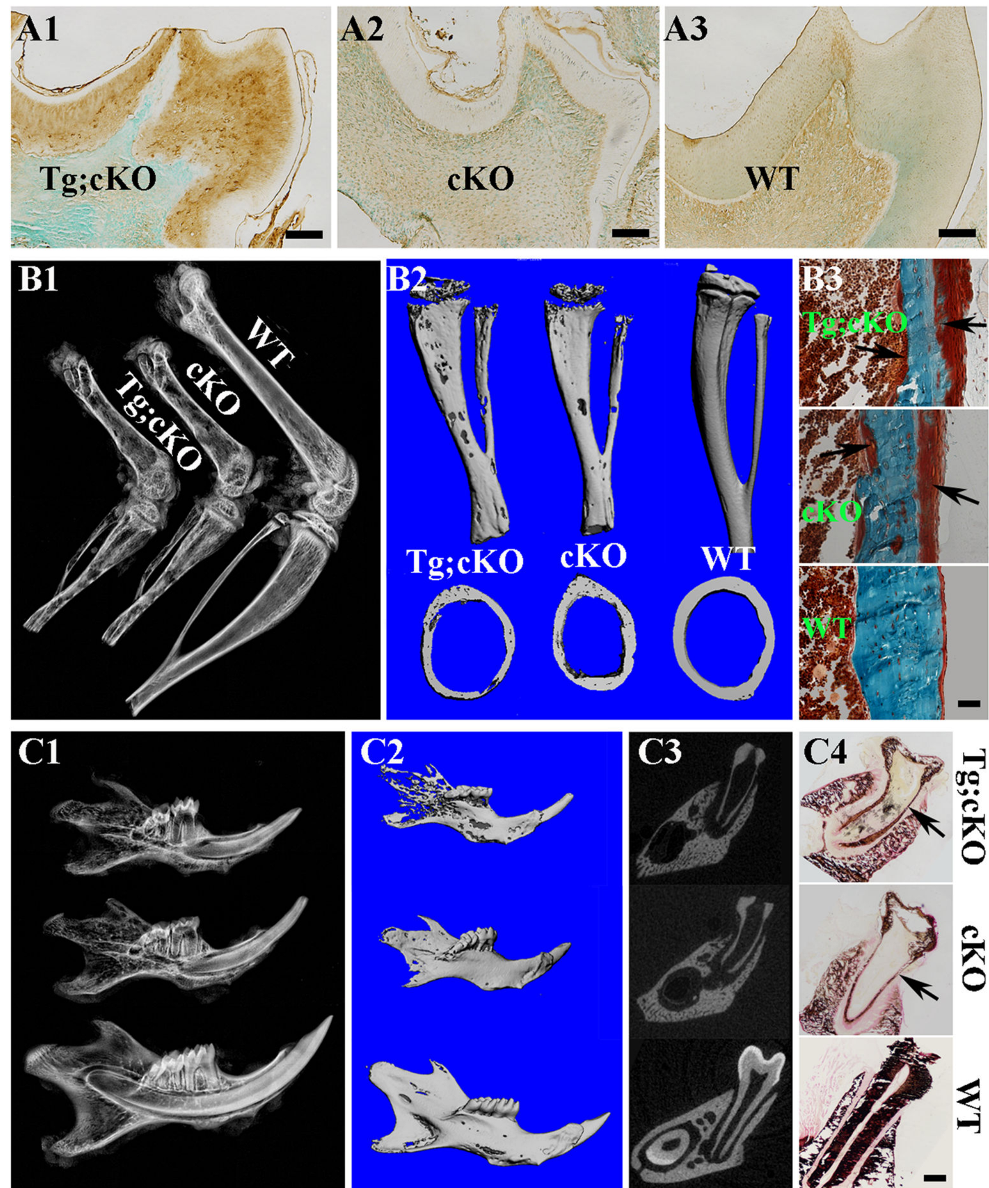

Figure 1. Transgenic Expression of *Dmp1* Failed to Rescue the Bone and Dentin Defects in the *Fam20C*-cKO Mice

(A) Validation of DMP1 overexpression in the *Dmp1*-Tg;*Fam20C*-cKO mice: IHC on the first lower molars of 4-week-old mice showed stronger DMP1 staining (brown) in the *Dmp1*-Tg;*Fam20C*-cKO mice (A1) and a lower expression level of DMP1 in the *Fam20C*-cKO mice (A2) compared with their WT littermates (A3).

(B) The transgenic expression of *Dmp1* failed to rescue the bone defects in the *Fam20C*-cKO mice:

(B1) Plain X-ray showed similar defects having shorter length, a thinner cortex and generalized hypomineralization in the long bones of the *Fam20C*-cKO mice with (left) or without (middle) *Dmp1*-Tg, in comparison with their WT littermates (right).

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(B2) Micro-CT of tibias. Upper panel: The *Dmp1*-Tg;*Fam20C*-cKO mice (left) had shorter and more porous tibia with less mineralized metaphysis, defects that were similar to those in the *Fam20C*-cKO mice (middle), compared with their WT littermates (right); Lower panel: Cross section of middle shafts indicated similar porosity and thickness of the cortex in the *Dmp1*-Tg;*Fam20C*-cKO mice (left) and the *Fam20C*-cKO mice (middle), in contrast to the well-mineralized and thicker cortex in their WT littermates (right).

(B3) Goldner's Masson trichrome staining displayed more osteoids (red stained, arrows) in the cortical bone of the *Dmp1*-Tg;*Fam20C*-cKO mice (upper) and the *Fam20C*-cKO mice (middle), in comparison with the well-mineralized cortex (blue stained) in the WT mice (lower).

(C) The transgenic expression of *Dmp1* failed to rescue the dentin defects in the *Fam20C*-cKO mice:

(C1) Plain X-ray showed similar features of smaller jaws, shorter teeth and generalized hypomineralization in the mandibles of both the *Fam20C*-cKO mice with (upper) and without (middle) *Dmp1*-Tg, compared to their WT littermates (lower).

(C2) Micro-CT assessment of the mandibles of *Dmp1*-Tg;*Fam20C*-cKO mice (upper) and the *Fam20C*-cKO mice (middle) showed that they were smaller and more porous than those of the WT mice (lower).

(C3) Micro-CT cross sections of the first lower molar showed extremely thin dentin (arrows) and porous alveolar bone in the *Dmp1*-Tg;*Fam20C*-cKO mice (upper) and the *Fam20C*-cKO mice (middle), in contrast to their WT littermates (lower).

(C4) *Von Kossa* staining on the cross sections of the first lower molar displayed thinner dentin and less mineralization (less black staining or more red staining) in the *Dmp1*-Tg;*Fam20C*-cKO mice (upper) and the *Fam20C*-cKO mice (middle), compared with their WT littermates (lower).

Scale bars: 100 μm in A1–3, B3; 200 μm in C4.

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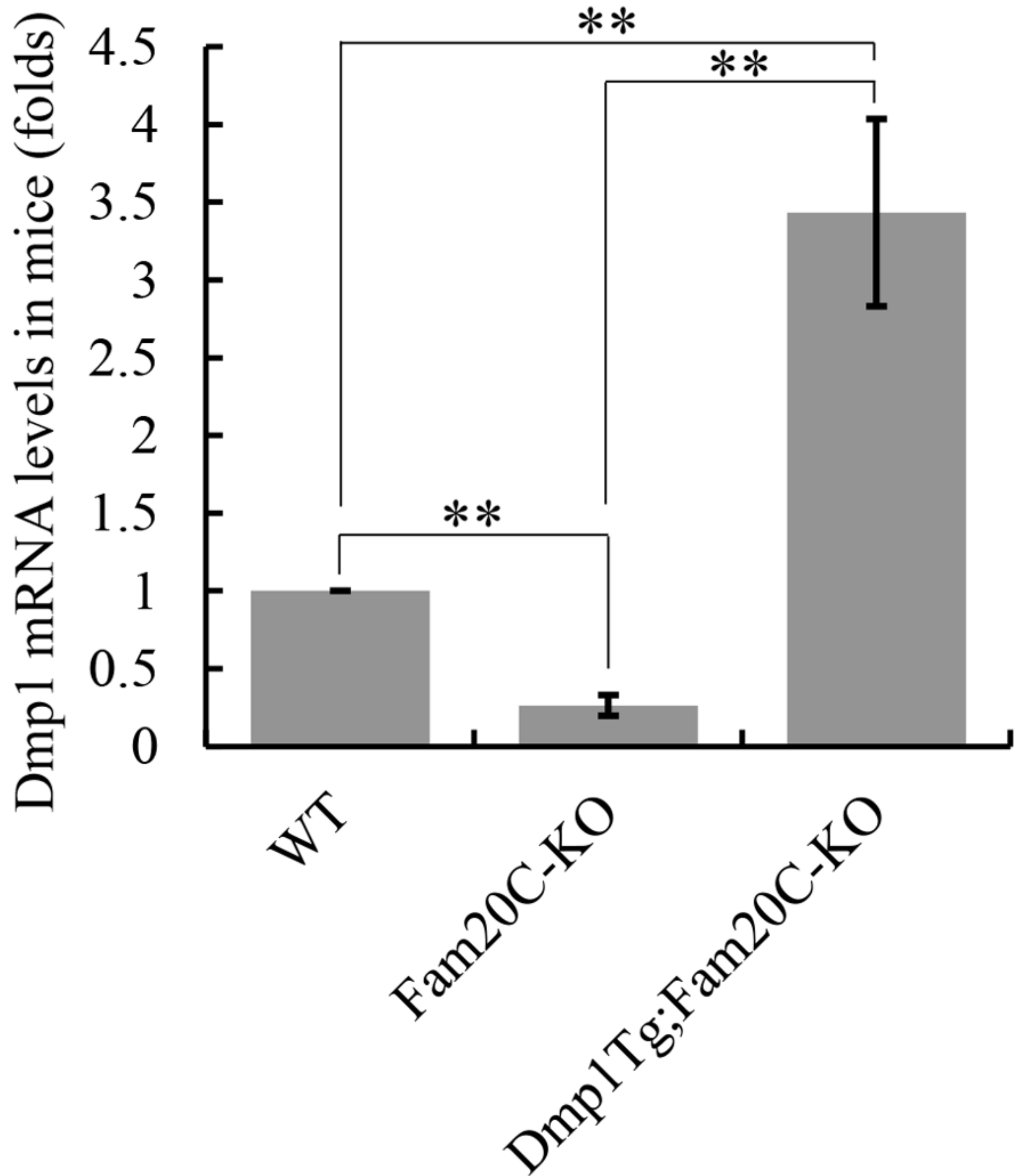

Figure 2. *Dmp1* mRNA levels in the long bone of WT, *Fam20C*-cKO and *Dmp1*-Tg;*Fam20C*-cKO mice

The *Dmp1* mRNA levels in the long bone of WT revealed by real-time PCR was taken as 1, while those of the *Fam20C*-cKO and the *Dmp1*-Tg; *Fam20C*-cKO mice were expressed as fold of change to the WT. **: significant difference ($p < 0.01$).

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Table 1

Quantitative μ-CT analyses of the cortical bone (the midshaft region)of the tibias from 4-week-old *Dmp1*-Tg;*Fam20C*-cKO mice, *Fam20C*-cKO mice and WT mice.

Variables (Mean±SD)	Tg;cKO (n=6)	cKO (n=6)	WT (n=6)
BV/TV	0.78±0.07*	0.81±0.06*	0.93±0.11
Apparent density (mg/cm^3)	655±14*	662±16*	913±15
Material density (mg/cm^3)	793±21*	781±19*	968±14

BV/TV: ratio of bone volume (BV) to total volume (TV); Apparent density: average mineral density within the segmented area of all voxels thresholded as bone; Material density: average mineral density of all voxels segmented as bone including voids; The parameters of Tg;cKO and cKO mice had no statistical difference, while they were significantly different from those of WT mice (*: $p<0.05$).

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Table 2

Serum biochemistry results in the 4-week-old *Dmp1*-Tg;*Fam20C*-cKO mice, *Fam20C*-cKO mice and WT mice.

Serum biochemistry (mean±SD)	Tg;cKO (n = 6)	cKO (n = 6)	WT (n = 6)
FGF23 (pg/ml)	45291±6685**	41653±10437**	269±64
Phosphorus (mg/dl)	6.25±2.41**	6.36±3.15**	16.36±3.28
Calcium (mg/dl)	8.28±0.60*	8.31±0.64*	9.30±0.54

* A p value of <0.05 was taken as statistically significant difference.

** significant difference from the WT mice (p<0.001). The parameters of Tg;cKO and cKO mice had no statistical difference, while they were significantly different from those of WT mice.