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Skeletal FGFR1 signaling is necessary for regulation of serum phosphate level by FGF23 and normal life span

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ABSTRACT

Fibroblast growth factor (FGF) 23 produced by the bone is the principal hormone to regulate serum phosphate level. Serum FGF23 needs to be tightly regulated to maintain serum phosphate in a narrow range. Thus, we hypothesized that the bone has some phosphate-sensing mechanism to regulate the production of FGF23. Previously we showed that extracellular phosphate induces the phosphorylation of FGF receptor 1 (FGFR1) and FGFR1 signaling regulates the expression of *Galnt3*, whose product works to increase FGF23 production *in vitro*. In this study, we show the significance of FGFR1 in the regulated FGF23 production and serum phosphate level *in vivo*. We generated late-osteoblast/osteocyte-specific *Fgfr1*-knockout mice (*Fgfr1*$^{fl/fl}$; *Ocn*$^{Cre/+}$) by crossing the *Ocn-Cre* and the floxed *Fgfr1* mouse lines. We evaluated serum phosphate and FGF23 levels, the expression of *Galnt3* in the bone, the body weight and life span. A selective ablation of *Fgfr1* aborted the increase of serum active full-length FGF23 and the enhanced expression of *Galnt3* in the bone by a high phosphate diet. These mice showed more pronounced hyperphosphatemia compared with control mice. In addition, these mice fed with a control diet showed body weight loss after 23 weeks of age and shorter life span. These results reveal a novel significance of FGFR1 signaling in the phosphate metabolism and normal life span.

1. Introduction

Circulating level of phosphate needs to be maintained in a narrow range. Hypophosphatemia and hyperphosphatemia cause rickets/osteomalacia and ectopic calcification, respectively [1]. While several hormones such as fibroblast growth factor (FGF) 23, parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] are involved in phosphate metabolism, FGF23 is the principal hormone to regulate serum phosphate level [2,3]. FGF23 is produced by the bone, especially by the osteoblasts and osteocytes [4], and works to reduce serum phosphate level by acting mainly in the kidney [3]. Namely, FGF23 inhibits renal phosphate reabsorption in the renal proximal tubules and decreases 1,25(OH)$_2$D level, which enhances intestinal phosphate absorption [3]. These facts suggest that the bone has some phosphate-sensing mechanism to regulate the production of FGF23 and serum phosphate level by FGF23. However, the precise mechanism of phosphate-sensing has remained to be clarified.

We recently reported that the activation of unliganded FGF receptor 1 (FGFR1) by extracellular phosphate regulates the production of FGF23 [5]. We found that high extracellular phosphate induces the phosphorylation of FGFR1 examined by phospho-proteomic method using osteoblastic UMR106 cells [5]. In addition, we demonstrated that the posttranslational O-glycosylation of FGF23 protein by a gene product of *Galnt3* inhibits the proteolytic cleavage of FGF23 protein [6]. This posttranslational modification seemed to be the main regulatory mechanism of increased active full-length FGF23 level by a high phosphate diet in wild-type mice. FGFR1 signaling, including the phosphorylation of FGFR substrate (FRS) 2α and extracellular signal-regulated kinase (ERK), regulated the expression of *Galnt3* in *in vitro* experiments [5]. Therefore, FGFR1 seemed to be a phosphate-sensing molecule in the regulation of FGF23 production and serum phosphate level.

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Here we show the significance of FGFR1 in the regulated FGF23 production and serum phosphate level *in vivo*. This study was initiated to generate late-osteoblast/osteocyte-specific *Fgfr1*-deficient mice. As a result, a selective ablation of *Fgfr1* in bone aborted the increase of serum FGF23 level by a high phosphate diet. In addition, these mice showed more pronounced hyperphosphatemia compared with control mice by a high phosphate diet. Furthermore, these knockout mice fed with a control diet also showed shorter life span. Together with our previous report, these results reveal a novel unrecognized significance of FGFR1 signaling in the phosphate metabolism and normal life span.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Animal Research Committee at Tokushima University (T27-89). The *Ocn-Cre* mouse line was from the Jackson Laboratory (Bar Harbor, ME, USA) [7]. The floxed *Fgfr1* mouse line was kindly provided form Dr. Juha Partanen (University of Helsinki, Helsinki, Finland) [8]. The mouse lines were maintained in C57BL/6 N background. All mice were housed in a specific-pathogen-free facility under climate-controlled conditions with a 12 h light/dark cycle. Late-osteoblast/osteocyte-specific *Fgfr1*-deficient mice ($Fgfr1^{fl/fl}$; $Ocn^{Cre/+}$) were obtained by crossing these mice. Offspring was genotyped by PCR using a specific pair of primers. The floxed *Fgfr1* allele was detected by PCR with specific primers 5′-AATAGGTCCCTCGACGGTATC-3′ and 5′-CTGGGTCAGTGTGGACAGTGT-3’. The wild-type *Fgfr1* allele was detected with primers 5′-CCCCATCCCATTTCCTTACCT-3′ and 5′-TTCTGGTGTGTCTGAAAACAGCT-3’. The *Ocn-Cre* transgenes were detected with specific primers 5′-CAAATAGCCCTGGCAGATTC-3′ and 5′-TGATACAAGGGACATCTTCC-3’. Eight-week-old male *Fgfr1*-cKO mice were fed with a control phosphate diet containing 0.6 % phosphate (16012703, Research Diets, New Brunswick, NJ, USA) or a high phosphate diet containing 1.2 % phosphate (11101203, Research diets, New Brunswick, NJ, USA) for 10 days. Each diet included 0.5 % calcium and there were no differences in any other component. Animals were sacrificed and blood was collected. Before sacrifice, 24-h urine samples were collected using metabolic cages. The femur was removed, the epiphysis and adherent soft tissues were cut away, and bone marrow was flushed with saline. The cleaned femur was rapidly frozen in liquid nitrogen.

2.2. Real-time RT-PCR

Tissue samples were soaked in RNAiso Plus (Takara, Otsu, Japan) and homogenized with TissueLyser II (Qiagen, Venlo, Netherlands). Total RNA was extracted with a NucleoSpin RNA system (Machrey-Nagel, Duren, Germany). First-strand cDNA was synthesized from total RNA with PrimeScript RT Master Mix (Takara, Otsu, Japan). Real-time RT-PCR was performed using FastStart Essential DNA Green Master (Roche, Basel, Switzerland) and LightCycler 96 System (Roche, Basel, Switzerland). The gene expression level was normalized to that of β-actin (*Actb*). The specific primers were designed as shown in **Supplemental Material**.

2.3. Analysis of skeletal morphology

Bone radiographs of the femur were taken with a soft X-ray apparatus. BMD was measured by DXA using a bone mineral analyzer. These analyses were performed in a commercial laboratory (Kureha, Fukushima, Japan).

2.4. Serum biochemistry

Serum levels of phosphate, calcium, urea nitrogen, and creatinine were measured in a commercial laboratory (SRL, Tokyo, Japan). Serum full-length FGF23 concentrations were measured by the FGF23 ELISA kit (Kainos, Tokyo, Japan) [9].

2.5. Statistical analysis

Data were analyzed by two-tailed Student’s *t*-test or ANOVA with a post hoc Tukey’s test. Survival rates were evaluated by log rank test. For all graphs, data are represented as means ± SEMs. Statistical significance was accepted at $P < 0.05$.

3. Results

To address the significance of FGFR1 in *in vivo* experiments, we first generated late-osteoblast/osteocyte-specific *Fgfr1*-deficient mice (*Fgfr1*-cKO, $Fgfr1^{fl/fl}$; $Ocn^{Cre/+}$) by crossing *Osteocalcin (Ocn)-Cre* transgenic mice [7] with mice harboring a floxed allele of *Fgfr1* [8] (Fig. 1A). Deletion of *Fgfr1* allele was observed in the calvaria and femur, but not in the brain, spleen, kidney, duodenum, and skin (Fig. 1B). The expression level of *Fgfr1* in the cKO mice was almost 80 % lower than control in the femur, but not in the kidney (Fig. 1C). Similar expression level of *Fgfr1* to control mice in the kidney suggested the similar FGFR1 and α-Klotho signaling as kidney function was the same between *Fgfr1*-cKO mice and control mice (Fig. 2D and E). *Fgfr1*-cKO mice were born according to the expected Mendelian ratio and showed no change in the length of the body at 8 weeks of age (Fig. 1D). There was no difference in the increase of body weight, soft X-ray images, and bone mineral density (BMD) of the femur by dual-energy X-ray absorptiometry (DXA) between *Fgfr1*-cKO mice and control mice, either (Fig. 1E–G). Together, there was no growth and skeletal phenotypes in *Fgfr1*-cKO mice at this age.

Our *in vitro* study suggested that phosphate regulates FGF23 level through FGFR1 signaling [5]. To test this hypothesis *in vivo*, *Fgfr1*-cKO mice at 8 weeks of age were fed with a control phosphate diet with 0.6 % phosphate or a high phosphate diet with 1.2 % phosphate for 10 days (Fig. 2A). After 10 days, we analyzed serum and urine parameters, and gene expression in the femur. Serum phosphate level was higher in control mice fed with a high phosphate diet than that in mice fed with a control phosphate diet (Fig. 2B). On the other hand, serum phosphate level in *Fgfr1*-cKO mice fed with a control phosphate diet was as high as that in control mice fed with a high phosphate diet (Fig. 2B). When we evaluated fractional excretion of phosphate (FE_{Pi}) using 24-h urine samples, a high phosphate diet significantly increased FE_{Pi} independent of serum full-length FGF23 level in this model (Fig. 2B). Serum calcium level was slightly higher in *Fgfr1*-cKO mice fed with both a control and a high phosphate diet than that in control mice fed with a high phosphate diet (Fig. 2C). Serum urea nitrogen and creatinine levels were not different among these groups (Fig. 2D and E). While a high phosphate diet increased serum active full-length FGF23 level in control mice, a selective ablation of *Fgfr1* in bone aborted this increase by a high phosphate diet (Fig. 2F). In addition, the baseline level of serum FGF23 was lower in *Fgfr1*-cKO mice than that in control mice with a control diet (Fig. 2F). We also analyzed gene expression of *Fgf23* and *Galnt3*, whose product works to increase active full-length FGF23 level, in the femur. The expression level of *Fgf23* was suppressed in *Fgfr1*-cKO mice fed with a control phosphate diet (Fig. 2G). A selective ablation of *Fgfr1* aborted the increase of *Galnt3* expression in the femur by a high phosphate diet (Fig. 2G).

Although the body size and appearance of *Fgfr1*-cKO mice were not different from those of control mice until 22 weeks of age, marked body weight loss of *Fgfr1*-cKO mice fed with a control phosphate diet was observed after 23 weeks of age (Fig. 3A). Moreover, the life span of *Fgfr1*-cKO mice fed with a control phosphate diet was significantly shorter than that of control mice (Fig. 3B).

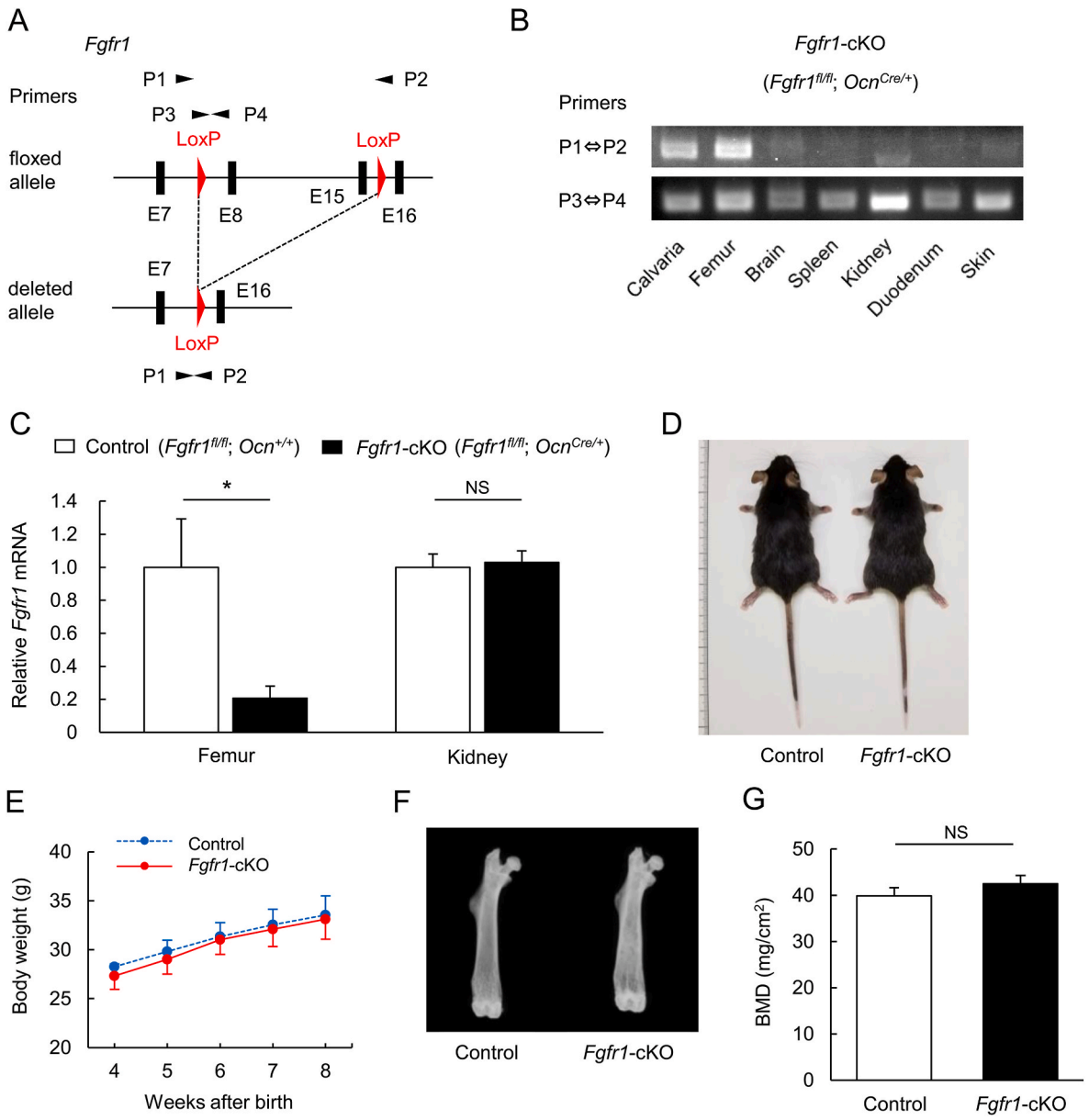

Fig. 1. Late-osteoblast/osteocyte-specific ablation of *Fgfr1* in mice and their phenotypes. (A) Genomic structure of the floxed and deleted *Fgfr1* alleles. Primers used in PCR experiments are indicated by arrows (P1, 5′-AGGTTCCCTCCTCTTGGATGA-3’; P2, 5′-CTGCATGCCAGCAGTCCCGCATC-3’; P3, 5′-AATAGGTCCCTCGACGG-TATC-3’; P4, 5′-CTGGGTCAGTGTGGACAGTGT-3′). (B) PCR analysis of genomic DNA from each tissue in *Fgfr1*-cKO mice. (C) Real-time RT-PCR analysis of *Fgfr1* mRNA expression in the femur and kidney of 8-week-old control and *Fgfr1*-cKO mice. $n = 4$ mice per group. (D) Body length of 8-week-old control and *Fgfr1*-cKO mice. (E) Growth curves of control and *Fgfr1*-cKO mice. $n = 4$ mice per group. (F) Soft X-ray images of the femur of 8-week-old control and *Fgfr1*-cKO mice. (G) Bone mineral density (BMD) of the femur of 8-week-old control and *Fgfr1*-cKO mice. $n = 4$ mice per group. (B, D, and F) Data are presented as representative images. (C, E, and G) Data represent the mean ± SEM. $^*P < 0.05$ by Student’s *t*-test; NS, not significant.

4. Discussion

This study revealed that FGFR1 signaling in the bone is necessary for the increase of FGF23 by a high phosphate diet and thus for the regulation of serum phosphate level. On the other hand, this study also showed that FGFR1 in the late-osteoblasts/osteocytes is not necessary for skeletal development and growth. However, deletion of FGFR1 signaling in the bone induced the body weight loss and shorter life span of mice in long-term observations.

According to our previous report, we hypothesized that a selective ablation of *Fgfr1* in the bone aborts the increase of serum active full-length FGF23 level and enhanced *Galnt3* expression in the bone by a high phosphate diet [5]. This study supported this hypothesis. Therefore, we propose that extracellular phosphate activates FGFR1 and increases active full-length FGF23 level through proteolytic protection of FGF23 protein by the gene product of *Galnt3 in vivo*. The identification of FGFR1 as a component of at least one phosphate-sensing mechanism is supported by previous reports suggesting the involvement of FGFR in the production of FGF23 in the bone [10,11]. In addition, local factors such as phosphate-regulating endopeptidase homolog, X-linked (PHEX) and dentin matrix protein 1 (DMP1), which are produced by genes responsible for FGF23-related hypophosphatemic rickets [1], were reported to alter the production of FGF23 through FGFR signaling in the bone [12]. Furthermore, some activating mutations in *FGFR1* cause osteoglophonic dysplasia associated with by high FGF23 level and hypophosphatemia [13]. On the other hand, it was reported that a patient with Hartsfield syndrome caused by an inactivating *FGFR1* mutation showed hyperphosphatemia [14]. Our hypothesis is further

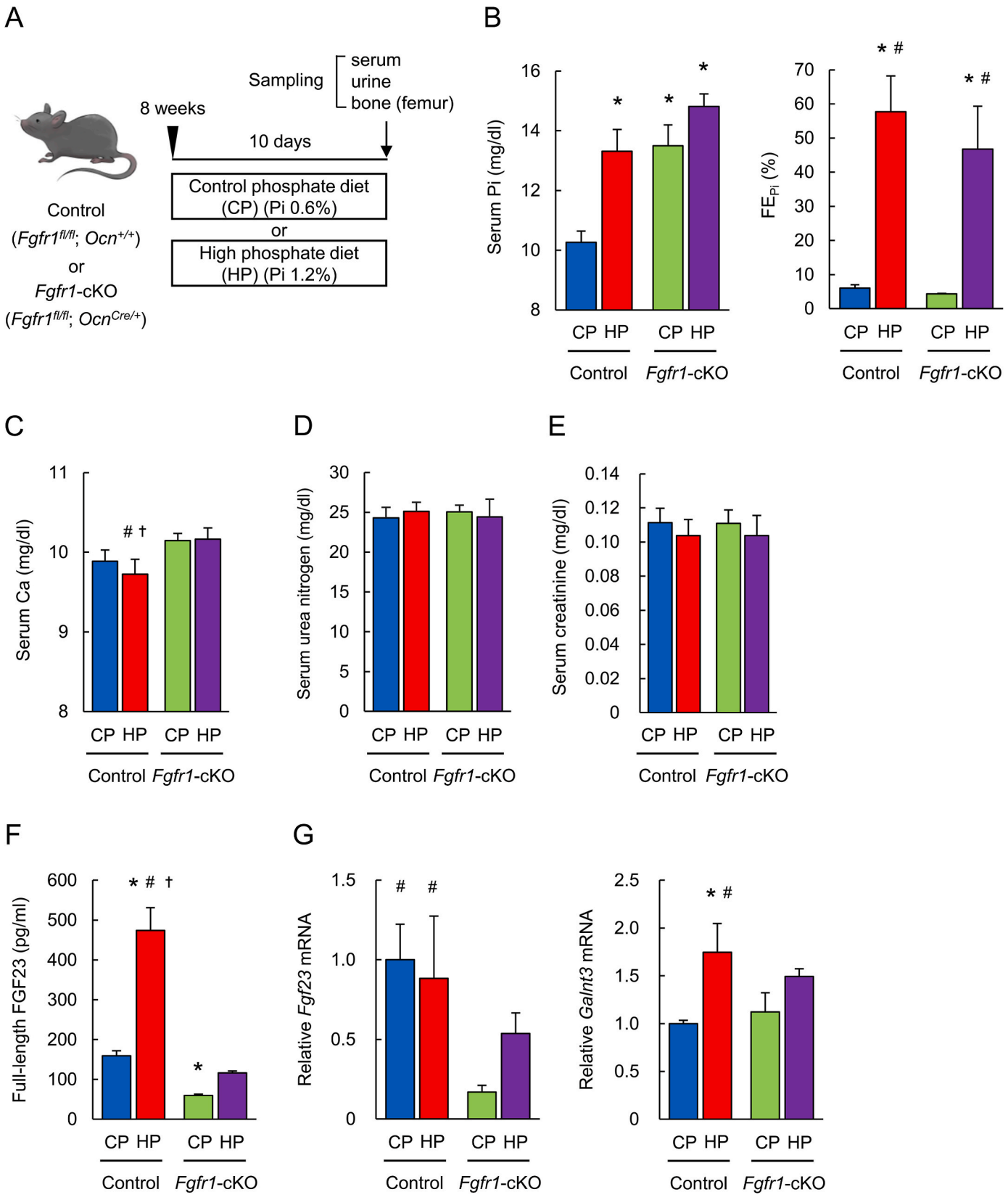

Fig. 2. Responses to a high phosphate diet in control and *Fgfr1*-cKO mice. (A) Schema of the experimental procedure. (B) Serum phosphate (Pi) and fractional excretion of phosphate (FE_{Pi}). (C) Serum calcium (Ca). (D) Serum urea nitrogen. (E) Serum creatinine. (F) Serum full-length FGF23. (G) *Fgf23* and *Galnt3* mRNA expression in the femurs were evaluated by real-time RT-PCR. (B–G) Data represent the mean ± SEM in control and *Fgfr1*-cKO mice fed a control phosphate diet (CP) or a high phosphate diet (HP) for 10 days. $n = 8$–11 mice per group. $^{*}P < 0.05$ compared with control mice fed a CP diet, $^{\#}P < 0.05$ compared with *Fgfr1*-cKO mice fed a CP diet, $^{\dagger}P < 0.05$ compared with *Fgfr1*-cKO mice fed a HP diet by ANOVA with a post hoc Tukey's test.

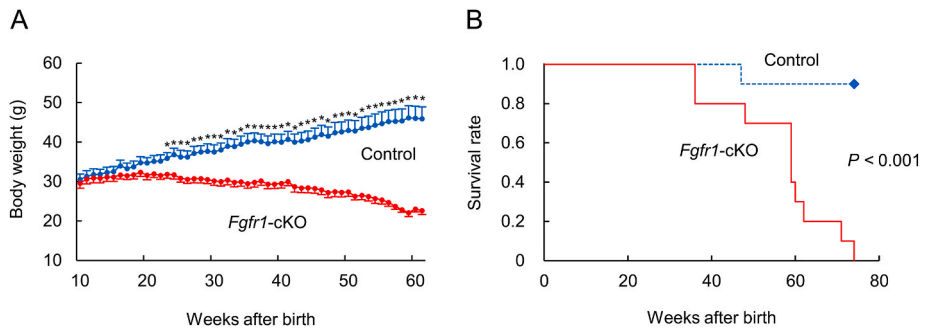

Fig. 3. Long-term observations in control and *Fgfr1*-cKO mice. (A) Changes of body weights in control and *Fgfr1*-cKO mice fed with a control phosphate diet. $n = 10$ mice per group at the start. Data represent the mean ± SEM. $^*P < 0.05$ by Student's *t*-test. (B) Survival rates for control and *Fgfr1*-cKO mice fed with a control phosphate diet. $n = 10$ mice per group at the start. *P* value was evaluated by log rank test.

supported by the biochemical characteristics of patients with these human diseases. While there are some other diseases caused by *FGFR1* mutations, such as Kallmann syndrome [15] and Pfeiffer syndrome [16], it is unknown whether these patients show the disturbance of serum phosphate and FGF23 levels. We have previously shown that extracellular phosphate and a canonical FGFR1 ligand, FGF2, induce different signals from FGFR1 [5]. It is possible that some specific signal from FGFR1 is essential for regulating the production of FGF23.

Serum phosphate level was significantly increased by a high phosphate diet in control mice. While it is unlikely that a high phosphate diet causes clear increase in serum phosphate in human, oral loading of high phosphate seemed to exceed the excretion capacity of phosphate in the kidney at least in this mouse model. It was also indicated in a previous report that mice fed with a high phosphate diet show significant increase in serum phosphate level [17]. Serum phosphate level in *Fgfr1*-cKO mice fed with a high phosphate diet was not significantly different from that in *Fgfr1*-cKO mice fed with a control phosphate diet. One may expect much higher serum phosphate in *Fgfr1*-cKO mice fed with a high phosphate diet because FGF23 did not increase by this diet. However, FGF23 is not the only determinant of serum phosphate level as shown by the similar phosphate level with quite different FGF23 in control mice with a high phosphate diet and *Fgfr1*-cKO mice with a control diet. In addition, *Fgfr1*-cKO mice fed with a high phosphate diet still showed enhanced renal excretion of phosphate. Because we used 24-h urine samples to analyze FE_{Pi} in this study, FE_{Pi} reflects daily oral intake of phosphate. Severe hyperphosphatemia is harmful, and it is likely that there is some FGF23-independent mechanism to enhance renal excretion of phosphate in order to prevent severe hyperphosphatemia. However, the detailed mechanism has not been uncovered yet.

The increase of serum phosphate level does not seem to have an acute effect on the production of FGF23. In our previous study, intravenous administration of phosphate into healthy volunteers for 4 h clearly increased serum phosphate level, while no increase was observed in serum full-length FGF23 level [18]. In addition, in the reports by the other groups, the increase in serum FGF23 level by a high phosphate diet was seen only after a few days in human [19,20]. Furthermore, *Galnt3* induction by high phosphate peaked at 48 h in our previous *in vitro* experiments using osteoblastic UMR106 cells [5]. Thus, we presume that the change of serum FGF23 in response to phosphate is a rather slow one in contrast to rapid fluctuation of serum PTH in response to alterations of serum calcium level.

Serum calcium level in control mice fed with a high phosphate diet was slightly lower than that in *Fgfr1*-cKO mice fed with both a control and high phosphate diet. This may be explained by the increase of serum FGF23 level, which works to decrease serum $1,25(OH)_2D$ [3], while we could not measure $1,25(OH)_2D$ level because of the limited amount of sera. *Fgfr1*-cKO mice fed with a control phosphate diet showed higher serum phosphate level and lower FGF23 level due to suppressed *Fgf23* expression level without changing *Galnt3* expression compared with control mice fed with a control phosphate diet. In addition, a high phosphate diet showed a trend to increase *Fgf23* expression in *Fgfr1*-cKO mice. A high phosphate diet did not increase the expression level of *Fgf23* in control mice as we previously showed using ICR mice [5]. While the enhanced expression of *Galnt3* is the main reason for the increase of FGF23 in response to a high phosphate diet, it is suggested that FGFR1 signaling in the bone is involved in the basal *Fgf23* expression. A previous report indicated that FGF23 promoter activity was stimulated by FGFR1 activation in *in vitro* study [21]. Therefore, one explanation is that the reduction of FGF23 promoter activity due to the ablation of FGFR1 in the bone induces this phenotype. There is also a possibility that some other molecules than the gene product of *Galnt3* are also involved in the regulation of *Fgf23*. There was non-significant trend of increase of *Fgf23* by a high phosphate diet in *Fgfr1*-cKO mice. It is possible that a high phosphate diet affects *Fgf23* expression in the absence of FGFR1.

In addition, it is considered that persistently higher serum phosphate level in *Fgfr1*-cKO mice throughout the life is the reason for the body weight loss and shorter life span of *Fgfr1*-cKO mice like *Fgf23*-KO mice [2]. As there is a term "phosphate toxicity", hyperphosphatemia is also well known to be associated with increased cardiovascular disease risk and higher mortality in both general population and patients with chronic kidney disease [22]. A high phosphate diet in *Fgfr1*-cKO mice is expected to increase the mortality earlier. This indicate that the maintenance of serum phosphate level by skeletal FGFR1 is critical for normal life. However, we could observe only the mortality in *Fgfr1*-cKO mice fed with a control phosphate diet and we do not have any data including serum level of phosphate and FGF23 in aging *Fgfr1*-cKO mice and the cause of death at this moment.

A recent report demonstrated that extracellular phosphate induces type III sodium-phosphate cotransporters heterodimerization, PiT1 and PiT2, and mediates the activation of ERK independent of phosphate uptake in *in vitro* experiments [23]. Another recent report indicated that extracellular phosphate stimulates the secretion of PTH through calcium-sensing receptor (CaSR) on the parathyroid cells [24]. CaSR is also expressed in the bone [25]. Phosphate has been reported to be involved in several other biological responses including chondrocyte apoptosis [26] and development of vascular diseases [27] other than regulating FGF23 level. Therefore, it is possible that several molecules other than FGFR1 can work as phosphate-sensing mechanisms regulating cell-specific functions.

This study identified that FGFR1 is at least one of the phosphate-sensing molecules to regulate serum phosphate and FGF23 level in the bone. Our findings uncover an unrecognized function of FGFR1 and provide a new molecular basis of phosphate-sensing in the regulated FGF23 production in the bone. As the identification of CaSR has come to fruition of new drug discoveries for patients with primary and secondary hyperparathyroidism, the elucidation of the phosphate-sensing mechanism may lead to the identification of novel druggable molecules for

patients with abnormal phosphate metabolism.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101107.

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