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Brief Communication

MOLECULAR METABOLISM

Phosphate-dependent FGF23 secretion is modulated by PiT2/Slc20a2

Nina Bon [1,2], Giulia Frangi [1,2], Sophie Source [1,2], Jérôme Guicheux [1,2,3], Sarah Beck-Cormier [1,2], Laurent Beck [1,2,*]

ABSTRACT

Objective: The canonical role of the bone-derived fibroblast growth factor 23 (Fgf23) is to regulate the serum inorganic phosphate (Pi) level. As part of a feedback loop, serum Pi levels control Fgf23 secretion through undefined mechanisms. We recently showed *in vitro* that the two high-affinity Na^+-Pi co-transporters PiT1/Slc20a1 and PiT2/Slc20a2 were required for mediating Pi-dependent signaling. Here, we addressed the contribution of PiT1 and PiT2 to the regulation of Fgf23 secretion.
Methods: To this aim, we used *PiT2* KO and *DMP1Cre; PiT1$^{lox/lox}$* fed Pi-modified diets, as well as *ex vivo* isolated long bone shafts. Fgf23 secretion and expression of Pi homeostasis-related genes were assessed.
Results: *In vivo, PiT2* KO mice responded inappropriately to low-Pi diets, displaying abnormally normal serum levels of intact Fgf23. Despite the high iFgf23 level, serum Pi levels remained unaffected, an effect that may relate to lower αKlotho expression in the kidney. Moreover, consistent with a role of PiT2 as a possible endocrine Pi sensor, the iFGF23/cFGF23 ratios were suppressed in *PiT2* KO mice, irrespective of the Pi loads. While deletion of *PiT1* in osteocytes using the *DMP1-Cre* mice was inefficient, adenovirus-mediated deletion of *PiT1* in isolated long bone shafts suggested that PiT1 does not contribute to Pi-dependent regulation of Fgf23 secretion. In contrast, using isolated bone shafts from *PiT2* KO mice, we showed that PiT2 was necessary for the appropriate Pi-dependent secretion of Fgf23, independently from possible endocrine regulatory loops.
Conclusions: Our data provide initial mechanistic insights underlying the Pi-dependent regulation of Fgf23 secretion in identifying PiT2 as a potential player in this process, at least in high Pi conditions. Targeting PiT2, therefore, could improve excess FGF23 in hyperphosphatemic conditions such as chronic kidney disease.
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Keywords FGF23 secretion; Phosphate sensing; PiT1/Slc20a1; PiT2/Slc20a2; Bone

1. INTRODUCTION

Fibroblast growth factor 23 (Fgf23) is a bone-derived hormone, which acts in concert with its co-receptor αKlotho to regulate the serum inorganic phosphate (Pi) concentration and vitamin D metabolism [1,2]. Mutations in humans and mice preventing normal FGF23 bioactivity result in elevated serum Pi concentrations due to impaired urinary Pi excretion. Prolonged hyperphosphatemia can lead to life-threatening situations resulting from an inappropriate deposition of calcium-Pi crystals in cardiovascular tissue that occurs at high prevalence in diabetes, chronic kidney disease (CKD) and cardiovascular diseases (CVD) [3,4]. Conversely, elevated levels of biologically active FGF23 observed in inherited or acquired disorders of Pi homeostasis lead to renal Pi wasting, low serum Pi levels, and abnormal vitamin D metabolism [5,6]. The regulation of Fgf23 expression and/or secretion is controlled by numerous factors, including FGFR1 signaling [5,7], iron deficiency [8], and pro-inflammatory stimuli [9,10]. The intracellular cleavage of the biologically active intact form of FGF23 into N- and C-terminal fragments is also an important regulatory step. This regulation involves a balance between O-glycosylation of Thr^{178} by polypeptide N-acetylgalactosaminyltransferase 3 (GalNT3), providing a protection of furin-mediated cleavage, and phosphorylation of Ser^{180} by Family with sequence similarity 20, member C (Fam20C) that counteracts O-glycosylation [11].
Interestingly, the presence of regulatory feedback loops operating between FGF23 and Pi/vitamin D has been suggested [1,12]. In humans, serum FGF23 levels have been associated with the amount of dietary Pi [13,14], while intravenous infusion or acute duodenal Pi load were found to increase FGF23 levels in healthy humans [15]. Similarly, *in vivo* animal models have shown a relationship between dietary Pi loads and circulating Fgf23 levels [16,17], indicating that extracellular Pi induces the secretion of Fgf23. However, the mechanism by which Pi might regulate FGF23 production remains insufficiently defined and difficult to elucidate.
An interesting and possible mechanism is the direct regulation of FGF23 expression by Pi, in view of the recently accumulated data indicating that extracellular Pi acts as a signaling molecule [18,19]. While binding and/or cellular uptake of Pi by specialized Pi transporters are involved in

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the Pi signal processing in lower organisms, no Pi-receptor or 'sensor' has yet been identified in mammals [20]. Remarkably, we recently investigated the role of the two high-affinity mammalian Na^+-Pi co-transporters PiT1/Slc20a1 and PiT2/Slc20a2 as Pi sensors in bone and showed that, *in vitro,* both PiTs were required for mediating Pi-dependent signaling [21], raising the question of their role in the modulation of the Pi-dependent FGF23 secretion.

To gain insights into the mechanism by which Pi could regulate FGF23 production, we explored the contribution of PiT1 or PiT2 to the regulation of Fgf23 expression and secretion by using *in vivo* and *ex vivo* murine models.

2. METHODS AND MATERIALS

2.1. Animals

The generation of *PiT1*$^{lox/lox}$ mice has been described previously [22]. Osteocyte-specific deletion of *PiT1* was performed by crossing *PiT1*$^{lox/lox}$ (129sv genetic background) to *DMP1-Cre* mice, generated using the 9.6 kb Dmp1 promoter [23]. *Slc20a2*$^{tm1a(EUCOMM)Wtsi}$ mice (thereafter named *PiT2* KO mice) on the C57BL/6 genetic background were obtained from the European Mouse Mutant Archive (EMMA). The mutant allele contains an IRES:lacZ trapping cassette and a splicing site disrupting *PiT2* gene expression [24]. Genomic DNA from tail was used for PCR genotyping. Animal care and maintenance were provided through the University of Nantes accredited animal facility at the Unité de Thérapeutique Expérimentale. Mice were housed under specific pathogen-free conditions and were fed with RM1 diet for maintenance and with RM3 diet for breeding (SDS Special Diets Services, France). This study conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All procedures were approved by the Animal Care and Use Committee of the Région Pays de la Loire and conducted according to the French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on 6 June 2001) and the National Institutes of Health Animal Welfare (project #02286.02). The mice were weaned at post-natal day 21 (P21) to a normal Pi diet containing 0.7% calcium and 0.55% phosphorus. To induce Fgf23 regulation, the mice were fed at P28 with either a customized low-Pi ($<$0.05% phosphorus) or normal-Pi (0.55% phosphorus) or high-Pi (1.65% phosphorus) diet for 7 days (ssniff Spezialdiäten GmbH, Soest, Germany).

2.2. Serum and urine parameters

Serum phosphorus and calcium were analyzed using the Phosphorus Liqui-UV Test and the Calcium CPC LiquiColor Test kits according to the manufacturer's protocol (Stanbio Laboratory, Boerne, TX). Serum intact Fgf23 concentrations were assessed using ELISA kit according to the manufacturer's protocol (Kainos Laboratories, Tokyo, Japan). Serum PTH and C-terminal Fgf23 levels were determined using ELISA kits for mouse intact PTH and mouse/rat C-term Fgf23, respectively (Immutopics). Urine Pi, calcium, and creatinine concentrations assays were performed with Olympus AU400 Chemistry Analyzer.

2.3. Gene expression analysis

Total RNA was isolated from tissues using the Trizol® Reagent (Thermo Fisher Scientific) (for diaphysis) or using the Nucleospin® RNA II kit (Macherey—Nagel, Germany) (for soft tissues) according to the manufacturer's instructions. RNA was reverse transcribed using SuperScript® VILO™ (for diaphysis) or SuperScript®III (for soft tissues) (Thermo Fisher Scientific) as per manufacturer's instructions. Real-time PCR (qPCR) was performed on a Bio-Rad CFX96 using SYBR®Select Master Mix (Thermo Fisher Scientific). Primer efficiency was determined using a standard curve with a 1:4 dilution and specificity of amplification was verified from the melting curve analysis. Expression of target genes were normalized to pinin (*Pnn*) and beta-glucuronidase (*GusB*) expression levels, and the relative gene expression levels were calculated as previously described [25].

2.4. Immunohistochemistry (IHC)

Paraffin embedded kidney sections were processed for IHC as previously described [22]. Primary antibody for Klotho (1:100, KM2076, TransGenic Inc.) was incubated overnight at 4 °C. Secondary anti-mouse biotinylated goat antibody (1:500, Dako) was used and staining was performed using 3,3-diaminobenzidine chromogen (Dako) for 5 min and counterstaining with Mayer's hematoxylin. Stained sections were then mounted with Eukitt® and scanned using a Hamamatsu NanoZoomer HT (Hamamatsu Photonics KK) digital scanner at a 20× magnification.

2.5. Long bone shafts preparation

Femur, tibia, and humerus bones from P35 mice were dissected free of the surrounding soft tissue and flushed. After stripping away the periosteum, the long bone shafts were trimmed into two pieces for weighing. One half of each bone type was cultured overnight at 37 °C in 5% CO_2 humidified incubator in a well of a 96-well plate with 100 μl of culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) high glucose GlutaMAX™ (catalogue no 31966, ThermoFisher Scientific, Saint-Aubin, France) supplemented with 0.2% Bovine Serum Albumine (Sigma—Aldrich, St Louis, MO, USA), 10 mM HEPES, and 50 μg/ml gentamycin. To study Pi-dependent Fgf23 secretion, the long bone shafts were washed three times with pre-warmed 0.9% NaCl solution and incubated during 24 h in stimulation medium. The stimulation medium consisted of phosphate-free high glucose GlutaMAX™ DMEM (catalogue no 11971-025, ThermoFisher Scientific) supplemented with 0.2% BSA, 10 mM HEPES, 50 μg/ml gentamycin and 0, 1, 3, 7, or 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4. Intact and C-terminal Fgf23 concentrations were assessed in the supernatants using ELISA kits according to the manufacturer's protocol (Kainos Laboratories and Immuntopics, respectively) and normalized by bone mass.

2.6. Transduction

Adeno-CMV-iCre (catalogue no 1045 Vector Biolabs) was added to the media of *PiT1*$^{lox/lox}$ long bone shafts culture at a concentration of 10^7 virus particles per mL of media. Control samples were treated with Ad-CMV-GFP (catalogue no 1060 Vector Biolabs) at the same concentration. The media were replaced 24 h after transduction, and, 72 h post-transduction, the long bone shaft were harvested and analyzed for PiT1 deletion.

2.7. Statistics

Data are expressed as mean ± S.E.M. GraphPad 5.0 software was used to perform Mann—Whitney tests or analysis of variance. A p value of less than 0.05 was considered statistically significant. Unless otherwise stated, experiments were repeated at least three times (exact numbers are indicated in the figures).

3. RESULTS

3.1. Deletion of *PiT2* in mice results in impaired Pi-dependent Fgf23 secretion

To investigate the role of PiT2 in the Pi-dependent regulation of Fgf23 secretion *in vivo*, we fed four-week-old *PiT2* KO mice with low

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(0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. RT-qPCR analysis confirmed the deletion of *PiT2* in tibias, while *PiT1* expression was increased in mutant tibias under low-Pi diet condition (Figure 1A). The expression of the Pi exporter *Xpr1* [26] in tibias and kidneys was not changed, nor was the expression of the Na-Pi cotransporters *Npt2a* and *Npt2c* in kidneys (not shown). When *WT* and *PiT2* KO were fed a low Pi diet, we observed the expected decrease in serum Pi levels (Figure 1B). However, feeding a high Pi diet failed to reveal an increase in serum Pi levels, an observation that was already made previously, which may relate to the existence of compensatory endocrine loops [27]. The urine Pi/creatinine ratio was consistent with the feeding scheme and revealed a massive increase when mice were fed a high Pi diet, and a massive decrease when mice were fed a low Pi diet. This was true for both mouse strains, with no observed difference between genotypes (Figure 1B).

In WT mice fed a low Pi diet, circulating intact Fgf23 (iFgf23) levels were decreased compared to normal Pi diets (Figure 1C), a finding that was consistent with the decrease in serum Pi and urine Pi/creatinine levels (Figure 1B). Remarkably, in *PiT2* KO mice fed low-Pi diet, the circulating iFgf23 levels were abnormally high, being similar to the iFgf23 levels in the normal-Pi diet condition (Figure 1C). Although the C-terminal Fgf23 (cFgf23) levels were not statistically different between *WT* and *PiT2* KO mice, we could observe a similar trend (Figure 1D), suggesting that increased iFgf23 does not originate from a decrease in Fgf23 cleavage. In support of this, we observed no difference in *Galnt3* and *Fam20C* gene expressions between *WT* and *PiT2* KO mice (not shown). Accordingly, the *Galnt3/Fam20C* mRNA expression ratio was similar in *WT* and *PiT2* KO mice (Figure 1E). Moreover, RT-qPCR analysis of *Fgf23* gene expression in the diaphysis (Figure 1A) and of *Fgfr1*, *Fgfr2*, *Fgfr3*, and *Fgfr4* in the kidneys revealed no statistical difference between *WT* and *PiT2* KO mice (not shown). Interestingly, when iFgf23/cFgf23 ratios were calculated for the different Pi diet conditions, no change was observed for *PiT2* KO mice, while this ratio increased as a function of Pi load in *WT* mice (Figure 1F). Overall, the response of *PiT2* KO mice to the variation of Pi load was blunted compared to the response of WT mice, further suggesting a role of PiT2 in the setting of Pi-dependent Fgf23 secretion.

Of note, although the serum parathyroid hormone (PTH) levels increased as a function of Pi diet content as expected, these levels were similar between *WT* and *PiT2* KO mice in all Pi diet conditions (Figure 1G). Therefore, the normal serum Pi level observed in *PiT2* KO in the low-Pi condition despite a high iFgf23 serum level cannot be attributed to abnormal serum PTH levels. Similarly, the expression of *Cyp27b1*, *Cyp24a1*, and *Vdr* genes was similar between *WT* and *PiT2* KO mice (not shown). Interestingly however, the gene expression of α*Klotho*, the mandatory co-receptor for mediating Fgf23 renal effects, was lower in *PiT2* KO mice in the low-Pi diet condition (Figure 1H). This was accompanied by a drastic reduction of the protein expression of αKlotho in the kidney (Figure 1I).

3.2. Deletion of *PiT1* in long bone shafts *ex vivo* does not support a role of PiT1 in Pi-dependent Fgf23 secretion from bone

We next investigated the role of PiT1 in the Pi-dependent regulation of Fgf23 secretion by crossing conditional *PiT1*$^{lox/lox}$ mice [22] with *DMP1-Cre* transgenic mice [23] to generate *DMP1-Cre; PiT1*$^{lox/lox}$ mutant mice. When analyzing the deletion of *PiT1* in *PiT1*$^{lox/lox}$*;DMP1-Cre* mice, we observed that *PiT1* was deleted in a number of non-skeletal tissues (Supplemental Figure 1A), revealing unintended deletions as was shown in a recent report [28]. In addition, although the deletion of *PiT1* was visible in bone tissues at the genomic level, the deletion at the mRNA level was very low (Supplemental Figure 1B). This latter result was confirmed using RT-qPCR analysis showing no decrease in *PiT1* gene expression in mutant mice (Supplemental Figure 1C). Accordingly, no difference was observed in circulating Pi and iFgf23 levels between *WT* and *DMP1-Cre; PiT1*$^{lox/lox}$ mice (Supplemental Figure 1D). Similarly, no difference was observed in urine Pi, urine calcium or serum calcium, and PTH levels in any Pi diet condition (not shown).

To overcome the inefficient targeting of *PiT1* using the *DMP1Cre* mice, we used *ex vivo* organ cultures of long bone shafts from *WT* and *PiT1*$^{lox/lox}$ mice transduced with Cre-expressing adenoviruses (*AdVCre*) (Figure 2A). We observed a 61—71% deletion of *PiT1* mRNA without upregulation of *PiT2* mRNA (Figure 2B), allowing us to assess the role of PiT1 in the Pi-dependent regulation of Fgf23 secretion. Despite this efficient deletion of PiT1, results showed that following Pi challenge, secreted iFgf23 and cFgf23 levels (Figure 2C), as well as the iFgf23/cFgf23 ratio (Figure 2D), were similar in *WT* and *PiT1*$^{lox/lox}$*; AdVCre* bones. Consistently, the expression of *Fgf23* in the diaphysis was similar in all conditions tested as were the expression of *Galnt3* and *Fam20C* and the *Galnt3/Fam20C* expression ratio (not shown). These data showed that despite an efficient deletion of PiT1 in bone, the role of PiT1 in the Pi-dependent regulation of Fgf23 could not be demonstrated, suggesting that more appropriate *in vivo* tools are required to successfully address this question.

3.3. The Pi-dependent modulation of Fgf23 secretion by PiT2 is bone-autonomous

We used the *ex vivo* long bone shaft approach to determine whether the role of PiT2 in modulating the Pi-dependent Fgf23 secretion was bone-autonomous. RT-qPCR analysis confirmed the deletion of *PiT2* in diaphyses, while *PiT1* expression was higher in mutant than *WT* tibias under high-Pi diet condition (Figure 3A). In *WT* bones, while the expression of *Fgf23* remained unchanged when extracellular Pi was changed (Figure 3B), the secretion of iFgf23 in the extracellular medium increased as a function of extracellular Pi concentration, as expected (Figure 3C). Strikingly, this was not the case anymore in *PiT2* mutant bones, where secreted iFgf23 remained low (Figure 3C). Interestingly, the secreted cFgf23 from *PiT2* KO mice displayed an inverse profile than the secreted iFgf23 and appeared higher in mutant than in *WT* bones (Figure 3C). Accordingly, the iFgf23/cFgf23 ratios were blunted in *PiT2* KO tibias (Figure 3D), as was observed in *in vivo* experiments (Figure 1F). The low iFgf23 secretion and iFgf23/cFgf23 ratios were consistent with the lower expression of *Fgf23* in mutant bones (Figure 3B). Finally, no differences in *Fam20C* and *Galnt3* gene expression, nor in *Galnt3/Fam20C* mRNA expression ratios, were evident between WT and mutant bones (not shown). Altogether, our data indicate that the expression of *PiT2* is mandatory for a normal regulation of Fgf23 secretion following changes in extracellular Pi concentrations. Interestingly, we recently showed that PiT2 was necessary for the Pi-dependent activation of the ERK1/2 MAPK pathway [21], a finding that may be relevant with the activation of the ERK1/2 MAPK pathway through FGFR1 that is known to stimulate Fgf23 secretion [29]. However, when blocking the Pi-dependent ERK1/2 phosphorylation with the MAPK inhibitor U0126 (Figure 3E) in isolated bone shafts, no change was observed in the Pi-dependent Fgf23 secretion, irrespective of the Pi load (Figure 3F). Moreover, similar expression of *Fgfr1* was found in WT and PiT2 KO mice (Figure 3G). These data suggested that mechanistic links other than the ERK1/2 pathway are likely at work between PiT2 and Fgf23 secretion.

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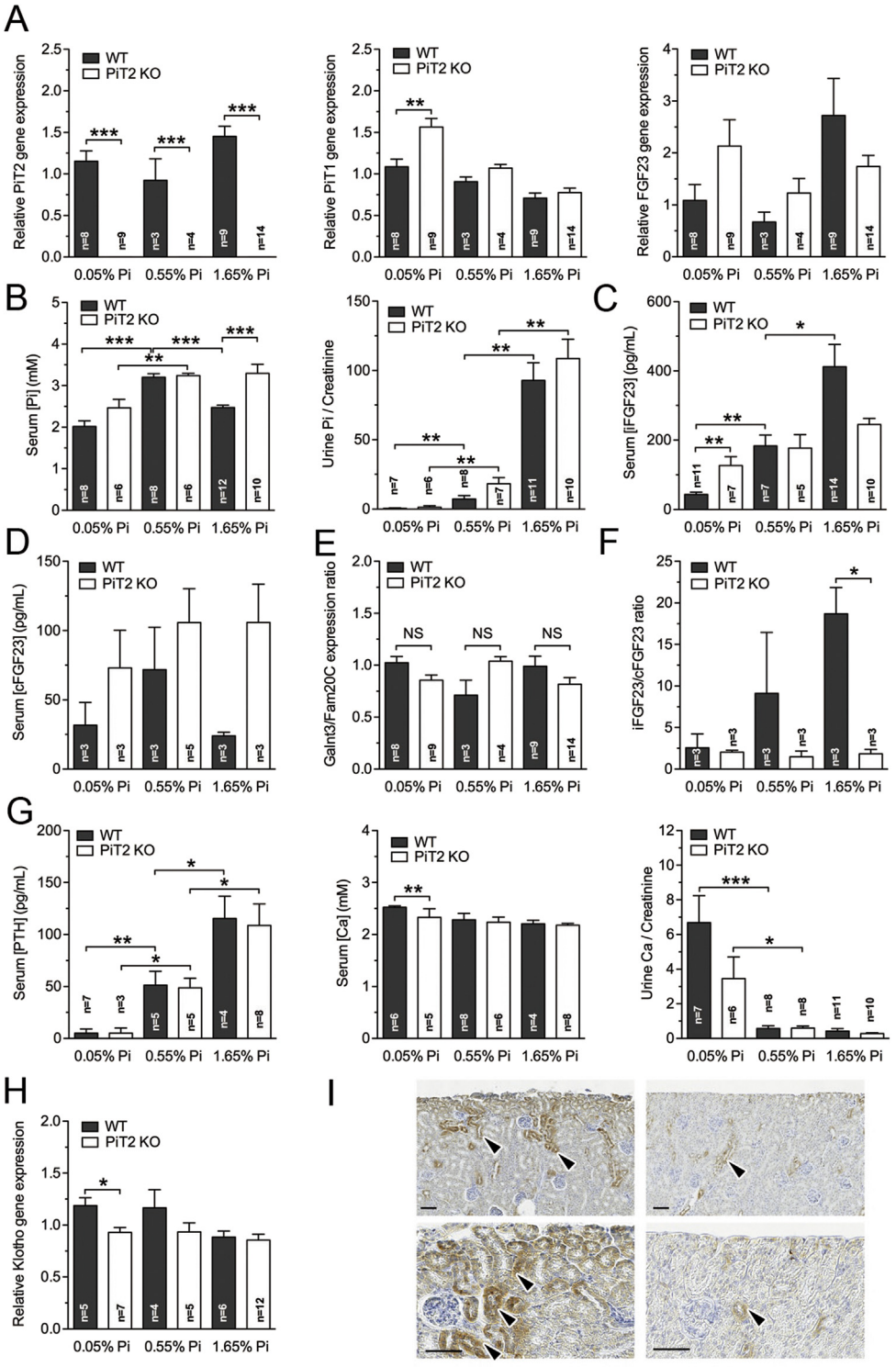

Figure 1: Deletion of *PiT2 in vivo* leads to inappropriate serum Fgf23 levels in low-Pi condition. *(A)* RT-qPCR analysis of *PiT2*, *PiT1* and *Fgf23* mRNA levels in flushed tibias isolated from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. *(B)* Serum and urine Pi values from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%), and high (1.65%) Pi diets for one week. *(C-D)* Serum intact Fgf23 (iFgf23) *(C)* and C-terminal Fgf23 (cFgf23) *(D)* levels (ELISA) from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *(E-F) Galnt3/Fam20C* mRNA ratios as determined by RT-qPCR analysis *(E)* and iFgf23/cFgf23 ratios *(F)* from flushed tibias from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. *(G)* Serum PTH levels *(Left)* and serum and urine Ca values *(Center and Right)* from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *(H)* RT-qPCR analysis of α*Klotho* mRNA levels in kidneys isolated from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. *(I)* Immunohistochemistry for αKlotho of kidney sections from *WT* or *PiT2* KO mice fed with low (0.05%) Pi diet for one week. *Black arrowheads*: αKlotho-positive staining. *Top,* low magnification; *Bottom,* high magnification, scale bar, 50 μm. Data are means ± S.E.M. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS: not significant.

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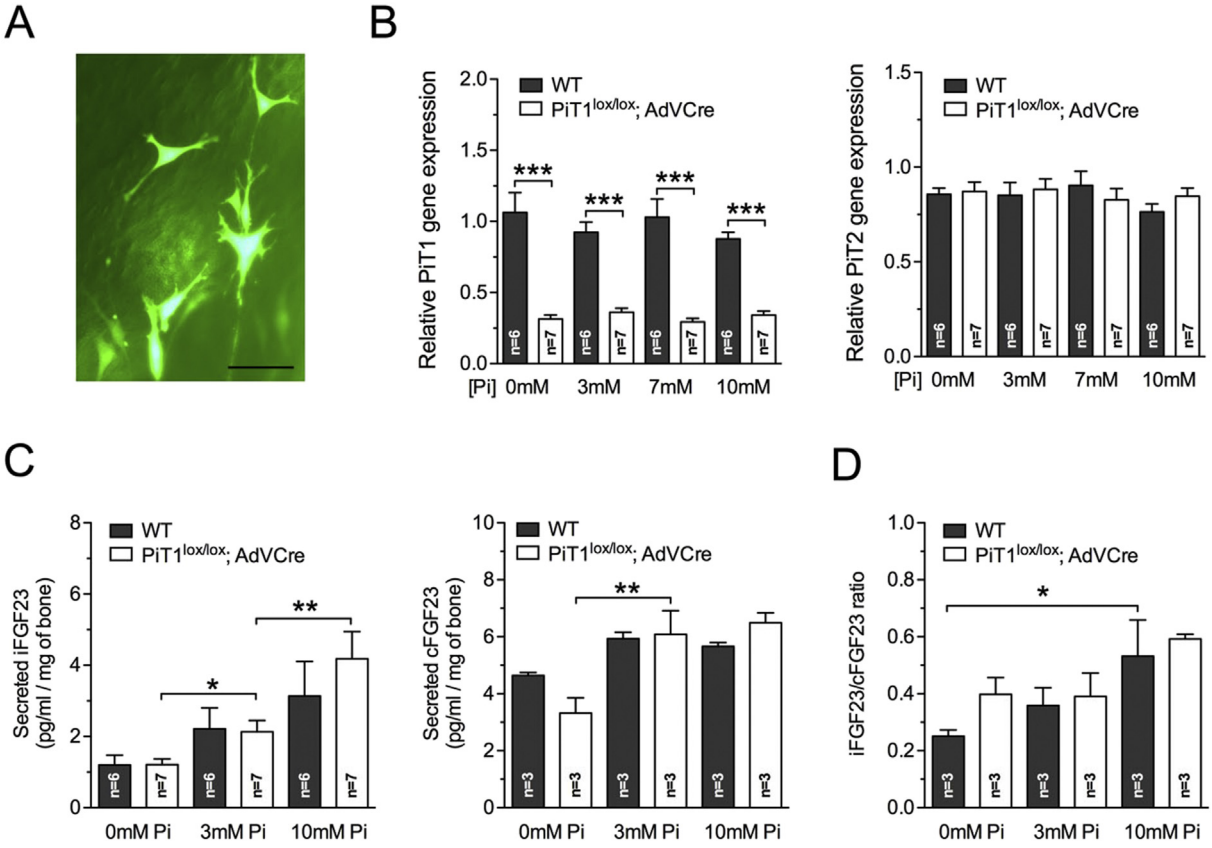

Figure 2: *Ex vivo* deletion of *PiT1* in long bone shafts does not support a role of PiT1 in Pi-dependent Fgf23 secretion from bone. *(A)* Expression of GFP in Ad-CMV-GFP-transduced bone shafts 48 h after transduction. *(B)* RT-qPCR analysis of *PiT1* and *PiT2* mRNA levels in flushed tibias isolated from *WT* or *PiT1*$^{lox/lox}$ transduced with Adeno-CMV-iCre (10^7 virus particles per mL), and stimulated for 24 h in medium containing 0, 3, 7, or 10 mM Pi, as indicated. *Pnn* and *GusB* genes were used as internal controls. *(C)* Secreted levels of intact Fgf23 (iFgf23) and C-terminal Fgf23 (cFgf23) from flushed tibias isolated from *WT* or *PiT1*$^{lox/lox}$ transduced with Adeno-CMV-iCre and stimulated for 24 h in medium containing 0, 3, or 10 mM Pi. *(D)* iFgf23/cFgf23 ratios from *WT* or *PiT1*$^{lox/lox}$ transduced with Adeno-CMV-iCre and stimulated for 24 h in medium containing 0, 3, or 10 mM Pi. Data are means ± S.E.M. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. DISCUSSION

This study presents the first direct evidence that PiT2 expression *in vivo* and *ex vivo* is essential for normal secretion of Fgf23 following dietary Pi changes. While iFgf23 levels were correlated to dietary Pi loads *in vivo* or extracellular Pi concentrations *ex vivo* in *WT* animals, the Pi-dependent regulation of Fgf23 secretion was blunted in *PiT2* KO mice and isolated bones, as was clearly showed when calculating iFgf23/cFgf23 ratios. Since similar Pi-dependent regulation of Fgf23 secretion was observed in whole mutant animals and isolated bone shafts from *PiT2* KO mice, the contribution of other organs or possible endocrine loops to this regulation appears unlikely. However, while this illustrates that Pi-dependent regulation of Fgf23 is dependent upon normal bone *PiT2* expression and highlights PiT2 as an important putative Pi sensor, the underlying mechanism linking PiT2 to the secretion of Fgf23 remains to be deciphered. Notably, the similar expression of *Fam20C* and *Galnt3* in *WT* and *PiT2* KO mice was not consistent with the difference observed in the Fgf23/cFgf23 ratios, suggesting that the regulation of Fgf23 cleavage by PiT2 might involve *Fam20C* and *Galnt3* independent mechanisms, as was suggested recently [30]. Moreover, although FGFR1-mediated stimulation of FGF23 secretion was illustrated through the activation of the ERK1/2 MAPK pathway [29], our data showed that the Pi-dependent activation of the ERK1/2 pathway was not involved in Fgf23 secretion from bone, further indicating that other mechanistic links are at work between PiT2 and Fgf23. One possibility is the involvement of PiT2-specific protein partners linking Pi signaling to Fgf23 secretion, as was shown recently for other PiT2-or PiT1-specific physiological functions [31,32]. Moreover, while our data clearly showed a role of PiT2 in the setting of Fgf23 secretion, most of the physiological parameters remained normal. This finding is consistent with the absence of identified PiT2 mutations in humans that would cause a disturbed Pi homeostasis and suggests the existence of mechanisms compensating the lack of PiT2. One of the possible compensatory mechanism involves the negative regulation of αKlotho that we uncovered in the *PiT2* KO kidneys, which may explain why mutant mice fed a low Pi diet and exhibiting abnormally normal Fgf23 levels retained normal serum and urine Pi levels. Other compensatory mechanisms, such as the existence of other Pi sensors, may also be involved. Along this line, although we could efficiently down-regulate PiT1 in isolated bone shafts and illustrated no role in Pi-dependent regulation of Fgf23 in this model, its actual role *in vivo* remains to be determined.

As an illustration of the complexity of these regulations, the effect of Pi-modified diets on FGF23 secretion in humans still remains a matter of debate [33,34]. Much of the recent focus on FGF23 has been driven by human studies showing that even a mild loss of kidney function is associated with considerable elevations of serum FGF23 levels, preventing hyperphosphatemia early in the course of

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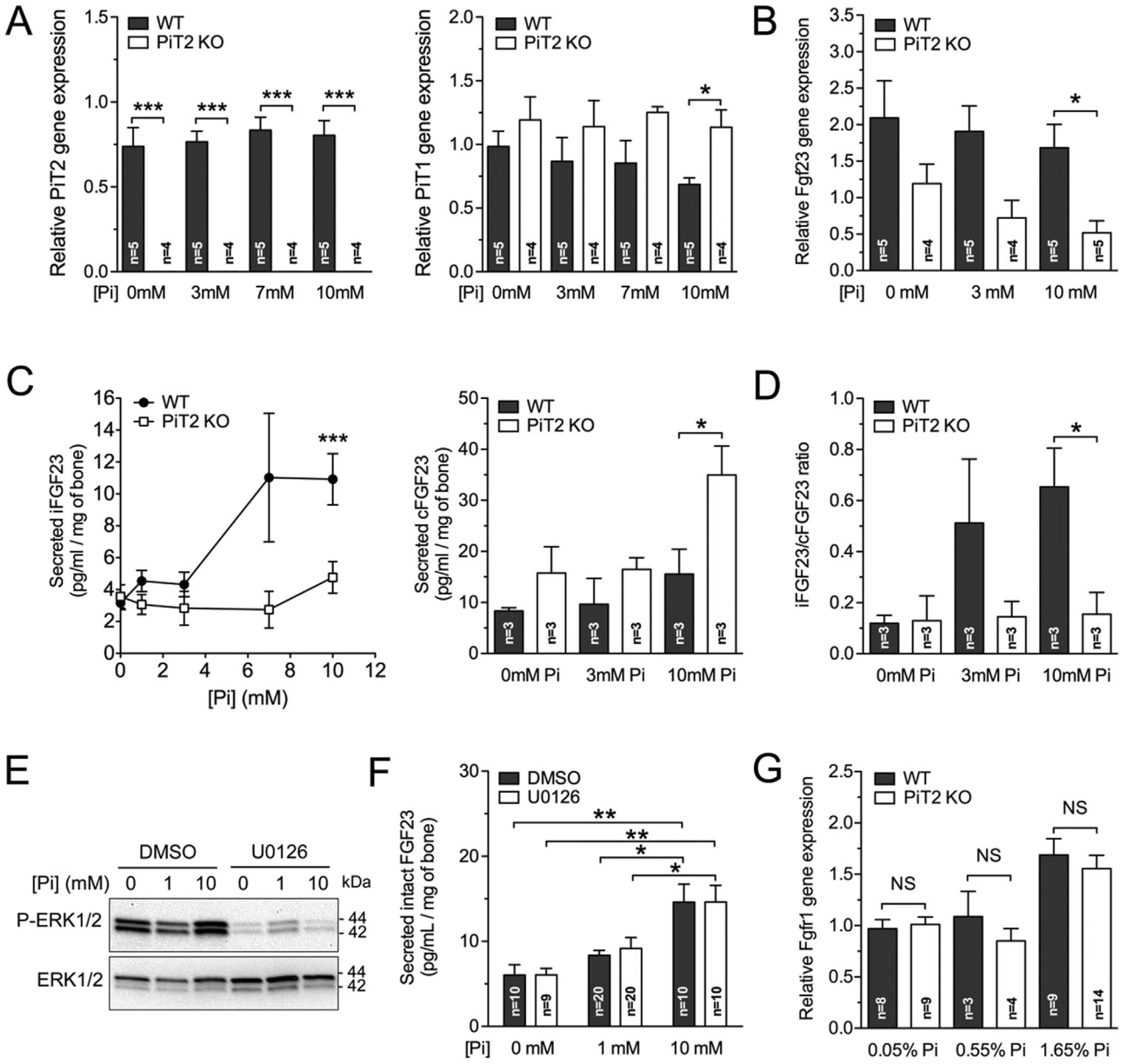

Figure 3: *Ex vivo* organ culture of long bone shafts from *PiT2* KO mice illustrates the bone-autonomous Pi-dependent role of PiT2 in regulating the FGF23 secretion. *(A)* RT-qPCR analysis of *PiT2* and *PiT1* mRNA levels in flushed tibias isolated from *WT* or *PiT2* KO mice and stimulated for 24 h in medium containing 0, 3, 7, or 10 mM Pi, as indicated. *Pnn* and *GusB* genes were used as internal controls. *(B)* RT-qPCR analysis of *Fgf23* mRNA levels in flushed tibias isolated as in *A*. *(C)* Secreted levels of intact (*Left*) and C-terminal (*Right*) Fgf23 from flushed tibias isolated from *WT* or *PiT2* KO and stimulated for 24 h in medium containing indicated Pi concentrations. *(D)* iFgf23/cFgf23 ratios from flushed tibias isolated from *WT* or *PiT2* KO and stimulated for 24 h in medium containing indicated Pi concentrations. *(E)* Western-blot analysis of ERK1/2 phosphorylation (P-ERK1/2) in flushed tibias from *WT* mice (C57BL/6 background) stimulated for 30 min with 0, 1 mM, or 10 mM extracellular Pi concentration, in the presence of DMSO or U0126, as indicated. Total ERK1/2 proteins were used as a loading control. *(F)* Secreted iFgf23 levels from flushed tibias from *WT* mice (C57BL/6 background) stimulated for 30 min with 0, 1 mM, or 10 mM extracellular Pi concentration, in the presence of DMSO or U0126, as indicated. *(G)* RT-qPCR analysis of *Fgfr1* mRNA levels in diaphysis from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%), and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. Data are means ± S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS: not significant.

the disease. Hence, several studies were designed to restrict dietary Pi in CKD patients to decrease circulating FGF23 levels. Although most studies showed a reduction in Pi excretion, the effects on FGF23 levels have been mixed, with some studies showing mild reduction, particularly when measuring intact FGF23 [33], and other studies revealing no change in FGF23 levels [34]. The role of PiT2 and possible other players in the setting of high FGF23 concentration following the early phase of CKD characterized by hyperphosphatemia, remains to be determined. This is particularly important in view of the detrimental effects of FGF23 in the later stages of the disease, which has fueled the search of strategies to lower serum FGF23 levels or block its unwanted actions [33]. In view of the emerging cross-talk between inflammation, iron deficiency, and bone mineral metabolism in controlling FGF23 secretion and/or expression [8–10,12,35], the possible involvement of PiT2 in these processes should now be considered.

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In summary, here we provide the first experimental evidence that PiT2 regulates synthesis and secretion of FGF23 in response to high Pi load *in vivo* and in bone organ cultures *ex vivo*. These findings may identify PiT2 as a target for novel therapies to improve the excessive FGF23 secretion in hyperphosphatemic disorders such as chronic kidney disease.

AUTHOR'S CONTRIBUTION

N.B., S.B.C., and L.B. designed experiments, N.B., G.F., S.B.C., and S.S. performed experiments, N.B., S.B.C., and L.B. analyzed data, N.B. and L.B. wrote the manuscript, N.B., J.G., S.B.C., and L.B. made adjustments to the final paper version. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.02.007.

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