Original Article

Short-term dietary phosphate restriction up-regulates ileal fibroblast growth factor 15 gene expression in mice

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Members of the fibroblast growth factor (FGF) 19 subfamily, including FGF23, FGF15/19, and FGF21, have a role as endocrine factors which influence the metabolism of inorganic phosphate (Pi) and vitamin D, bile acid, and energy. It has been reported that dietary Pi regulates circulating FGF23. In this study, the short-term effects of dietary Pi restriction on the expression of FGF19 subfamily members in mice were analyzed. An initial analysis confirmed plasma FGF23 levels positively correlated with the amount of dietary Pi. On the other hand, ileal *Fgf15* gene expression, but not hepatic *Fgf21* gene expression, was up-regulated by dietary Pi restriction. In addition, we observed the increase of plasma 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] levels by dietary Pi restriction, and the up-regulation of ileal *Fgf15* mRNA expression by 1,25(OH)$_2$D$_3$ and vitamin D receptor (VDR). Importantly, dietary Pi restriction-induced *Fgf15* gene expression was prevented in VDR-knockout mice. Furthermore, diurnal variations of plasma triglyceride concentrations and hepatic mRNA expression of the bile acid synthesis enzyme *Cyp7a1* as one of *Fgf15* negative target genes was influenced by dietary Pi restriction. These results suggest that dietary Pi restriction up-regulates ileal *Fgf15* gene expression through 1,25(OH)$_2$D$_3$ and VDR, and may affect hepatic bile acid homeostasis.

***Key Words*:** **fibroblast growth factor 15, gene regulation analysis, inorganic phosphate, 1,25-dihydroxyvitamin D, mice**

Inorganic phosphorus (Pi) plays a critical role in skeletal development, mineral metabolism, and diverse cellular functions involving intermediary metabolism and energy-transfer mechanisms. Serum Pi concentration is maintained through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption.[1,2] Pi transport in the kidney and intestine is mediated by several sodium-dependent phosphate cotransporters (NaPis).[2–4] Pi metabolism is regulated by many factors, such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$], fibroblast growth factor (FGF) 23, insulin, thyroid hormone, and other factors.[5] FGF23 was identified as a gene responsible for tumor-induced osteomalacia (TIO) and autosomal dominant hypophosphatemic rickets (ADHR).[6,7] It has been shown that FGF23 suppresses the expression of type 2a and 2c sodium-phosphate cotransporters (NaPi-2a and NaPi-2c) in the brush border membrane (BBM) of proximal tubules which mediates physiological phosphate reabsorption. In addition, FGF23 reduces serum 1,25(OH)$_2$D$_3$ concentration by suppressing the expression of 25-hydroxyvitamin D [25(OH)D]-1α-hydroxylase (CYP27b1) and also enhancing the expression of 25(OH)D-24-hydroxylase (CYP24a1).[8–11] Circulating FGF23 is regulated by dietary Pi and 1,25(OH)$_2$D$_3$.[9,12] In fact, circulating FGF23 is decreased in vitamin D receptor (VDR) knockout (KO) mice.[13–15] Dietary Pi deficiency stimulates renal 1,25(OH)$_2$D$_3$ synthesis and leads to an increase in Pi absorption in the small intestine. Intestinal absorption of Pi is mediated primarily via the type 2b sodium-phosphate cotransporter (NaPi-2b).[2] Segawa *et al.*[16] demonstrated an elevation of intestinal sodium-dependent Pi transport activity and BBM NaPi-2b protein content in mice fed a low-Pi diet.

Phylogenetic and sequence analyses have been used to group FGF15 (the mouse ortholog of human FGF19), FGF19, FGF21, and FGF23 from the other FGF family members, forming the FGF19 subfamily. FGF15 produced by distal intestine inhibits the expression of cholesterol 7α-hydroxylase Cyp7a1 in the liver where it functions as the key rate-limiting enzyme for the biosynthesis of bile acid through the Fgf receptor 4 (FgfR4)/β Klotho complex.[9,17] Additionally, *Fgf15* gene expression is positively regulated by bile acids which bind to the farnesoid X receptor (FXR), thus indicating that regulation of *Fgf15* gene expression is important in the maintenance of bile acid homeostasis.[9,18] Furthermore, *Fgf21* gene expression is regulated by free fatty acids (FFA) through peroxisome proliferator-activated receptor α (PPARα) and is associated with energy homeostasis.[9] Previously, we have revealed the effect of Pi intake on circulating FGF23, PTH, and vascular endothelial function in humans and animals.[19,20] In this study, we have focused on the effect of dietary Pi on the ileal *Fgf15* and hepatic *Fgf21* gene expression known as FGF19 subfamily members.

Materials and Methods

Animals. Eight week old C57BL/6J male mice (24–27 g) were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained on 12 h light-12 h dark cycles (lights on from 8:00 to 20:00) with free access to distilled water and food. Zeitgeber time (ZT), the standardized notation for the time during an entrained circadian cycle, was used in this study. ZT0 is coincides with the onset of light, while ZT12 coincides with the onset of darkness. An egg white-based AIN-93 experimental diet formula-

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Table 1. Composition of experimental diets

Ingredient (g)	Pi				
	0.02%	0.10%	0.20%	0.60%	1.20%
Egg-white	20.0	20.0	20.0	20.0	20.0
L-Cystein	0.3	0.3	0.3	0.3	0.3
Cornstarch	39.7	39.7	39.7	39.7	39.7
α-Cornstarch	13.2	13.2	13.2	13.2	13.2
Sugar	10.44	10.00	9.56	7.80	5.16
Soybean Oil	7.0	7.0	7.0	7.0	7.0
Cellulose	5.0	5.0	5.0	5.0	5.0
Vitamin mix	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Tert-butylhydroquinone	0.0014	0.0014	0.0014	0.0014	0.0014
$CaCO_3$	1.4894	1.4894	1.4894	1.4894	1.4894
KH_2PO_4	0	0.4394	0.8789	2.6366	5.2731
Mineral mix changed	1.5645	1.5645	1.5645	1.5645	1.5645

Table 2. Oligonucleotides used for real-time PCR

Gene name	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Gene Accession No.
Mouse *Fgf15*	CCAGAGAACAGCTCCAGGAC	TCCATGCTGTCACTCTCCAG	NM008003
Mouse *Fgf21*	CTACCAAGCATACCCCATCC	GCCTACCACTGTTCCATCCT	NM020013
Mouse *Cyp7a1*	GAGCCCTGAAGCAATGAAAG	GCTGTCCGATATTCAAGGA	NM007824
Mouse *β-actin*	AGCAGCATCCTCTCCACACGA	GGGCATGCTGTTGCTGATAC	NM007397
Mouse *Cyclophilin*	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT	NM011149

tion, without casein, was fed to the mice. From this base diet, five diets containing 0.6% calcium plus 0.02%, 0.1%, 0.2%, 0.6%, or 1.2% Pi were prepared (Table 1). Groups of mice received one of the five diets for 1 to 5-day. Mice received $1,25(OH)_2D_3$ (Solvay Pharmaceuticals, Marietta, GA) (0.5 μg/kg body weight) i.p. and were sacrificed 6-h after treatment and subsequently compared with saline-treated controls. The 7 to 8 week old male VDR KO mice used in the study were generated by heterozygous crosses; VDR genotypes were determined by analyzing the DNA obtained from each mouse.[21] Study mice were mainly sacrificed between ZT5 and ZT7. The mice were anesthetized using diethyl ether and killed by exsanguinations. Protocols were approved by the Guidelines for Animal Experimentation of the Tokushima University School of Medicine.

Plasma parameters. Plasma concentrations of Pi, total cholesterol (TC), and total triglycerides (TG) were determined using the Phospho C-test Wako, T-cholesterol E-test Wako and Triglyceride E-test Wako kits, respectively (Wako Pure Chemical Industries, Osaka, Japan). Concentrations of plasma intact FGF23 were measured using the FGF23 ELISA kit (Kinos, Tokyo, Japan). Plasma $1,25(OH)_2D$ was measured with a RIA kit (TFB, Tokyo, Japan).

Quantitative RT-PCR analysis. First-strand cDNA was synthesized from DNase I-treated total RNA templates that were primed with oligo-dT using the Moloney-murine-leukaemia virus-reverse transcriptase kit from Invitrogen (San Diego, CA). Quantitative RT-PCR was performed using the Light Cycler system (Roche Diagnostics, Mannheim, Germany) or StepOnePlus™ Real-Time PCR System with FastStart SYBR green master mix (Applied Biosystems, Foster City, CA). The primer sequences for PCR amplification are shown in Table 2. The PCR products were quantified by fit-point analysis, and results were normalized to β-*actin* or *cyclophilin*.

Western blot analysis. Fresh ileal mucosa was homogenized in Lysis buffer (10 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) with Protease Inhibitor Cocktail (SIGMA-ALDRICH, St. Louis, Missouri) and 1 mM DDT. This suspension was centrifuged for 20 min at $10,000 \times g$ and the cytoplasmic fraction protein was collected from the supernatant fraction. Protein samples were heated at 95°C for 5 min in sample buffer in the presence of 5% 2-mercaptoethanol and subjected to SDS-PAGE. The separated proteins were transferred by electrophoresis on to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA). The membranes were treated with diluted anti-FGF15 antibody (1:200) (Santa Cruz Biotechnology, CA). Mouse anti-β-actin monoclonal antibody (SIGMA-ALDRICH) was used as an internal control. HRP-labeled anti-IgG (BIO-RAD Hercules, CA) was utilized as a secondary antibody, and signals were detected using the ECL Prime system (GE Healthcare, Buckinghamshire, UK).

Statistical analysis. Data are expressed as means ± SEM. The Student's unpaired *t* test and 1-way ANOVA was performed. Differences between experimental group means were analyzed using either the Tukey-Kramer or Fisher's protected least significant difference (PLSD) post hoc tests. $p<0.05$ was considered significant.

Results

Effects of dietary Pi on the expressions of FGF19 subfamily. Firstly, to examine the effect of dietary Pi on *Fgf15* and *Fgf21* gene expression, mice were fed diets with different Pi contents. As reported previously,[12] plasma Pi is significantly decreased in mice fed a 0.02% Pi diet, and the concentration of plasma FGF23 was decreased following restriction of dietary Pi (Fig. 1A and B). Quantitative RT-PCR analysis showed that *Fgf15* mRNA expression in the ileum was significantly increased in the mice fed the 0.1% Pi diet compared with groups fed the 0.6% or 1.2% Pi diet (Fig. 1C). Conversely, hepatic *Fgf21* mRNA was decreased in the mice fed 0.6% Pi compared with those fed 1.2% Pi. However, the change in *Fgf21* mRNA expression was independent of the amount of dietary Pi (Fig. 1D). Secondly, the

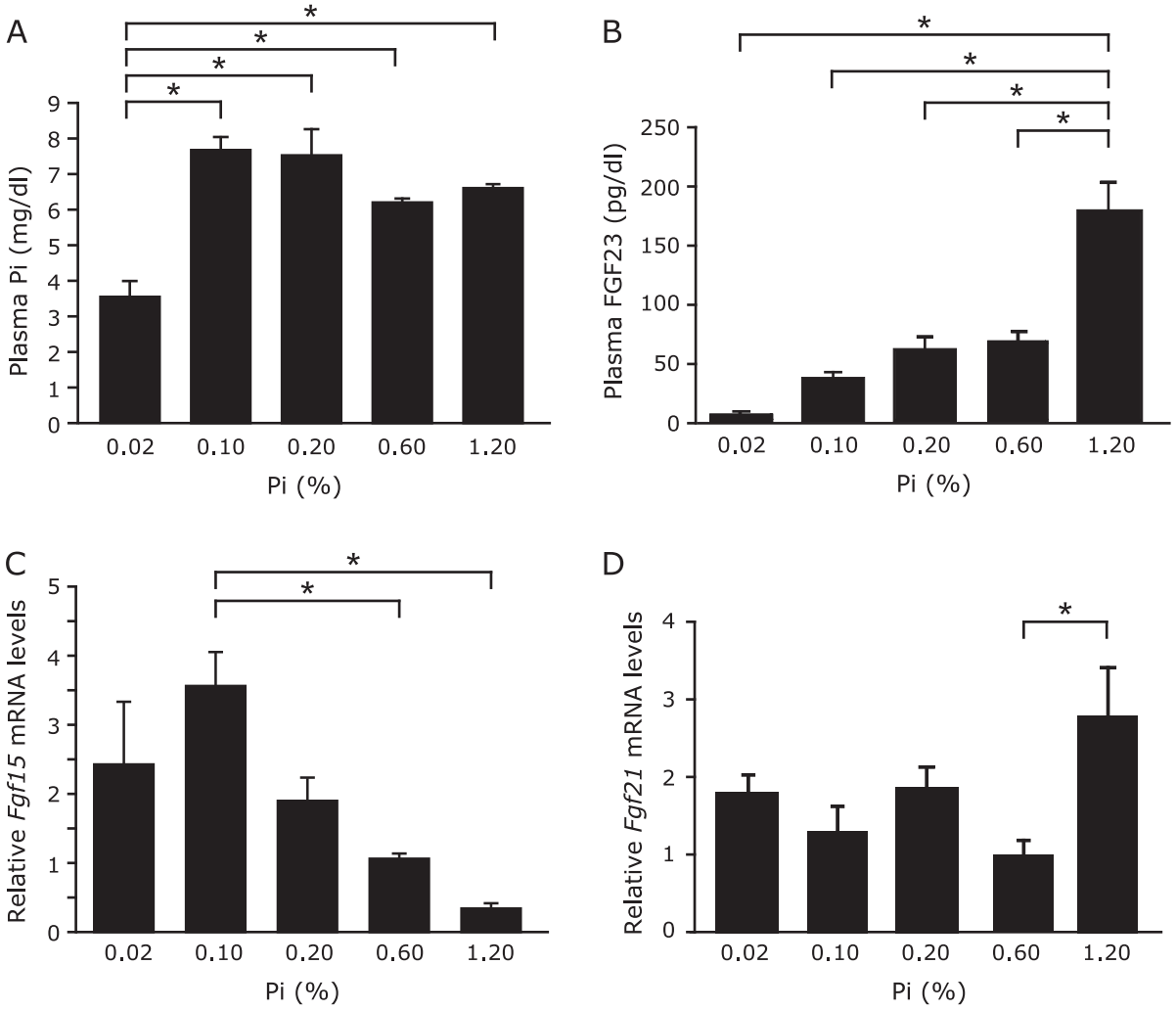

Fig. 1. Effects of dietary Pi on the expressions of FGF19 subfamily. Groups of 8-week-old C57BL/6 male mice were fed diets containing different amounts of Pi (0.02–1.2% Pi) for 5-day. (A) Plasma Pi concentrations. (B) Plasma FGF23 concentrations. (C) *Fgf15* mRNA expression in the ileum. (D) *Fgf21* mRNA expression in the liver. Total mRNA was prepared from the ileum and liver of each mouse, and gene expression was measured by quantitative RT-PCR. Results were normalized to β-*actin* mRNA expression. The data are represented as the mean ± SEM (n = 3–6). *p<0.05.

effect of the Pi-restricted (0.02% Pi) and Pi-sufficient diet (1.2% Pi) on *Fgf15* gene expression was studied over time. As a result, *Fgf15* mRNA expression increased with time in the Pi-restricted group (Fig. 2A). The Western blot analysis suggested that Fgf15 protein expression of the Pi-restricted group was higher than it was in the Pi-sufficient group (Fig. 2B).

The up-regulation of ileal *Fgf15* mRNA expression by 1,25(OH)$_2$D$_3$ and VDR. As previously reported,[22] dietary Pi restriction increased the concentration of 1,25(OH)$_2$D in blood (Fig. 3A), so we investigated the effect of 1,25(OH)$_2$D$_3$ on *Fgf15* gene expression. Figure 3B shows that ileal *Fgf15* mRNA expression in mice was increased nearly 2.5-fold by the administration of 1,25(OH)$_2$D$_3$. *Fgf15* gene expression in VDR KO mice was approximately 20% that of WT mice (Fig. 3C).

The effect of dietary Pi restriction on ileal *Fgf15* gene expression in VDR KO mice. To understand the role of VDR in the regulation of *Fgf15* gene expression by dietary Pi, WT and VDR KO mice were fed the Pi-restricted (0.02% Pi) or Pi-sufficient diet (1.2% Pi) for 5-day. As a result, dietary Pi flux significantly changed the plasma Pi concentrations in both WT and VDR KO mice. A previous report showed there plasma Pi concentrations were lower in VDR KO mice.[16] The plasma Pi concentrations of VDR KO mice were observed to be lower than those of WT mice fed the Pi-restricted diet; however, there was no difference between WT and VDR KO mice in the group fed the Pi-sufficient diet (Fig. 4A). In WT mice, *Fgf15* mRNA expression was increased 20-fold in the Pi-restricted group relative to Pi-sufficient group, whereas there was no significance difference between both groups of VDR KO mice, despite the 3-fold increase in the Pi-restricted vs Pi-sufficient group (Fig. 4B).

The effect of dietary Pi restriction on diurnal variations of plasma lipid parameters, ileal *Fgf15,* and hepatic *Cyp7a1* gene expression. It has been reported that the concentration of blood TG and gene expression of *Cyp7a1*, which is negatively regulated by *Fgf15*, exhibits diurnal variations.[23–25] Therefore, our aim was to elucidate the effect of dietary Pi on the diurnal variations of plasma lipid parameters and *Fgf15* and *Cyp7a1* gene expression. Plasma Pi concentrations were significantly decreased in the Pi-restricted group compared with the Pi-sufficient group at all times (data not shown). Fig. 5A showed that the plasma TG concentrations exhibited diurnal variation and significantly increased 1.7-fold at ZT5 and increased 2.7-fold at ZT13 in the Pi-restricted group relative to the Pi-sufficient group. In contrast, there was no effect of dietary Pi on plasma cholesterol throughout

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the day (Fig. 5B). *Fgf15* mRNA expression in the ileum decreased between ZT9 and ZT13, and *Fgf15* expression was higher in the Pi-restricted than in the Pi sufficient group throughout the day (Fig. 5C). Contrary to *Fgf15*, the expression pattern of the *Cyp7a1* gene was increased between ZT9 and ZT13. Interestingly, a Pi-restricted diet increased *Cyp7a1* mRNA levels at ZT9 and decreased them at ZT13 (Fig. 5D), showing that the effect of dietary Pi on *Cyp7a1* expression varied according to time of day. These results suggest that dietary Pi regulates hepatic *Cyp7a1* expression through *Fgf15*-dependent and -independent pathways, and may affect on the diurnal variations of plasma TG levels.

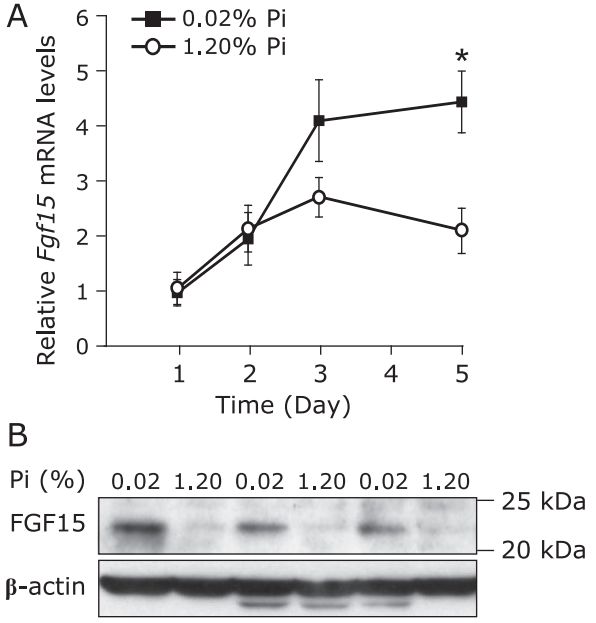

Fig. 2. The effects of a Pi-restricted diet on the ileal *Fgf15* mRNA and protein expression. Groups of 8-week-old C57BL/6 male mice were fed 0.02% Pi-restricted (0.02% Pi) or 1.2% Pi-sufficient (1.2% Pi) diets and then sacrificed at 1, 2, 3 and 5-day. (A) *Fgf15* mRNA expression in the ileum. Total mRNA was prepared from the ileum and liver of each mouse, and gene expression was quantified by quantitative RT-PCR. Results were normalized to β-*actin* mRNA expression. The data represent the mean ± SEM (n = 4–6). *p<0.05 vs 1.2% Pi-sufficient group. (B) FGF15 protein expression in the ileum. 0.02% Pi-restricted (0.02% Pi) or 1.2% Pi-sufficient (1.2% Pi) diets were fed to mice for 5-day. Cytoplasmic extracts were prepared from the lower ileum of each mouse. Protein (50 μg) was loaded per lane for Western blot analyses and probed with FGF15 antibody. β-actin was used as an internal control.

Discussion

This study examined the effect of dietary Pi on FGF19 subfamily gene expression in mice. While Pi restriction decreased plasma FGF23 concentrations, it increased *Fgf15* mRNA expression in the ileum. Hepatic *Fgf21* mRNA concentrations were increased in mice fed a diet containing 1.2% Pi relative to those fed the 0.6% Pi diet (Fig. 1). Elevated concentrations of FGF21 have been observed, not only during fasting, but also in obese individuals, people with type 2 diabetes, and in peritoneal dialysis patients. In addition, FGF21 was expressed not only in the liver but also in white adipose tissue (WAT) and the pancreas, suggesting that FGF21 expression might be associated with the state of nutritional metabolism.[26–30] To assess the effect of dietary Pi on *Fgf21* gene expression requires an examination of the blood concentration and the expression in WAT and the pancreas. We considered the possibility that dietary Pi restriction induced *Fgf15* mRNA expression, and Fig. 2B shows that FGF15 protein concentrations are also increased by a Pi-restricted diet. It has been reported that the mechanism of *Fgf23* gene expression includes not only transcriptional regulation, but also post-translational regulation via glycosylation and protease degradation, for example.[31] However, the glycosylation and degradation of FGF15/19 and FGF21 are not yet fully elucidated. It is proposed that *Fgf15* gene expression is positively regulated by dietary Pi restriction.

Previous reports show that a dietary Pi restriction is associated with decreased circulating FGF23 and elevated $1,25(OH)_2D_3$ concentrations. Through the VDR, $1,25(OH)_2D_3$ induced *Fgf23* mRNA expression in bone and increased FGF23 concentrations in the blood,[9] so we investigated whether $1,25(OH)_2D_3$ and VDR regulate *Fgf15* gene expression. Fig. 3B shows that *Fgf15* mRNA expression in the ileum is increased by the administration of

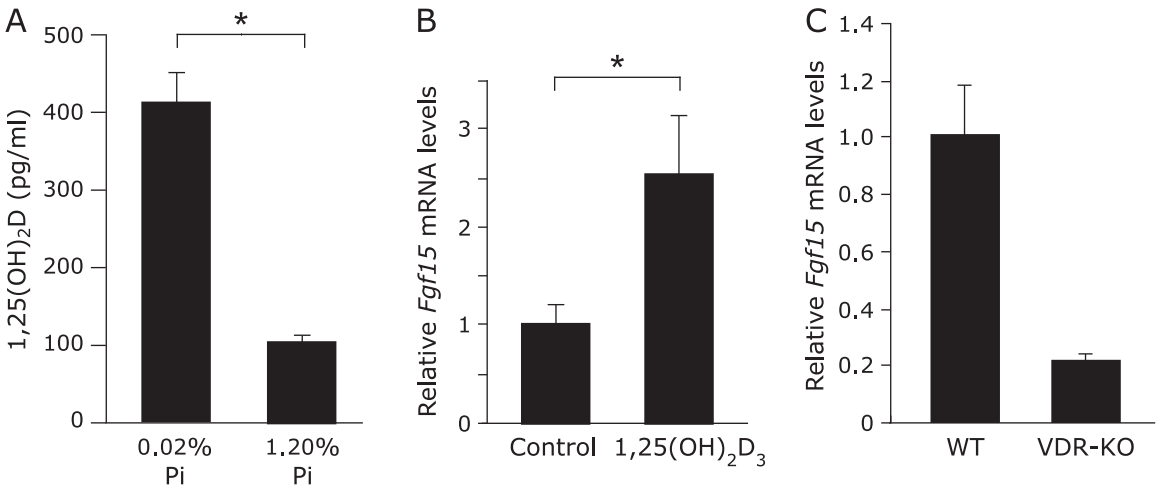

Fig. 3. The up-regulation of ileal *Fgf15* mRNA expression by $1,25(OH)_2D_3$ and VDR. (A) Plasma $1,25(OH)_2D$ concentration. Groups of 8-week-old C57BL/6J male mice were fed 0.02% Pi-restricted (0.02% Pi) or 1.2% Pi-sufficient (1.2% Pi) diets and sacrificed at 5-day (n = 5). (B) *Fgf15* expression in the ileum 6-h after treatment with $1,25(OH)_2D_3$. Groups of 8-week-old male C57BL/6J mice received saline i.p. (control) or $1,25(OH)_2D_3$ i.p. (0.5 μg/kg body weight) (n = 4–6). (C) Expression of *Fgf15* in the ileum of WT and VDR KO male mice (n = 2). Total mRNA was prepared from the ileum of each mouse, and gene expression was measured by quantitative RT-PCR using β-*actin* as the internal control. The data represent the mean ± SEM. * p<0.05 vs control.

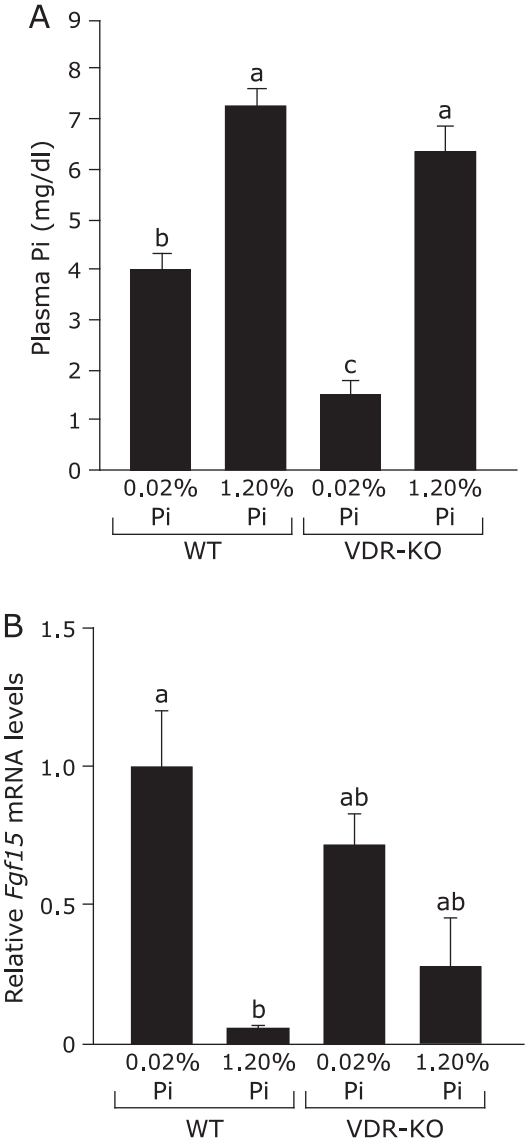

Fig. 4. The effect of a Pi-restricted diet on the ileal *Fgf15* mRNA expression in VDR KO mice. (A) Plasma Pi concentrations. (B) The effect of a Pi-restricted diet on *Fgf15* mRNA expression in VDR KO mice. Groups of 7 to 8-week-old WT or VDR KO male mice were fed 0.02% Pi-restricted (0.02% Pi) or 1.2% Pi-sufficient (1.2% Pi) diets for 5-day. Total mRNA was prepared from the ileum and liver of each mouse, and gene expression was measured by quantitative RT-PCR using *β-actin* as the internal control. The data represent the mean ± SEM (n = 4–9). Means without a common letter are significantly different. p<0.05.

$1,25(OH)_2D_3$. In addition, *Fgf15* mRNA expression is decreased in VDR KO mice compared with WT mice. It is well known that $1,25(OH)_2D_3$/VDR is regulated by transcriptional gene expression through the vitamin D response element (VDRE) in the promoter of target genes.[32] Indeed, we observed that $1,25(OH)_2D_3$ stimulates mouse *Fgf15* gene promoter activity in several cells overexpressing VDR and RXR (data not shown). More importantly, the results of this study using VDR KO mice revealed that a Pi-restricted diet regulates *Fgf15* gene expression through the VDR (Fig. 4B). In the past, it has been reported that some genes associated with bile acid metabolism are regulated by $1,25(OH)_2D_3$ and VDR *in vitro* and *in vivo*.[33,34] VDR has dual functions, as an endocrine receptor for $1,25(OH)_2D_3$ and as a metabolic sensor for secondary bile acids such as lithocholic acid. It is hypothesized that $1,25(OH)_2D_3$ suppresses bile acid synthesis thereby averting competition with bile acid.[35,36] Both vitamin D and bile acid are the metabolic products of cholesterol, and interestingly, CYP27a1 that identified as vitamin D 25-hydroxyrase has a role as a bile acid synthesis enzyme.[37,38] There is a strong connection between vitamin D and bile acid metabolism, and it is suggested that dietary Pi regulates the vitamin D and bile acid metabolism through the FGF23 and FGF15.

FGF15 was shown to bind and activate the FgfR4/β-klotho complex, leading to the down regulation of *Cyp7a1* expression, and inhibiting synthesis of bile acid from cholesterol.[9] Bile acids facilitate intestinal absorption and transport of lipids,[39] and it has been reported that FgfR4 KO and FXR KO mice exhibit elevated *Cyp7a1* gene expression in their liver and high concentrations of blood TG and cholesterol.[40,41] A recent study has reported that hepatic *Cyp7a1* gene expression and blood lipid parameters exhibit diurnal variations. These diurnal variations are caused by the regulation of some clock genes.[23–25] As shown in Fig. 5, we observed that plasma TG concentration show diurnal variation, and this variation is affected by dietary Pi. Although dietary Pi restriction increased *Fgf15* gene expression throughout the day, it also increased *Cyp7a1* expression at ZT9 and decreased it at ZT13. This result suggested that *Cyp7a1* is regulated by dietary Pi through an *Fgf15*-dependent and -independent pathway. Our recent study demonstrated that 12 days of restriction of dietary Pi increased high cholesterol diet-induced hepatic lipid accumulation and decreased hepatic *Cyp7a1* mRNA expression.[42] Therefore, we suggest that elevation of *Fgf15* expression induced by dietary Pi restriction may inhibit hepatic *Cyp7a1* gene expression and accelerate the development of high cholesterol diet-induced fatty liver.

In the chronic kidney disease (CKD) patient, hyperphosphatemia and dyslipidemia might be risk factors for the development of CKD and the pathogenesis of cardiovascular disease. A Pi-restricted diet may be a useful treatment for a CKD patient to ameliorate their hyperphosphatemia and to reduce their risk of CKD.[43–46] This study strongly suggests that dietary Pi is involved in lipid metabolism, and proper control of dietary Pi in CKD might contribute to amelioration of lipid metabolism abnormalities. Future investigations of the effect of a Pi-restricted diet on bile acid and lipid metabolism in the CKD patient are warranted.

In summary, we revealed that dietary Pi restriction increased ileal *Fgf15* gene expression through $1,25(OH)_2D_3$ and VDR in mice. Furthermore, it was shown that dietary Pi affects diurnal variations in plasma TG concentrations and hepatic *Cyp7a1* gene expression.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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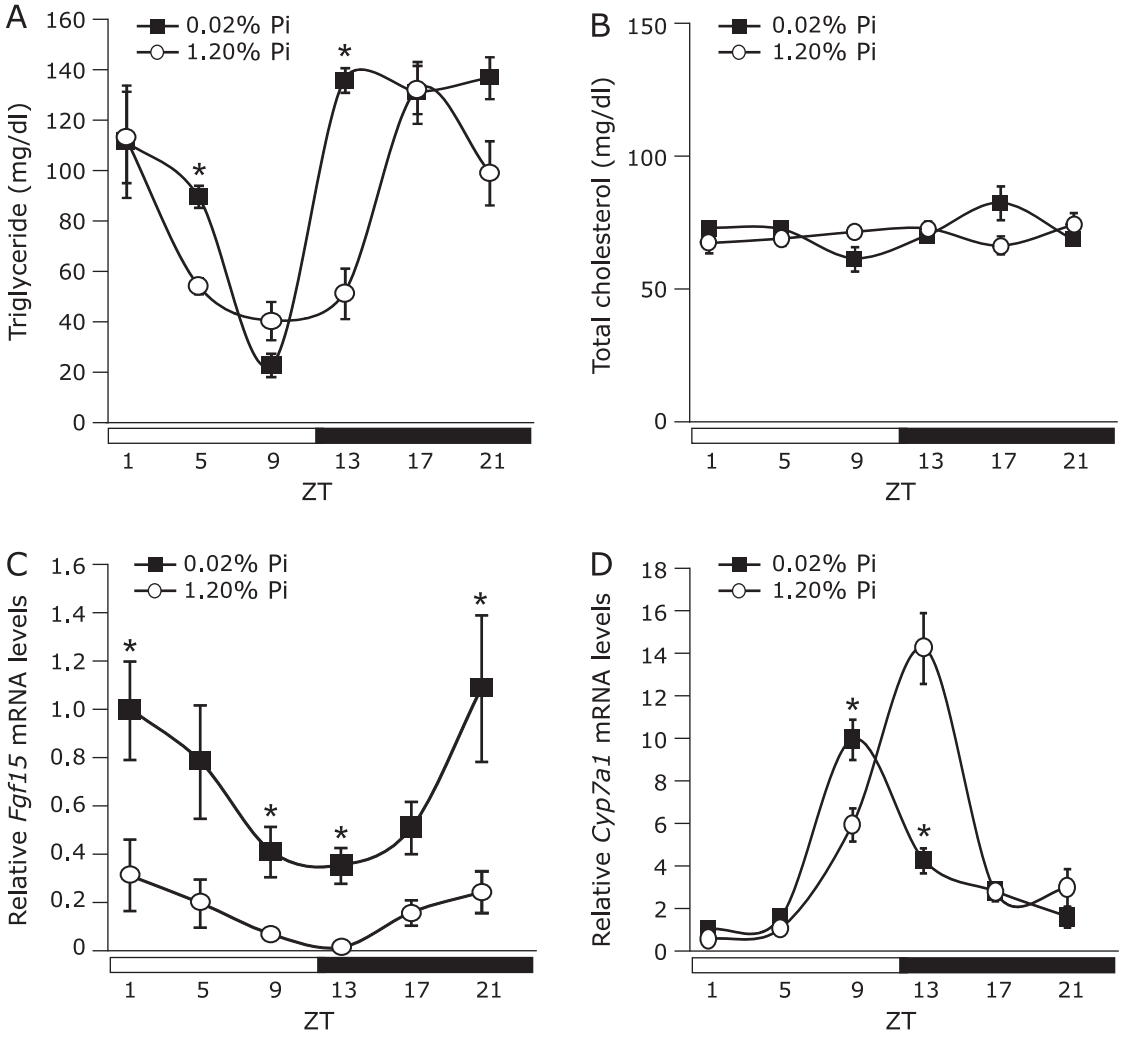

Fig. 5. The effects of a Pi-restricted diet on diurnal variations of plasma lipid concentrations and ileal *Fgf15* and hepatic *Cyp7a1* mRNA expression. Groups of 8-week-old C57BL/6J male mice were fed 0.02% Pi-restricted (0.02% Pi) or 1.2% Pi-sufficient (1.2% Pi) diets for 5-day and sacrificed at ZT1, 5, 9, 13, 17 and 21. (A) Plasma triglyceride concentrations. (B) Plasma cholesterol concentrations. (C) *Fgf15* mRNA expression in the ileum. (D) *Cyp7a1* mRNA expression in liver. Total mRNA was prepared from the ileum and liver of each mouse, and gene expression was measured by quantitative RT-PCR using *β-actin* or *cyclophilin* as the internal control. The data represent the mean ± SEM (n = 4–5). *p<0.05 vs 1.2% Pi-sufficient group.

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