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Maternal excess dietary phosphate intake in the periconceptional period is a potential risk for mineral disorders in offspring mice

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Growing consumption of processed foods may cause a greater risk of excessive dietary phosphate intake. The increased dietary phosphate intake as a food additive in the periconceptional period may affect the children's future health. Here, we investigated the effects of maternal excess dietary phosphate intake on offspring in C57BL/6J mice. Female mice were fed a control diet (CP, 0.8% phosphate) or a high-phosphate diet (HP, 1.5% phosphate) for either 21 days during pre-pregnancy or almost 20 days during pregnancy. After weaning, offspring were raised on the CP diet. Relative to the CP groups, offspring from dams fed HP during pre-pregnancy or pregnancy showed decreased urinary phosphate excretion without significant changes in either plasma phosphate level or renal sodium-dependent phosphate transporter mRNA expression at 3 or 10 weeks. However, mRNA expression of intestinal sodium-dependent phosphate transporter was decreased, suggesting that the reduced urinary phosphate excretion was due to decreased absorption of intestinal phosphate. Interestingly, offspring in the HP groups also demonstrated significant differences in plasma levels of parathyroid hormone, fibroblast growth factor 23, and vitamin D. To our knowledge, this is the first report to show that maternal excess intake of dietary phosphate in the periconceptional period disturbs phosphate metabolism in offspring.

Keywords DOHaD hypothesis, Maternal nutrition, Intestinal phosphate absorption, Vitamin D

Phosphate (Pi) is essential for maintaining various biological functions, growth, skeletal development, and energy metabolism. Its homeostasis is regulated by intestinal absorption, reabsorption, excretion via the kidney, and mobilization from the bone. These processes are regulated by various phosphate (Pi)-regulating factors such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], and[1]fibroblast growth factor 23 (FGF23), a potent phosphaturic hormone that is secreted from the bone in response to elevated serum Pi or increased dietary Pi intake.

In recent decades, excess dietary Pi intake has emerged as a public health concern because of the growing consumption of processed foods/ultra-processed foods containing phosphate compounds as food additives[1]. Some epidemiological studies have reported that high dietary Pi intake and/or serum Pi levels can be positively associated with the risk of cardiovascular disease (CVD) and mortality in both general population and chronic kidney disease (CKD) patients, as well as bone and mineral disorders[2,3]. Although the underlying mechanisms between excess dietary Pi intake and CVD or mortality have not been clarified, increased serum PTH and/or FGF23 levels must play a key role. Both primary and secondary hyperparathyroidism cause cardiovascular disease via endothelial dysfunction, left ventricular hypertrophy, hypertension, ectopic calcification, and arrhythmia[4]. High serum FGF23 levels also cause left ventricular hypertrophy, cardiac fibrosis, activating the renin-angiotensin-aldosterone system, and anemia[5].

Another issue is that high Pi intake decreases the expression of α-klotho, which is identified initially as an aging-suppressor gene and works as a co-receptor for FGF23 to suppress Pi reabsorption and 25-hydroxyvitamin D-1 α-hydroxylase (CYP27B1) in the kidney[9–13]. Secreted FGF23 binds to the FGF receptor and its co-receptor α-klotho in the kidney and suppresses the expression of CYP27B1 and type II sodium-dependent

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Pi cotransporters (NaPi2a and NaPi2c)[6–10]. Both α-klotho mutant (kl/kl) mice and FGF23 knock-out mice exhibit premature aging-like phenotypes such as shorter life-span, arteriosclerosis and ectopic calcification with hyperphosphatemia and higher serum 1,25-$(OH)_2$D levels[11]. Notably, α-klotho expression is suppressed by high dietary Pi intake, inflammation, oxidative stress, and chronic kidney disease (CKD)[12–15]. In other words, high dietary Pi intake may factor in pathological processes leading to aging-related diseases.

At least one-half of the U.S. population in all age groups, including periconceptional period except adolescents, consumes phosphorus more than their Estimated Average Requirement, which is 400 mg/d for all age groups except rapidly growing adolescents[16]. A few studies investigated the effect of a high Pi diet in mice's periconceptional period on maternal and fetal Pi homeostasis[17–19]. However, these studies focused on the role of FGF23 and PTH on the abnormal Pi homeostasis in the fetus. They concluded that different mechanisms from FGF23 may regulate serum Pi levels in the fetus.

Thus, the increased intake of dietary Pi in the periconceptional period may affect the children's future health. We hypothesized that excessive Pi intake in pregnancy or pre-pregnancy may cause abnormal Pi metabolism in offspring and may predispose adult offspring to CKD. In this study, we examined the effects of maternal dietary Pi intake during the pre-pregnancy and pregnancy periods on development and Pi metabolism in offspring.

Materials and methods

Animals and experimental diet

C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). All animals were kept on a 12-h:12-h light-dark cycle with unlimited access to distilled water. The control Pi diet (CP) was MF with 0.8% Pi (Oriental Yeast Co., ltd., Tokyo, Japan); the high Pi diet (HP) was MF modified by adding K_2HPO_4 as Pi (final Pi concentration was 1.5%) (Oriental Yeast Co., ltd., Tokyo, Japan). This study was approved by the Animal Experimentation Committee of Tokushima University (T2022-28). We conducted in accordance with Fundamental Guidelines for Proper Conduct of Animal Experiment under the jurisdiction of the Japanese government for the management and handling of experimental animals. We also confirmed this experiment conducted in accordance with the ARRIVE guidelines.

Study design

Study 1: effects of dietary Pi intake during pre-pregnancy on offspring

Eight-week-old female mice were randomly divided into two groups, which were fed either CP or HP (Figure S1), and statistical analysis revealed no significant difference in body weight between the two groups. After 21 days, each mouse was housed with a male (8–19 weeks of age) for 3 days to achieve pregnancy and both groups were fed CP. All offspring were given breast milk until 3 weeks of age. After weaning, they were maintained on CP and distilled water *ad libitum*. At the end of the experimental period, mice were euthanized under anesthesia by intraperitoneal administration of pentobarbital, and blood, urine, breast milk, kidney, duodenum, and femur bone samples were collected for analysis. After blood collection, the sample was centrifuged (5400 rpm, 15 min), and the supernatant was collected as plasma for analysis. All samples were stored at -80 °C until analysis. To control for the nutritional environment until weaning, the number of littermates was normalized with 7 or 8 offspring from each of dam (we prepared 5 dams for the CP group and 5 for the HP group), and at 10 weeks of age, only samples from male offspring mice were analyzed to eliminate the effects of sex hormones.

Study 2: effects of dietary Pi intake during pregnancy on offspring

Eight-week-old female mice were randomly divided into two groups and statistical analysis revealed no significant difference in body weight between two groups. Each mouse was housed with a male mouse (8–19 weeks of age) for 3 days to achieve pregnancy. They were then fed either CP or HP throughout pregnancy (almost 20 days) (Figure S1). After delivery, both groups were given CP. All offspring were given breast milk until 3 weeks of age. After weaning, they were maintained on CP and distilled water *ad libitum*. Mice were euthanized and samples collected as described in Study 1. To control for the nutritional environment until weaning, the number of littermates was normalized with 6–9 offspring from each of dam (we prepared 7 dams for CP group and 9 for HP group), and at 10 weeks of age, only samples from male offspring mice were analyzed to eliminate the effects of sex hormones.

Biochemical analysis of plasma and urine

Plasma and urine concentrations of Pi, Ca, and creatinine were determined using Phosphide-C, Calcium-E, and LabAssay™ Creatinine tests (all Wako, Osaka, Japan). Concentrations of plasma intact-FGF23 and intact PTH were determined using FGF23 (Kainos, Tokyo, Japan) and Mouse PTH 1–84 (Immutopics, San Clemente, CA) ELISA kits. Plasma 1,25$(OH)_2$D was measured using a radioimmunoassay kit (TFB, Tokyo, Japan) by SRL Co., Ltd. (Tachikawa, Japan).

Determination of Ca and Pi in breast milk

Four hours before collecting breast milk, dams and offspring were separated. The dams were anesthetized and given 0.06 IU/g BW of oxytocin (Sigma-Aldrich Co. LLC, St. Louis, USA) subcutaneously. Breast milk was obtained by using a single-hand milking machine for mice and rats (Natsume Seisakusho Co., Ltd., Tokyo, Japan). The breast milk was heated at 350 °C for 3 h, 450 °C for 3 h, and 550 °C for 24 h; the ash was then dissolved in 10 mL of 1% HCl for analysis. Phosphate was determined by the vanado-molybdate method and calcium by the succinylation method.

Real-time PCR analysis

Total RNA was isolated from homogenized kidneys and duodenum using RNAiso Plus (Takara Bio Inc., Shiga, Japan), and from femur bone using ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 2.5 μg of total RNA and primed with oligo (dT) using M-MLV Reverse Transcriptase (Promega Corporation, WI, USA). Real-time quantitative polymerase chain reaction (RT-PCR) analysis was performed by using a StepOne Plus™ Real-time PCR System (Applied Biosystems, Foster, CA, USA). The prepared first-strand cDNA was amplified by PCR using Fast SYBR®Green Master Mix (Applied Biosystems) in a 10 μl reaction volume, containing 10 pmol of each primer (Table 1). The amplification program was 95 °C for 20 s, followed by 40–50 cycles of 95 °C for 3 s, 60 °C for 30 s, and 60 °C for 1 min. The PCR products were quantified by fit-point analysis and mRNA expression was normalized to β-actin as an internal control. The value of the CP group was considered to be 1.00.

Statistical analysis

Results are expressed as the mean ± standard error of mean (SEM). Differences between groups were assessed for statistical significance by Student's t-test, Welch's t-test, or Mann-Whitney's U test. Student's t-test was applied if a Kolmogorov-Smirnov test indicated that data were distributed normally and an F test indicated homogeneity of variance. Welch's t-test was used if the data were normally distributed with non-homogeneous variance. Mann-Whitney's U test was used to compare data with a non-normal distribution. A *P* value of less than 0.05 was considered to be significant.

Results

Study 1: effects of maternal dietary Pi intake during pre-pregnancy

Biochemical parameters in maternal plasma, urine, and breast milk

First, we measured Pi, Ca, and FGF23 concentrations in plasma, urine at 11 weeks of age, and breast milk from 17 weeks of age female mice fed the CP or HP for 21 days during pre-pregnancy. As summarized in Table 2, plasma FGF23 was significantly higher in the HP group than in the CP group. Although plasma Pi and Ca concentrations did not differ significantly between the two diet groups, plasma Pi concentration in tended to be higher in the HP group than in the CP group. Urinary Pi excretion was significantly higher, and urinary Ca excretion was significantly lower in the HP group. Concentrations of Pi and Ca in breast milk did not differ significantly between the two groups.

Maternal renal mRNA expression of genes related to Pi and vitamin D metabolism

To examine the effect of dietary Pi intake during pre-pregnancy on the 11 weeks of age maternal kidney, we evaluated the expression of genes related to Pi and vitamin D metabolism by measuring renal mRNA expression using RT-PCR. As shown in Fig. 1A, renal α-klotho mRNA expression was significantly lower in the HP group than in the CP group. mRNA expression of the sodium-dependent Pi transporters NaPi2a and NaPi2c tended to be lower in the HP group. Regarding vitamin D metabolism, renal CYP27B1 mRNA expression tended to decrease, while CYP24A1 mRNA expression tended to increase in the HP group.

Gene name	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)	Accession number
Mouse α-klotho	CAAAAGCTGATAGAGGACAATGGC	GGCAGAGAAATCAACACAGTAAGG	NM_013823
Mouse β-actin	CTGACCCTGAAGTACCCCATTGAACA	CTGGGGTGTTGAAGGTCTCAAACATG	NM_007393
Mouse NaPi2a	AGAGCCCTTCACAAGACTCATCAT	TACCCTGGACATAGAAGTGGAAGC	NM_011392
Mouse NaPi2c	TGAAGAACGCTGACCAACTGA	AGCAGAGCTGAGGATGTCCAG	NM_080854
Mouse CYP27B1	ATGGTGAAGAATGGCAGAGG	TAGTCGTCGCACAAGGTCAC	NM_010009
Mouse CYP24A1	TGCCATTCACAACTCGGACCCT	TCAAGCCAGCGTTCGGGTCTAA	NM_009996
Mouse VDR	CACCTGGCTGATCTTGTCAGTTAC	GACTTAAGCAGGACAATCTGGTCA	NM_009504
Mouse FGF23	ATGCTAGGGACCTGCCTTAGA	AGCCAAGGAATGGGGAAGTG	NM_022657
Mouse PHEX	TATGTGTCCCCTGATGACAAGG	GCAGTGTCCACCATGAATTTGT	NM_011077
Mouse DMP-1	TCGCATCCCAATATGAAGACTG	CCTCTGGGCTAGCTTGACTTC	NM_016779
Mouse GALNT3	GTTGCTAGGGGCAGCTGTAG	TCACCACGGCAGTGTAGTTC	NM_015736
Mouse Fam20c	AGCAGACGAGAGAGCAGGAG	CGGATCTCCTTGGTCATGTT	NM_001359593
Mouse Runx2	TGCACCTACCAGCCTCACCATAC	GACAGCGACTTCATTCGACTTCC	NM_006523548
Mouse DNMT1	CCTAGTTCCGTGGCTACGAGGAGAA	TCTCTCTCCTCTGCAGCCGACTCA	NM_011242393
Mouse DNMT3a	GCCGAATTGTGTCTTGGTGGATGACA	CCTGGTGGAATGCACTGCAGAAGGA	NM_006514956
Mouse DNMT3b	CCAAAAGGAGGCCCATTAGAG	GTACCCCGTTGCAATTCCAT	NM_006498689
Mouse Tet1	TGGAGACTAGGTTTGGCCAGAA	CCCCGTGAACACTATCTTCTCAAT	NM_001253857
Mouse Tet2	TCTCAGGAGTCACTGCATGTTTG	GCTCCGACTTCTCGATTGTCTT	NM_001346736
Mouse Tet3	ACCTGCGATTGTGTCG	GTGAGTGTAATATGGGCCTTCATCT	NM_001347313

Table 1. Sequences of primers used for PCR amplification.

	CP	HP
Pre pregnancy		
Pi (mg/dl)	5.76 ± 0.50	7.09 ± 0.28
Ca (mg/dl)	8.30 ± 0.31	7.95 ± 0.20
FGF23 (pg/ml)	78.83 ± 5.89	270.98 ± 39.06*
Urine		
Pi (mg/mgCre)	0.02 ± 0.01	9.44 ± 0.78*
Ca (mg/mgCre)	1.42 ± 0.17	0.31 ± 0.06*
Breast milk		
Pi (mg/g)	2.89 ± 0.42	2.59 ± 0.11
Ca (mg/g)	4.20 ± 0.46	3.53 ± 0.32
Pregnancy		
Plasma		
Pi (mg/dl)	7.86 ± 0.64	8.87 ± 0.68
Ca (mg/dl)	5.99 ± 0.39	6.67 ± 0.32
Urine		
Pi (mg/mgCre)	0.03 ± 0.02	0.06 ± 0.04
Ca (mg/mgCre)	0.28 ± 0.11	0.21 ± 0.06
Breast milk		
Pi (mg/g)	3.03 ± 0.35	2.93 ± 0.21
Ca (mg/g)	5.60 ± 1.12	6.00 ± 0.64

Table 2. Effects of dietary Pi intake during pre-pregnancy or pregnancy on biochemical parameters in maternal plasma, urine, and breast milk. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for either 21 days during pre-pregnancy or almost 20 days during pregnancy. Plasma Pi, Ca, and FGF23 ($n = 5$/per diet group), urine Pi and Ca ($n = 5$/per diet group) at 11 weeks of age, and breast milk Pi and Ca (CP: $n = 7$, HP: $n = 3$) from 17 weeks of age in Study 1, or plasma Pi, and Ca (CP: $n = 6$, HP: $n = 9$), urine Pi and Ca (CP: $n = 6$, HP: $n = 9$), and breast milk Pi and Ca (CP: $n = 8$, HP: $n = 9$) from 14 weeks of age in Study 2 were analyzed. Data are given as mean ± SEM. *$p < 0.05$ vs. CP.

Food intake and body weight of offspring

To examine the effects of pre-pregnancy intake of Pi on offspring, we first monitored food intake from 3 to 10 weeks of age and body weight from 1 to 10 weeks of age in offspring. Average daily food intake did not differ significantly between the two groups (Fig. 1B). However, body weight was significantly lower in the HP group than in the CP group at 6, 9, and 10 weeks (Fig. 1C).

Biochemical parameters in offspring plasma and urine

Among various biochemical parameters, plasma Pi and Ca concentrations were not significantly different in the two diet groups. Plasma FGF23 concentration was significantly elevated in the HP group offspring at 3 weeks of age, and plasma PTH, and $1,25(OH)_2D$ concentration were significantly elevated in the HP group offspring at 3 or 10 weeks of age (Table 3). Urinary Pi and Ca excretion did not differ significantly between the two groups at 3 weeks of age. However, urinary Pi excretion was significantly lower in the HP group at 10 weeks of age.

Renal mRNA expression of genes related to Pi and vitamin D metabolism in offspring

We also examined the effects of maternal dietary Pi intake during pre-pregnancy on the kidney of offspring by measuring the renal expression of genes related to Pi and vitamin D metabolism. No differences between HP and CP offspring were observed in renal α-klotho, NaPi2a, and NaPi2c mRNA expression. At 3 weeks, renal CYP27B1 and CYP24A1 mRNA expression were significantly higher in the HP group than in the CP group. On the other hand, renal CYP27B1 mRNA expression was the same in the two groups at 10 weeks, while CYP24A1 mRNA expression was significantly lower in the HP group at 10 weeks. Renal vitamin D receptor (VDR) mRNA expression did not differ between the two groups at either age (Fig. 2A, B).

Renal mRNA expression of genes related to DNA methylation in offspring

DNA methylation, in which a methyl group is attached to the cytosine base in CpG islands, suppresses gene expression[20]. Therefore, we investigated whether dietary Pi intake during pre-pregnancy affects renal DNA methylation-related genes. As shown in Fig. 2C, D, however, there were no differences in mRNA expression of DNA methyltransferases (DNMTs)[21] or Ten-eleven translocation (Tet) demethyltransferases[22] between the two diet groups at 3 or 10 weeks of age.

Duodenal mRNA expression of genes related to Pi and vitamin D metabolism in offspring

We also examined the effects of dietary Pi intake during pre-pregnancy on the expression of phosphate transporters in the duodenum of offspring, including the type II sodium-dependent Pi cotransporter NaPi2b and

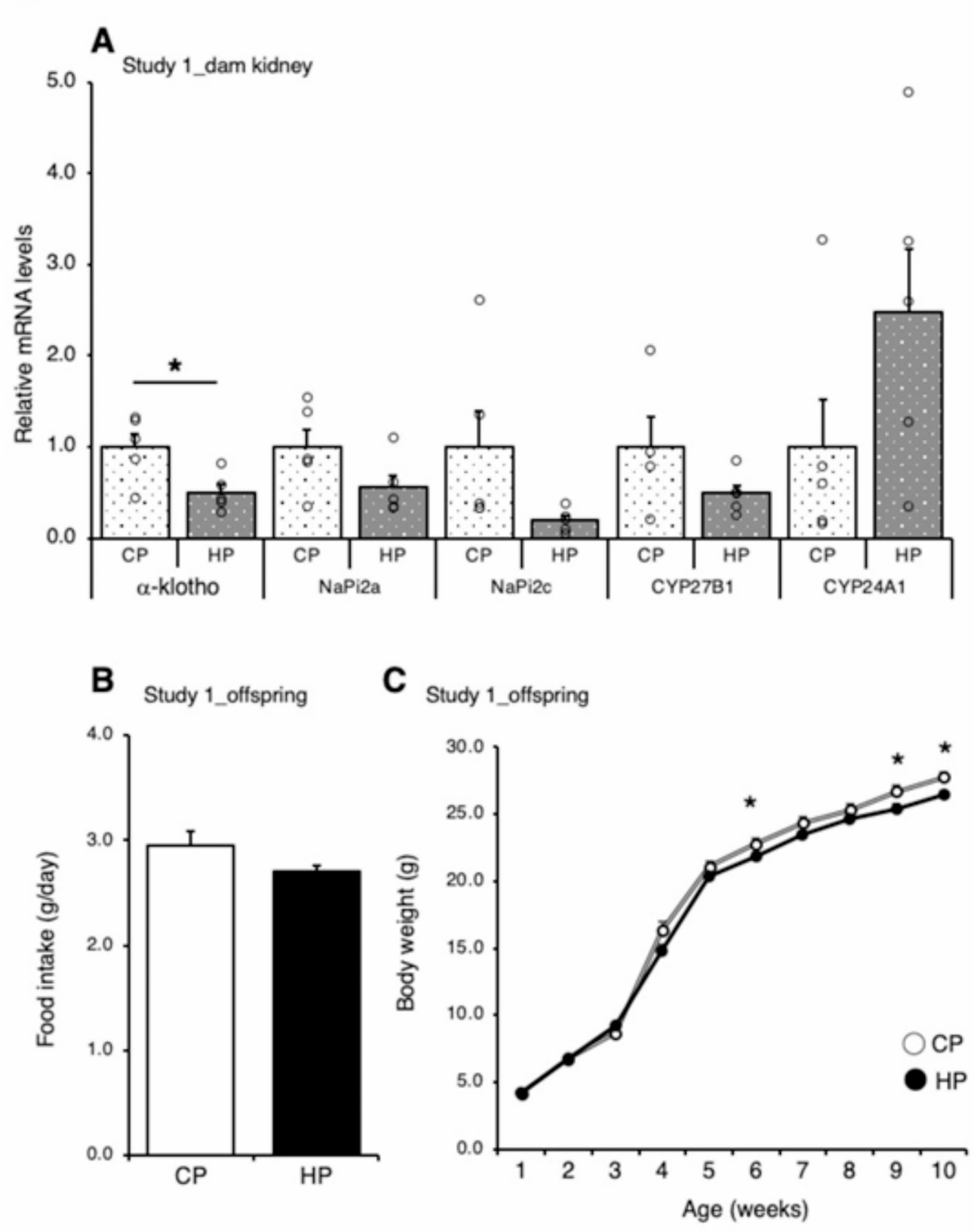

Fig. 1. Effect of dietary Pi intake during pre-pregnancy on maternal renal mRNA expression of Pi and vitamin D metabolism related genes and intake volume or body weight of offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for 21 days during pre-pregnancy (Study 1). (**A**) Total RNA was prepared from maternal kidney at 11 weeks of age. mRNA expression of renal α-klotho, NaPi2a, NaPi2c, CYP27B1, and CYP24A1 was analyzed ($n = 4–5$ per group). (**B**) The offspring's food intake and body weight were measured once a week: average daily food intake in 3 to 10 weeks of offspring age (CP: $n = 11$, HP: $n = 23$). (**C**) Average body weight of 1 to 10 weeks of age offspring (CP: $n = 16$, HP: $n = 22$). Data are given as mean ± SEM. $^*p < 0.05$ vs. CP at the same week of age.

	3week-old offspring		10-week-old offspring	
	CP	HP	CP	HP
Pre-pregnancy				
Plasma				
Pi (mg/dl)	8.61 ± 0.44	7.86 ± 0.41	5.98 ± 0.27	6.87 ± 0.28
Ca (mg/dl)	9.23 ± 0.90	8.84 ± 0.90	7.65 ± 0.49	7.40 ± 0.20
FGF23 (pg/ml)	69.89 ± 5.35	99.09 ± 5.01*	74.64 ± 8.35	72.40 ± 3.50
PTH (pg/ml)	22.04 ± 5.42	35.53 ± 4.13*	27.76 ± 1.56	98.42 ± 20.82*
$1,25(OH)_2D$ (pg/ml)	90.92 ± 10.68	157.6 ± 20.26*	43.52 ± 4.37	51.88 ± 1.90*
Urine				
Pi (mg/mgCre)	0.50 ± 0.12	0.40 ± 0.11	0.12 ± 0.057	0.04 ± 0.0078*
Ca (mg/mgCre)	1.04 ± 0.19	1.34 ± 0.18	0.25 ± 0.079	0.55 ± 0.11
Pregnancy				
Plasma				
Pi (mg/dl)	7.19 ± 0.48	6.65 ± 0.21	7.10 ± 0.84	6.06 ± 1.63
Ca (mg/dl)	5.92 ± 0.19	6.53 ± 0.28	6.33 ± 0.51	6.11 ± 0.26
FGF23 (pg/ml)	65.53 ± 17.67	46.43 ± 5.29	53.86 ± 7.53	65.71 ± 30.16
PTH (pg/ml)	29.50 ± 9.12	44.59 ± 12.3	54.38 ± 11.40	60.88 ± 11.13
$1,25(OH)_2D$ (pg/ml)	139.80 ± 4.16	153.80 ± 10.42	111.70 ± 12.56	99.06 ± 7.33
Urine				
Pi (mg/mgCre)	1.91 ± 0.61	0.37 ± 0.16*	0.062 ± 0.011	0.038 ± 0.0052*
Ca (mg/mgCre)	1.24 ± 0.27	1.77 ± 0.22	0.54 ± 0.10	0.93 ± 0.11*

Table 3. Effects of maternal dietary Pi intake during pre-pregnancy or pregnancy on plasma and urine biochemical parameters in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for either 21 days during pre-pregnancy or almost 20 days during pregnancy. After weaning, offspring were fed with CP. Plasma Pi and Ca (3 wk-CP: $n = 22$, 3 wk-HP: $n = 33$, 10 wk-CP: $n = 16$, 10 wk-HP: $n = 22$), plasma FGF23, PTH, and $1,25(OH)_2D$ (3 wk-CP: $n = 5$–8, 3 wk-HP: $n = 5$–15, 10 wk-CP: $n = 16$, 10 wk-HP: $n = 22$), urinary Pi and Ca (3 wk-CP: $n = 22$, 3 wk-HP: $n = 33$, 10 wk-CP: $n = 16$, 10 wk-HP: $n = 22$) in Study 1, and plasma Pi and Ca (3 wk-CP: $n = 28$, 3 wk-HP: $n = 28$, 10 wk-CP: $n = 13$, 10 wk-HP: $n = 19$), plasma FGF23,PTH, and $1,25(OH)_2D$ (3 wk-CP: $n = 7$, 3 wk-HP: $n = 7$, 10 wk-CP: $n = 8$, 10 wk-HP: $n = 19$), urinary Pi and Ca (3 wk-CP: $n = 14$, 3 wk-HP: $n = 26$, 10 wk-CP: $n = 13$, 10 wk-HP: $n = 19$) in Study 2 were analyzed. Data are given as mean ± SEM. *$p < 0.05$ vs. CP at the same week of age.

two type III sodium-dependent Pi cotransporters (Pit1, Pit2). Although mRNA expression did not significantly differ between the HP and CP group at 3 or 10 weeks (Fig. 3A, B), NaPi2b and Pit2 mRNA expression levels tended to be lower in the HP group at 3 weeks of age.

Femur mRNA expression of FGF23-related genes in offspring

Because FGF23 is secreted from the bone in response to elevated serum Pi levels or increased dietary Pi intake, we measured the mRNA expression of FGF23-related genes in the femur of offspring. mRNA levels of PHEX and DMP-1, two negative regulators of FGF23[23,24], were significantly lower in the HP group at 10 weeks of age. In addition, mRNA levels of UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 3 (GALNT3), which inhibits inactivation of FGF23 and is important in the secretion of active FGF23[25], and family with sequence similarity 20, member C (Fam20c), which inhibits glycosylation of GALNT3[26], were also significantly lower in the HP group at 10 weeks. Runt-related transcription factor 2 (Runx2), related to bone transcription factors[27,28], also showed significantly lower mRNA expression in the HP group at 10 weeks (Fig. 4A, B).

Femur mRNA expression of genes related to DNA methylation in offspring

Lastly, we evaluated the expression of DNA methylation-related genes in the femur of offspring. The mRNA levels of DNMT1, DNMT3a, and DNMT3b were significantly lower in the HP group at 10 weeks. Furthermore, Tet1 mRNA expression at 3 weeks and Tet2 mRNA expression at 10 weeks were significantly decreased in the HP group. However, there were no differences in Tet3 mRNA expression between the two diet groups at any age (Fig. 4C, D).

Study 2: effects of maternal dietary Pi intake during pregnancy

Biochemical parameters in maternal plasma, urine, and breast milk

In Study 2, we measured Pi, and Ca concentrations in plasma, urine, and breast milk from 14-weeks-old female mice fed the CP or HP during pregnancy (almost 20 days). As summarized in Table 2, Pi and Ca concentrations in plasma and urine did not differ significantly between the groups. Similarly, Pi and Ca concentrations in breast milk were not significantly different between the two groups.

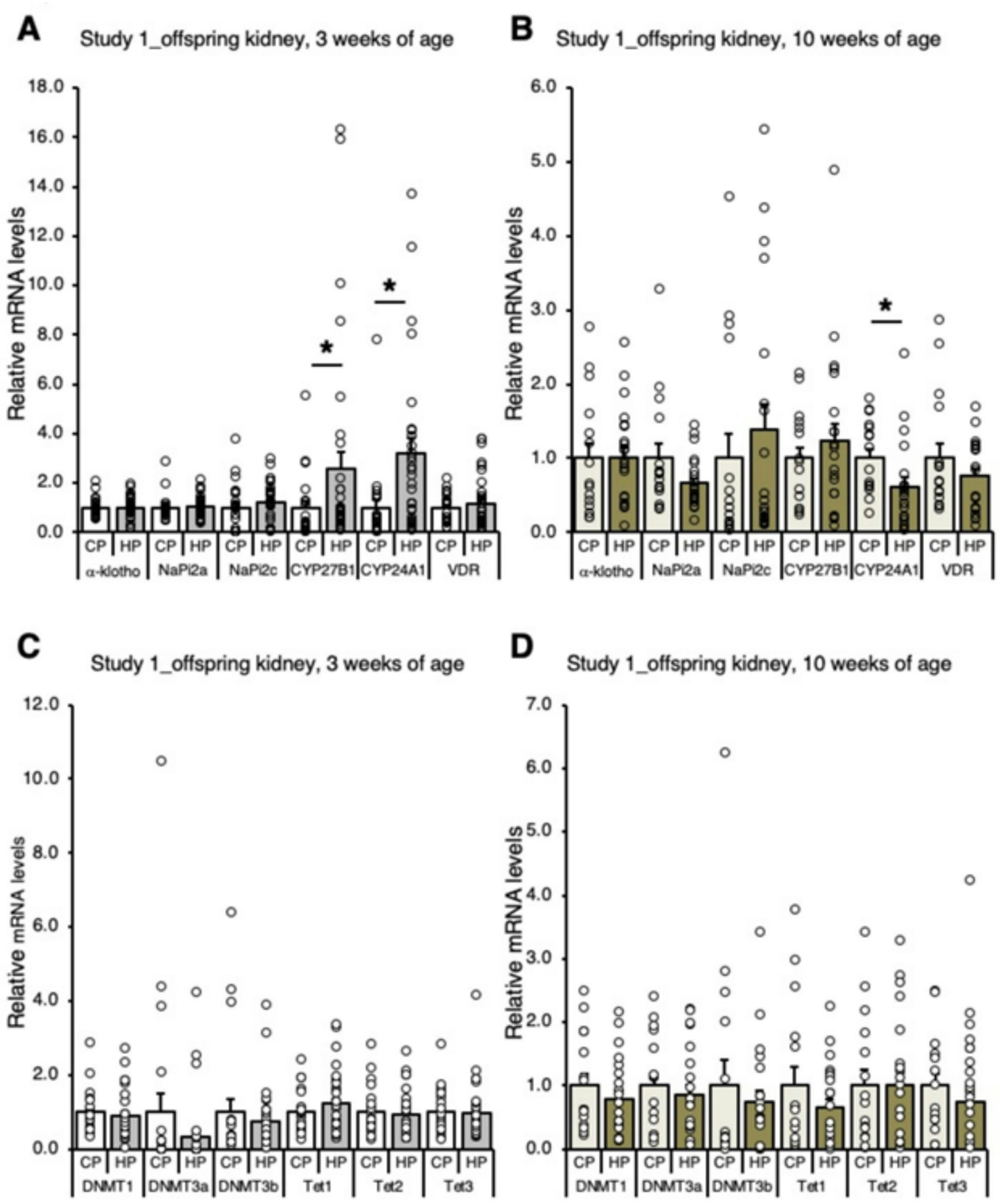

Fig. 2. Effect of maternal dietary Pi intake during pre-pregnancy on renal mRNA expression of genes related to Pi and vitamin D metabolism and DNA methylation in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for 21 days during pre-pregnancy (Study 1). (**A**–**D**) Total RNA was prepared from offspring kidney at 3 or 10 weeks of age and mRNA expression of α-klotho, NaPi2a, NaPi2c, CYP27B1, CYP24A1, and VDR (3 weeks of age, (**A**)) (10 week of age, ((**B**) and DNMT1, DNMT3a, DNMT3b, Tet1, Tet2, and Tet3 (3 week of age, (**C**)) (10 week of age, (**D**)) was measured by quantitative RT-PCR (3 wk-CP: $n = 22$, 3 wk-HP: $n = 32$–33, 10 wk-CP: $n = 16$, 10 wk-HP: $n = 22$). Data are given as mean ± SEM. *$p < 0.05$ vs. CP at the same week of age.

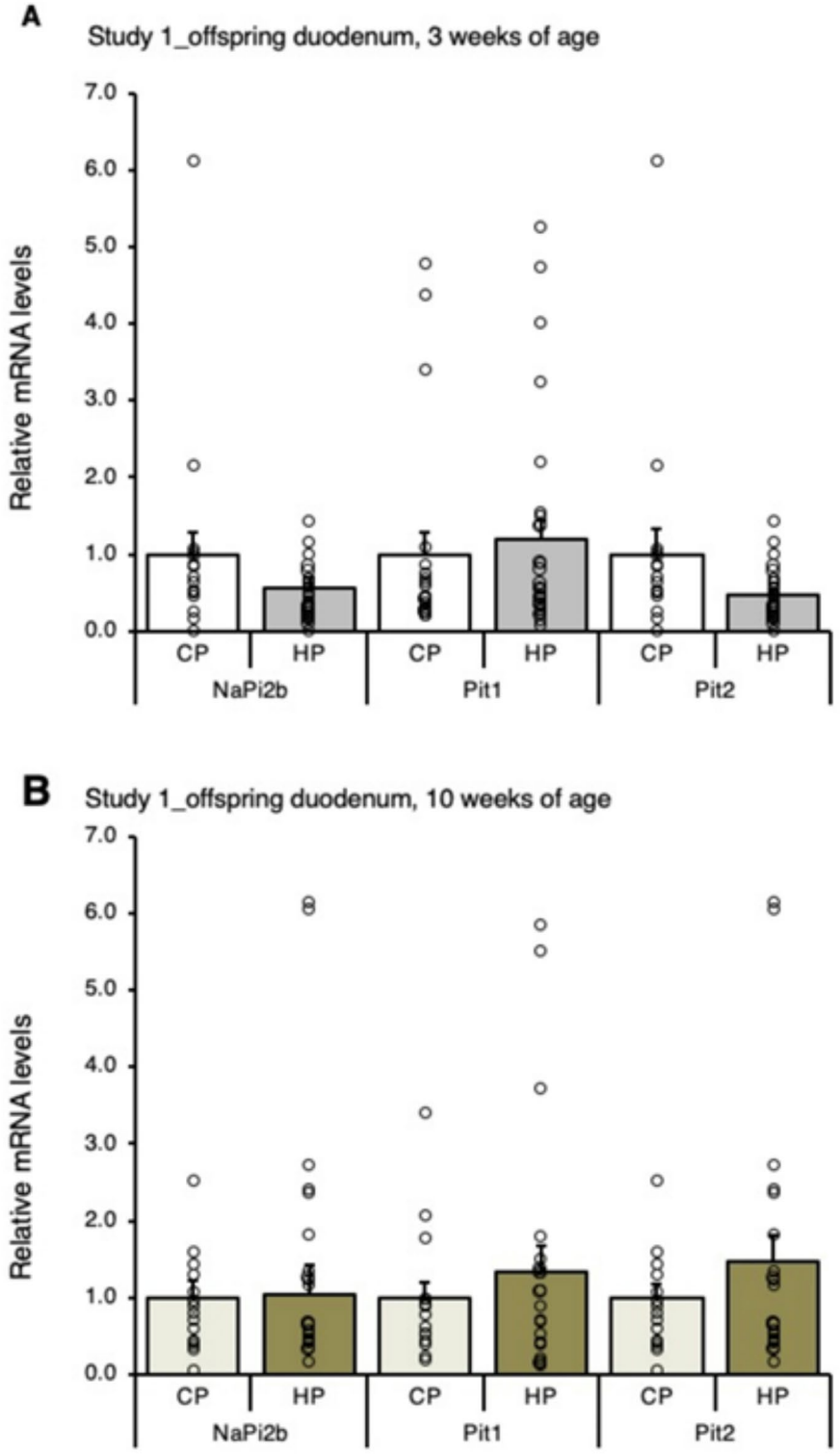

Fig. 3. Effects of maternal dietary Pi intake during pre-pregnancy on duodenum mRNA expression of genes related to Pi and vitamin D metabolism in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for 21 days during pre-pregnancy (Study 1). (**A**,**B**) Total RNA was prepared from the duodenum of offspring at 3 or 10 weeks of age, and mRNA expression of NaPi2b, Pit1, and Pit2 (3 weeks of age, ((**A**) (10 weeks of age, (**B**)) was measured by quantitative RT-PCR (3 wk-CP: $n = 21–22$, 3 wk-HP: $n = 33$, 10 wk-CP: $n = 15–16$, 10 wk-HP: $n = 22$). Data are given as mean ± SEM.

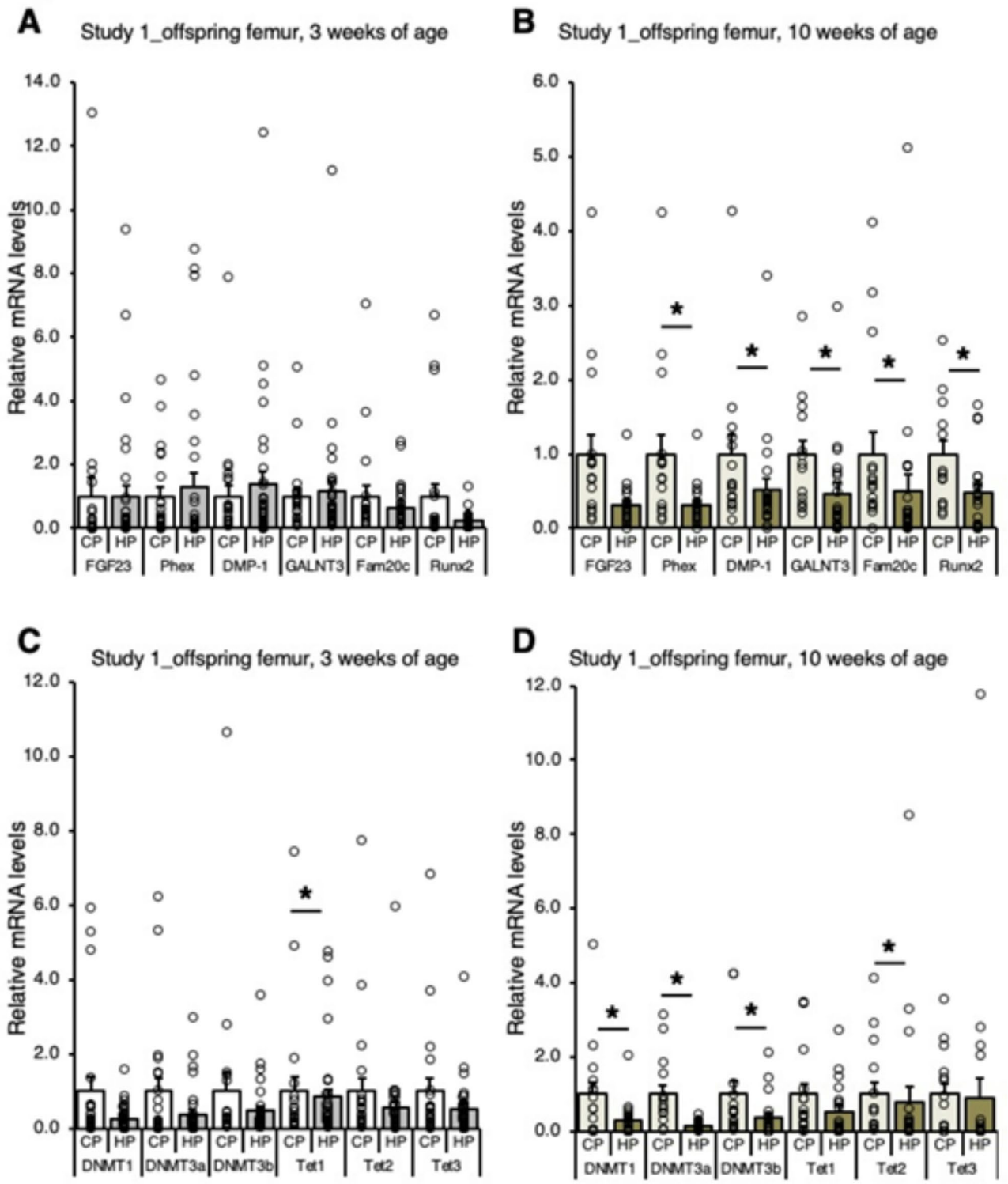

Fig. 4. Effects of maternal dietary Pi intake during pre-pregnancy on femur mRNA expression of genes related to FGF23 and DNA methylation in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for 21 days during pre-pregnancy (Study 1). (**A**-**D**) Total RNA was prepared from the femur of offspring at 3 or 10 weeks of age, and mRNA expression of FGF23, PHEX, DMP1, GALNT3, Fam20c and Runx2 (3 weeks of age, (**A**)) (10 weeks of age, (**B**)), and DNMT1, DNMT3a, DNMT3b, Tet1, Tet2, and Tet3 (3 weeks of age, (**C**)) (10 weeks of age, (**D**)) was measured by quantitative RT-PCR (3 wk-CP: $n = 21–22$, 3 wk-HP: $n = 33$, 10 wk-CP: $n = 15–16$, 10 wk-HP: $n = 22$). Data are given as mean ± SEM. $^*p < 0.05$ vs. CP at the same week of age.

Maternal renal mRNA expression of genes related to Pi and vitamin D metabolism
To examine the effects of dietary Pi intake during pregnancy on the 14-weeks-old maternal kidney, we measured the mRNA expression levels of renal genes related to Pi and vitamin D metabolism using RT-PCR. The mRNA levels of all genes tested did not differ significantly between the two groups (Fig. 5A).

Food intake and body weight of offspring
To examine the effects of maternal intake of Pi during pregnancy on offspring, food intake from 3 to 10 weeks of age and body weight from 1 to 10 weeks of age were measured in offspring born from dams fed the CP diet or HP diet. Although average daily food intake did not differ significantly between the groups (Fig. 5B), body weight was significantly lower in the HP group at 9 and 10 weeks (Fig. 5C).

Biochemical parameters in offspring plasma and urine
Plasma concentrations of Pi, Ca, FGF23, PTH, and $1,25(OH)_2D$ did not differ between the two diet groups. Urinary Pi excretion was significantly lower in the HP group at 3 and 10 weeks of age, while urinary Ca excretion was significantly higher in the HP group at 10 weeks (Table 3).

Renal mRNA expression of genes in offspring
As in Study 1, we examined the effects of maternal dietary Pi intake during pregnancy on the kidney of offspring by measuring the renal mRNA expression of genes related to Pi and vitamin D metabolism and DNA methylation. Dietary Pi intake during pregnancy led to lower mRNA levels of renal α-klotho in the HP group at 3 weeks compared to the CP group at the same age (Fig. 6A). However, there was no significant difference in any genes at 10 weeks (Fig. 6B). Additionally, the mRNA expression of DNMTs and Tet did not differ significantly between the two diet groups at either age (Fig. 6C, D).

Duodenal mRNA expression of genes related to Pi and vitamin D metabolism in offspring
In the offspring duodenum, mRNA expression of the sodium-dependent Pi transporter NaPi2b was significantly lower in the HP group compared to the CP group at 3 and 10 weeks of age (Fig. 7A, B). There were no differences in mRNA expression levels of Pit1 or Pit2.

Femur mRNA expression of genes in offspring
Lastly, we examined the effects of maternal dietary Pi intake during pregnancy on the bone of offspring. As in Study 1, we measured mRNA expression levels of femur FGF23-related genes and DNA methylation-related genes using RT-PCR (Fig. 8A, B). Regarding FGF23-related genes, PHEX and GLANT3 mRNA expression was significantly lower in the HP group at 10 weeks of age (Fig. 8B).

Regarding genes related to DNA methylation, DNMT1 mRNA expression was significantly lower in the HP group at 10 weeks. Moreover, Tet1 and Tet2 mRNA expression were significantly higher, and Tet3 mRNA expression was significantly lower in the HP group at 3 weeks. On the other hand, Tet2 mRNA expression was significantly lower in the HP group at 10 weeks (Fig. 8C, D).

Cross-fostering study

To confirm the observed effects of high maternal intake of Pi on offspring, we repeated Study 1 and Study 2 with cross-fostering, where the offspring from dams fed CP during pre-pregnancy or pregnancy, respectively, were raised by dams fed HP during pre-pregnancy or pregnancy (mCP-HP group), and vice versa (mHP-CP group) (Figure S1).

Consistent with the results of Study 1, the mHP-CP group tended to exhibit higher plasma levels of FGF23, PTH, and $1,25(OH)_2D$, and decreased urinary Pi excretion as compared with the mCP-HP group (Table S1). mHP-CP offspring also demonstrated significantly higher renal CYP27B1 mRNA expression (Figure S3A). On the other hand, mHP-CP offspring did not exhibit the disturbance of Pi-regulating hormones observed in Study 2 (Table S1). However, mHP-CP offsprings demonstrated significantly lower renal α-klotho mRNA expression as in Study 2 (Figure S3B).

Discussion

Here, we examined whether consuming a high dietary Pi diet during pre-pregnancy or pregnancy affects P-regulating hormones, such as PTH, FGF23, vitamin D, and the mRNA expression of P-metabolism-related genes in offspring mice. Our findings showed that a high Pi diet during the periconceptional period causes dysregulation in Pi metabolism in their offspring.

Intestinal Pi absorption doubles during rodent pregnancy, but serum Pi levels remain normal during pregnancy in both rodents and human, while $1,25(OH)_2D$ and PTH-related peptide (PTHrP) levels increase[29]. Serum FGF23 concentration increases in dams during rodent pregnancy, but it is unclear in humans. The current study and previous study by Berit Sellars et al.[19] demonstrate that the high Pi diet al.so increased serum FGF23 levels in pregnant mice compared to the normal Pi diet. These results suggest that FGF23 can protect dams from hyperphosphatemia by increasing renal Pi wasting. On the other hand, the fetus should accumulate Ca and Pi as a skeleton before delivering so that dams adapt to the demand of fetal Ca and Pi while keeping serum Ca and P levels within the normal range. Berit Sellars et al. also reported that murine fetal serum Pi level is regulated independently by FGF23 and PTH[19]. Their further analysis revealed that FGF23 can defend against the development of fetal hyperphosphatemia induced by maternal high Pi loading. Our findings show that higher serum FGF23 and PTH levels were kept in the growing offspring from the dam fed with the HP diet before or during pregnancy. Higher plasma PTH and FGF23 levels are associated with cardiovascular disease, adverse prognosis of CKD, and all-cause mortality[2,3]. Therefore, appropriate control of dietary Pi intake in women of

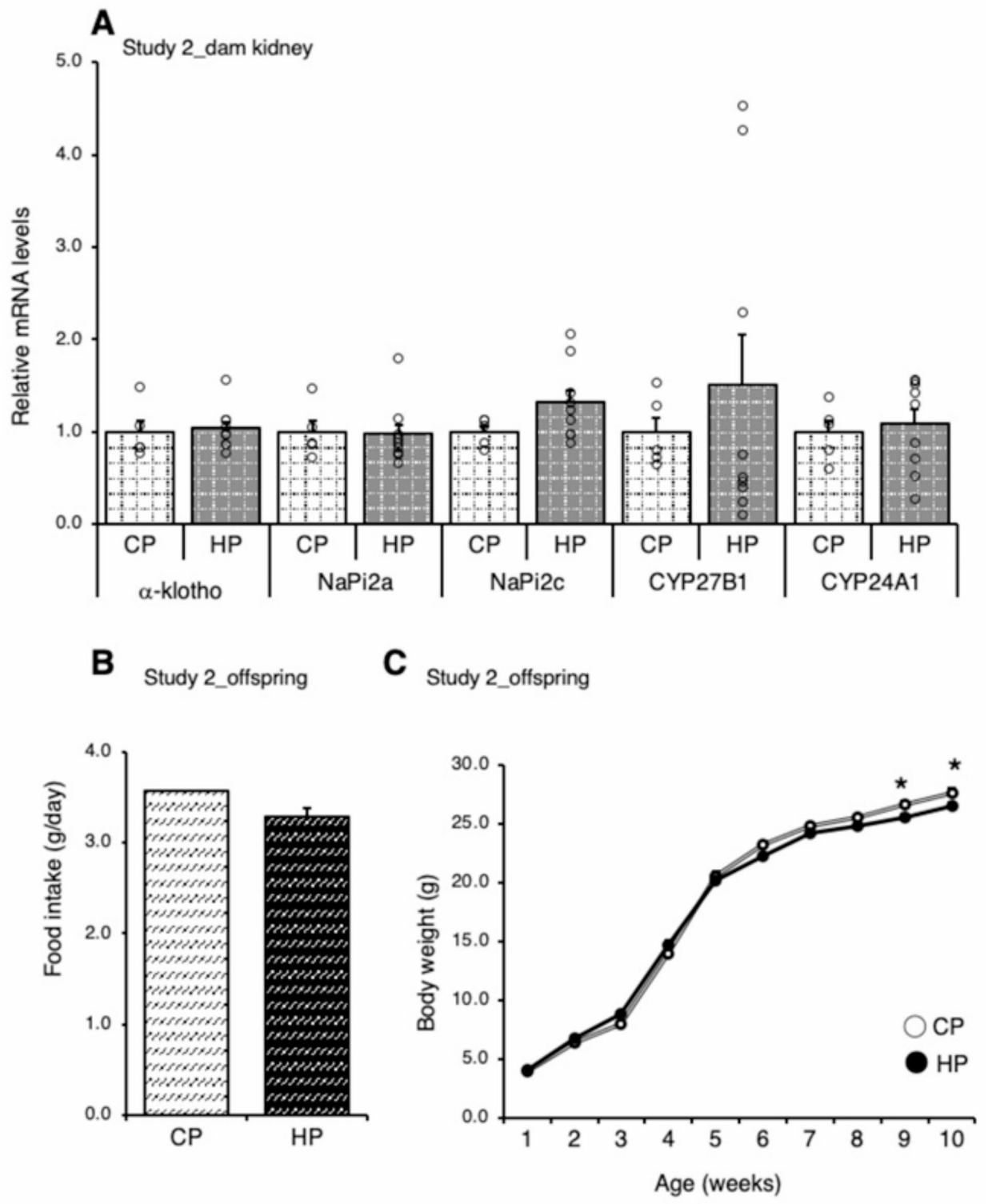

Fig. 5. Effect of dietary Pi intake during pregnancy on maternal renal mRNA expression of Pi and vitamin D metabolism-related genes and intake volume or body weight of offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for almost 20 days during pregnancy (Study 2). (**A**) Total RNA was prepared from maternal kidney at 14 weeks of age. mRNA expression of renal α-klotho, NaPi2a, NaPi2c, CYP27B1, and CYP24A1 was analyzed ($n = 7$–9 per group). (**B**) Food intake and body weight of offspring were measured once a week: average daily food intake in 3 to 10 weeks of age offspring (CP: $n = 11$, HP: $n = 23$). (**C**) Average body weight of 1 to 10 weeks of age offspring (CP: $n = 13$, HP: $n = 19$). Data are given as mean ± SEM. $^*p < 0.05$ vs. CP at the same week of age.

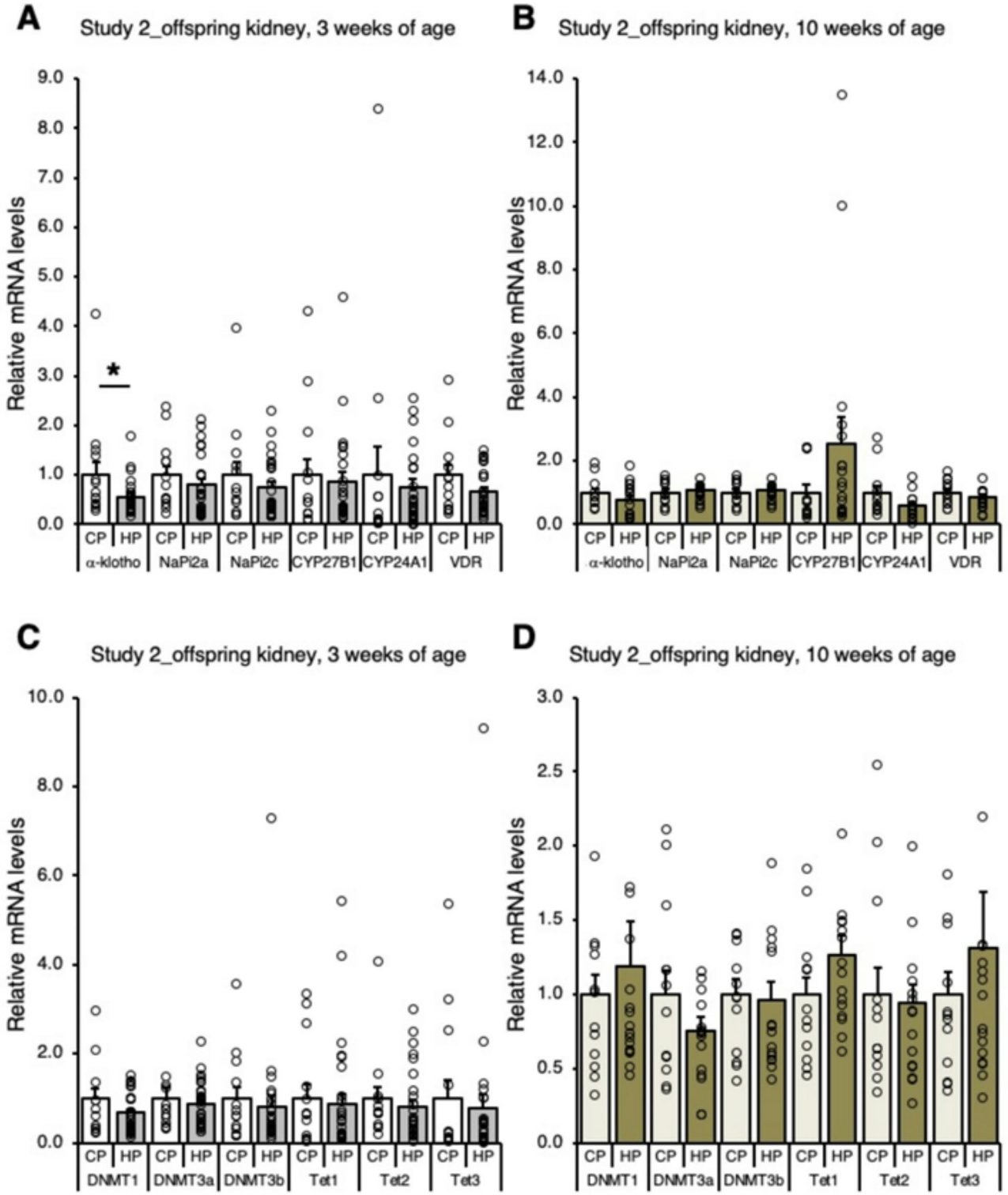

Fig. 6. Effect of maternal dietary Pi intake during pregnancy on renal mRNA expression of genes related to Pi and vitamin D metabolism and DNA methylation in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for almost 20 days during pregnancy (Study 2). (**A**-**D**) Total RNA was prepared from offspring kidney at 3 or 10 weeks of age and mRNA expression of α-klotho, NaPi2a, NaPi2c, CYP27B1, CYP24A1, and VDR (3 weeks of age, (**A**)) (10 weeks of age, (**B**)) and DNMT1, DNMT3a, DNMT3b, Tet1, Tet2, and Tet3 (3 weeks of age, (**C**)) (10 weeks of age, (**D**)) was measured by quantitative RT-PCR (3 wk-CP: $n = 14$, 3 wk-HP: $n = 29$, 10 wk-CP: $n = 12$–13, 10 wk-HP: $n = 16$–19). Data are given as mean ± SEM. $^*p < 0.05$ vs. CP at the same week of age.

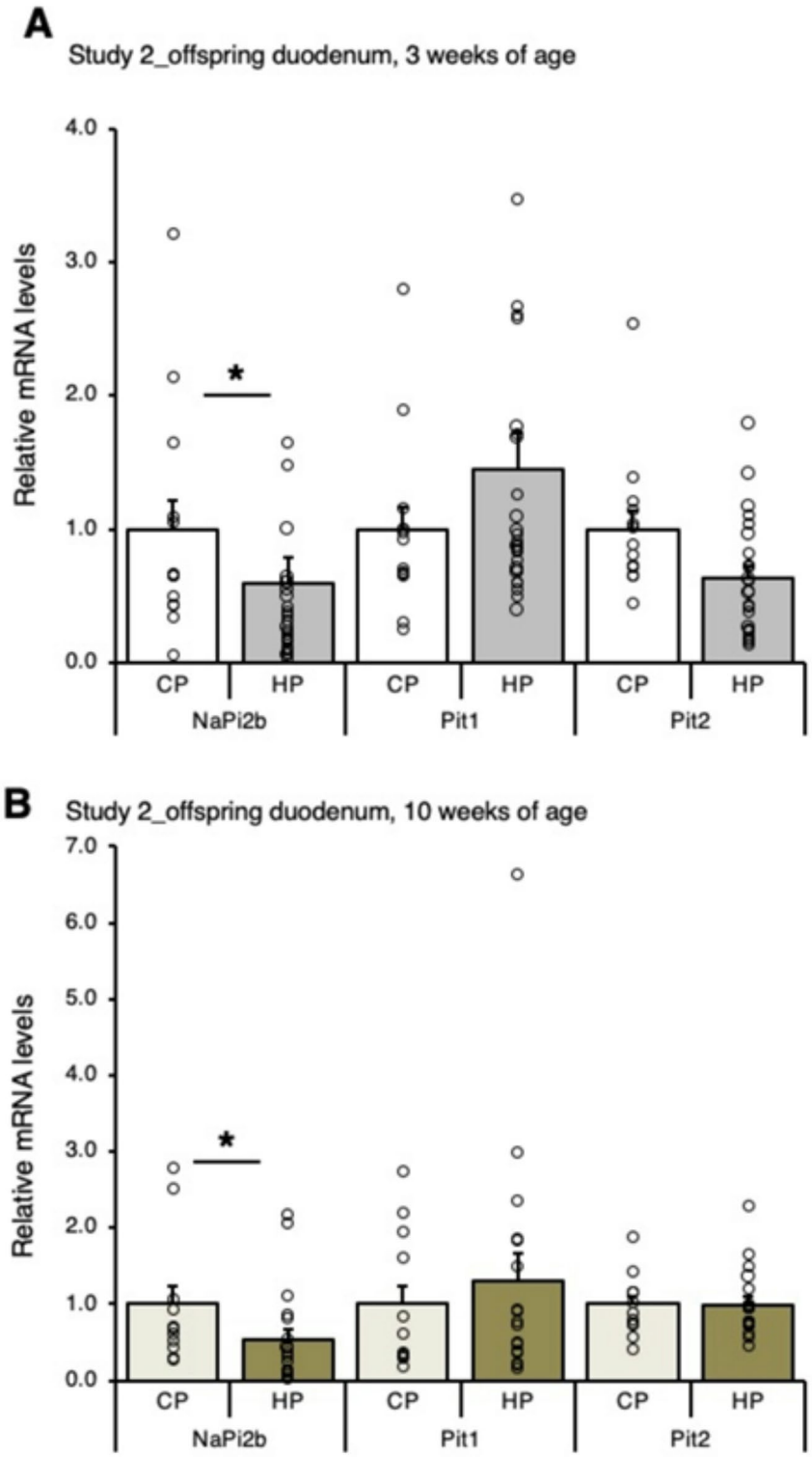

Fig. 7. Effects of maternal dietary Pi intake during pregnancy on duodenum mRNA expression of genes related to Pi and vitamin D metabolism in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for almost 20 days during pregnancy (Study 2). (**A**,**B**) Total RNA was prepared from the duodenum of offspring at 3 or 10 weeks of age, and mRNA expression of NaPi2b, Pit1, and Pit2 (3 weeks of age, (**A**)) (10 weeks of age, (**B**)) was measured by quantitative RT-PCR (3 wk-CP: $n = 14$, 3 wk-HP: $n = 27$, 10 wk-CP: $n = 11$, 10 wk-HP: $n = 18$–19). Data are given as mean ± SEM. $^*p < 0.05$ vs. CP at the same week of age.

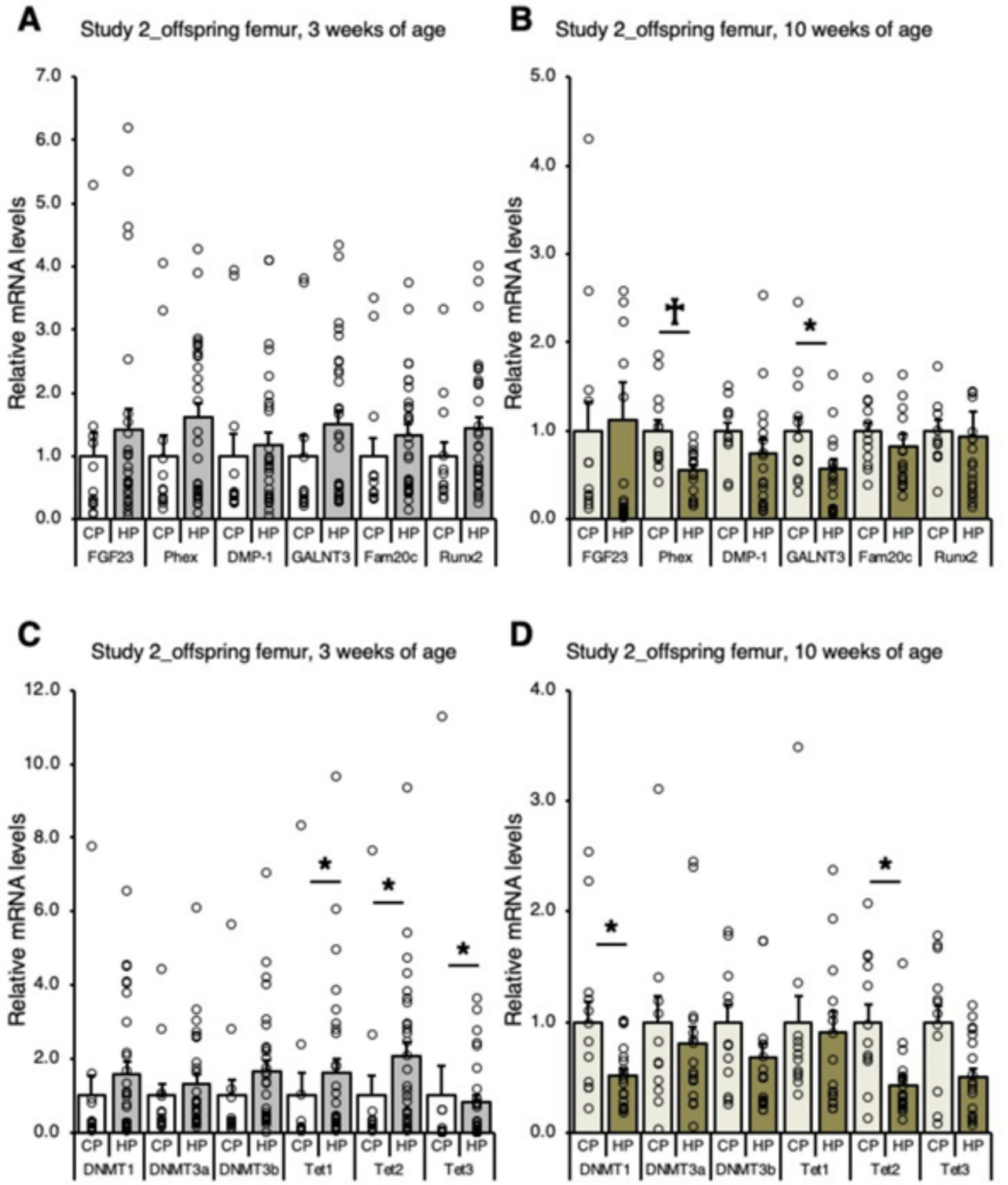

Fig. 8. Effects of maternal dietary Pi intake during pregnancy on femur mRNA expression of genes related to FGF23 and DNA methylation in offspring. Female mice were fed 0.8% Pi diet (CP) or 1.5% Pi diet (HP) for almost 20 days during pregnancy (Study 2). (**A**-**D**) Total RNA was prepared from the femur of offspring at 3 or 10 weeks of age, and mRNA expression of FGF23, PHEX, DMP1, GALNT3, Fam20c and Runx2 (3 weeks of age, (**A**)) (10 weeks of age, (**B**)), and DNMT1, DNMT3a, DNMT3b, Tet1, Tet2, and Tet3 (3 weeks of age, (**C**)) (10 weeks of age, (**D**)) was measured by quantitative RT-PCR (3 wk-CP: $n = 13$, 3 wk-HP: $n = 29$, 10 wk-CP: $n = 12$–13, 10 wk-HP: $n = 16$–19). Data are given as mean ± SEM. $^{*}p < 0.05$ and $\dagger p < 0.01$ vs. CP at the same week of age.

reproductive age as well as pregnant is likely to play a role in reducing the potential of offspring to develop cardiovascular disease, CKD, and other disorders in adulthood.

According to DOHaD, it is important to consider the impact of dietary Pi during pre-pregnancy and pregnancy on Pi homeostasis in the offspring because maternal nutritional status may influence the risk of disease in offspring. A previous study reported that offspring from dams malnourished during pre-pregnancy and pregnancy are predisposed to adult lifestyle-related diseases due to energy-saving characteristics developed through adaptation to a malnourished environment[30,31]. In addition, offspring from dams with slightly high blood pressure due to high sodium diet intake before and during pregnancy exhibited a predisposition to high blood pressure[32,33]. The present study has shown that offspring from dams fed the HP diet during the periconceptional period may also be predisposed towards increased phosphaturic hormones and decreased intestinal Pi absorption caused by adaptation to the high maternal Pi environment. We are also interested in the underlying mechanisms of the effects of high Pi diets during the fertility cycle on offspring. We found some differences in the mRNA expression patterns of the genes associated with DNA methylation, suggesting the involvement of epigenetic modifications. We could not identify the responsible modified DNA methylation at this time, and further studies are needed.

Our study has several limitations. The present study is an animal study, so epidemiological studies are needed to verify whether excessive dietary Pi intake during pre-pregnancy and/or pregnancy may increase the future risk of disease in offspring in humans. Second, our offspring study was carried out using male mice only. We are interested in the differences in outcomes between female and male offspring. In future studies, we would like to investigate more detail to compare the female and male offspring. Third, we investigated the mRNA expression of genes relating to Pi homeostasis but not protein levels and their activities. The mRNA expressions mostly correspond their protein activities, but future studies will bring us more detailed consideration.

In summary, this study has shown that high dietary Pi intake during pre-pregnancy and pregnancy affected Pi homeostasis, vitamin D metabolism, and development in offspring. To our best knowledge, this is the first report to document the effects of excess dietary Pi intake during pre-pregnancy and pregnancy on Pi homeostasis in offspring. Further investigation will be required to clarify the molecular mechanisms underlying the changes seen in offspring.

Data availability

All data and information are available in the main text or the Supplementary Material. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon reasonable request.

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Author contributions

M. H-S, S.F-T. and Y.T. conceived and designed research; M. H-S, S.F-T., M.K-O., M.E-K., H.O., and M.M performed experiments and analyzed data; M. H-S, S.F-T., M.E-K., H.O., M.M., and Y.T. interpreted results of experiments; M. H-S, S.F-T. prepared figures, M. H-S, S.F-T., and Y.T. drafted manuscript, M. H-S, S.F-T., H.O., M.M., and Y.T. edited and revised manuscript; M.H-S., and Y.T. approved final version of manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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