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Regulation of serum 1,25(OH)$_2$Vitamin D$_3$ levels by fibroblast growth factor 23 is mediated by FGF receptors 3 and 4

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Gattineni J, Twombley K, Goetz R, Mohammadi M, Baum M. Regulation of serum 1,25(OH)$_2$Vitamin D$_3$ levels by fibroblast growth factor 23 is mediated by FGF receptors 3 and 4. *Am J Physiol Renal Physiol* 301: F371–F377, 2011. First published May 11, 2011; doi:10.1152/ajprenal.00740.2010.—Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone implicated in the pathogenesis of several hypophosphatemic disorders. FGF23 causes hypophosphatemia by decreasing the expression of sodium phosphate cotransporters (NaPi-2a and NaPi-2c) and decreasing serum 1,25(OH)$_2$Vitamin D$_3$ levels. We previously showed that FGFR1 is the predominant receptor for the hypophosphatemic actions of FGF23 by decreasing renal NaPi-2a and 2c expression while the receptors regulating 1,25(OH)$_2$Vitamin D$_3$ levels remained elusive. To determine the FGFRs regulating 1,25(OH)$_2$Vitamin D$_3$ levels, we studied FGFR3$^{-/-}$FGFR4$^{-/-}$ mice as these mice have shortened life span and are growth retarded similar to FGF23$^{-/-}$ and Klotho$^{-/-}$ mice. Baseline serum 1,25(OH)$_2$Vitamin D$_3$ levels were elevated in the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with wild-type mice (102.2 ± 14.8 vs. 266.0 ± 34.0 pmol/l; $P = 0.001$) as were the serum levels of FGF23. Administration of recombinant FGF23 had no effect on serum 1,25(OH)$_2$Vitamin D$_3$ in the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice (173.4 ± 32.7 vs. 219.7 ± 56.5 pmol/l; vehicle vs. FGF23) while it reduced serum 1,25(OH)$_2$Vitamin D$_3$ levels in wild-type mice. Administration of FGF23 to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice resulted in a decrease in serum parathyroid hormone (PTH) levels and an increase in serum phosphorus levels mediated by increased renal phosphate reabsorption. These data indicate that FGFR3 and 4 are the receptors that regulate serum 1,25(OH)$_2$Vitamin D$_3$ levels in response to FGF23. In addition, when 1,25(OH)$_2$Vitamin D$_3$ levels are not affected by FGF23, as in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice, a reduction in PTH can override the effects of FGF23 on renal phosphate transport.

proximal tubule; phosphate; FGF23

FIBROBLAST GROWTH FACTOR 23 (FGF23) is a phosphaturic hormone that has been implicated in several inherited and acquired hypophosphatemic disorders (15). FGF23 increases urinary phosphate excretion by decreasing renal brush-border expression of the sodium phosphate cotransporters 2a and 2c (NaPi-2a and NaPi-2c) (14, 42). In addition, FGF23 decreases the expression of 25(OH)Vitamin D-1α-hydroxylase (CYP27B1) and increases the expression 24-hydroxylase (CYP24) resulting in low-serum 1,25(OH)$_2$Vitamin D$_3$ levels (37, 42). The inherited hypophosphatemic disorders where FGF23 levels are increased include X-linked hypophosphatemic rickets, autosomal dominant hypophosphatemic rickets, and autosomal recessive hypophosphatemic rickets (12, 14, 21, 24, 31, 36, 50). The increased FGF23 levels in these disorders result in severe hypophosphatemia, rickets/osteomalacia, bone pain, fractures, and growth failure in children. Serum FGF23 levels are also elevated in tumor-induced osteomalacia (8, 9).

The FGF family of ligands bind to FGF receptors (FGFRs) to mediate their actions (10, 17, 23, 34). Four FGFRs (FGFR1–4) are encoded by four genes and alternative splicing (b and c isoforms of FGFR1–3) results in tissue and ligand binding specificity (33, 35). The proximal tubule, the site of most renal phosphate reabsorption (4, 46) and 25(OH)Vitamin D-1α-hydroxylase activity (7, 13), has FGFR1, 3, and 4, but it does not express FGFR2 (14).

We previously utilized FGFR-null mice with the goal of determining which receptor was responsible for the FGF23-mediated decrease in renal phosphate transport and vitamin D production. In these studies, FGF23 was administered to conditional FGFR1$^{-/-}$, FGFR3$^{-/-}$, and FGFR4$^{-/-}$ mice. FGFR1 was found to be the predominant receptor mediating the hypophosphatemic actions of FGF23 by decreasing brush-border membrane (BBM) NaPi-2a and NaPi-2c expression with FGFR4 playing an additional but relatively minor role (14). Liu et al. (29) also found that deletion of either FGFR3 or FGFR4 in a mouse model of X-linked hypophosphatemic rickets (*Hyp* mouse) did not correct the disturbances in phosphate homeostasis. Intriguingly, in the conditional FGFR1$^{-/-}$ mice, as well as FGFR3$^{-/-}$ and FGFR4$^{-/-}$ mice, 1,25(OH)$_2$Vitamin D$_3$ levels decreased comparably after administration of FGF23 (14). This implies that FGF23 regulates proximal tubular 1,25(OH)$_2$Vitamin D$_3$ biosynthesis using a different receptor than for the inhibition of phosphate transport in the proximal tubule. 1,25(OH)$_2$Vitamin D$_3$ is primarily synthesized in the proximal tubule and CYP27B1 is the rate-limiting enzyme in the synthesis of 1,25(OH)$_2$Vitamin D$_3$ (7, 13). CYP24 catalyzes the conversion of 1,25(OH)$_2$Vitamin D$_3$ to its inactive form, calcitroic acid (19). Recently, Li et al. (27) demonstrated that the deletion of FGFR3 and FGFR4 from *Hyp* mice resulted in increased 1,25(OH)$_2$Vitamin D$_3$ levels, partially correcting the hypophosphatemia.

FGF23$^{-/-}$ and Klotho$^{-/-}$ mice are growth retarded and have shortened life spans (25, 44, 45). In addition, FGF23$^{-/-}$ mice have hyperphosphatemia and increased levels of 1,25(OH)$_2$Vitamin D$_3$ levels. FGFR3$^{-/-}$FGFR4$^{-/-}$ mice also are growth retarded and have a decreased life span (49). We previously showed that serum 1,25(OH)$_2$Vitamin D$_3$ levels were significantly higher in the FGFR3$^{-/-}$ mice and 1,25(OH)$_2$Vitamin D$_3$ levels tended to be higher in FGFR4$^{-/-}$ mice than the wild-type mice. We, therefore, hypothesized that the loss of both of these receptors may impact 1,25(OH)$_2$Vitamin D$_3$ homeostasis. To this end, we examined the effect of FGF23 on phosphate transport and vitamin D levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice.

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METHODS

FGFR$^{-/-}$ mice. We previously studied FGFR3$^{-/-}$ and FGFR4$^{-/-}$ mice (14) and the generation of these individual receptor-null mice has been described previously (49). FGFR3$^{-/-}$FGFR4$^{-/-}$ mice were generated by breeding FGFR3$^{-/-}$ with FGFR4$^{-/-}$ mice. Wild-type, FGFR4$^{-/-}$, and FGFR3$^{-/-}$ mice are from the same 129/Black Swiss background (49). The mice were genotyped to ensure that *FGFR3* and *FGFR4* were deleted from the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. The mice were on a 12:12-h day-night cycle and had free access to water and standard rodent diet. The diet was obtained from Harlan Laboratories (Teklad Global 16% Protein Rodent Diet) and the phosphorus content was 0.7%, 0.4% nonphytate phosphorus, and 1% calcium. The studies were performed when the mice were ~3 mo of age. The mice were housed at the state of the art Animal Research Center at UT Southwestern Medical Center. All of the animal studies were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

Recombinant FGF23 administration. Recombinant human FGF23 (FGF23) with two mutations, R176Q and R179Q, found in patients with autosomal dominant hypophosphatemic rickets was used for these studies (18). The FGF23 protein was expressed in *Escherichia coli* and thus it is nonglycosylated. As the recombinant FGF23 has two known mutations at the proteolytic cleavage site ^{176}RXXR^{179}, this protein is more resistant to proteolytic degradation than the wild-type FGF23. We previously showed that injection of FGF23 intraperitoneally at the dose of 12 μg·injection^{-1}·mouse^{-1} every 12 h for 8 doses to wild-type mice showed significant hypophosphatemia and decreased 1,25(OH)$_2$Vitamin D$_3$ levels when studied 10–12 h after the last injection (14). We therefore injected 12 μg·injection^{-1}·mouse^{-1} and followed the same dosing regimen for the wild-type and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice in this study. Vehicle (buffer consisting of 25 mM HEPES-NaOH, pH 7.5, and 1 M NaCl) was injected as a control. Serum and tissue samples were collected at the time of death of the mice, 10–12 h after the last injection.

Serum biochemistry. Isoflurane was used as an anesthetic to sedate the mice before phlebotomy via retro-orbital venipuncture. The serum obtained was aliquoted for various biochemical measurements. Serum was immediately frozen in liquid nitrogen and stored at −80°C until the assays were performed for FGF23, PTH, and 1,25(OH)$_2$D$_3$. Intact mouse PTH kit (Immutopics, San Clemente, CA) was used to measure PTH employing the ELISA technique. Intact serum FGF23 levels were measured using the FGF23 kit from Kainos Laboratories (Tokyo, Japan). Radioimmunoassay technique was used to measure serum 1,25(OH)$_2$D$_3$ levels using γ-B 1,25-dihydroxy vitamin D kit (IDS, Tyne and Wear, UK). Serum phosphorus was measured using a Phosphorus Liqui-UV Test (Stanbio Laboratories, Boerne, TX). The manufacturer's instructions were followed for the kits used in this study.

BBM vesicle isolation. The renal cortex was quickly dissected after the kidneys were harvested and the dissected cortex was placed in ice-cold isolation buffer [5 mM ethylene glycol-bis (β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid (EGTA), 16 mM HEPES, and 300 mM mannitol titrated to pH 7.4 with Tris] containing protease inhibitor cocktail (1:1,000; Sigma Biochemicals, St. Louis, MO) and phenyl-methyl-sulfonyl fluoride (100 μg/μl; Sigma Biochemicals) as described previously (15, 16). The cortex was homogenized using a Potter Ejevhem homogenizer at 4°C. BBM vesicles (BBMV) were isolated using two consecutive magnesium precipitations and differential centrifugation as described previously (3). Isolation buffer was used to suspend the final BBMV pellet.

Na-dependent BBMV phosphate transport activity. Using the rapid millipore filtration technique, sodium-dependent phosphate (^{32}P) transport was measured in freshly isolated BBMVs. One hundred micrograms (10 μl) of BBMVs were preloaded in an intravesicular buffer (300 mM mannitol and 16 mM HEPES titrated to pH 7.5 using Tris) and then thoroughly mixed by vortexing with extravesicular buffer (90 μl; 150 mM NaCl and 16 mM HEPES, 0.1 mM KH_2PO_4 titrated to pH 7.5 with Tris) as previously described (1, 40). Uptake was terminated by using ice-cold stop solution (135 mM NaCl, 16 mM HEPES, 10 mM sodium arsenate and pH 7.5) at 10 s and the contents were filtered using 0.45-μm filters (Millipore, Billerica, MA). All the uptake measurements were performed in triplicate.

SDS-PAGE and immunoblotting. BBM protein was assayed using the Bradford method with bovine serum albumin as the standard. Equal amounts of protein (25 μg) were denatured at 37°C after diluting the samples with SDS-PAGE loading buffer. BBM proteins were fractionated on a 8% SDS-polyacrylamide gel as described previously (14). Proteins were then transferred at 350–450 mA over 1 h to a polyvinylidene difluoride membrane. The blot was blocked with Blotto (0.05% Tween 20 and 5% nonfat milk in PBS) for 1 h and then probed using primary antibody to NaPi-2a (1:1,000) overnight at 4°C (generous gifts from Drs. J. Biber and H. Murer, University of Zürich, Switzerland). The blots were washed extensively with Blotto and then incubated with the secondary anti-rabbit antibody at 1:10,000 dilution for 1 h. The blots were further washed with PBS containing 0.05% Tween 20. Enhanced chemiluminescence (Amersham Life Sciences) was used to detect bound antibody. Antibody to β-actin at 1:15,000 dilution was used to validate equal loading of the protein (Sigma Biochemicals). NaPi-2a protein abundance was quantified in relation to β-actin using Scion Image software (Scion).

RNA isolation and quantitative PCR. Kidneys were quickly harvested and the renal cortex was dissected. Total RNA was extracted using the GenElute mammalian total RNA kit as per their instruction manual (Sigma). Two micrograms of RNA were used to synthesize cDNA (reverse transcription) in a volume of 40 μl after DNase treatment (Invitrogen). The reverse transcription step was verified by GAPDH check. Quantitative PCR was performed using the iCycler PCR Thermal cycler (Bio-Rad). 28s was used as a housekeeping gene and the relative mRNA expression of 1α-hydroxylase (CYP27B1) and CYP24 genes was quantified using the method described by Vandesomple et al. (48). SYBR green master mix (Bio-Rad) was used for 28s and the primer sequence for 28S is (forward) 5-TTG AAA ATC CGG GGG AGA G-3 and (reverse) 5-ACA TTG TTC CAA CAT GCC AG-3. *Taq*Man gene expression assays and universal master mix (Applied Biosystems) were used for quantification of CYP27B1 and CYP24 genes per the manufacturer's protocol.

Statistical analysis. All the data are expressed as means ± SE. Student's *t*-test was used to assess the difference between two groups. Differences between multiple groups were assessed using ANOVA followed by a post hoc Student-Newman-Keuls test. A P value <0.05 was considered significant.

RESULTS

Baseline characteristics. We first studied the baseline characteristics of wild-type and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. We weighed the mice at 4 wk of age and compared their weights with their wild-type counterparts (19 ± 1 vs. 11 ± 0.9 g; wild-type vs. FGFR3$^{-/-}$FGFR4$^{-/-}$ mice; $P < 0.001$). The mice were weighed at 4 wk to ensure that FGFR3$^{-/-}$FGFR4$^{-/-}$ mice were indeed small in size as described previously (49). We next measured the baseline serum phosphorus, FGF23, PTH, and 1,25(OH)$_2$Vitamin D$_3$ levels in wild-type and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. Compared with the wild-type mice, FGFR3$^{-/-}$FGFR4$^{-/-}$ mice had lower serum phosphorus levels and lower renal cortical BBM NaPi-2a expression (Table 1 and Fig. 1). The lower serum phosphorus and NaPi-2a expressions were not due to PTH as the levels were comparable in the two groups of mice (Table 1). The serum 1,25(OH)$_2$Vitamin D$_3$ levels were elevated in the FGFR3$^{-/-}$

Table 1. *Baseline serum parameters in wild-type and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice*

	Wild-Type	FGFR3$^{-/-}$FGFR4$^{-/-}$
Serum phosphorus, mg/dl	8.7 ± 0.3 (n = 18)	6.9 ± 0.2* (n = 18)
Serum FGF23, pg/ml	88.5 ± 6.1 (n = 8)	221.5 ± 23.0* (n = 10)
Serum 1,25(OH)$_2$D$_3$, pmol/l	102.2 ± 14.8 (n = 8)	266.0 ± 34.0† (n = 11)
Serum PTH, pg/ml	141.9 ± 43.3 (n = 8)	141.0 ± 45.6 (n = 11)

Values are means ± SE. FGFR, fibroblast growth factor receptor; PTH, parathyroid hormone. *P < 0.001. †P = 0.001.

FGFR4$^{-/-}$ mice compared with the wild-type mice. We previously showed that FGFR3$^{-/-}$ mice had significantly elevated levels of 1,25(OH)$_2$Vitamin D$_3$ and the 1,25(OH)$_2$Vitamin D$_3$ levels tended to be higher in FGFR4$^{-/-}$ mice than the wild-type mice (14). These data are consistent with the hypothesis that both FGFR3 and FGFR4 are involved in the regulation of serum 1,25(OH)$_2$Vitamin D$_3$ levels (table 1) and compensatory mechanisms exit when one FGF receptor is deleted. As a likely result of elevated 1,25(OH)$_2$Vitamin D$_3$ levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice, FGF23 levels were elevated in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with the wild-type mice (table 1). The lower serum phosphorus levels were thus due to the elevated baseline FGF23 despite the higher levels of 1,25(OH)$_2$Vitamin D$_3$ levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with the wild-type mice. In summary, at baseline, FGFR3$^{-/-}$FGFR4$^{-/-}$ mice have lower serum phosphorus levels, elevated FGF23 levels, and elevated 1,25(OH)$_2$Vitamin D$_3$ levels compared with wild-type mice.

To further examine whether the effect of FGF23 on 1,25(OH)$_2$Vitamin D$_3$ was mediated via FGFR3 and FGFR4, we studied the effects of 4-day treatment with pharmacological doses of FGF23 on serum phosphorus, PTH, and 1,25(OH)$_2$Vitamin D$_3$ as well as renal cortical BBM NaPi-2a expression and renal cortical BBMV phosphate uptake. We first measured FGF23 levels to verify that the group that received FGF23 indeed had higher levels of FGF23. The levels of serum FGF23 ~12 h after the last dose were at least 10-fold higher in the group that received FGF23 compared with the vehicle group (wild-type, vehicle vs. FGF23, 105.6 ± 9.3 vs. 1,252 ± 140; FGFR3$^{-/-}$FGFR4$^{-/-}$ mice, vehicle vs. FGF23, 163.5 ± 18.4 vs. >1,680).

As expected, the serum phosphorus levels (Table 2) decreased after FGF23 administration in the wild-type mice, which was mediated in large part by the decrease in renal cortical BBM NaPi-2a expression (Fig. 2) and the resultant decrease in BBMV phosphate transport (Fig. 3). FGF23 administration to wild-type mice also caused the expected decrease in serum 1,25(OH)$_2$Vitamin D$_3$ levels (Table 2). The serum PTH levels did not change in the wild-type mice with FGF23 administration (Table 2). These results are comparable to the effects of FGF23 on wild-type mice in our previous studies (14).

FGF23 exhibited different effects on FGFR3$^{-/-}$FGFR4$^{-/-}$ mice than on the wild-type mice. The serum phosphorus levels were in fact higher in the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice after FGF23 administration (Table 2). Renal cortical BBM NaPi-2a expression and renal cortical BBMV phosphate transport increased after FGF23 administration in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice (Figs. 2 and 3). To determine the cause for the elevated serum phosphorus levels and increased BBMV phosphate transport after FGF23 administration, serum PTH and 1,25(OH)$_2$Vitamin D$_3$ levels were measured. There was no effect of FGF23 on serum 1,25(OH)$_2$Vitamin D$_3$ levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice as shown in Table 2. However, serum PTH levels were significantly decreased in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice in response to FGF23 (Table 2). Thus, the increased phosphorus levels after FGF23 administration to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice are likely due to the decrease in serum PTH levels.

Finally, we examined the effects of FGF23 on CYP27B1 and CYP24 mRNA expression (Table 2). FGF23 administration to wild-type mice resulted in a decrease in CYP27B1 mRNA expression while mRNA expression of CYP24 increased. In response to FGF23, mRNA expression of both CYP27B1 and CYP24 increased in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. Thus, FGF23 administration to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice resulted in an increase in serum phosphorus levels and increased renal cortical BBM NaPi-2a expression, an increase in BBM phosphate transport, and a decrease in serum PTH levels, but it had no effect on serum 1,25(OH)$_2$Vitamin D$_3$ levels.

DISCUSSION

The current study was designed to examine the FGF receptors responsible for the FGF23-mediated decrease in serum 1,25(OH)$_2$Vitamin D$_3$ levels. We compared wild-type mice to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice and at baseline found elevated levels of 1,25(OH)$_2$Vitamin D$_3$ levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice despite higher baseline serum FGF23 levels. In addition, there was no decrease in serum 1,25(OH)$_2$Vitamin D$_3$ levels

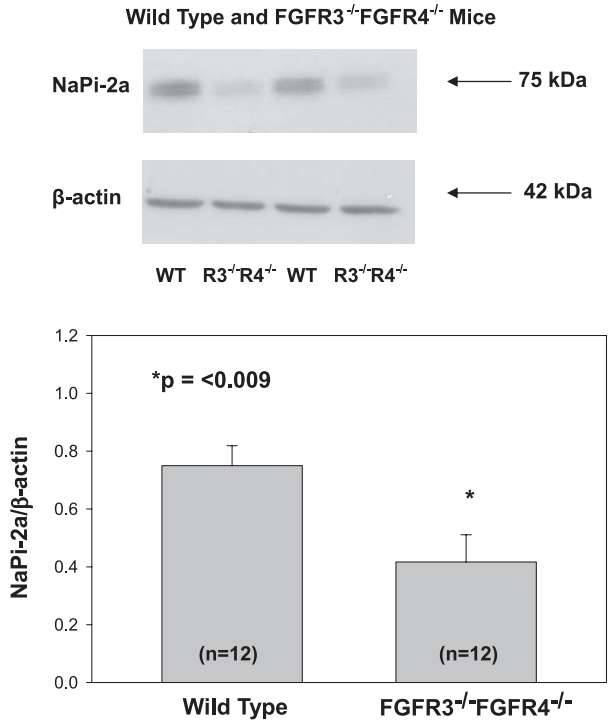

Fig. 1. Renal cortical brush-border membrane (BBM) NaPi-2a expression in wild-type and fibroblast growth factor receptor (FGFR)3$^{-/-}$FGFR4$^{-/-}$ mice at baseline. NaPi-2a expression normalized to β-actin analyzed by immunoblotting is shown here. BBM NaPi-2a expression was lower in the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. Bars represent means and SE.

Table 2. *Serum parameters in wild-type mice and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice in response to pharmacological doses of FGF23*

	Wild-Type Mice		FGFR3$^{-/-}$FGFR4$^{-/-}$ Mice	
	Vehicle	FGF23	Vehicle	FGF23
Serum phosphorus, mg/dl	8.3 ± 0.4 (n = 12)	5.6 ± 0.2* (n = 12)	6.8 ± 0.4§ (n = 12)	11.5 ± 0.6* (n = 12)
Serum 1,25(OH)$_2$D$_3$, pmol/l	77.4 ± 13.0 (n = 11)	19.1 ± 3.7† (n = 11)	173.4 ± 32.7§ (n = 11)	219.7 ± 56.5 (n = 11)
Serum PTH, pg/ml	86.8 ± 16.2 (n = 9)	88.3 ± 9.5 (n = 9)	126.8 ± 25.6 (n = 9)	21.3 ± 5.1† (n = 9)
1α-Hydroxylase (CYP27B1) mRNA expression/28 s	15.6 ± 3.3 (n = 9)	4.3 ± 0.5† (n = 9)	27.4 ± 7.5§ (n = 9)	108.4 ± 31† (n = 9)
24-Hydroxylase (CYP24) mRNA expression/28 s	9.6 ± 2.1 (n = 9)	182.9 ± 47.1† (n = 9)	70.3 ± 6.1§ (n = 9)	512.9 ± 63.9† (n = 9)

Values are means ± SE. *P < 0.001 vehicle vs. FGF23. †P < 0.05 vehicle vs. FGF23. §P < 0.05 wild-type postvehicle vs. FGFR3$^{-/-}$FGFR4$^{-/-}$ postvehicle.

in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice with administration of pharmacologic doses of FGF23 while serum 1,25(OH)$_2$Vitamin D$_3$ levels decreased in the wild-type mice. We also found that administration of FGF23 caused the expected decrease in serum phosphorus levels in wild-type mice (14, 42, 43) but caused an increase in serum phosphorus in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice due to a decrease in serum PTH levels. Thus, this study shows that FGFR3 and FGFR4 are the critical receptors involved in the FGF23-mediated decrease in serum 1,25(OH)$_2$Vitamin D$_3$ levels and a decrease in PTH can overcome pharmacologic doses of FGF23 to regulate serum phosphorus levels.

In the present study, we found that administration of FGF23 to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice resulted in a decrease in PTH levels while PTH levels remained unchanged in wild-type mice. Parathyroid glands express both FGFR1 and FGFR3 and FGF23 has been shown to inhibit PTH secretion (6). In wild-type mice, PTH levels remain unchanged due to the opposing effects of an increase in FGF23 and a decrease in 1,25(OH)$_2$Vitamin D$_3$ levels on PTH secretion. However, in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice, the 1,25(OH)$_2$Vitamin D$_3$ levels were not affected by FGF23 administration and the inhibitory effect of FGF23 on PTH was unopposed.

In wild-type mice, we found that FGF23 administration resulted in a decrease in CYP27B1 mRNA and an increase in CYP24 mRNA expression as previously shown (37). In FGFR3$^{-/-}$FGFR4$^{-/-}$ mice, both CYP27B1 and CYP24 mRNA expression increased after FGF23 administration. Lack of correlation between CYP27B1 mRNA expression and 1,25(OH)$_2$Vitamin D$_3$ levels has been previously shown in *Hyp* mice (2, 27, 41). There might be other factors that regulate mRNA expression of CYP27B1 and CYP24 independent of FGF23 in our study. While we did not examine the enzyme activity, the net result of FGF23 administration to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice was that the serum 1,25(OH)$_2$Vitamin D$_3$ levels remain unchanged.

FGFs bind to FGFRs that are encoded by four *Fgfr* genes (*Fgfr1–4)* (5, 22). Alternative splicing of FGFR 1–3 results in "b" and "c" isoforms. Thus, seven FGFR proteins are present and they have distinct ligand-binding specificity (33, 35, 39). The FGF family of growth factors binds to FGFRs in a

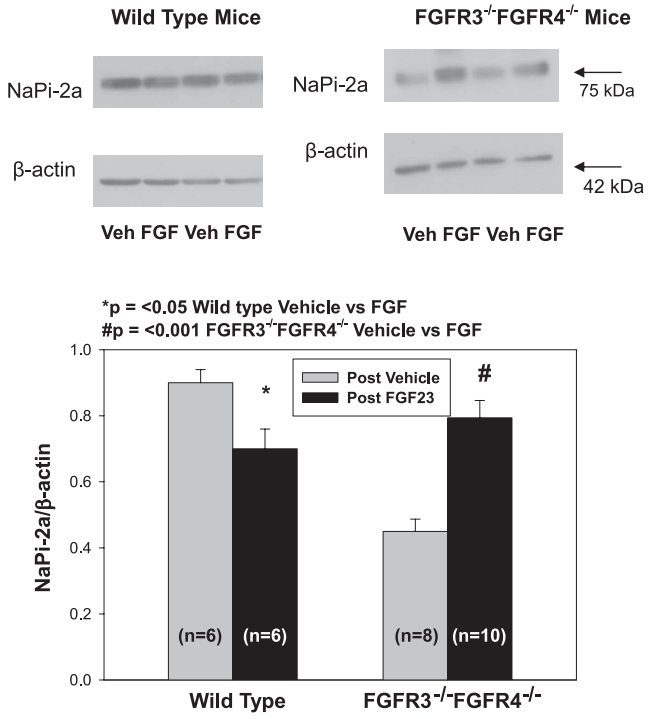

Fig. 2. Acute effects of FGF23 on renal cortical BBM NaPi-2a expression in wild-type and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. NaPi-2a expression normalized to β-actin performed by immunoblotting is shown here. In response to FGF23, as anticipated, renal cortical BBM NaPi-2a expression decreased significantly in the wild-type mice. In FGFR3$^{-/-}$FGFR4$^{-/-}$ mice, upon FGF23 administration, keeping with the serum phosphorus levels, renal cortical BBM NaPi-2a expression increased. Bars represent means and SE.

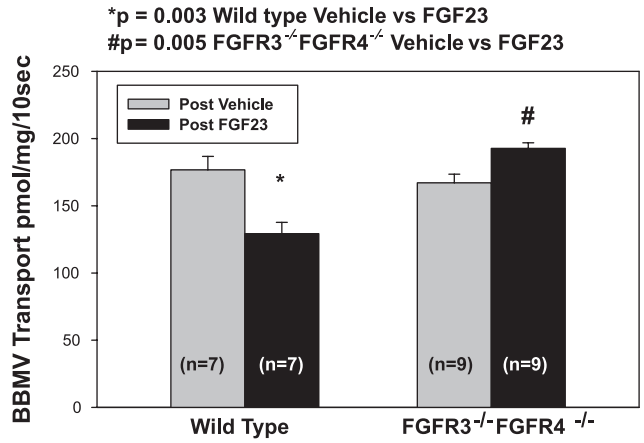

Fig. 3. Acute effects of FGF23 on renal cortical BBM vesicle (BBMV) phosphate transport in wild-type and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. Renal BBMVs were examined for phosphate transport using ^{32}P as described in METHODS. To 100 μg of BBMV loaded in an intravesicular buffer, an extravesicular buffer containing ^{32}P was added. Phosphate transport was terminated using ice-cold stop solution. As shown in this graph, the BBMV phosphate transport is decreased in response to FGF23 in the wild-type mice but the phosphate transport is increased in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. This is in keeping with the serum phosphorus levels and BBM NaPi-2a expression.

heparin/heparan sulfate-dependent manner and it is essential for their stable interaction to generate intracellular signaling (10, 17, 23). However, FGF23 is an endocrine FGF that has a low affinity to heparin/heparan sulfate thus facilitating its actions as an endocrine FGF (18, 51).

The similarity of *Klotho*-null mice and *FGF23*-null mice established that both Klotho and FGF23 act in a common metabolic pathway in regulating phosphate homeostasis (25, 44, 45). Recent in vitro studies demonstrated the need for Klotho as an important coreceptor in the interaction of FGF23 and FGFRs. Controversy exists regarding specific receptor(s) for FGF23. One group of investigators demonstrated that FGF23 binds to FGFR1c, FGFR3c, and FGFR4 in the presence of Klotho employing cell lines transfected with Klotho and different FGFRs (26). Another study showed that FGF23 binds effectively only to the FGFR1c Klotho complex (47). These studies were primarily in vitro studies that did not provide information regarding the FGFRs responsible for the various actions of FGF23 in vivo including phosphate homeostasis and regulation of 1,25(OH)$_2$Vitamin D$_3$ levels. Previously, it was thought that Klotho expression was primarily in the distal convoluted tubule, choroid plexus, pituitary gland, parathyroid gland, and reproductive organs (28, 32). Recently, Hu et al. (20) showed that Klotho mRNA is present in the proximal tubule and that Klotho protein is not only expressed in the proximal tubular cells, but also in the lumen. Thus, Klotho made by the proximal tubule presumably acts as the coreceptor for FGF23 signaling in the proximal tubule to regulate NaPi-2a and NaPi-2c. It is also possible that there is a distal convoluted tubule to proximal tubule cross talk as there is a higher expression of Klotho in the distal convoluted tubule, but the cross talk processes/factors still need to be elucidated (11).

We have been interested in identifying the receptors responsible for the various actions of FGF23 in vivo. Using FGFR-null mice, we previously showed that FGFR1 is the predominant receptor mediating the hypophosphatemic action of FGF23 in vivo (14). We showed that only FGFR1, 3, and 4 are present in the proximal tubule and thus we studied FGFR1$^{-/-}$, FGFR3$^{-/-}$, and FGFR4$^{-/-}$ mice. As FGFR1$^{-/-}$ mice are embryonically lethal, we studied FGFR1 conditional null mice where FGFR1 is deleted from the metanephric mesenchyme (38). Baseline serum phosphorus levels were not different in the individual receptor-null mice. This contrasts the low-serum phosphorus levels seen in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with the wild-type mice. This suggests that compensatory mechanisms exist to maintain normal serum phosphorus levels at baseline in the individual FGFR-null mice but deletion of these two receptors alters the compensatory mechanisms. However, upon administration of pharmacological doses of FGF23, wild-type, FGFR3$^{-/-}$, and FGFR4$^{-/-}$ mice demonstrated a decrease in serum phosphorus along with a decrease in the expression of NaPi-2a and NaPi-2c. In response to FGF23, serum phosphorus levels were unchanged in the conditional FGFR1$^{-/-}$ mice and the expression of NaPi-2a and NaPi-2c was also unchanged. This indicated that FGFR1 is the principal receptor that regulates NaPi-2a and NaPi-2c in the proximal tubule. Liu et al. (29) also found that the deletion of either FGFR3 or FGFR4 in a mouse model of X-linked hypophosphatemic rickets (*Hyp* mouse) did not correct the disturbances in phosphate homeostasis in the *Hyp* mouse, suggesting that FGFR3 and FGFR4 individually were not the receptors regulating phosphate homeostasis.

Recently, Li et al. (27) showed that the deletion of FGFR3 and FGFR4 in *Hyp* mice resulted in a partial rescue of the *Hyp* phenotype. *Hyp* mice have elevated levels of FGF23, hypophosphatemia, inappropriately low levels of 1,25(OH)$_2$Vitamin D$_3$ and have rickets/osteomalacia (30). As in the current study, Li et al. compared FGFR3$^{-/-}$FGFR4$^{-/-}$ mice with wild-type mice. At baseline, Li et al. found elevated serum FGF23 levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with wild-type mice but unlike the current study, their results were not statistically significant (27). The higher FGF23 levels in our study were likely responsible for the decrease in serum phosphorus levels in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with the wild-type mice in our study. Li et al. found that FGF23 mRNA expression in the bone also tended to be higher in the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with wild-type mice although not statistically significant. Li et al. also found that FGFR3$^{-/-}$FGFR4$^{-/-}$ mice have comparable PTH levels but elevated 1,25(OH)$_2$Vitamin D$_3$ levels compared with wild-type mice at baseline. The elevated levels of 1,25(OH)$_2$Vitamin D$_3$ in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with wild-type mice and their results showing that 1,25(OH)$_2$Vitamin D$_3$ levels were comparable in Hyp/FGFR3$^{-/-}$FGFR4$^{-/-}$ mice and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice are consistent with our results demonstrating that FGFR3 and FGFR4 together are the two FGF receptors involved in the regulation of serum 1,25(OH)$_2$Vitamin D$_3$ levels. However, there were differences in comparing the Hyp/FGFR3$^{-/-}$FGFR4$^{-/-}$ mice in Li et al.'s study to our study where we administered exogenous FGF23 to FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. Li et al. found an increase in NaPi-2a gene and protein expression and increased serum phosphorus levels in Hyp/FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with *Hyp* mice but not compared with FGFR3$^{-/-}$FGFR4$^{-/-}$ mice (27). In addition, Li et al. showed increased PTH levels in Hyp/FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with wild-type, *Hyp*, and FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. These results differ from our findings where we find that FGF23-treated FGFR3$^{-/-}$FGFR4$^{-/-}$ mice had an increase in serum phosphorus and NaPi-2a as well as BBMV phosphate uptake likely due to the reduction in PTH.

We previously showed that upon administration of FGF23 to the individual FGFR-null mice and their wild-type counterparts, all groups of mice had a 70–90% reduction in serum 1,25(OH)$_2$Vitamin D$_3$ levels. These data established that a single receptor is not responsible for the regulation of 1,25(OH)$_2$Vitamin D$_3$ levels by FGF23. In addition, these data demonstrated that one receptor compensates for the deletion of the other in FGFR single knockout mice. In the present study, PTH levels were unchanged at baseline in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice compared with wild-type mice and thus did not contribute to the elevated levels of 1,25(OH)$_2$Vitamin D$_3$. In addition, upon FGF23 administration, serum 1,25(OH)$_2$Vitamin D$_3$ levels did not decrease in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice even though PTH levels were suppressed, once again demonstrating that PTH did not contribute to the elevated levels of 1,25(OH)$_2$Vitamin D$_3$ in FGFR3$^{-/-}$FGFR4$^{-/-}$ mice. In summary, this study finds that baseline serum phosphorus levels are lower in the FGFR3$^{-/-}$FGFR4$^{-/-}$ mice due to elevated FGF23 levels confirming the role of FGFR1 in regulating phosphate homeostasis. Furthermore, the current study indicates that

FGF23 regulates the decrease in 1,25(OH)$_2$Vitamin D$_3$ via FGFR3 and FGFR4. Finally, these data show that even in the presence of elevated FGF23 levels and unchanged 1,25(OH)$_2$Vitamin D$_3$ levels, serum phosphorus levels are higher when PTH levels are suppressed. Thus, a decrease in PTH can overcome a 10-fold increase in FGF23 levels to increase phosphate reabsorption. In conclusion, FGF23 regulates its different actions via different FGF receptors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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