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FGF23-Induced Hypophosphatemia Persists in *Hyp* Mice Deficient in the WNT Coreceptor *Lrp6*

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Abstract

Deregulated phosphate homeostasis can lead to a wide range of disorders, including myopathy, cardiac dysfunction, and skeletal abnormalities. Therefore, characterization of the molecular regulation of phosphate metabolism is of pathophysiological and clinical significance. *Hyp* mouse is the model for human X-linked hypophosphatemia which is due to mutations that inactivate the endopeptidases of the X chromosome (PHEX). PHEX inactivation leads to increased serum levels of fibroblast growth factor 23 (FGF23), a phosphaturic hormone that induces excessive renal phosphate excretion and severe hypo-phosphatemia. The expression of WNT signaling components is increased in *Hyp* mice. To determine the potential role of WNT signaling in FGF23-mediated hypophosphatemia, we cross-bred *Hyp* mice with mice deficient in the WNT coreceptor low-density lipoprotein receptor-related protein 6 (*Lrp6*) to generate *Hyp* and *Lrp6* double mutant mice (*Hyp/Lrp6*). Like *Hyp* mice, *Hyp/Lrp6* double mutants maintained high serum levels of FGF23, and accordingly exhibited hypophosphatemia to the same degree as the *Hyp* mice did, indicating that genetically reducing WNT signaling does not impact FGF23-induced phosphaturia. Moreover, similar to *Hyp* mice, the *Hyp/Lrp6* double mutants also exhibited reduced mineralization of the bone, further supporting that reduced WNT signaling does not affect the chronic phosphate wasting caused by excess FGF23 in these mice. In further support of our finding, injection of bioactive FGF23 protein into *Lrp6* mutant mice reduced serum phosphate levels to a similar degree as FGF23 injection into wild-type mice. Our in vivo studies provide genetic and pharmacological evidence for a WNT-independent function of FGF23 in the regulation of phosphate homeostasis.

Determining the molecular regulation of phosphate metabolism is of great pathophysiological and clinical significance, as altered phosphate balance can induce a wide range of disorders, including myopathy, cardiac and skeletal dysfunctions [1–6]. Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone that plays a key role in the regulation of phosphate metabolism [7–12]. This is reflected by the profound clinical manifestations of either abnormally high or abnormally low bioactivity of FGF23. Increased FGF23 activity causes renal phosphate wasting and leads to the development of hypophosphatemic rickets and osteomalacia, whereas reduced FGF23 activity results in enhanced renal phosphate retention and manifests in hyperphosphatemic rickets and ectopic calcifications, among

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others [6–9]. Recent studies have pointed to a possible role of WNT signaling in FGF23-mediated functions, and have also suggested that WNT signaling can stimulate the promoter activity of the FGF23 gene in osteoblasts [13, 14].

The canonical pathway of WNT signaling has been implicated in numerous essential biological functions, ranging from embryogenesis to organogenesis [15–17]. In mammals, canonical WNT signaling is initiated through the binding of WNT to Frizzled receptors and the low-density lipoprotein receptor-related protein-5 or protein-6 (Lrp5/6), to stabilize β-catenin, which induces downstream target genes via the T cell factor and lymphoid enhancer binding factor [14–18]. According to the cellular requirement, the secreted molecule Dickkopf 1 (Dkk-1) can fine-tune the WNT signaling activity by a negative feedback mechanism [18]; Dkk-1 antagonizes WNT signaling by disrupting the molecular interaction between WNT and Lrp5/6 [19–21]. Studies using genetic or pharmacological approaches have convincingly shown that inactivating the function of either of the two WNT coreceptors, Lrp5 or Lrp6, can significantly reduce WNT activities to influence bone growth and maintenance [22, 23]. Disruption of *Lrp6* activity in mice resulted in developmental anomalies [24], including limb patterning defects. Importantly, some of the *Lrp6* mutant phenotypes are similar to the changes observed in embryos lacking *Wnt1*, *Wnt3a* and *Wnt7a* activity [25–27]. Since homozygous *Lrp6*^{−/−} mice are embryonic lethal and display numerous phenotypes including limb defects and axial truncation [24], we used heterozygous *Lrp6*^{+/-} mice as a model system to reduce WNT activities from *Hyp* mice.

PHEX is a phosphate-regulating gene with homology to endopeptidases of the X chromosome, and is a membrane-bound ectoenzyme expressed in bone cells. Mutations in the *PHEX* gene are associated with X-linked hypophosphatemia, a renal phosphate wasting disorder in humans [28–30]. *Hyp* mice are the murine model of X-linked hypophosphatemia, with an inactivating mutation in the *PHEX* gene. Similarly as in the human disease, increased activity of FGF23 in the *Hyp* mice leads to hypophosphatemia, due to excessive urinary loss of phosphate [29, 31–33]; such renal loss of phosphate eventually leads to rickets and/or osteomalacia. In this study, we generated and characterized *Hyp/Lrp6* double mutant mice to determine the in vivo role of WNT signaling in FGF23-mediated systemic regulation of phosphate homeostasis.

Materials and Methods

Generation of *Hyp/Lrp6* Double Mutant Mice

We crossbred *Hyp* mutants (Jackson Laboratory, Me., USA) with heterozygous *Lrp6* mutants to obtain *Hyp/Lrp6* double mutants. Routine PCR using genomic DNA extracted from tail clips was performed for genotyping the various groups of mice [31, 34, 35]. Since homozygous *Lrp6*^{−/−} are embryonic lethal and display numerous phenotypes including limb defects and axial truncation [24], we used heterozygous *Lrp6*^{+/-} mice for our study. The *Lrp6*^{+/-} mice have a reduction in bone density and are viable, fertile and usually do not display any obvious phenotype, except occasional kinked/curly tail, spina bifida and imperforated vagina. The following primer sequences were used to genotype *Lrp6* mutant mice (LRP6 forward primer: CAG GCA TGT AGC CCT TGG AG; LRP6 reverse primer: ACT ACA AGC CCT GCA CTG CC and LRP6 Neo reverse primer: GTA GAG TTC CCA GGA GGA GCC). The PCR product for wild-type *Lrp6* mice was detected as 285 bp, for heterozygous *Lrp6* as 385 and 285 bp, and for homozygous *Lrp6* as 385 bp. Mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the institution's subcommittee on animal care (IACUC). The total body weight of wild-type, *Hyp*, *Lrp6*^{+/-} and *Hyp/Lrp6* double mutant mice were taken weekly starting at 3 weeks of age until 20 weeks. At least five or more mice from each genotype were sacrificed at around 8 weeks and long bones, including

femur and tibia were isolated for radiological imaging. Plain radiographs were taken (29 kV for 10 s) using an X-ray apparatus (Micro 50; Micro Focus Imaging). Films were scanned (scanjet 5530; Hewlett-Packard) and saved.

Serum Measurements

Blood was obtained from wild-type, *Hyp*, *Lrp6*^{+/-} and *Hyp/Lrp6* double mutant mice by cheek pouch bleeding at different time points. Serum was isolated by centrifugation at 3,000 *g* for 10 min and was stored at -80°C until use. Serum phosphorus and calcium were determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test and the Calcium (Arsenazo) LiquiColor Test, respectively. The serum level of FGF23 was measured with ELISA using a commercial kit (Kainos Laboratories, Tokyo, Japan), as described in a previous publication [31].

Quantitative Real-Time PCR

Total RNA was isolated from the kidneys of wild-type, *Hyp*, *Lrp6*^{+/-} and *Hyp/Lrp6* double mutant mice, and used to measure relative expression of *NaPi-2a* mRNA by real-time PCR, as described earlier. Real-time PCR was performed in duplicate, and all reactions were controlled by standards, as detailed in earlier studies [36, 37]. The relative quantity of mRNA was calculated by normalizing the CT (threshold cycle value) of *NaPi-2a* to the CT of the housekeeping gene GAPDH.

Treatment of Mice with Recombinant Bioactive FGF23

Wild-type and *Lrp6*^{+/-} mutant mice were injected intraperitoneally with vehicle, or recombinant bioactive FGF23 protein (10 µg per mouse) [38]. Blood was collected by cheek-pouch bleeding prior to the injections and at 9 h postinjection. Isolated serum was used to determine phosphate levels in an animal pre- and postinjection, using the Stanbio Liqui-UV kit [39].

Statistics

Statistically significant differences between groups were evaluated by either Student's *t* test or Mann-Whitney *U* test for a comparison between the groups. All values are expressed as mean ± SE. *p* < 0.05 was considered to be statistically significant. All analyses were performed using StatView.

Results

Generation of Hyp/Lrp6 Double Mutant Mice

To elucidate the consequences of reducing WNT activity from *Hyp* mice in vivo, we generated a new *Hyp* mouse model deficient in *Lrp6* function (*Hyp/Lrp6* double mutant mice) (fig. 1a). The generated double mutant mice were viable throughout their adulthood but were smaller than wild-type mice (fig. 1a). At birth, *Hyp/Lrp6* double mutant mice were indistinguishable from littermates of other genotypes. However, at 8 weeks of age, double mutant mice (24.1 ± 0.3 g) were smaller significantly (*p* < 0.01) than wild-type mice (29.2 ± 0.8 g) and *Lrp6*^{+/-} mice (28.5 ± 2.2 g), but similar in weight as *Hyp* mice (23.5 ± 0.5 g) (fig. 1a, b). At 16 weeks of age, *Hyp/Lrp6* double mutant mice (28.9 ± 0.4 g), like *Hyp* mice (26.6 ± 0.5 g), still lagged behind their wild-type (38.9 ± 2.0 g) and *Lrp6*^{+/-} (35.1 ± 3.8 g) littermates with regard to body weight (fig. 1b).

Serum Phosphate and Calcium Levels of Hyp/Lrp6 Double Mutant Mice

We measured serum phosphate and calcium levels in the various genotypes. As expected, *Hyp* mice showed reduced serum phosphate levels (4.3 ± 0.2 mg/dl) compared to wild-type

mice (6.8 ± 0.3 mg/dl) at around 6–10 weeks of age. Serum phosphate of *Hyp/Lrp6* double mutant mice (3.9 ± 0.4 mg/dl) was similarly low as that of *Hyp* mice at this age, whereas serum phosphate concentrations of age-matched *Lrp6*^{+/-} mice (6.0 ± 0.2 mg/dl) were within the normal range (fig. 2a, left panel). A similar pattern of serum phosphate levels was also noted in mice of 11 to 16 weeks of age. Compared to wild-type mice (7.0 ± 0.4 mg/dl), *Hyp/Lrp6* double mutant mice were hypophosphatemic at 11 to 16 weeks of age, as were *Hyp* mice. By contrast, *Lrp6*^{+/-} mice remained normophosphatemic (6.6 ± 0.3 mg/dl). Importantly, the low serum phosphate levels in *Hyp/Lrp6* double mutant mice (4.4 ± 0.1 mg/dl) were similar to those seen in *Hyp* mice (5.0 ± 0.5 mg/dl) at 11–16 weeks of age (fig. 2a, right panel). As in *Hyp* mice, renal expression of NaPi-2a was reduced in *Hyp/Lrp6* double mutant mice compared to wild-type littermates, which likely accounted for the hypophosphatemia (fig. 5). There was no change in NaPi-2a expression in the kidneys of *Lrp6*^{+/-} mice compared to wild-type mice (fig. 5).

Concomitant with the hypophosphatemia, *Hyp/Lrp6* double mutant mice (7.3 ± 0.3 mg/dl), like *Hyp* mice (7.5 ± 0.2 mg/dl), had slightly lower serum calcium levels than wild-type mice (8.1 ± 0.1 mg/dl) at around 6–10 weeks of age (fig. 2b, left panel). By contrast, the serum calcium level of age-matched *Lrp6*^{+/-} mice was within the normal range (8.3 ± 0.1 mg/dl; fig. 2b, left panel), as expected based on their normophosphatemic status. Reduced serum calcium and phosphate levels both in *Hyp/Lrp6* double mutant mice and in *Hyp* mice were associated with defective skeletal mineralization compared to wild-type littermates (fig. 3). In *Lrp6*^{+/-} mice, bone mineralization was somewhat reduced compared to wild-type mice (fig. 3). Compared to wild-type mice, the bones of *Hyp/Lrp6* double mutant mice were short and thick, and showed the typical features of rickets such as widening of the epiphysis and cupping of the metaphysis, all of which resembling the phenotype of *Hyp* mice, except that the skeletal anomalies were more marked in *Hyp/Lrp6* double mutant mice than in *Hyp* mice (fig. 3). No such bone phenotype was seen in the mice deficient in *Lrp6* only (fig. 3).

Serum FGF23 Levels in *Hyp/Lrp6* Double Mutant Mice

As expected, serum FGF23 levels were extremely high in the *Hyp* mice ($1,989.43 \pm 139.6$ pg/ml) at 8 weeks of age compared to wild-type littermates (137.2 ± 9.1 pg/ml; fig. 4). *Hyp/Lrp6* double mutant mice showed a similar increase of serum FGF23 ($1,997.2 \pm 139.7$ pg/ml) as *Hyp* mice at this age (fig. 4). In mice deficient in *Lrp6* only, serum levels of FGF23 were within normal range (111.9 ± 11.7 pg/ml; fig. 4). We have previously shown that increased serum levels of FGF23 are associated with the hypophosphatemia in *Hyp* mice, since serum phosphate can be increased by blocking FGF23 activity in these mice [40]. Thus, the lack of a difference between *Hyp/Lrp6* double mutant mice and *Hyp* mice in regard to serum FGF23 and concomitant hypophosphatemia suggests that the phosphaturic activity of FGF23 is a WNT-independent process.

Effects of FGF23 Protein Injection into *Lrp6*-Deficient Mice

To further explore whether suppressing WNT signaling activity affects FGF23-mediated regulation of phosphate metabolism, we injected recombinant bioactive FGF23 protein into *Lrp6*-deficient mice. FGF23 administration to wild-type mice served as a control. As expected, exogenous FGF23 significantly reduced serum phosphate levels in wild-type mice (pre-injection 6.7 ± 0.2 mg/dl vs. postinjection 5.0 ± 0.4 mg/dl; $p < 0.05$; fig. 6). Similarly, FGF23 treatment led to a significant decrease of serum phosphate levels in *Lrp6*-deficient mice (preinjection 6.8 ± 0.3 mg/dl vs. postinjection 5.5 ± 0.3 mg/dl; $p < 0.05$; fig. 6). There was no major difference in the magnitude of the effect between *Lrp6* mice and wild-type mice, which suggests that suppressing WNT signaling activity by genetically reducing *Lrp6* activity does not affect the phosphaturic activity of FGF23.

Discussion

The expression of WNT signaling components is increased in *Hyp* mice [14], suggesting a potential role for WNT signaling in the regulation of phosphate homeostasis by FGF23 [31, 32]. We have utilized two complementary approaches to determine the potential role of WNT signaling in the phosphaturic activity of FGF23 in vivo. The first is the genetic approach of generating *Hyp/Lrp6* double mutant mice by ablating *Lrp6* function from *Hyp* mice. The second is the pharmacological approach of treating *Lrp6*^{+/-} mice with bioactive FGF23 protein and measuring its effects on phosphate metabolism in reduced of WNT signaling activity.

Hyp mice produce excessive amounts of FGF23 in the bone, leading to accumulation of FGF23 in serum. Such increased serum levels of FGF23 induce chronic hypophosphatemia in *Hyp* mice, owing to persistent suppression of phosphate reabsorption in the renal proximal tubule [29, 31–33]. Suppressing WNT signaling by reducing *Lrp6* function from *Hyp* mice did not change the systemic phosphate metabolism. Specifically, the hypophosphatemia was not altered by ablating *Lrp6* function from *Hyp* mice, suggesting that genetic manipulation of WNT signaling cannot affect phosphate metabolism in these mice. Further studies are needed to rule out the compensatory effects of other components of WNT signaling in *Hyp/Lrp6* double mutant mice. We then tested whether suppressing WNT signaling by ablating *Lrp6* function from otherwise normal mice affected the phosphaturic activity of exogenous FGF23. Remarkably, exogenous FGF23 exhibited a similar phosphaturic effect in *Lrp6*-deficient mice as in wild-type littermates, which clearly suggests that suppressing WNT signaling activity does not affect the regulation of systemic phosphate metabolism by FGF23. Taken together, our in vivo results suggest that the phosphaturic activity of FGF23 is a WNT-independent process.

The in vivo mechanism by which FGF23 exerts its phosphate lowering functions requires further investigation, although α -Klotho plays a vital role in such regulation. In support of such a role, the severe FGF23-induced hypophosphatemic phenotype of *Hyp* mice can be reversed to hyperphosphatemia when α -Klotho function is eliminated from *Hyp* mice. In other words, in spite of significantly increased serum levels of FGF23, without α -Klotho function, FGF23 activity is disabled [31]. Similarly, injection of bioactive FGF23 protein into either α -*klotho* knockout mice or *Fgf23*/ α -*klotho* double knockout mice fails to lower serum phosphate levels, whereas in wild-type and *Fgf23* knockout mice, exogenous FGF23 reduces serum phosphate levels [35]. Together, these results provide compelling evidence that FGF23 does not have an α -Klotho-independent role in the systemic regulation of phosphate homeostasis. Although recent studies have established that FGF23 forms a ternary complex with cognate FGF receptor and α -Klotho to exert its phosphate-lowering effects [31, 35, 40–42], the signaling network involved in mediating the phosphaturic activity of FGF23 requires additional study. Using *Hyp* mice as an in vivo model system, our results of this study indicate that FGF23 can exert its phosphate lowering effects without involving WNT signaling. Despite the crucial importance of maintaining physiological mineral ion balance, our knowledge of the in vivo coordinated regulations of the FGF23- α -Klotho system is limited and requires additional carefully designed studies.

In summary, our pharmacological and genetic manipulation studies demonstrate that the phosphate-lowering effect of FGF23 is a WNT-independent process, particularly in *Hyp* mice lacking *PHEX* activities. Additional studies are needed to determine the signaling network involved in the regulation of FGF23-mediated phosphate metabolism. The *Hyp/Lrp6* double mutant mice we have generated provide a unique in vivo tool to study WNT-independent downstream signaling events exerted by FGF23. Detailed studies to determine the molecular interactions among FGF23, FGF receptor and α Klotho, and downstream

signaling targets will provide important insights into the physiologic regulation of phosphate metabolism and pathologic complications, arising from its abnormal regulation [43–45].

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Fig. 1. Gross phenotype of *Hyp/Lrp6* double mutant mice. **a** Gross appearance of male wild-type (WT), *Hyp*, *Lrp6*^{+/-}, and *Hyp/Lrp6* double mutant mice at 8 weeks of age. **b** Growth curve of WT (n = 6), *Hyp* (n = 8), *Lrp6*^{+/-} (n = 7) and *Hyp/Lrp6* double mutant mice (n = 5). Note that the physical appearance of *Hyp/Lrp6* double mutant mice is identical with that of *Hyp* mice; in particular, mice of these two genotypes are smaller in size than WT or *Lrp6*^{+/-} mice. The statistical analysis of each time point is provided in figure 5.

Fig. 2.

Serum phosphate and serum calcium in *Hyp/Lrp6* double mutant mice. **a** Serum phosphate levels of wild-type (WT), *Hyp*, *Lrp6*^{+/-}, and *Hyp/Lrp6* double mutant mice at 6–10 weeks and 11–16 weeks of age. Note that the serum phosphate levels of *Hyp/Lrp6* double mutant mice are significantly below the normal level, and are as low as those of *Hyp* mice. For example, at 6–10 weeks of age, serum phosphate of *Hyp/Lrp6* double mutant mice is 3.9 ± 0.4 (n = 7) compared to 4.3 ± 0.2 in *Hyp* mice (n = 9) and 6.8 ± 0.3 in WT mice (n = 7). **b** Serum calcium levels of WT, *Hyp*, *Lrp6*^{+/-}, and *Hyp/Lrp6* double mutant mice at 6–10 weeks and 11–16 weeks of age. Note that the serum calcium of *Hyp/Lrp6* double mutant mice (7.3 ± 0.3 , n = 7) is slightly lower than normal (8.1 ± 0.14 , n = 7) at 6–10 weeks of age, as also seen in *Hyp* mice (7.5 ± 0.2 , n = 9). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. WT; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. *Lrp6*^{+/-}.

Fig. 3.

X-ray analysis of bone tissue in *Hyp/Lrp6* double mutant mice. Shown are X-ray images of femur and tibia from male wild-type (WT), *Hyp*, *Lrp6*^{+/-}, and *Hyp/Lrp6* double mutant mice at 8 weeks of age. Like *Hyp* mice, the *Hyp/Lrp6* double mutant mice show reduced skeletal mineralization of bone with the ricketic widening of the growth plate. Please note that the skeletal anomalies are more pronounced in *Hyp/Lrp6* double mutant mice than in *Hyp* mice. Scale bar = 5 mm.

Fig. 4.

Serum FGF23 in *Hyp/Lrp6* double mutant mice. Average levels of serum FGF23 in wild-type (WT), *Hyp*, *Lrp6*^{+/-} and *Hyp/Lrp6* double mutant mice at 8 weeks of age are shown. Note that serum FGF23 of *Hyp/Lrp6* double mutant mice (1,997 ± 139 pg/ml, n = 5) is elevated by nearly 20-fold above normal (137 ± 9 pg/ml, n = 5). The increase in serum FGF23 is similar in magnitude to that seen in *Hyp* mice (1,989 ± 139 pg/ml, n = 5). ***p < 0.001 vs. WT, ###p < 0.001 vs. *Lrp6*^{+/-}.

Fig. 5.

Renal NaPi-2a gene expression in *Hyp/Lrp6* double mutant mice. Shown are mRNA levels of NaPi-2a in the kidneys of *Hyp*, *Lrp6*^{+/-}, and *Hyp/Lrp6* double mutant mice relative to those of wild-type (WT) mice. For each tissue sample, NaPi-2a mRNA expression was normalized to that of the house-keeping gene GAPDH. Note that mRNA levels for NaPi-2a are reduced in *Hyp/Lrp6* double mutant mice similarly as in *Hyp* mice when compared to WT or *Lrp6*^{+/-} mice. [#] $p < 0.05$, vs. *Lrp6*^{+/-}.

Fig. 6. Hypophosphatemic effect of exogenous FGF23 in *Lrp6*-deficient mice. Shown are serum phosphate levels before and 9 h after the last of two injections of recombinant bioactive FGF23 protein. Note that exogenous FGF23 lowers serum phosphate in *Lrp6*-deficient mice to a similar degree as in wild-type (WT) mice [*Lrp6*+/- mice (n=6): pre-injection 6.8±0.3 mg/dl vs. post-injection 5.5±0.3 mg/dl; WT mice (n=5): pre-injection 6.7±0.2 mg/dl vs. post-injection 5±0.4 mg/dl]. *p<0.05 vs. corresponding pre-injection value.