Altered renal FGF23-mediated activity involving MAPK and Wnt: effects of the *Hyp* mutation

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Abstract

Fibroblast growth factor-23 (FGF23), a hormone central to renal phosphate handling, is elevated in multiple hypophosphatemic disorders. Initial FGF23-dependent Erk1/2 activity in the kidney localizes to the distal convoluted tubule (DCT) with the co-receptor α -Klotho (KL), distinct from Npt2a in proximal tubules (PT). The *Hyp* mouse model of X-linked hypophosphatemic rickets (XLH) is characterized by hypophosphatemia with increased Fgf23, and patients with XLH elevate FGF23 following combination therapy of phosphate and calcitriol. The molecular signaling underlying renal FGF23 activity, and whether these pathways are altered in hypophosphatemic disorders, is unknown. To examine Npt2a *in vivo*, mice were injected with FGF23. Initial p-Erk1/2 activity in the DCT occurred within 10 min; however, Npt2a protein was latently reduced in the PT at 30–60 min, and was independent of *Npt2a* mRNA changes. *KL*-null mice had no DCT p-Erk1/2

staining following FGF23 delivery. Under basal conditions in *Hyp* mice, c-Fos and Egr1, markers of renal Fgf23 activity, were increased; however, *KL* mRNA was reduced 60% ($P < 0.05$). Despite the prevailing hypophosphatemia and elevated Fgf23, FGF23 injections into *Hyp* mice activated p-Erk1/2 in the DCT. FGF23 injection also resulted in phospho- β -catenin (p- β -cat) co-localization with KL in wild-type mice, and *Hyp* mice demonstrated strong p- β -cat staining under basal conditions, indicating potential crosstalk between mitogen-activated protein kinase and Wnt signaling. Collectively, these studies refine the mechanisms for FGF23 bioactivity, and demonstrate novel suppression of Wnt signaling in a KL-dependent DCT-PT axis, which is likely altered in XLH. Finally, the current treatment of phosphate and calcitriol for hypophosphatemic disorders may increase FGF23 activity.

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Introduction

Fibroblast growth factor-23 (FGF23) has been identified as the key regulator of phosphate metabolism. The importance of FGF23 in phosphate metabolism is underscored by disorders of hypophosphatemia associated with increased circulating FGF23 including autosomal dominant hypophosphatemic rickets (ADHR), X-linked hypophosphatemic rickets (XLH), autosomal recessive hypophosphatemic rickets, tumor-induced osteomalacia, as well as through syndromes of reduced FGF23, primarily hyperphosphatemic tumoral calcinosis (TC). In situations of elevated FGF23, renal phosphate wasting occurs through down-regulation of the proximal tubule (PT) membrane type-IIa (Npt2a) and type-IIc (Npt2c) sodium phosphate co-transporters (Larsson *et al.* 2004, Shimada *et al.* 2004c). In parallel, FGF23 decreases the expression of PT vitamin D 1- α -hydroxylase (CYP27b) and increases the expression of 24-hydroxylase (CYP24), thereby reducing the serum concentrations of 1,25(OH)₂

vitamin D (Shimada *et al.* 2001). These changes in gene expression account for the paradoxical low-circulating 1,25(OH)₂ vitamin D in the face of prevailing hypophosphatemia in syndromes associated with increased FGF23.

The co-receptor α -Klotho (KL) has been identified as necessary for FGF23 activity (Kuro-o 2006, Urakawa *et al.* 2006). The hypothesized mechanism for FGF23 bioactivity with KL is the recruitment of canonical FGF receptors (FGFRs) to form heteromeric complexes that then signal through the mitogen-activated protein kinase (MAPK) cascade and phospho-ERK1/2 (p-ERK1/2; Kuro-o 2006, Urakawa *et al.* 2006). In support of FGF23–KL interactions, the *Fgf23*- and *KL*-null animals have highly similar biochemical and skeletal phenotypes (Shimada *et al.* 2004b, Sitara *et al.* 2004), and a recessive, inactivating mutation in the *KL* gene resulted in impaired KL expression and a severe human TC phenotype, most likely due to end-organ resistance to FGF23 (Ichikawa *et al.* 2007). Although it has been shown that interactions between KL and a canonical

FGFR are necessary to mediate FGF23 signaling *in vitro*, the mechanisms underlying FGF23 bioactivity *in vivo* are unclear, as KL localizes to the renal distal convoluted tubule (DCT) and FGF23 mediates its effects on NPT2a and vitamin D metabolizing enzymes within the PT (Shimada *et al.* 2001, Larsson *et al.* 2004). We previously demonstrated that initial renal FGF23-dependent activity, as measured by p-ERK1/2 expression, occurs in the DCT localizing with KL expression (Farrow *et al.* 2009). These findings indicate the presence of an intra-renal signaling axis controlling the biological effects of FGF23 on renal phosphate and vitamin D metabolism. Previous studies demonstrated a maximal decrease in serum phosphate concentrations at 8 h following FGF23 injection into mice (Shimada *et al.* 2004a). Additionally, acute administration of parathyroid hormone (PTH) results in a decrease in Npt2a expression in the apical membrane 30 min after injection (Bacic *et al.* 2003). Although initial FGF23 activity occurs in the renal distal tubule within 10 min (Farrow *et al.* 2009), the temporal control of Npt2a and the downstream mediators of FGF23-dependent effects between the DCT and PT remain unclear.

XLH is the most common form of heritable hypophosphatemia (HYP-Consortium 1995), and most patients have chronically elevated serum FGF23 (Jonsson *et al.* 2003). The current therapy for XLH includes an oral regimen of phosphate and calcitriol; however, the treatment itself can have significant side effects including hyperparathyroidism (Lyles *et al.* 1982). Of significance, phosphate and 1,25(OH)₂ vitamin D stimulate the production of Fgf23 in animals (Azam *et al.* 2003, Shimada *et al.* 2004c), and this therapeutic regimen has recently been shown to increase circulating FGF23 concentrations in XLH patients (Imel *et al.* 2010). The *Hyp* mouse model of XLH, in parallel with the human condition, has significantly elevated Fgf23 serum concentrations and *Fgf23* bone mRNA expression (Liu *et al.* 2006b). These animals also increase FGF23 in response to dietary phosphate supplementation and vitamin D injections (Liu *et al.* 2006a). However, the molecular effects of chronically elevated FGF23 on the renal mechanisms of phosphate handling are unknown, and whether FGF23 is capable of further signaling effects in the face of elevated Fgf23 in *Hyp* is also unclear. Therefore, the goals of the present studies were to identify novel pathways involved in renal FGF23 bioactivity as a portion of a multinephron segment process, and to test whether FGF23-dependent signaling is altered in syndromes associated with FGF23 excess.

Materials and Methods

Animal studies

All animal studies were performed according to the Indiana University Institutional Animal Care and Use Committee guidelines. Wild-type (WT) C57/Bl6, *Hyp* (Jackson Laboratories, Bar Harbor, ME, USA), or *KL*-null (Lexicon Genetics,

Inc., The Woodlands, TX, USA) mice were injected with either vehicle (saline) or 10 µg recombinant FGF23 (gift from Genzyme Corp., Framingham, MA, USA) either i.v. or i.p. The *KL*-null mice carry the β -galactosidase gene cassette in place of mouse KL exons 2 and 3. Human FGF23 with the R176Q ADHR mutation was produced in insect cells (d-Mel-2), and is fully glycosylated. Animals were killed at 10, 30, and 60 min following injection. Kidneys were harvested for the preparation of RNA and immunofluorescence (IF) analyses, and serum was collected for serum FGF23 analysis.

Cell culture

HEK293 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Hudson, NH, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, and 25 mM penicillin-streptomycin at 37 °C and 5% CO₂. Cells stably expressing mKL were generated as described previously (Farrow *et al.* 2009). For bioactivity assays, mKL cells (1.5×10^5) were seeded in 12-well plates. Cells were serum starved for a minimum of 2 h before treatment with vehicle or 100 ng/ml FGF23 for 0, 30, or 60 min.

Immunofluorescence

Kidneys were fixed with 4% paraformaldehyde, mounted in OCT (Sakura Finetek, Torrance, CA, USA), and sliced into 10-µm sections. Sections were treated with pepsin (Biocare Medical, Concord, CA, USA) for antigen retrieval, and were probed with anti-mouse KL (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-ERK1/2 (Cell Signaling, Danvers, MA, USA), phospho- β -catenin (p- β -cat; Cell Signaling), or a highly specific antibody to mouse Npt2a (Custer *et al.* 1994) followed by incubation with the appropriate fluorescent secondary antibodies (Alexa Fluor, Invitrogen). Where indicated, immunofluorescent signal amplification was performed using the Tyramide signal amplification systems kit according to the manufacturer's directions (Perkin Elmer, Waltham, MA, USA). Slides were mounted using DAPI (Vector Laboratories, Burlingame, CA, USA). Imaging was performed using a Leica DM5000B fluorescent microscope (Leica Microsystems, Inc., Bannockburn, IL, USA), and SPOT camera and computer program (RTKE Diagnostic Instruments, Inc., Sterling Heights, MI, USA). A minimum of 3–4 animals per time point and 8–12 sections per animal were examined for each primary antibody set.

Quantitative RT-PCR

Kidneys were harvested for RNA using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was harvested from the cellular lysates using the RNeasy kit (Qiagen, Inc.). MAPK signal (PAMM-061) and signal transduction pathway finder (PAMM-014) array plates were obtained from SABiosciences (Frederick, MD, USA) and used according to

the manufacturer's protocol. To confirm array results, RNA samples were tested with intron-spanning primers specific for mouse *Egr1* mRNA (forward 5'-agccgagcgacaaccctat-3', reverse 5'-cgccttctcattattcagagcg-3', probe 5'-agcacctgaccacagagtccttttctgaca-3'), mouse *c-Fos* (Applied Biosystems, Carlsbad, CA, USA), and mouse *KL* (forward 5'-cagctc-caggctcgggta-3', reverse 5'-aggtgttagagatgccagacttt-3', probe 5'-ttgccacaacctatttggctcatg-3'), and mouse *actin* (forward 5'-ggctcctagcaccatgaag-3', reverse 5'-cgccttctcattattcagagcg-3', probe 5'-agcacctgaccacagagtccttttctgaca-3') was used as an internal control. Intron-spanning primers specific for human *EGR1* mRNA (forward 5'-ggacacgggcgagcag-3', reverse 5'-cgtgttcagagagatgtcagga-3', probe 5'-cctacgag-cacctgaccgcagagtct-3'), human *c-FOS* (Applied Biosystems), and human *actin* (forward 5'-ggcaccagcacaatgaag-3', reverse 5'-gccgatccacacggagtact-3', probe 5'-tcaagatcattgtcctcctcgagcgc-3') were used for *in vitro* experiments with HEK293 cells. The TaqMan One-Step RT-PCR kit (Applied Biosystems) was used to perform quantitative RT-PCR (qPCR). PCR conditions for all experiments were 30 min 48 °C, 10 min 95 °C, followed by 40 cycles of 15 s 95 °C and 1 min 60 °C. The data were collected and analyzed by the 7500 Real-Time PCR system and software (Applied Biosystems). The expression levels of *Egr1*, *c-Fos*, *Klotho*, and β -*actin* mRNAs were calculated relative to WT littermates or vehicle-treated controls. All primer sets were tested for specific amplification of mRNA by parallel analyses of controls that included omitting RT or template, and resulted in no fluorescent signal detection. Each RNA sample was analyzed in at least triplicate, and each experiment was performed independently at least three times. The $2^{-\Delta\Delta C_T}$ method described by Livak was used to analyze the data (Livak & Schmittgen 2001).

FGF23 serum measurements

Serum intact FGF23 concentrations were assessed using an ELISA according to the manufacturer's protocol (Kainos Laboratories Int'l, Tokyo, Japan). This two-site, monoclonal antibody ELISA has previously been shown to recognize rodent *Fgf23* (Yamazaki *et al.* 2002).

Statistical analysis

Statistical analysis of the data was performed by Student's *t*-test, and significance for all the tests was set at $P < 0.05$. Data are presented as means $\pm$ S.E.M.

Results

Temporal control of *Npt2a* by FGF23

We previously demonstrated that initial FGF23 bioactivity, as assessed by ERK1/2 phosphorylation (p-ERK1/2), co-localizes with KL in the renal DCT within 10 min after injection (Farrow *et al.* 2009). However, it has been shown that FGF23

delivery results in a latent reduction in serum phosphate concentrations, with a maximum decrease apparent at 8 h (Shimada *et al.* 2004a). Thus, the temporal control of *Npt2a* in the renal PT by FGF23 is not clear. To determine the effect of FGF23 on *Npt2a* expression, WT mice were injected with vehicle or 10 μ g recombinant human FGF23 for 10, 30, and 60 min, and the kidneys were harvested for RNA and for IF analyses. *Npt2a* expression was diminished in the PT apical membrane within 30 min following FGF23 injection, and remained decreased 60 min after injection (Fig. 1a). No change in *Npt2a* protein expression was observed in vehicle-injected animals at any time point (Fig. 1a). Kidney sections were co-stained with actin to delineate the apical membrane, which confirmed that the majority of *Npt2a* was removed from the apical membrane by 60 min (Fig. 1a). Robust p-ERK1/2 staining was confirmed to localize to the renal DCT at 10 min (Fig. 1b). Immunofluorescent signal amplification was used to test for p-ERK1/2 expression in the PT at 30 and 60 min. However, we were unable to identify a p-ERK1/2 signal co-localized with *Npt2a* at these time points (Fig. 1b).

The change in *Npt2a* protein localization within the apical membrane was independent of a transcriptional change, as a reduction in *Npt2a* mRNA was not detected between vehicle- and FGF23-injected animals ($P = 0.7$; Fig. 1c). In contrast, the positive control for FGF23 activity, early growth response gene-1 (*Egr1*) mRNA was dramatically increased (~ 50 -fold; $P < 0.05$; not shown). Thus, initial FGF23 activity in the DCT through p-ERK1/2 occurs within 10 min, whereas *Npt2a* protein expression in the PT is a latent effect (30–60 min) that does not require changes in *Npt2a* mRNA levels.

To test whether KL was required for initial FGF23 activity through MAPK in the DCT, *KL*-null mice were injected with 10 μ g FGF23 and killed at 10 min. These analyses indicated that ERK1/2 was not activated in the tubule segments of vehicle-injected WT mice (Fig. 1d, left), or in the *KL*-null mice that express a surrogate marker for KL expression, β -galactosidase (Fig. 1d, right). The positive control WT mice showed strong p-ERK1/2 activity (Fig. 1d, middle).

FGF23-mediated signaling under normal conditions and in disease

To identify genes responsive to FGF23, we tested for changes in transcription factors other than *Egr1* that are known to be downstream of canonical FGF signaling, including *c-Fos* (Kang *et al.* 2005). In this regard, WT mice were injected with either vehicle or 10 μ g recombinant human FGF23 i.p. ($n = 3$ –4/group) and killed at 60 min. qPCR was performed for *c-Fos* and *Egr1* on RNA isolated from the kidneys of injected animals. *c-Fos* showed a 13-fold increase in mRNA expression. The positive control, *Egr1*, increased over 70-fold in FGF23-injected mice when compared with the vehicle-treated controls ($P < 0.02$; Fig. 2a). To confirm these results, HEK293 cells stably expressing mKL were treated with

Figure 1 Control of Npt2a by FGF23. (a) Mice were injected with FGF23 or vehicle for 10, 30, or 60 min. Under standard staining conditions, Npt2a expression was tested. Npt2a was reduced 30–60 min following injection with FGF23 (top row). Sections were co-stained with actin (red), to mark the apical membrane, and co-localization of Npt2a in the apical membrane was detected (yellow). By 60 min, Npt2a was not detected in the apical membrane (middle row). Higher magnification of the proximal tubule shows the progressive removal of Npt2a from the apical membrane (bottom row). (b) Sections were co-stained for Npt2a and p-ERK1/2. p-ERK1/2 activity was absent in the vehicle-injected animals. At the 10-min time point, p-ERK1/2 was confirmed in the animals, and localization was distinct from Npt2a expression in the PT; no p-ERK1/2 activity was detected in the PT at any time point tested. At the 30- and 60-min time points, Npt2a staining was not detected as reduced (as in 1a) due to the effects of signal amplification for Npt2a and p-ERK1/2. (c) The reduction in Npt2a at 60 min was independent of transcription, as no change in *Npt2a* mRNA was detected ($P=0.7$). (d) WT or KL-null mice were injected with vehicle or 10 μ g FGF23 (WT vehicle, left panel; WT FGF23, middle panel) to test for renal MAPK activity. In the KL-null animals, the *LacZ* gene is inserted into the Klotho locus, which enabled β -galactosidase (β -gal) immunofluorescence to be used as a surrogate marker for KL (green, right panel). Following FGF23 delivery to KL-null mice, p-ERK1/2 activity was absent in the DCT labeled with β -gal (KL) (right panel). As confirmed, strong p-ERK1/2 activity co-localized with KL in WT animals injected with FGF23 (middle). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0181>.

100 ng/ml recombinant human FGF23 for 0, 30, and 60 min. A time-dependent increase was observed for both *c-Fos* and *EGR1* mRNAs with a maximum increase of 30- and 200-fold respectively ($P<0.05$; Fig. 2b). *c-Fos* is known to be

downstream of ERK1/2 in canonical FGF signaling; however, it has not been reported previously as responsive to FGF23. Taken together, these *in vivo* and *in vitro* results confirm the utilization of *c-Fos* as a marker of FGF23 bioactivity.

Patients with XLH have increased circulating FGF23; however, the molecular consequences in renal phosphate handling due to this elevation are unclear. Building upon the results directly above, we next tested basal levels of genes downstream of FGF23 activity in the *Hyp* model of XLH. These animals are characterized by hypophosphatemia and significantly increased serum Fgf23 levels (~ 12 -fold over WT littermates $P<0.05$; Fig. 3a); however, whether this leads to alterations in markers of FGF23 activity are unknown. Using *c-Fos* and *Egr1* to test FGF-dependent activity in kidneys from *Hyp* mice, we observed a 200% increase in the expression of these genes in *Hyp* mice in the basal state compared with sex- and age-matched WT littermates ($P<0.05$; Fig. 3b), suggesting that FGF23-dependent activity is enhanced in *Hyp* mice. Furthermore, *KL* mRNA was reduced by 60% in these mice compared with WT littermates ($P<0.05$; Fig. 3b), indicating that a potential compensatory mechanism is induced in an attempt to down-regulate FGF23-dependent signaling.

MAPK activity in the *Hyp* mouse model

The standard treatment for patients with XLH is a combination therapy of phosphate and calcitriol, which is used to raise serum phosphate. This regimen has recently been associated with increasing circulating FGF23

Figure 2 *In vivo* and *in vitro* MAPK signaling. (a) To test for novel markers of renal activity, mice were injected with 10 μ g FGF23 i.p. for 1 h, and the kidneys were harvested for RNA preparation and qPCR. *Egr1*, measured as a positive control, was increased 70-fold over vehicle-injected mice. *c-Fos* was increased 13-fold ($*P<0.05$). (b) HEK293 cells stably expressing mKL were treated with 100 ng/ml FGF23 for 30 and 60 min. *EGR1* mRNA increased 80- and 200-fold at 30 and 60 min respectively in the FGF23-treated mKL cells compared with vehicle-treated control ($*P<0.05$). *c-Fos* increased 20- and 30-fold at 30 and 60 min respectively ($*P<0.05$).

Figure 3 MAPK signaling in Hyp mice. (a) *Hyp* mice had intact serum FGF23 levels ~12-fold higher than WT controls (* $P < 0.05$); (b) *Hyp* mice had a 200% increase in *Egr1* and *c-Fos* mRNAs, and *Klotho* expression was reduced by 60% compared with wild-type controls (* $P < 0.05$).

concentrations in patients with XLH (Imel *et al.* 2010); however, the molecular effects of elevated FGF23 on the renal mechanisms of phosphate handling in this disorder are unknown. To test whether *Hyp* mice are able to respond to increased levels of FGF23, *Hyp* mice were injected with vehicle or FGF23 (10 μ g/animal). Despite the decreased mRNA levels of KL, KL was detectable by IF (Fig. 4); however, no p-ERK1/2 activity was detected in the basal state in *Hyp* mice. In contrast, *Hyp* mice injected with FGF23 demonstrated strong p-ERK1/2 staining (Fig. 4), and this activity co-localized with KL in the renal DCT. These findings demonstrate that *Hyp* mice are capable of responding to increased serum FGF23 levels, consistent with the idea that the current treatment for XLH could be associated with elevated renal FGF23 bioactivity.

FGF23-dependent effects on renal Wnt signaling

To screen for novel pathways involved in renal FGF23-dependent signaling, PCR-based array technology was utilized in initial experiments. This Pathway-Finder array contains primers recognizing 84 genes from 18 signaling pathways, including the positive control for Fgf23 activity, *Egr1*. WT mice were injected with 10 μ g recombinant human FGF23 and killed at 60 min, and the kidneys were harvested for RNA preparation. Consistent with our earlier results, *Egr1* mRNA was elevated ~20-fold in the array following injection. Unexpectedly, these initial analyses also identified *Engrailed-1* (*En1*) and *Wnt1* as elevated following injection with 3.5- and 5-fold increases in mRNA of FGF23-injected mice compared with vehicle-injected mice (not shown). *En1* is a well-characterized repressor of Wnt signaling (Bachar-Dahan *et al.* 2006). The role of Wnts as well as the

localization of this signaling cascade in FGF23-dependent renal activity has not been previously defined.

To build upon the mRNA results at the protein level, FGF23-dependent suppression of renal Wnt signaling was tested. In this regard, kidney sections from vehicle- and FGF23-injected mice (10 μ g/animal for 10 min) were co-stained for p- β -cat, a well-characterized marker of suppression of Wnt signaling, as well as for KL. A strong p- β -cat signal was only detected in the renal glomeruli of the vehicle-injected animals (Fig. 5a). In contrast, p- β -cat signaling was observed in the glomeruli and also co-localized with KL in the renal DCT in the FGF23-injected animals (Fig. 5b). Thus, in a similar manner to p-ERK1/2, p- β -cat activity was spatially separate from PT Npt2a expression.

Owing to the strong Wnt signaling response in WT mice, we next tested for the presence of p- β -cat signaling in *Hyp* mice, which would be consistent with the increased *Egr1* and *c-Fos* mRNAs indicating elevated MAPK signaling in the basal state (see Fig. 3b). Indeed, uninjected *Hyp* mice expressed robust p- β -cat in the DCT co-localized with KL (Fig. 6). Furthermore, p- β -cat was specifically co-localized with the sodium chloride co-transporter (NCC), a reliable DCT marker (Bostanjoglo *et al.* 1998). Importantly, the DCT-specific staining pattern for p- β -cat paralleled that of WT mice injected with FGF23, i.e. being distinct from Npt2a in the PT (Fig. 6). Thus, these findings revealed that FGF23 acts to suppress Wnt signaling in the renal DCT, further supporting an intra-renal axis for FGF23 activity.

Figure 4 Response of *Hyp* to FGF23 delivery. Results obtained from vehicle-injected *Hyp* mice are shown in the upper panels, and from FGF23-injected *Hyp* mice shown in the lower panels at 10 min post injection. Phospho-ERK1/2 staining was only observed in the FGF23-injected animals; Klotho (KL) was positive in both vehicle- and FGF23-injected animals. p-ERK1/2 staining (red) localized to the nucleus in the same nephron segment as KL (green) in FGF23-injected mice as shown by p-ERK1/2 and KL co-staining ('Merge' column; arrows show positive nuclear p-ERK1/2 co-localized with KL). Nuclei were stained blue using DAPI in the Merge column. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0181>.

Figure 5 Wnt pathway in renal FGF23 signaling. (a) Strong p- β -catenin signaling was detected in the glomeruli of all animals (green), separate from the DCT, which was demarcated using anti-NCC IF (red). (b) p- β -catenin signaling was tested by IF in vehicle- and FGF23-injected mice at 10 min following injection. p- β -catenin signaling was not detected in the vehicle-injected animals. In the FGF23-injected animals, p- β -catenin (red) was detected in the distal tubule, co-localized with Klotho (green). This activity did not co-localize with Npt2a in the renal PT (right column). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0181>.

Discussion

FGF23 controls renal phosphate handling by directing the membrane localization of Npt2a and Npt2c (Larsson *et al.* 2004, Shimada *et al.* 2004c) in the PT. Previously, we demonstrated that initial FGF23-dependent MAPK activity occurs within minutes in the renal DCT (Farrow *et al.* 2009). It has also been shown that serum phosphate levels are maximally decreased in mice ~ 9 h after FGF23 injection (Shimada *et al.* 2004a). Herein, we show that following FGF23 delivery, in a manner similar to PTH (Bacic *et al.* 2003), Npt2a protein expression is decreased in the PT by 30 min and fully at 60 min (Fig. 1a), and is associated with p-ERK1/2 activity in the DCT (Fig. 1b). This relatively rapid reduction in Npt2a is likely due to the endocytic retrieval of the co-transporter from the apical membrane, as no transcriptional changes in Npt2a were detected at 60 min (Fig. 1c). Although FGF23

rapidly down-regulates Npt2a, changes in serum phosphate concentrations and 1,25(OH) $_2$ vitamin D are known to be latent (Shimada *et al.* 2004a). Our findings support the idea that elevations in FGF23 may also be associated with indirect endocrine changes that manifest over longer time intervals, as opposed to balancing minute to minute serum phosphate changes. Importantly, FGF23 activity through MAPK in the DCT was dependent upon the presence of KL. In this regard, p-ERK1/2 activity was not detected that co-localized with DCT segments expressing a surrogate marker for KL, β -galactosidase (Fig. 1d) in KL-null animals injected with FGF23. These results are consistent with the fact that KL and a canonical FGFR are required for FGF23-dependent MAPK signaling (Urakawa *et al.* 2006). Furthermore, the lack of FGF23-dependent p-ERK1/2 activity in the DCT in the absence of KL provides mechanistic support for a report demonstrating a likely end-organ deficiency in FGF23-dependent signaling in a patient with severe TC harboring a homozygous KL loss-of-function missense mutation (His193Arg; Ichikawa *et al.* 2007).

We have now demonstrated the presence of early FGF23 signaling in the DCT with both p-ERK1/2 and Wnt involvement (see below). Nonetheless, the mechanisms underlying a potential transfer of FGF23 bioactivity across a spatial separation to the PT remain unclear. Although the second step or process following initial FGF23 signaling is unknown, our results support the fact that it likely occurs relatively quickly (under 30 min) after initial signaling (5 min), although physiological changes in serum phosphate are known to occur over at least 8–9 h (Shimada *et al.* 2004a). Collectively, these findings provide a narrowed time frame for the identification of candidate pathways guiding intra-renal FGF23-dependent activity. At this time, however, the possibility that FGF23 has a direct effect in the PT that does not involve p-ERK1/2 (or Wnts) cannot be ruled out.

To test for additional markers of FGF23 bioactivity, the transcription factor c-Fos was identified as being upregulated following FGF23 delivery *in vivo* and *in vitro* (Fig. 2b). Although c-Fos is known to be downstream of the MAPK

Figure 6 Renal p- β -catenin expression in *Hyp* mice. (a) p- β -catenin (red) was readily detectable in kidneys from *Hyp* mice in the basal state, localized to the renal DCT with KL (green staining; left panel); (b) p- β -catenin (red) was spatially separate from Npt2a (green; middle panel). (c) In the *Hyp* basal state, strong p- β -catenin signal (green) was co-localized with NCC, a marker for the renal DCT (red; right panel). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0181>.

pathway initiated by canonical FGF signaling (autocrine and paracrine FGFs), it had not previously been associated with renal FGF23 bioactivity. *Hyp* mice have significantly elevated serum FGF23 and hypophosphatemia, and are a well-established model of the human syndrome XLH. The standard treatment for XLH currently includes supplemental oral phosphate and high-dose calcitriol (Tenenhouse & Econs 2001). This therapeutic regimen has now been realized to result in further increases in circulating FGF23 in this patient group (Imel *et al.* 2010). Similarly, administration of phosphate or vitamin D potentially increases FGF23 production in WT mice and in *Hyp* mice (Azam *et al.* 2003, Shimada *et al.* 2004c). Although the *Hyp* mouse has been well studied, the levels of relative renal FGF23 bioactivity under a state of chronic hypophosphatemia in this model remained unclear. We therefore examined FGF23 signaling in *Hyp* mice in the basal state and found that the markers of MAPK signaling, *Egr1* and *c-Fos* mRNAs, were elevated in kidneys from *Hyp* mice compared with WT littermates (Fig. 3b). These findings are consistent with the idea that FGF23-dependent signaling is increased in *Hyp* mice under 'normal' circumstances, consistent with their significantly increased circulating Fgf23 (Fig. 3a; Liu *et al.* 2006b). Although renal *KL* mRNA concentrations were reduced by 60% in *Hyp* mice compared with WT littermates, when *Hyp* mice were injected with FGF23, robust p-ERK1/2 signal was detected in the DCT with KL, similar to the response observed in WT mice (Fig. 1d). Consistent with our findings in *Hyp* mice, *KL* mRNA was reported to be the most significant downregulated kidney gene in a markedly hypophosphatemic transgenic mouse over-expressing human FGF23 in osteoblasts and osteocytes (Marsell *et al.* 2008). Conversely, *KL* transcripts were upregulated in a model of hyperphosphatemic TC, the *GALNT3*-null mouse (Ichikawa *et al.* 2009), which suggests that FGF23 may potentially be regulated at the level of its receptor complex in kidney depending upon phosphate status. Taken together, these results indicate that in the basal state, *Hyp* mice have altered renal MAPK activity, but overall, FGF23-dependent signaling remains intact. These findings have implications for XLH, as increased FGF23 following treatment could suppress renal 1,25(OH)₂ vitamin D production by decreasing 25-vitamin D 1- α -hydroxylase and increasing 24-hydroxylase expression, or have direct actions on the parathyroid, further leading to a scenario for secondary hyperparathyroidism, a common and often severe complication of XLH (Tenenhouse & Econs 2001).

To screen for novel pathways associated with FGF23 renal activity, we used an array approach and identified markers of suppressed Wnt signaling. Wnt signaling is critical for proper kidney development (Herzlinger *et al.* 1994, Stark *et al.* 1994), and altered Wnt activity has been implicated in the development of several renal diseases including renal cell carcinoma and cystic kidney disease (Kim *et al.* 1999, 2000). Importantly, following FGF23 delivery, p- β -cat signaling co-localized to the DCT with KL

and with NCC (Figs 5b and 6) within the same time course for DCT p-ERK1/2 expression (10 min). In *Hyp* mice in the basal state, p-ERK1/2 was not detected by IF (Fig. 4), consistent with the modest increases in *Egr1* and *c-Fos* mRNAs (Fig. 3b). However, in the basal state, strong p- β -cat signaling was detected in the DCT of *Hyp* mouse, indicating that p- β -cat may be a sensitive marker in XLH and in disease models of altered FGF23 activity.

The localization of Wnt signaling has not been defined in terms of renal phosphate homeostasis *in vivo*, and its role in the DCT for normal phosphate handling is unclear. It was reported that GSK3- β phosphorylation was increased *in vitro* in cells treated with FGF23 and a recombinant KL (Medici *et al.* 2008) in parallel with p-ERK1/2 (Medici *et al.* 2008, Farrow *et al.* 2009). In canonical Wnt signaling, GSK3- β is upstream of p- β -cat, consistent with our results that p- β -cat is localized to the DCT with p-ERK following FGF23 delivery, spatially distinct from Npt2a in the PT. Complementary signaling between the FGF-dependent MAPK and Wnt pathways has been well documented during development (ten Berge *et al.* 2008); however, the molecules at the origin of the branching point between these two pathways is less clear. Recent reports indicated that GSK3- β may serve as a mediator between FGF and non-canonical Wnt signaling during carcinogenesis (Katoh 2006), and during osteoblast proliferation AKT may signal between MAPK and Wnts (Raucci *et al.* 2008). Thus, our identification of FGF23-dependent control of Wnt activity in the kidney may also be useful for the further understanding of MAPK-Wnt interactions. Genes known to be involved in Wnt and FGF signaling are also altered in *Hyp* mouse bone (Liu *et al.* 2009); thus in addition to renal pathogenesis, these systems may also be associated with increased skeletal Fgf23 (Liu *et al.* 2003).

In summary, we have identified novel markers of renal FGF23 bioactivity, have defined the temporal control of Npt2a by FGF23, and have localized alterations in MAPK and Wnt signaling to the renal DCT in the *Hyp* mouse. These studies have important implications for understanding the altered FGF23-dependent signaling in XLH, as well as in other disorders associated with elevated FGF23.

Declaration of interest

E G F, L J S, J A M, and D H E have nothing to declare. S C S is employed by Genzyme, Corp. K E W receives research funding from Genzyme and royalties for licensing the *FGF23* gene to Kyowa Hakko Kirin, Ltd.

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