Osteocyte-Specific Deletion of Fgfr1 Suppresses FGF23

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Abstract

Increases in fibroblastic growth factor 23 (FGF23 or Fgf23) production by osteocytes result in hypophosphatemia and rickets in the Hyp mouse homologue of X-linked hypophosphatemia (XLH). Fibroblastic growth factor (FGF) signaling has been implicated in the pathogenesis of Hyp. Here, we conditionally deleted FGFR1 (FGFR1 or Fgfr1) in osteocytes of Hyp mice to investigate the role of autocrine/paracrine FGFR signaling in regulating FGF23 production by osteocytes. Crossing dentin matrix protein 1 (Dmp1)-Cre;Fgfr1null mice with female Hyp;Fgfr1Dmp1-cre;null mice created Hyp and Fgfr1 (Fgfr1Dmp1-cre)-null mice (Hyp;Fgfr1Dmp1-cre;null) with a 70% decrease in bone Fgfr1 transcripts. Fgfr1Dmp1-cre;null mice exhibited a 50% reduction in FGF23 expression in bone and 3-fold reduction in serum FGF23 concentrations, as well as reductions in sclerostin (Sost), phosphate regulating endopeptidase on X chromosome (PHEX or Phex), matrix extracellular phosphoglycoprotein (Mepe), and Dmp1 transcripts, but had no demonstrable alterations in phosphate or vitamin D homeostasis or skeletal morphology. Hyp mice had hypophosphatemia, reductions in 1,25(OH)2D levels, rickets/osteomalacia and elevated FGF2 expression in bone. Compared to Hyp mice, compound Hyp;Fgfr1Dmp1-cre;null mice had significant improvement in rickets and osteomalacia in association with a decrease in serum FGF23 (3607 to 1099 pg/ml), an increase in serum phosphate (6.0 mg/dl to 9.3 mg/dl) and 1,25(OH)2D (121±23 to 192±34 pg/ml) levels, but only a 30% reduction in bone FGF23 mRNA expression. FGF23 promoter activity in osteoblasts was stimulated by FGFR1 activation and inhibited by overexpression of a dominant negative FGFR1(TK-) in PLCγ and MAPK inhibitors. FGF2 also stimulated the translation of an FGF23 cDNA transfected into osteoblasts via a FGFR1 and PI3K/Akt-dependent mechanism. Thus, activation of autocrine/paracrine FGF pathways is involved in the pathogenesis of Hyp through FGFR1-dependent regulation of FGF23 by both transcriptional and post-transcriptional mechanisms. This may serve to link local bone metabolism with systemic phosphate and vitamin D homeostasis.

Introduction

The FGF family consists of canonical FGFs, intracellular FGFs, and hormone-like FGF gene products (i.e., FGF19, FGF21 and FGF23). The earliest evolved family members are likely intracellular FGFs, exemplified by high molecular weight FGF2 (HMW-FGF2), which interact with intranuclear FGFR1 to directly activate gene transcription (i.e., integrative nuclear FGFR1 signaling) [1]. Canonical or FGFs evolved later to serve autocrine/paracrine functions. These secreted FGFs have high heparin affinity, limited diffusion capacity and act locally on cell surface FGF receptors [2]. More recently, circulating FGFs emerged with the capability to diffuse from tissues and specifically target FGF receptor/Klotho complexes at distal sites due to their unique C-terminal domain [3]. For example, FGF23 is a ~32 kDa hormone with an N-terminal FGF-homology domain and a novel 71 amino acid C-terminus [4] that allows it to be released into the circulation and to interact with α-Klotho, a type I membrane, β-glycosidase to form a trimeric complex with FGFRs in specific tissues [4–9].

FGF23 is produced and secreted by osteoblasts/osteocytes in bone, FGF23 activates FGFR/α-Klotho complexes in the kidney to decrease Npt2a co-transporters leading to inhibition of renal tubular phosphate reabsorption and to reduce circulating 1,25(OH)2D levels by inhibiting enzymes regulating vitamin D metabolism (i.e., inhibiting Cyp27b1 and stimulating Cyp24) [6,7,9–13]. The biological functions of FGF23 are essential for maintenance normal mineral metabolism. Ablation of FGF23 in mice is lethal in the early postnatal period due to hyperphosphatemia and excessive 1,25(OH)2D production [14,15]. On the other hand, excess FGF23 causes hypophosphatemia, aberrant vitamin D metabolism and rickets/osteomalacia. Increases in FGF23 underlie acquired and hereditary forms of hypophosphatemic rickets and are involved in the pathogenesis of mineral metabolism abnormalities in chronic kidney diseases [16]. Consequently, understanding the factors that regulate FGF23 is of high clinical importance.

FGF23 gene transcription in bone is complex and poorly understood. FGF23 is regulated by systemic factors, including 1,25-(OH)2D, PTH, and calcium [17] and local bone derived factors [14,17–29]. Systemic regulators have variable and often opposite effects on FGF23 expression that are possibly explained by their differential effects on bone mineralization and/or the presence of hypocalcaemia. Local bone-derived factors that
regulate bone mineralization are important regulators of FGF23 expression, although the mechanisms are poorly understood. For example, high circulating FGF23 and increased FGF23 gene transcription occur in XLH rickets and its Hyp mouse homologue. XLH and Hyp are caused by inactivating mutations of PHEx, one of several genes regulating bone mineralization and FGF23 production. Disruption of the extracellular matrix (ECM) mineralization by mutations of DMP1, PHEx, and ENPP1 leads to increased FGF23 gene expression in osteoblasts/osteocytes [29–33]. How these mutations lead to elevated FGF23 remains unclear.

Recent studies implicate a role of FGFR1 activation in regulating FGF23 gene transcription [29,34–36]. In this regard, FGF23 is increased in osteophytic chondroplasia, which is caused by activating mutations in FGFR1 [35,36]. In addition, ligands for FGFR1, including FGF1, FGF2, and FGF7 are significantly increased in the Hyp and Dmp1 knockout mice [37–39]. Pharmacological inhibition of FGFR1 also blocks FGF23 transcription in bone both in vitro and ex vivo [29,40,41]. Moreover, recent studies have reported that administration of monoclonal FGFR1 activating antibodies stimulates FGF23 production and induces hypophosphatemia [42]. These findings suggest that autocrine/paracrine FGF/FGFR1 signaling pathways may be involved in regulation of hormonal FGF23. The gain-of-function studies implicating FGFR1 in the regulation of FGF23, however, are confounded by the generalized effects to activate FGF receptors in multiple tissues. Interpretation of the inhibitor studies are confounded by actions to block FGF23 end-organ effects and other potential systemic actions that could lead to feedback regulation of FGF23. These limitations preclude establishing a direct cause and effect relationship between FGFR1 function and FGF23 gene transcription in osteoblasts/osteocytes.

In the current study, to test the central role of FGFR1 in mediating the increased expression of FGF23 in bone, we use a mouse genetic approach to conditionally deleted Fgfr1 from osteocytes of Hyp mice. We find an important role of FGFR1 signaling in osteocytes in mediating the increase of FGF23 caused by Phex mutations in Hyp mice, thereby linking alterations autocrine/paracrine functions of FGF/FGFR1 pathways in the bone microenvironment with the secretion of circulating FGF23 that activates FGFRs in distal tissues to coordinate bone mineralization with renal regulation of phosphate and vitamin D metabolism.

Materials and Methods

Animals breeding and genotyping
All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The University of Tennessee Health Science Center’s Animal Care and Use Committee approved all animal studies (Protocol number: 12-2551, 12-2552). CMV-Cre and Hyp mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in C57BL/6j background. The floxed Fgfr1 mice (Fgfr1<sup>flox/cKO</sup>) were obtained from Dr. Chuxia Deng at National Institute of Diabetes and Digestive and Kidney Diseases and maintained in C57BL/6j background for at least six generations. 9.6-kb Dmp1-Cre mice were used to delete the floxed Fgfr1 in bone as described previously [43] and maintained in C57BL/6j background for at least five generations. The 9.6-kb Dmp1-Cre has been widely utilized to delete a gene specifically in osteocytes [43,44], where it has been shown to be active in 77% of osteocytes and only ~1% of surface osteoblasts in long bone [45,46]. All mice were maintained on a standard diet (7912, Harlan Teklad, Madison, WI, USA). First, we crossed Fgfr1<sup>flox/cKO</sup> to CMV-Cre to obtain a germine-specific deletion of Fgfr1 (Fgfr1<sup>flox/flox</sup>). The Fgfr1<sup>flox/cKO</sup> mice were crossed to Dmp1-Cre mice to obtain double heterozygous Dmp1-Cre:Fgfr1<sup>flox/flox</sup> mice. Second, heterozygous female Hyp (C57BL/6j<sup>Hyp/+</sup>) mice were crossed to Fgfr1<sup>flox/flox</sup>/Cre males to obtain Hyp:Fgfr1<sup>flox/flox</sup> mice. Finally, Hyp:Fgfr1<sup>flox/flox</sup> females were crossed to Dmp1-Cre:Fgfr1<sup>flox/flox</sup> mice to obtain the osteocyte-specific deletion of Fgfr1 in Hyp mice. These mice are on a mixed genetic background. For the entire study, samples were collected from 6-week-old Fgfr1<sup>flox/cKO</sup> (wild-type equivalent) control, conditional Dmp1-Cre:Fgfr1<sup>flox/flox</sup> heterozygous, conditional Dmp1-Cre:Fgfr1<sup>flox/flox</sup> (Fgfr1<sup>flox/flox</sup>/Cre null), Hyp (C57BL/6j<sup>Hyp</sup>), and compound Hyp:Fgfr1<sup>flox/flox</sup>-null male littermates as well as Fgfr1<sup>flox/flox</sup> (equivalent to Fgfr1<sup>flox/flox</sup>/Cre null) and Hyp:Fgfr1<sup>flox/flox</sup>-null (equivalent to Hyp:Fgfr1<sup>flox/flox</sup>/Cre null) male mice. Tail clips were collected to genotype the mice. REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) was used for DNA extraction and PCR amplification. Mice were genotyped for Phex mutations and Dmp1-Cre using previously described primers [43,44], for the Fgfr1<sup>flox</sup> allele using forward primer 5’-TGTCGGAGTTTTGCTTGTGGGAC-3’ and reverse primer 5’-CAACCTGCCATATGAAAGAC-3’ (325 bp product for the Fgfr1<sup>flox</sup> wild-type allele, 400 bp product for the Fgfr1<sup>flox</sup> floxed allele), and for the Fgfr1<sup>flox</sup> allele using forward primer 5’-GGATCGTTTGTCCATTTGAGGA-3’ and reverse primer 5’-CCATCATGATGACTTCC-3’ (250 bp product for the Fgfr1<sup>flox</sup> null allele).

Serum Biochemistry
Serum samples were collected by intracardiac exsanguinations and urine samples were collected overnight in metabolic cages. Calcium was measured using a Calcium CPG Liquicolor Kit (Stanbio Laboratories, Boerne, TX, USA) and phosphorus was measured using the phosphomolybdate-ascorbic acid method, as previously described [47]. Serum parathyroid hormone (PTH) levels were measured using the Mouse Intact PTH ELISA kit (Immunotopics, Carlsbad, CA, USA). Serum full-length FGF23 levels were measured using the FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan), and serum C-terminal Fgf23 levels were measured using the FGF23 (C-Term) ELISA kit (Immunotopics, Carlsbad, CA, USA).

Bone and kidney RNA isolation and real-time reverse transcriptase (RT)-PCR
For quantitative real-time RT-PCR, 1.0 μg total RNA isolated from tibias without bone marrow and kidney of 6-week-old four genotypes mice was reverse transcribed as previously described [48]. PCR reactions contained 20 ng template (cDNA or RNA), 375 nM each forward and reverse primers, and 1× SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA, USA) in a total of 10 μl reaction volume. The threshold cycle (Ct) of tested gene product from the indicated genotype was normalized to the Ct for cyclophilin A. Expression of total Fgfr1 transcripts was performed using the following Fgfr1-allele-specific primers in exons 9 and 10: forward primer of normal Fgfr1<sup>+</sup> transcripts (Fgfr1<sup>+</sup>) plus Fgfr1<sup>flox</sup>; 5’- ACC AAC AAG AGG GAG GCC TGG CTT CA-3’ and reverse primer: 5’- AAC CAG GAG AAG CCC AGA GT-3’. The Normal Fgfr1<sup>+</sup> vs. cyclophilin A was normalized to the mean ratio of five control mice, which was set to 1. The percentage of Fgfr1<sup/null</sup> (Fgfr1<sup>flox</sup>) and/or conditional deleted (Fgfr1<sup>flox/cKO</sup>)

PLOS ONE | www.plosone.org 2 August 2014 | Volume 9 | Issue 8 | e104154
transcripts was calculated from the relative levels of the normal Fgfr1* transcripts in different Fgfr1-deficient mice [49]. All primer information of other genes used in real-time RT-PCR can be found in our previous report [44].

High resolution 3D microtomography

The femurs were collected, fixed and dehydrated in 70% ethanol. High-resolution micro-Computed Tomography (μCT40, Scanco Medical, Basserdorf, Switzerland) was used to scan and evaluate the metaphyseal trabecular bone microarchitecture and the midshaft cortical bone parameters. The entire femurs were scanned in a 12.5 mm diameter sample holder at 6 μm resolution; energy level of 55 KeV and intensity of 145 μA. Evaluation of the bone growth was obtained by measuring the length of the scanned femur. The trabecular bone volume (BV/TV%) was measured within the secondary spongiosa on a set of 50 sections (0.6 mm) underneath the growth plate at a threshold of 200 as previously described [50]. The cortical bone thickness (ClTh, mm) was analyzed from 100 sections chosen at the midshaft of each femur at a threshold of 350.

Bone histology and histomorphometry

Evaluation of the bone growth was obtained by measuring the length of the femur of 6-week-old mice with a slide caliper. Femurs were fixed and dehydrated in 70% ethanol, and embedded in methylmethacrylate at low temperature. Nonserial longitudinal frontal slices (5 μm) were cut from the embedded bones with a microtome (Polycut-S, Reichert-Jung, Wetzlar, Germany) and were either left unstained or used for modified Goldner staining. Alizarin complexone dehydrate (Sigma, St. Louis, MO) double labeling of bone and histomorphometric analyses of periosteal mineral apposition rate (MAR) in femurs were performed using the osteomeasure analysis system (Osteometrics, Decatur, GA, USA).

In vitro promoter studies

We used the MC3T3-E1 osteoblastic cell line from Dr. Hiroko Sudo [51] that we previously characterized [52]. MC3T3-E1 osteoblasts were maintained in α-MEM containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). To perform plasmid transfection in MC3T3-E1 cells, 5×10⁴ cells were seeded in 6-well plates in α-MEM media (Life technologies, Grand Island, NY) with 10% FBS at 37°C in 5% CO₂ and humidified incubator. Cells were plated for 18 hours before transfection and fed with fresh medium 4 hours before transfection. Mouse 0.6 kb FGF23 promoter luciferase reporter construct (0.25 μg) along with pcDNA3.1-FGFR1 cDNA expression plasmids (0.5 μg) or pcDNA3.1-FGFR1(TK−) cDNA expression plasmids (0.5 μg) or pCMV-HMW-FGF2 (0.25 μg) (a gift from Dr. Michal K. Stachowiak at Neuroscience State University of New York at Buffalo) [53] and pRL Renilla reniformis luciferase control plasmids (0.1 μg) were co-transfected into MC3T3-E1 cells using cationic liposomes (LipofectAMINE2000, Life technologies, Grand Island, NY) for 16–18 hours, and then cells were washed twice with phosphate-buffered saline and incubated in fresh medium containing 10% FBS for 38 hours. Various doses of FGF2 (0–100 ng/ml) or FGF2 (50 ng/ml) in the presence and absence of Wortmannin (PI3K inhibitor, 1.0 μM), various doses of U73122 (PLC inhibitor, 1–10 μM), and various doses of U0126 (MEK inhibitor, 5–20 μM) were added to the cell culture media for 24 hours before cells were harvested. Cells were lysed in 50 μl of reporter lysis buffer (Promega, Madison, WI). A luciferase assay (20 μl of cell lysis) was performed using a dual luciferase assay kit (Promega, Madison, WI), and activity was measured with an Optiecump 1 luminometer (MGM Instruments, Inc., Hamden, CT).

Western blot analysis

Bone marrow-free femurs from four groups of mice were homogenized into a fine powder in liquid nitrogen using a porcelain mortar and pestle. The powder was transferred into T-PER Tissue Protein Extraction Reagent with 1× Halt protease inhibitor (Thermo Scientific, Rockford, IL) and 1 mM phenylmethylsulfonyl fluoride (PMSF). After three 30-second sonications, samples were centrifuged at 13,000× g for 10 minutes and supernatants were stored at −80°C until use.

To examine if Fgfr1 signaling has a role in translational control of FGF23 protein expression in vitro, a number of 1.2×10⁶ of MC3T3-E1 cells were transfected with pcDNA3.1-FGFR1 cDNA expression plasmids (3.0 μg) and human pcDNA3.1-FGFR3-V5-His cDNA expression plasmids (3.0 μg) conducted by electroporation using Cell Line Nucleofector Kit R according to the manufacturer’s protocol (Amaxa Inc, Gaithersburg, MD). 2.0×10⁴ cells were seeded in 6-well plates in α-MEM media (Life technologies, Grand Island, NY) with 10% FBS at 37°C in 5% CO₂ and humidified incubator for 8 hours. For time-course experiments, the cells were changed into fresh growth medium containing 5% FBS and FGF2 (50 ng/ml, Sigma-Aldrich, St. Louis, MO) and heparin (10 μg/ml, Sigma-Aldrich, St. Louis, MO) were added to the cell culture media for 8, 24, and 48 hours before cells were harvested. For signalization studies, the cells were changed into fresh growth medium containing 5% FBS and FGF2 (50 ng/ml) and heparin (10 μg/ml) in the presence and absence of Wortmannin (PI3K inhibitor, 1.0 μM) or Cycloheximide (0.5 μg/ml, Sigma-Aldrich, St. Louis, MO) were added to the cell culture media for 24 hours before cells were harvested. The cells were lysed with 150 μl of T-PER with 1× Halt protease inhibitor and 1 mM per well, After three 30-second sonications, total cell lysates were centrifuged at 13,000× g for 10 minutes and supernatants were stored at −80°C until use. Protein concentrations of the supernatant were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal quantities of protein were subjected to NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and analyzed with standard Western blot protocols (HRP-conjugated secondary antibodies from Santa Cruz Biotechnology and ECL from GE Healthcare Bio-Sciences (Pittsburgh, PA). Antibody against Fgfr2 (MAB2629) was obtained from R&D Systems, Inc. (Minneapolis, MN). Antibody against Fgfr2 (610073) was purchased from BD Biosciences (San Jose, CA). Anti-V5-HRP antibody (R961-25) was purchased from Santa Cruz Biotechnology (Paso Robles, CA). The intensity of bands was quantified using Image J software (http://rsb.info.nih.gov/ij/).

Statistics

We evaluated differences between two groups by unpaired t-test and multiple groups by one-way analysis of variance. All values are expressed as means ± SD. All computations were performed using GraphPad Prism5 (GraphPad Software Inc. La Jolla, CA, USA).

Results

Osteocyte specific deletion of Fgfr1 in mice

We selectively deleted Fgfr1 in osteocytes by crossing Dmp1-Cre;Fgfr1flo/flo mice with homozygous Hypr/Hypr mice. Conditional Fgfr1Dmp1-Cre;Fgfr1flo/flo mice. Conditional Fgfr1Dmp1-Cre;Fgfr1flo/flo mice. Conditional Fgfr1Dmp1-Cre;Fgfr1flo/flo;Hyp/fl mice with intact Phex but reduced Fgfr1 in osteocytes; Hypr (Xfr2Y)
mice with intact Fgfr1 but mutant Phex; compound Hyp [Xxyy;Dmp1-Cre;Fgfr1floxs/+ null mice with reduced Fgfr1 in osteocytes and mutant Phex, hereafter referred to as Hyp:Fgfr1Dmp1-KO null mice; and Fgfr1floxs/+ control mice with both intact Fgfr1 and Phex, were all born with the expected Mendelian frequency. There was no difference in survival between genotypes produced by this breeding strategy. All mice survived to adulthood.

To confirm Dmp1-Cre-mediated conditional deletion of Fgfr1 in bone, we performed PCR analysis of genomic DNA using a combination of primers that specifically detected floxed Fgfr1 alleles (Fgfr1floxs) and the excised floxed Fgfr1 alleles (Fgfr1Δlox), as well as wild type alleles (Fgfr1+) in Dmp1-Cre:Fgfr1floxs/+ heterozygous mice (Figure 1A). We found that Dmp1-Cre-mediated excision of the Fgfr1floxs alleles occurred in bone tissues, including calvaria and femur. Low levels of recombinase activity were detected in brain, muscle, and intestine, indicating that Dmp1-Cre promoter is not restricted to bone (Figure 1B). We found no evidence for the Fgfr1Δlox alleles in a wide range of other tissues tested, including the kidney. The floxed Fgfr1floxs alleles were detected in all tissues tested (Figure 1B). Real-time RT-PCR analysis revealed that Fgfr1 mRNA levels in bone tissues were reduced by about 30% from Dmp1-Cre:Fgfr1floxs/+ mice and about 70% from conditional Fgfr1Dmp1-KO null mice compared with control mice (Figure 1C), respectively. Consistent with the absence of Dmp1-Cre expression in kidney, there were no reduction in Fgfr1 transcripts in the kidney of Dmp1-Cre:Fgfr1floxs/+ mice. However, Fgfr1 transcripts were reduced by 50% in Fgfr1Dmp1-KO null mice, consistent with the presence of a null Fgfr1 allele (Figure 1D).

**Effects of osteocyte specific deletion of Fgfr1 on mouse bone phenotype**

Dmp1-Cre:Fgfr1floxs/+ mice were indistinguishable from Fgfr1floxs/+ littermates and wild-type mice. We focused our studies on Fgfr1Dmp1-KO null and compound Hyp:Fgfr1Dmp1-KO null mice. The overall appearance, body weight and femur length of Fgfr1Dmp1-KO null mice were indistinguishable from Fgfr1floxs/+ littermates, which we used as controls (Figure 2A and 3A). In spite of the known role of Fgfr1 in bone development [54], conditional deletion of Fgfr1 in osteocytes, which represent the terminal stage of osteoblasts differentiation, had minimal effects on the skeletal phenotype. Micro-CT 3D image analyses of the femur revealed that deletion of Fgfr1 in osteocytes resulted in no measurable changes in cortical or trabecular bone structural parameters. Indeed, Fgfr1Dmp1-KO null mice had normal femur length, trabecular bone volume, and cortical thickness (Figure 3). The histological appearance of bone was indistinguishable in Fgfr1Dmp1-KO null mice (i.e., normal growth plate, trabecular bone and osteoid volume and mineral apposition rate) compared with age-matched control mice (Figure 4).

Assessment of mRNA expression in bone, however, revealed that the conditional deletion of Fgfr1 in osteocytes did result in significant but selective reductions in osteocyte-specific gene expression, including Fgfr2, Sost, Phex, Dmp1, and Mepe, but not Osteopontin, Osteocalcin, Fgf1, Fgf2, Fgf3, Fgf4, and Mmp13 (Table 1). Consistent with the 50% decrease of Fgf23 transcripts, western blot analysis showed a significant reduction of FGF23 protein in bone from Fgfr1Dmp1-KO null mice (Figure 2B and 2C). Conditional deletion of Fgfr1 in osteocytes also resulted in a 2-fold reduction in serum FGF23 protein levels in the Fgfr1Dmp1-KO null mice (Table 2).

We also observed significant reductions in Sost and dickkopf WNT signaling pathway inhibitor 1 (Dkk1) but not frizzled class receptor 2 (Fzd2), which are antagonist of the Wnt signaling; however we failed to see an increase in either Wnt10b or Axin-2 in the Fgfr1Dmp1-KO null mice. Loss of Fgfr1 in osteocytes had no effect on osteoclast, chondrocyte, or adipocyte transcripts in bone (Table 1). There were no differences in the Fgf2 protein expression between control and Fgfr1Dmp1-KO null mice (Figure 2B and 2C).

In spite of this reduction in FGF23, there were no significant changes in serum PTH, 1,25(OH)2D, phosphorus, and calcium levels in Fgfr1Dmp1-KO null mice compared with age-matched control mice (Table 2). As expected due to the inclusion of an Fgfr1 null allele in the conditional knockout strategy, Fgfr1 transcripts were reduced by ~50% reduction in kidney of Fgfr1Dmp1-KO null mice. However, there were no significant differences in the FGF23-regulated genes, including Npnl2, Npnl2c, Cyp24a1, Cyp24a1 and Cyp27b1, or α-Klotho transcripts in the kidney of Fgfr1Dmp1-KO null mice (Table 3).

**Phex-mutant Hyp mice exhibit elevated FGF23 expression, hypophosphatemia and rickets/osteomalacia**

Hyp mice with inactivating Phex mutations have very high production of FGF23 in osteocytes and markedly elevated circulating FGF23 levels leading to hypophosphatemia and abnormalities in vitamin D metabolism, as well as impaired mineralization of cartilage and bone leading to rickets and osteomalacia [31,39,47]. These features were present in Hyp mice in this study. Hyp mice derived from the current breeding strategy were considerably smaller, had significantly lower body weight, and demonstrated reductions in tail and femur lengths due to the presence of rickets/osteomalacia (Figure 2A and 3A). Micro-CT 3D image analyses of the femur of Hyp mice revealed that loss of Phex function resulted in shortened bone length, widened growth plate and reduced cortical or trabecular bone structural parameters, consistent with the presence of rickets and osteomalacia. Consistent with the abnormal mineralization in Hyp mice, histological evaluation revealed a reduction in trabecular bone volume, cortical thickness, and mineral apposition rate as well as an apparent increase of un-mineralized osteoid in both trabecular and cortical bone areas (Figure 3 and 4).

Bone samples from Hyp mice showed a 2-fold increase of Fgfr23 transcripts (Table 1) and western blot analysis showed a 7-fold increment in FGF23 protein levels in bone (Figure 2B and 2C). Hyp bone also showed a significant increase in other osteocyte-specific markers, including Dmp1 and Mepe. Consistent with the observations in Hyp osteocytic cells [55], Fgf1, Fgf2, Fgf3, and Fgf4 as well as Osteopontin, Osteocalcin, Gabal3, Mmp13, OPG, and RankL mRNA levels were also increased in bone derived from Hyp mice (Table 1). In addition, Hyp mouse bone exhibited an increase in Fzd2, Wnt10b, and Axin-2 expression, as well as suppression of Sost and Dkk1, and Collagen II and Mmp9 were also increased in Hyp bone. We also observed more than 2-fold increase of Fgfg2 protein expression in Hyp mice compared to control Fgfr1floxs/+ mice (Figure 2B and 2C).

Similar to previous reports [39,47], Hyp mice displayed the anticipated elevations in serum Fgfg3 concentrations (~18-fold) that were associated with significant reductions in serum phosphate. There was also evidence for the previously reported diminished degradation of FGF23 in Hyp mice, as evidenced by alterations in the ratio of intact FGF23 to total FGF23, which was 0.31 in control mice and 0.65 in Hyp mice (Table 2). In addition, serum PTH levels were significantly elevated in Hyp mice, whereas 1,25(OH)2D levels were significantly decreased in Hyp mice compared with age-matched control mice. Consistent with known effects of FGF23 on the kidney, Hyp mice exhibited reduction of
Figure 1. Dmp1-Cre-mediated conditional deletion of Fgfr1 from the floxed Fgfr1 allele (Fgfr1<sup>lox+</sup>) in different tissues. (A) Schematic illustration of wild-type (Fgfr1<sup>+</sup>), floxed Fgfr1 allele before (Fgfr1<sup>lox+</sup>) and after deletion (Fgfr1<sup>loxnull</sup>) of the lox P cassette containing Exon 8–14 via Cre-mediated recombination. "/'" stands for all the Introns and Exons omitted between Exon 8 and Exon 14. (B) Genotype PCR analysis of different tissues that were harvested from heterozygous Dmp1-Cre:Fgfr1<sup>lox+/+</sup> mice at 6 weeks of age. Both Fgfr1 wild-type and floxed alleles existed in all tested tissues of heterozygous Dmp1-Cre:Fgfr1<sup>lox+/+</sup> mice. However, Dmp1-Cre-mediated recombination of excised floxed Fgfr1 (Fgfr1<sup>loxnull</sup>) allele occurred in bone tissues such as calvarias and femur, but also had a leakage in the brain, muscle, and intestine. (C and D) Real-time RT-PCR analysis of total Fgfr1 transcripts in bone and kidney. Expression of total Fgfr1 transcripts was performed using Fgfr1-allele-specific primers as described in Materials and Methods. The normal Fgfr1<sup>+</sup> vs cyclophilin A is normalized to the mean ratio of 5 control mice, which has been set to 1. Data are expressed as a relative abundance of wild-type (Fgfr1<sup>+</sup> and Fgfr1<sup>lox+</sup>) mRNA messages in control Fgfr1<sup>lox+/+</sup> mice, heterozygous Dmp1-Cre:Fgfr1<sup>lox+/+</sup> and homozgyous Dmp1-Cre:Fgfr1<sup>loxnull</sup> (Fgfr1<sup>loxnull</sup>) null mice. Values sharing the same superscript in different groups are not significantly different at P<0.05. doi:10.1371/journal.pone.0104154.g001

The conditional deletion of Fgfr1 in osteocytes of compound Hyp:Fgfr1<sup>Dmp1-KO</sup>-null mice resulted in partial rescue of the Hyp gross phenotype. In this regard, the tail length, a marker of rickets, was 2" shorter in Hyp mice compared to controls, but was only 0.5" shorter in compound Hyp:Fgfr1<sup>Dmp1-KO</sup>-null mice compared to Fgfr1<sup>lox+/+</sup> control mice. Similarly, compound Hyp:Fgfr1<sup>Dmp1-KO</sup>-null mice displayed body weight and femur lengths intermediate to those of Hyp and control mice (Figure 1A & 3A).

More importantly, both the elevated serum FGF23 and hypophosphatemia in Hyp mice were significantly improved in compound Hyp:Fgfr1<sup>Dmp1-KO</sup>-null mice. Conditional deletion of Fgfr1 in Hyp osteocytes resulted in about 70% reduction in serum FGF23 (from 3607 to 1099 pg/ml) and about 50% increase in serum phosphorus (from 6.0 to 9.3 mg/dl, values not significantly different from age-matched control mice). Ablation of Fgfr1 in Hyp osteocytes also resulted in reductions of PTH to the normal range, changes associated with significant increments of serum 1,25(OH)<sub>2</sub>D and phosphate concentrations. We observed no significant differences in serum calcium concentrations across the four genotypes (Table 2). Serum FGF23 and phosphorus concentrations (207±84 pg/ml and 9.8±0.63 mg/dl, respectively) were not significantly different in Fgfr1<sup>lox/null</sup> (equivalent to Fgfr1<sup>1/4null</sup>) mice compared to Fgfr1<sup>lox+/+</sup> controls (155±70 pg/ml and 9.6±1.34 mg/dl, respectively) (Table 3). In Hyp:Fgfr1<sup>lox/null</sup> mice, we observed an intermediate reduction of serum FGF23 (1881±230 pg/ml) and a corresponding increment of serum phosphorus (7.2±0.84 mg/dl) compared to Hyp:Fgfr1<sup>Dmp1-KO</sup>-null mice, indicating a gene dosage effect of Fgfr1 reductions in osteocytes on the Hyp phenotype.

In contrast to the nearly 70% reduction in both bone FGF23 proteins and circulating FGF23 concentrations in Hyp:Fgfr1<sup>Dmp1-KO</sup>-null mice, conditional deletion of Fgfr1 in Hyp osteocytes resulted in only a 28% reduction of Fgfr1 transcripts in bone of Hyp:Fgfr1<sup>Dmp1-KO</sup>-null compared with Hyp mice (Figure 3C & 3D).
Figure 2. Effects of conditional deletion of Fgfr1 in Hyp osteocytes on gross appearance and bone-related gene expressions in 6-week-old mice. (A) Gross appearance, tail length, and body weight. Compared with control mice, Fgfr1<sup>Dmp1-cKO</sup>-null mice had normal gross appearance and body weight. However, Hyp mice showed considerably shorter tail length and lower body weight, compound Hyp:Fgfr1<sup>Dmp1-cKO</sup>-null mice displayed intermediate tail length and body weight between control and Hyp mice. Data are mean ± S.D. from 5–6 individual mice. (B and C) Western blot analysis of total Fgf2 and Fgf23 protein levels in bone. A representative Fgf2, Fgf23, and β-Actin gel were shown in upper, middle, and lower panels of B, respectively. The intensity of bands was quantified using Image J software (http://rsb.info.nih.gov/ij/), and the data shown in C are mean ± S.D. from three independent experiments. Values sharing the same superscript in different groups are not significantly different at P<0.05. doi:10.1371/journal.pone.0104154.g002

Figure 3. Effects of conditional deletion of Fgfr1 in Hyp osteocytes on bone structure in 6-week-old mice. Representative μCT 3D images of (A) whole femur sagittal section, (B) the distal femoral metaphyses, and (C) femoral midshaft diaphyses in four genotypes of mice. Compared with control mice, Fgfr1<sup>Dmp1-cKO</sup>-null mice had normal bone structure. However, Hyp mice showed considerably shorter femur length, less trabecular bone volume, and thinner cortical thickness, compound Hyp:Fgfr1<sup>Dmp1-cKO</sup>-null mice displayed intermediate femur length and cortical thickness between control and Hyp mice, but no recovery was found in trabecular bone volume. Data are mean ± S.D. from 5–6 individual mice. Values sharing the same superscript in A, B, and C are not significantly different at P<0.05. doi:10.1371/journal.pone.0104154.g003
and Table 1 & 2). In Hyp mice the ratio of intact and C-terminal FGF23 in serum was consistent with impaired FGF23 degradation in Hyp mice, as previously described [56,57], however conditional deletion of Fgfr1 in Hyp osteocytes did not affect the ratio of intact and C-terminal Fgf23 in the compound Hyp:Fgfr1Dmp1-cKO-null mice (Table 2).

Conditional deletion of Fgfr1 in Hyp osteocytes normalized Npt2a and Klotho expression in the kidney. The alterations in Cyp27b1 and Cyp24 transcripts that regulate vitamin D metabolism were also corrected in the kidney from compound Hyp:Fgfr1Dmp1-cKO-null mice. These results are consistent with an effect of reduced circulating FGF23 to increase serum phosphate and 1,25(OH)2D levels in the compound Hyp:Fgfr1Dmp1-cKO-null mice.

In addition to the improvement in serum biochemistries in Hyp mice by conditional deletion of Fgfr1 in osteocytes, there was a  

Figure 4. Effects of conditional deletion of Fgfr1 in Hyp osteocytes on bone histology in 6-week-old mice. Representative images of (A) the distal femoral growth plate, (B) the femoral cortical bone by Goldner staining, and (C) femoral sagittal section by Alizarin Red double labeling in four genotypes of mice. Compared with control mice, Fgfr1Dmp1-cKO-null mice had normal growth plate, no osteomalacia in cortical bone, and normal mineral apposition rate. However, Hyp mice showed an unorganized growth plate, an apparent increase of un-mineralized osteoid in both trabecular and cortical bone areas, and significant reduction in mineral apposition rate, compound Hyp:Fgfr1Dmp1-cKO-null mice exhibited a recovery of organized growth plate, only fewer un-mineralized osteoid in cortical bone, and increased mineral apposition rate between control and Hyp mice, suggesting a partial rescue of the hypophosphatemic rickets phenotype in the compound Hyp:Fgfr1Dmp1-cKO-null mice. Data are mean ± S.D. from 5–6 individual mice. Values sharing the same superscript in C are not significantly different at P<0.05.

doi:10.1371/journal.pone.0104154.g004
### Table 1. Gene-expression profiles in bone in 6-week-old mice.

| Gene                  | Fgfr1^flox+/c | Fgfr1^Dmp1-cKO | Hyp | Hyp/Fgfr1^Dmp1-cKO | p-value |
|-----------------------|---------------|----------------|-----|-------------------|---------|
| **Osteoblast lineage**|               |                |     |                   |         |
| Fgfr1                 | 1.00 ± 0.12a  | 0.30 ± 0.15b   | 1.64 ± 0.12c | 0.31 ± 0.14b | <0.0001 |
| Fgfr2                 | 1.00 ± 0.22a  | 0.86 ± 0.29b   | 1.87 ± 0.77b | 1.02 ± 0.22a | 0.0078  |
| Fgfr3                 | 1.00 ± 0.13a  | 0.98 ± 0.43a   | 2.26 ± 0.85b | 1.53 ± 0.18b | 0.0029  |
| Fgfr4                 | 1.00 ± 0.18a  | 0.94 ± 0.38b   | 1.78 ± 0.55b | 1.24 ± 0.16a | 0.0096  |
| Pth                   | 1.00 ± 0.11a  | 0.73 ± 0.19b   | 0    | 0                 | <0.0001 |
| Fgf1                  | 1.00 ± 0.29a  | 0.94 ± 0.33b   | 1.83 ± 0.54b | 1.03 ± 0.11a | 0.0030  |
| Fgf2                  | 1.00 ± 0.36a  | 1.01 ± 0.29a   | 2.03 ± 0.54b | 1.11 ± 0.03a | 0.0007  |
| Fgf23                 | 1.00 ± 0.22a  | 0.53 ± 0.21b   | 23.12 ± 5.48b | 16.72 ± 0.67 | <0.0001 |
| Dmp1                  | 1.00 ± 0.26a  | 0.56 ± 0.15b   | 1.53 ± 0.36b | 0.84 ± 0.14b | 0.001   |
| Mepe                  | 1.00 ± 0.14a  | 0.42 ± 0.11b   | 1.65 ± 0.23b | 0.32 ± 0.10b | <0.0001 |
| Osteopontin           | 1.00 ± 0.19a  | 1.25 ± 0.11a   | 2.14 ± 0.34b | 0.95 ± 0.32a | <0.0001 |
| Osteocalcin           | 1.00 ± 0.12a  | 1.01 ± 0.13a   | 1.41 ± 0.14b | 0.79 ± 0.22a | <0.0001 |
| Galt3                 | 1.00 ± 0.35a  | 1.01 ± 0.38a   | 2.00 ± 0.91b | 1.02 ± 0.20a | 0.0217  |
| Mmp13                 | 1.00 ± 0.13a  | 1.31 ± 0.47a   | 2.57 ± 0.37b | 1.41 ± 0.46a | <0.0001 |
| Wnt10b                | 1.00 ± 0.19a  | 0.64 ± 0.18b   | 2.69 ± 0.85b | 1.09 ± 0.16b | 0.0002  |
| Axin-2                | 1.00 ± 0.18a  | 0.77 ± 0.13a   | 2.11 ± 0.39b | 1.08 ± 0.12b | <0.0001 |
| Fzd2                  | 1.00 ± 0.23a  | 0.97 ± 0.31b   | 2.43 ± 0.76b | 1.22 ± 0.15a | 0.0002  |
| Sost                  | 1.00 ± 0.17a  | 0.41 ± 0.09b   | 0.37 ± 0.08b | 0.36 ± 0.02b | <0.0001 |
| Dkk1                  | 1.00 ± 0.44a  | 0.42 ± 0.10b   | 0.61 ± 0.12b | 0.42 ± 0.06b | 0.0047  |
| Opg                   | 1.00 ± 0.21a  | 0.77 ± 0.11a   | 1.36 ± 0.22b | 0.79 ± 0.14a | 0.0002  |
| RanKl                 | 1.00 ± 0.28a  | 0.84 ± 0.19a   | 1.36 ± 0.28b | 0.90 ± 0.15a | 0.0117  |
| **Osteoclast**        |               |                |     |                   |         |
| Trap                  | 1.00 ± 0.27a  | 0.82 ± 0.21a   | 0.96 ± 0.32a | 0.84 ± 0.14a | 0.5591  |
| Mmp9                  | 1.00 ± 0.33a  | 0.87 ± 0.21a   | 1.35 ± 0.13b | 0.89 ± 0.14a | 0.0072  |
| **Chondrocyte**       |               |                |     |                   |         |
| Collagen II           | 1.00 ± 0.29a  | 0.74 ± 0.21a   | 3.66 ± 1.92b | 1.24 ± 0.52a | 0.0015  |
| VegfA                 | 1.00 ± 0.35a  | 1.07 ± 0.36a   | 1.26 ± 0.53a | 0.95 ± 0.21a | 0.6095  |
| **Adipocyte**         |               |                |     |                   |         |
| PPARγ                 | 1.00 ± 0.23a  | 0.91 ± 0.16a   | 1.22 ± 0.27a | 1.02 ± 0.15a | 0.1037  |
| aP2                   | 1.00 ± 0.17a  | 1.18 ± 0.16a   | 1.19 ± 0.23a | 0.98 ± 0.56a | 0.6206  |

Data are mean ± S.D. from 5–6 tibiae of 6-week-old individual mice and expressed as the fold changes relative to the housekeeping gene β-actin subsequently normalized to control mice. Values sharing the same superscript between two groups are not significantly different at P<0.05.

doi:10.1371/journal.pone.0104154.t001

### Table 2. Biochemistry analysis in 6-week-old mice.

| Parameters              | Fgfr1^flox+/c | Fgfr1^Dmp1-cKO | Hyp | Hyp/Fgfr1^Dmp1-cKO | p-value |
|-------------------------|---------------|----------------|-----|-------------------|---------|
| FGF23 (Intact, pg/ml)   | 155 ± 70a     | 67 ± 23a       | 3607 ± 870b | 1099 ± 272b | <0.0001 |
| FGF23 (C-term, pg/ml)   | 415 ± 94a     | 242 ± 51a      | 6524 ± 904b | 1952 ± 608b | <0.0001 |
| Ratio (Intact/C-term)   | 0.31 ± 0.08a  | 0.33 ± 0.10a   | 0.65 ± 0.11b | 0.64 ± 0.17b | <0.0001 |
| P (mg/dl)               | 9.6 ± 1.34a   | 9.8 ± 0.63a    | 6.0 ± 1.29b | 9.3 ± 1.17b | <0.0001 |
| Ca (mg/dl)              | 7.0 ± 0.45a   | 6.7 ± 0.12a    | 6.7 ± 0.63a | 6.9 ± 0.33a | 0.6382 |
| PTH (pg/ml)             | 229 ± 139a    | 170 ± 62a      | 477 ± 216b | 146 ± 72a | 0.0078  |
| 1,25(OH)2D (pg/ml)     | 178 ± 48a     | 163 ± 46a      | 121 ± 23b | 192 ± 34a | 0.0083  |

Data are mean ± S.D. from 5–6 serum samples of 6-week-old individual mice. Values sharing the same superscript between two groups are not significantly different at P<0.05.

doi:10.1371/journal.pone.0104154.t002
partially rescued the observed abnormalities of the Wnt signaling null mice compared to age-matched mineralized osteoid in cortical bone was reduced and an increase plate abnormalities were also qualitative improved, as evidenced c promoter activity; however, high dose of PLC
FGF2 to stimulate FGFR1-dependent activation of FGF23-
U73122 (15

thickness in the compound c abnormalities remained, consistent with an intrinsic mineralization bone volume was not corrected in compound mutant mice skeletal Sost transcripts were reduced in compound Hyp messages were elevated in Hyp c

FGFR1-mediated stimulation of FGF23 promoter activity (Figure 5E). In addition, we observed that overexpression of HMW-FGFR2, which activates intracrine dependent FGFR1 signaling [61], stimulated FGF23 promoter activity (Figure 5F).

Fgfr1 signaling regulates FGF23 protein expression in vitro via a PI3K-AKT dependent pathway
Since we found disproportionate reductions in serum circulating FGF23 protein and bone Fgf23 mRNA levels in compound Hyp:Fgfr1Dmp1-cKO mice, and Fgfr1 is known to enhance recruitment of RNA to polysomes to increase in protein expression in other systems [62], we examined if Fgfr1 regulate FGF23 protein expression through an effect to stimulate mRNA translation. For these studies we transfected human FGF23-V5-His cDNA expression plasmid into mouse MC3T3-E1 osteoblasts and assessed epitope tagged FGF23 protein production before and after stimulation with FGF22. We found that FGF2 resulted in a time-dependent induction of FGF23-V5-His protein expression, achieving a maximal 3-fold stimulation at 24 hours after FGF2 stimulation (Figure 6A and 6B). Both the PI3K inhibitor Wortmannin and protein synthesis inhibitor Cycloheximide completely blocked FGF2-induced FGF23 protein increase (Figure 6C and 6D), consistent with an effect of FGF2 to stimulate FGF23 protein translation through activation of PI3K/Akt pathway.

Discussion
Osteocytes, the most abundant cells in bone, play a central role in regulating bone remodeling and mineralization through the release of paracrine factors and systemic phosphate and vitamin D metabolism through the release of the hormone FGF23 [63]. FGF23 is known to be involved in the regulation of bone development and remodeling [64,65]. Inactivating mutations of FGF23 in autosomal dominant Kallmann syndrome [66] and the Pfeiffer craniosynostosis [67] and conditional deletion of Fgfr1 gene in osteo-chondro-progenitors using Col1a1(2.3)-Cre enhances osteoblast differentiation and endochondral bone formation [65], suggesting that FGF23 has osteoblast-stage specific effects. The specific role of FGF23 in osteocytes, however, has not been previously defined. The current study is the first to selectively ablate Fgfr1 gene in osteocytes in bone.

We found that Dmp1-Cre-mediated loss of Fgfr1 in osteocytes resulted in selective alterations of multiple genes involved in bone remodeling and mineral metabolism. Fgfr1Dmp1-cKO-null mice exhibited reductions in Fgf23 transcripts in bone and circulating levels of FGF23. In addition, targeted disruption of Fgfr1 in osteocytes resulted in reductions of Dmp1 and Mepe, two members

| Table 3. Gene-expression profiles in kidney in 6-week-old mice. |
| --- |
| Gene | Fgfr1Fkz1- | Fgfr1Dmp1-cKO | Hyp | Hyp:Fgfr1Dmp1-cKO | p-value |
| --- | --- | --- | --- | --- | --- |
| Fgfr1 | 1.00±0.11a | 0.50±0.09b | 1.04±0.08a | 0.50±0.06b | 0.0001 |
| Npt2a | 1.00±0.14a | 1.04±0.30b | 0.48±0.15b | 0.83±0.21a | 0.0013 |
| Npt2c | 1.00±0.08a | 0.99±0.09a | 0.76±0.28b | 0.98±0.32a | 0.2417 |
| Klotho | 1.00±0.41a | 1.41±0.29a | 0.46±0.17b | 0.92±0.30a | 0.0008 |
| Cyp24a1 | 1.00±0.22a | 0.60±0.34a | 1.57±0.54b | 0.84±0.27a | 0.0049 |
| Cyp27b1 | 1.00±0.17a | 1.25±0.22a | 0.67±0.21b | 0.96±0.15a | 0.0014 |

Data are mean ±S.D. from 5–6 kidneys of 6-week-old individual mice and expressed as the fold changes relative to the housekeeping gene β-actin subsequently normalized to control mice. Values sharing the same superscript between two groups are not significantly different at P<0.05.

doi:10.1371/journal.pone.0104154.t003

FGF1 regulates FGF23 gene transcription in vitro via a MAPK dependent pathway
To test the role of FGFR1 activation on FGF23 gene transcription, we co-transfected MC3T3-E1 osteoblasts with a mouse Fgfr2 6 kb promoter-luciferase reporter construct (p0.6 kb-Fgfr2-luciferase) and either a wild-type FGFR1 or a dominant negative Fgfr1(TK-) cDNA construct [59,60]. Addition of FGF2 (5–100 ng/ml), dose dependently, stimulated FGF23 promoter activity in MC3T3-E1 osteoblasts co-transfected with Fgfr1 (Figure 5A), achieving a maximal response at an FGF2 of 50 ng/ml. Co-transfection of the dominant negative Fgfr1(TK-) completely inhibited FGF2 stimulation of FGF23 promoter activity in MC3T3-E1 osteoblasts overexpressing FGFR1 (Figure 5B). To investigate which of the FGFR1-dependent signaling pathway (i.e., PI3K/AKT, RAS/MAPK, and PLCγ) mediating the actions of FGF1 on FGF23 gene transcription, we investigated the effects of a Wortmannin, a PI3K inhibitor, U73122, a PLCγ inhibitor, and U0126, an inhibitor of MAPK pathway, on FGF2-stimulated FGF23 promoter activity. Neither Wortmannin (1 μM, Figure 5C) or low dose of the PLCγ inhibitor U73122 (10 μM, Figure 5D) inhibited the effects of FGF2 to stimulate FGFR1-dependent activation of FGF23-promoter activity; however, high dose of PLCγ inhibitor U73122 (15–20 μM, Figure 5D) and U0126, which inhibits both MEK1 and MEK2, exhibited a dose-dependent inhibition of FGFR1-mediated stimulation of FGF23 promoter activity (Figure 5E).
of the SIBLING family of proteins that are expressed in osteocytes as well as Phex, an endopeptidase, that are involved in the regulation of bone mineralization. We also found in the Fgfr1Δmsg1−KO-null mice evidence for reductions in Sost and Dkk1, which are produced by osteocytes and function as antagonists of the Wnt-signaling pathway, which is an important anabolic signal for bone. In contrast, Fgfr1Δmsg1−KO−null mice exhibited no changes in more general markers of osteoblast differentiation, such as Osteopontin and Osteocalcin, and bone expression of Wnt10b and Axin-2 were decreased, not increased, in conditional the Fgfr1Δmsg1−KO−null mice, as would be expected from reductions in Sost and Dkk1. In spite of these changes in osteocyte gene expression, we observed no abnormalities of bone structure or mineralization (i.e., no changes in bone volume or mineral apposition rates) in the Fgfr1Δmsg1−KO-null mice under normal conditions. This may be due to the complexity of FGFR1 signaling in bone and cross-talk between downstream molecular targets affecting bone remodeling [68] that may have offset the effects of the loss-of-FGFR1 in osteocytes.

Indeed, additional pathological perturbations caused by Phex mutations in Hyp mice uncovered a more evident role of Fgfr1 in osteocytes on bone structure and gene expression [29]. We found

![Figure 5. Enhancement of mouse FGF23 promoter activity by FGF2 via FGFR1 signaling pathway.](image-url)
that conditional deletion of Fgfr1 in osteocytes of Hyp mice decreased Fgf23 transcripts in bone, as well as a lead to a striking reduction in circulating FGF23 levels in compound Hyp:Fgfr1\textsuperscript{Dmp1-\textminus KO}-null mice. The reductions in FGF23 in Hyp mice partially rescued the hypophosphatemic rickets phenotype, as evidenced by increased serum phosphate levels, improved bone parameters and bone-related gene expression as well as improved kidney gene expression and 1,25(OH)\textsubscript{2}D metabolism. In addition, alterations in Fgfr1 and Fgfr2, as well as Fgfr3, and Fgfr4 expression that are known to be increased in osteocytes derived from Hyp bone [55] were normalized in Hyp:Fgfr1\textsuperscript{Dmp1-\textminus KO}-null mice (Table 1), indicating that Fgfr1 regulates other members of the FGF/FGFR signaling family in Hyp osteocytes. Since FGF23 has dose-dependent effects on the kidney [69,70], the 3-fold reduction in circulating FGF23 in Hyp:Fgfr1\textsuperscript{Dmp1-\textminus KO}-null mice likely accounts for the improvement in the hypophosphatemic rickets phenotype, whereas the persistent elevations of FGF23 would explain the residual hypophosphatemia and bone abnormalities in Hyp:Fgfr1\textsuperscript{Dmp1-\textminus KO}-null mice. Alteration in matrix mineralization caused by loss of Phex function are persistent [41,58,71] and could contribute to persistent skeletal abnormalities are present in the compound Hyp:Fgfr1\textsuperscript{Dmp1-\textminus KO}-null mice.

Studies of FGF23 gene transcription and translation in osteoblast cultures confirmed an important role of FGFR1 in regulating FGF23 production. In this regard, we demonstrated that a dominant negative FGFR1(TK−) construct inhibits FGF2-levels of FGFR1-mediated control of FGF23 by FGFR1 may explain discrepancies between FGF23 message and circulating levels of this hormone [75], as well as provide another therapeutic target, along with regulation of transcription and degradation [76], to modify circulating FGF23 levels in disease states.

Previous data have implicated FGFR1 signaling in the regulation of FGF23 expression, but these observations lack the cell- and organ- specificity of our studies [77,78]. For example, pharmacological inhibition of FGFR1 in vitro in Hyp mice [78] results in inhibition of both FGF23 end-organ effects as well as variable effects on FGF23 production by bone. Similarly, Y372C missense gain-of-function mutation in FGFR1 in osteoglophonic dysplasia (OGD) and systemic administration of activating antibodies to FGFR1 in mice elevate circulating FGF23 levels [79], but have the confounding effects of activation of FGFRs in
both bone and kidney. Thus, use of non-specific inhibitors of FGFR1 or FGFR1 activating antibodies in vivo are limited by generalized effects that prevent distinguishing between actions on FGFR1 in osteocytes or indirect effects through other actions on FGFs in the kidney or other tissues. In contrast, the selective deletion of Fgfr1 in osteocytes provides in vivo confirmation of prior in vitro studies showing that pan-FGFR inhibitors directly suppress FGF23 expression in Hyp-derived osteoblasts and FGFR1 and FGFR2-mediated FGFR1 activation directly stimulates FGF23 promoter activity in osteoblasts cultures [39,77].

Perhaps more importantly, we have discovered that the evolutionarily linked canonical (or autocrine/paracrine) and intracellular (or intracrine) FGFS are also physiologically coupled to the hormone-like FGF23 in bone through FGFR1-dependent mechanisms. FGFR1 and FGFR2 (both low and HMW forms), produced by the osteoblast lineage and stored in bone matrix, are increased in Hyp bone [37–39]. Osteocytes also participate in a peri-cellular demineralization process called osteolytic osteolysis that involves matrix degradation and growth factor activation by enzymes, such as Matrix metalloproteinase-9 and 13 (MMP-9 and –13), which are also increased in Hyp mice [80]. It is tempting to speculate that either the local release of stored FGFs from the extracellular matrix, or other factors regulating, FGFR1, FGFR2 and HMW-FGF2, are regulating osteocyte functions through FGFR signaling. Further studies are needed to define how alterations in bone metabolism and mineralization caused by Phex mutations regulate FGF ligands and FGFR signaling pathways in osteocytes. Since hormonal-like FGFs emerged early in vertebrate evolution, our findings raise the possibility that the mineralized skeleton developed a means to coordinate local and systemic functions by the linkage of autocrine/paracrine and intracrine FGF signaling with hormonal FGF23.

Our study has several limitations. First, conditional deletion of Fgfr1 failed to completely restore FGF23 expression to normal in the compound Hyp:Fgfr1<sup>Dmp1<sup>-Cre</sup></sup></sub>+null mice. The persistent elevations of FGF23 might be due to incomplete ablation of Fgfr1 in osteocytes or continued production of FGF23 from osteoblasts not affected by Dmp1-Cre (e.g., 30% of Fgfr1 transcripts persisted in bone of Fgfr1<sup>Dmp1<sup>-Cre</sup></sub></sup></sub> mice and Dmp1-Cre targets ~1% of osteoblasts in long bone). Alternatively, FGFR1-independent pathways known to regulate FGF23 expression may account for the elevations of FGF23. The possibility that other FGFRs in osteocytes might mediate the residual stimulation of Fgfr1 in Hyp mice appears unlikely, since we observed a decrease in Fgfr2, Fgfr3, and Fgfr4 in Hyp:Fgfr1<sup>Dmp1<sup>-Cre</sup></sub></sup></sub>-null mice. In addition, prior studies indicate that conditional deletion of Fgfr2 in mice leads to no abnormalities of serum FGF23 or phosphate [81], and global loss of Fgfr3 and Fgfr4 enhances, rather than inhibits, FGF23 expression in bone, due to impaired end organ sensing of FGF23 by the kidney [92,93]. Second, although Dmp1-Cre had no kidney expression, to achieve the 70% reduction of Fgfr1 expression in osteocytes using Dmp1-Cre required using a strategy that paired a flox with a null Fgfr1 allele, which led to a 50% reduction in Fgfr1 expression in the kidney, and other tissues. Meaningful conclusions can be made about FGFR1 function in bone, however, because heterozygous Fgfr1<sup>Flox/null</sup> (equivalent to Fgfr1<sup>+/null</sup>) mice had no demonstrable renal phenotype and the reduction in FGF23 expression was dose-dependently related to reductions in Fgfr1 expression in bone. We lack evidence that end-organ resistance to FGF23 actions on the kidney in Hyp:Fgfr1<sup>Dmp1<sup>-Cre</sup></sub></sub> mice, since this would lead to hyperphosphatemia and elevated FGF23 levels, a phenotype different from the one observed in these mice.

In conclusion, conditional deletion of Fgfr1 in osteocytes from Hyp mice reduced circulating FGF23 concentrations through regulation of both the transcription and translation of FGF23. These studies establish a function of FGFR1 in osteocytes and show a physiological coupling between canonical FGFRs and hormone-like FGFs that may provide a mechanism to link local regulation of bone metabolism with systemic phosphate and vitamin D homeostasis. This evolutionary relation may permit local changes in bone metabolism regulated by FGFs-FGFRs to communicate with the kidney to permit FGFR1 regulation of bone formation and mineralization to be coordinated with phosphate absorption and/or vitamin D metabolism to match the bone needs for mineral with the renal handling of these minerals.

**Author Contributions**

Conceived and designed the experiments: ZSX XBJ LDQ. Performed the experiments: ZSX JSH LC YJL XBJ. Analyzed the data: ZSX XBJ LDQ. Contributed reagents/materials/analysis tools: ZSX JSH LC YJL XBJ. Wrote the paper: ZSX XBJ LDQ.

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