Pathogenic role of Fgf23 in Hyp mice

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Liu, Shiguang, Jianping Zhou, Wen Tang, Xi Jiang, David W. Rowe, and L. Darryl Quarles. Pathogenic role of Fgf23 in Hyp mice. Am J Physiol Endocrinol Metab 291: E38–E49, 2006. First published January 31, 2006; doi:10.1152/ajpendo.00008.2006.—Inactivating mutations of the PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) endopeptidase, the disease-causing gene in X-linked hypophosphatemia (XLH), results in increased circulating levels of fibroblast growth factor-23 (FGF23), a bone-derived phosphaturic factor. To determine the causal role of FGF23 in XLH, we generated a combined Fgf23-deficient enhanced green fluorescent protein (eGFP) reporter and Phex-deficient Hyp mouse model (Fgf23<sup>+/−</sup>/Hyp<sup>−/−</sup>). eGFP expression was expressed in osteocytes embedded in bone that exhibited marked upregulation of eGFP in response to Phex deficiency and in CD31-positive cells in bone marrow vessels that expressed low eGFP levels independently of Phex. In bone marrow stromal cells (BMSCs) derived from Fgf23<sup>+/−</sup>/Hyp<sup>−/−</sup> mice, eGFP expression was also selectively increased in osteocyte-like cells within mineralization nodules and detected in low levels in CD31-positive cells. Surprisingly, eGFP expression was not increased in cell surface osteoblasts, indicating that Phex deficiency is necessary but not sufficient for increased Fgf23 expression in the osteoblast lineage. Additional factors, associated with either osteocyte differentiation and/or extracellular matrix, are necessary for Phex deficiency to stimulate Fgf23 gene transcription in bone. Regardless, the deletion of Fgf23 from Hyp mice reversed the hypophosphatemia, abnormal 1,25(OH)2D3 levels, rickets, and osteomalacia associated with Phex deficiency. These results suggest that Fgf23 acts downstream of Phex to cause both the renal and bone phenotypes in Hyp mice.

X-linked hypophosphatemia; hypophosphatemia; osteomalacia; fibroblastic growth factor-23

X-LINKED HYPOPHOSPHATEMIA (XLH) is characterized by a skeletal and renal phenotype. The skeletal abnormalities are characterized by defective calcification of cartilage and bone, leading to rickets, osteomalacia, and growth retardation. Disorders of renal function include impaired renal tubular reabsorption of phosphate and aberrant regulation of 1,25(OH)2D3 production, leading to hypophosphatemia that is resistant to phosphorus and vitamin D therapy (31). Inactivating mutations of PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) (31), a member of the M13 family of the type II cell surface zinc-dependent proteases (32), is the cause of X-linked hypophosphatemic rickets (XLH). The mouse Phex cDNA sequence is highly homologous to that of humans, and a 3'-deletion of the Phex gene in the Hyp mouse results in an animal model of XLH (4, 29).

The mechanisms whereby inactivation of Phex leads to renal phosphate wasting, abnormal vitamin D metabolism, and rickets/osteomalacia are not clear. PHEX is predominantly expressed in osteoblasts and osteocytes in bone (4, 6, 10). Several observations suggest the existence of putative PHEX substrates that regulate systemic phosphate homeostasis, called phosphatonin (20), and the mineralization process, called minhibins (36). Both cross-transplant and parabiosis studies in mice support the presence of a phosphaturic factor in Hyp mice that stimulates renal phosphate wasting and impairs production of 1,25(OH)2D3 (20). The evidence for minhibin is derived from both in vivo and in vitro studies demonstrating that osteoblasts and bone marrow stromal cell (BMSC) cultures from Hyp mice have an intrinsic defect in the mineralization of extracellular matrix due to the production of a mineralization inhibitory factor (36), although recent findings suggest that hypophosphatemia and alterations in the physicochemical milieu may be sufficient to account for the defective mineralization (19). Regardless, the presence of PHEX in bone, the importance of phosphate in regulating the mineralization process, and the presence of a circulating phosphaturic factor in PHEX-deficient states have given rise to the possibility of a bone-kidney axis involving PHEX and the production of systemic and local factors by bone that regulate phosphate homeostasis as well as mineralization of extracellular matrix (21).

Fibroblastic growth factor-23 (FGF23) is a leading candidate for phosphatonin (8). In this regard, FGF23 has phosphaturic activity in vivo (25), is also predominantly expressed in bone, and has been shown to be coexpressed with PHEX in osteoblast cultures (15). Moreover, the essential role of FGF23 in regulating phosphate levels is supported by the fact that overexpression of FGF23 in mice (2, 13, 26) or mutations that prevent its degradation (1, 3, 35) cause hypophosphatemia and suppress 1,25(OH)2D3 production by the kidney, whereas FGF23 deficiency (3, 24) or mutations increasing FGF23 degradation (12) result in hyperphosphatemia, increased serum 1,25(OH)2D3 levels, and soft tissue calcifications. In addition, serum levels of FGF23 are elevated in XLH (37) and Hyp mice (15), and the renal and skeletal phenotypes of Hyp mice resemble those of mice with excess Fgf23. Finally, recent reports indicate that crossing Fgf23 null mice onto the Hyp background reverses the hypophosphatemia (27), and blocking antibodies for Fgf23 ameliorates hypophosphatemia and rickets in Hyp mice (1a).

However, several questions remain about the regulation of Fgf23 by Phex and the role of Fgf23 in mediating the phenotype of Hyp mice. For example, the mechanism whereby inactivation of Phex results in increased Fgf23 levels is not clear. Although initial studies suggested that FGF23 might be a substrate for PHEX (28), other studies have been unable to confirm that FGF23 is metabolized by PHEX (5, 15). Furthermore, Fgf23 transcripts are increased in bone and osteoblasts.
derived from Hyp mice, suggesting that inactivation of Phex results in increased synthesis of Fgf23. In addition, the role that Fgf23 plays in the skeletal abnormalities in Hyp mice has not been determined. Fgf23 excess might directly contribute to the apparent intrinsic mineralization defect observed in Hyp-derived osteoblasts (7, 36), or, alternatively, Phex may have other substrates or actions in osteoblasts that impact upon bone independently of Fgf23.

In the present investigations, we examined the mechanism whereby Fgf23 is regulated by Phex and evaluated the contribution of elevated Fgf23 levels to the systemic and skeletal abnormalities observed in Hyp mice, namely hypophosphatemia, abnormal 1,25(OH)2D3 production, and rickets/osteomalacia. To accomplish this, we created a reporter mouse line where enhanced green fluorescent protein (eGFP) was inserted into the Fgf23 locus to replace the first exon of the Fgf23 gene with eGFP sequence, thereby creating a null allele called Fgf23−/− that was crossed onto the Hyp background to create combined Fgf23 and Phex-deficient mice. We found that Fgf23 was expressed predominately in osteocytes, but also identified a novel location in endothelial cells in the venous sinusoids of the bone marrow and thymus. Inactivating mutations of Phex resulted in increased expression of Fgf23 at these sites, presumably from the production of intrinsic or extrinsic factors stimulating Fgf23 gene transcription. In addition, we found that Fgf23−/− mice, despite profound hyperphosphatemia, lacked active mineralization, as assessed by fluorescent labeling of bone. Moreover, the Fgf23 null phenotype was dominant to that of Hyp, resulting in conversion of hypophosphatemia and inappropriately low 1,25(OH)2D3 levels in Hyp to hyperphosphatemia and elevated 1,25(OH)2D3 levels. With regard to the skeleton, superimposed Fgf23 deficiency corrected the rickets but not the defective mineralization in Hyp mice. Rather, there was a conversion of osteomalacia to a complete absence of mineralization in combined Fgf23−/−/Hyp mice. In addition, the presence of accentuated metaphyseal sclerosis in combined Fgf23−/−/Hyp compared with Fgf23−/− mice also suggests an independent effect of Phex on bone.

METHODS

Generation of Fgf23 knockout mice. The Fgf23-deficient/reporter mouse model was created by knocking in an eGFP construct following the ATG in exon 1 of the Fgf23 gene (Fig. 1A). A bacteria artificial chromosome (BAC) clone containing the full-length Fgf23 gene from C57BL/6J mouse genomic DNA was obtained from the

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**Fig. 1.** Generation of combined Fgf23- and Phex-deficient mice. A: schematic representation of Fgf23-targeted disruption strategy. Partial restriction map of the Fgf23 locus (top), gene-targeting vector (middle), and the expected structure of the mutated locus (bottom) are shown. S, SpeI; H, HindIII; eGFP, enhanced green fluorescent protein; Neo, neomycin resistance gene; TK, thymidine kinase. B: genotyping of mice by PCR. Representative PCR analysis of genomic DNA for the Fgf23 gene and neomycin cassette (top) and Phex gene (bottom) in wild-type (Fgf23+/+), heterozygous (Fgf23+/−), homozygous (Fgf23−/−), Phex-deficient Hyp, and combined Fgf23−/−/Hyp and homozygous Fgf23−/−/Hyp mice. C: gross appearance of mice. Fgf23+/+, Fgf23+/−, Fgf23−/−, Fgf23+/−/Hyp, Fgf23+/−/Hyp, and Fgf23−/−/Hyp mice were 6 wk of age.
BACAPC Resource Center at the Children’s Hospital Oakland Research Institute (Oakland, CA). In the targeting vector, we replaced exon 1 of Fgf23 with eGFP so that we could assess the tissue-specific regulation of endogenous Fgf23 promoter activity as well as delete the functional Fgf23 product. To make the targeting construct, pKO Scrambler NTKV-1903 (Stratagene, La Jolla, CA) was used as a backbone vector. A 1.3-kb region of the 5’ flanking region containing an NcoI site at start condon ATG was amplified from the BAC DNA described above with Pfx polymerase (Invitrogen, Carlsbad, CA). eGFP sequence from the first codon ATG to the polyA signal was amplified from the pIRE2-eGFP plasmid (Clontech, Palo Alto, CA) The eGFP coding sequence was cloned downstream of the 1.3-kb 5’ flanking region of Fgf23 (short arm) and cloned into the deletion vector pKO Scrambler NTKV-1903 (Stratagene, La Jolla, CA) at the EcoRI and KpnI sites. For the long arm, the BAC clone was digested with HindIII. An 8-kb fragment containing exon 2 and exon 3 was cloned into the HindIII site in the deletion construct containing short arm. The nucleotide sequences of the eGFP coding region were confirmed by DNA sequence analysis. The pKO-Fgf23 construct was linearized using XhoI and transfected embryonic stem cells. The EF1 embryonic stem cell line was kindly provided by Dr. Frederick W. Alt (Harvard University, Cambridge, MA). Targeted clones were identified by PCR and confirmed by Southern blot analysis. We used four embryonic stem cell clones with disruptions in Fgf23 to generate Fgf23-deficient mice. The correctly mutated embryonic stem cells were injected into blastocysts of C57BL/6J and implanted into 2.5-day-old pseudopregnant females by the Duke Transgenic Facility. The resulting chimera animals were bred with C57BL/6J mice. All four independent clones resulted in germline transmission. Homozygous founders were generated by mating these heterozygous mice. The successful targeting of Fgf23 in embryonic stem (ES) cells was determined by Southern blot analysis of the genomic DNA from ES cell clones. We observed no apparent differences in the founders generated from different ES cell clones. We focused our studies on founder line D.

All mice were fed a standard rodent diet (8604 Harlan Teklad Rodent Diet; Harlan Teklad, Madison, WI) containing 1.01% phosphorus and 1.36% calcium and tap water. All mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985).

Transfer of Fgf23 deficiency onto Phex-deficient Hypl background. We crossed male heterozygous Fgf23-deficient mice (Fgf23+/−/XY) with Hypl mice to generate female heterozygous Fgf23 deficient Hypl mice (Fgf23+/−/HyplX). We then crossed Fgf23+/−/XY with Fgf23+/−/HyplX to generate Fgf23 and Phex-double-deficient mice. Cross between Fgf23+/−/XY with Fgf23+/−/HyplX generated 12 genotypes at the predicted frequency. Wild-type (WT; Fgf23+/+), heterozygous (Fgf23+/−), and homozygous (Fgf23−/−) Fgf23-deficient mice were genotyped by PCR (see below) and were found to be born at the expected Mendelian frequency (Fig. 1B). Because of the inability to genotype female Hypl mice, we examined only male mice in these studies. Genotyping. Genomic DNA tissue was extracted and purified from the tail of each mouse by use of a Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA). Genotypes were determined by PCR using the following primers: Fgf23F (5’-CTGACCTCTGTAGGCACTGA-3’), Fgf23R (5’-GAAGATTGTTCGACAAGCAA-3’), NeoF (5’-ATTCGGCAAGACGAGCTAC-3’), and NeoR (5’-CTTGCTTCCCTCTCCTCATCTC-3’) for Fgf23 null mice; and Phex19F 5’-GCTTGGGCATGTGCTATCT-3’ and Phex19R 5’-TGAGGGTTGGTGATTACACGGAG-3’ for Hypl mice (14).

Serum biochemical measurements. Serum calcium was measured using a Calcium kit (Sigma-Aldrich, St. Louis, MO), and serum phosphorus was measured by the phosphomolybdate-ascorbic acid method, as previously described (16). Serum parathyroid hormone (PTH) levels were measured by a mouse intact PTH ELISA kit (Immutopics, Carlsbad, CA). Serum 1,25-(OH)2D3 levels were measured using a Gamma-B 1,25-Dihydroxy Vitamin D Kit 4. Serum FGF23 levels were measured by using an FGF23 ELSIA kit (Kainos Laboratories, Tokyo, Japan), following the manufacturer’s protocol.

Bone marrow harvest and stromal cell culture. BMSCs from long bones isolated from 6-wk-old male animals were cultured as previously described (14). Adherent cells were grown in the differentiation medium (α-MEM containing 10% FBS supplemented with 5 mmol/l β-glycerophosphate and 25 μg/ml ascorbic acid) to induce osteoblastic differentiation. During the culture period, we checked the cells under an inverted microscope with both bright field and fluorescent every day. On day 14, the cell’s pictures were taken under bright field and fluorescent light microscopy and then fixed in 4% paraformaldehyde dissolved in PBS-4% formaldehyde for immunohistochemical analysis. Fluorescent imaging and immunohistochemical analysis. eGFP fluorescent imaging in tissues was performed using modifications of previously described methods (11). Briefly, mouse bones were quickly dissected and fixed in 4% paraformaldehyde dissolved in PBS (pH 7.4). The fixed samples were then embedded in frozen embedding medium (Thermo Shandon). Cryosectioning was performed on a Leica CM1900 Cryostat (D-69226; Leica, Nussloch, Germany) equipped with a Cryo-Jane freezing sectioning kit (Instrumedics, Hackensack, NJ). Five-micrometer sections were obtained from the femurs and the other organs. eGFP was examined with a Zeiss Axioplan 2 inverted microscope equipped with epifluorescence and a Zeiss AxioCam color digital camera. The filter set 51019 with wavelength of excitation 475/25, dichroic 495, and emission 525/45 was used to observe eGFP. To see the tissue structure of the slide, hematoxylin staining was used in the serial sections next to the section for eGFP image.

For immunohistochemical analysis of frozen section, 5-μm slides were rinsed with PBS and incubated for 30 min at room temperature with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity. After being washed in PBS, the slides were blocked for 20 min in 1× Universal Blocking Reagent (BioGenex, San Ramon, CA) and rinsed with PBS. The frozen sections were incubated overnight at 4°C with a 1:100 dilution of purified rat anti-mouse CD31 monoclonal antibody (BD Biosciences, San Diego, CA). After being washed in PBS, the sections were incubated with 1:200 dilution of biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature and then washed and incubated for 30 min at room temperature with Vector ABC reagent. The slides were rewashed and developed with a DAB kit (Vector Laboratories). To further characterize the eGFP-positive cells in bone marrow, 15-μm sections were used for fluorescent labeling and confocal microscopy analysis. Alexa 594-conjugated goat anti-rat IgG (1:200; Molecular Probes, Eugene, OR) was used to achieve fluorescent labeling, and a ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole special packaging (Invitrogen, Carlsbad, CA) was used to stain the nuclei. Photomicrographs were taken with a Zeiss LSM 510 confocal microscope using Zeiss LSM 510 software (Carl Zeiss MicroImaging, Thornwood, NY). Immunofluorescence staining for CD31 in BMSCs was performed similarly to the above protocol for tissues. The primary rat anti-mouse CD31 monoclonal antibody (1:100) and the Alexa 594-conjugated secondary antibody (1:200) were used in the staining. Endothelial cells were identified by red fluorescence under fluorescent microscope with the filter set 51019.

High-resolution radiography of femurs, bone densitometry, and three-dimensional analysis of the femurs by microcomputed tomography. The femurs from 6-wk-old mice were collected and fixed in 70% ethanol. The radiography of femurs was performed using a Faxitron MX-20 Specimen Radiography system with a digital camera attached (Faxitron X-Ray, Buffalo Grove, IL). Bone mineral density (BMD) of femurs was measured using a PIXImus bone densitometer (Lunar, Madison, WI). High-resolution microcomputed tomography (μCT) was used to evaluate trabecular volume fraction and microar-
chitecture in the distal femur [microcomputed tomography (μCT40); Scanco Medical, Basserdorf, Switzerland] (14). The three-di-

imensional (3-D) structure was constructed and 3-D morphometric analysis conducted with the built-in software of the μCT system.

Histomorphometric analysis of nondecalcified bone. Skeletal patterns of mice were preabeled on day −6 with alizarin complexone (Acros Organics, Fair Lawn, NJ) and on day −1 with calcine (Sigma-Aldrich) by intraperitoneal injection before collection of bone sam-

tles. Bones were fixed in 70% ethanol and embedded in methyl methacrylate. Five-micrometer sections were stained with Goldner’s stain and analyzed under transmitted light, and 20-μm unstained sections were evaluated under fluorescent light.

Statistics. We evaluated differences between groups by one-way ANOVA. All values are expressed as means ± SE. A P value of <0.05 was considered statistically significant. All computations were performed using the Statgraphics statistical graphic system (STSC, Rockville, MD).

RESULTS

Gross appearance. Similar to previous reports (24, 27), Fgf23−/− mice displayed growth retardation and impaired survival, whereas the heterozygous Fgf23+/− deficient mice were indistinguishable in appearance from WT littermates (Fig. 1C and Table 1). Fgf23−/− mice became noticeably smaller and had significantly lower body length and body weight compared with WT littermates at 2 wk of age (Table 1). In addition, whereas WT mice displayed a progressive increase in body weight and length, growth was arrested in homozygous Fgf23−/− mice, as evidenced by no significant change in body weight or length between 2 and 6 wk of age (Table 1). In contrast, heterozygous Fgf23+/− mice were indistinguishable from WT mice in gross appearance and body measurements (Table 1). At 2 wk of age, H yp mice also displayed evidence of reduced body weight and length but showed age-dependent growth retardation and skeletal dysplasia consistent with the skeletal effects of rickets/osteomalacia. Superimposing heterozygous Fgf23+/− mice onto the H yp background resulted in mice that grossly resembled H yp mice (Fig. 1C), but with a slight yet significant increase in body weight and femur length compared with H yp mice (Table 1). In contrast, transfer of homozygous Fgf23−/− onto H yp mice resulted in gross appearance, body weight and length, and femur length identical to those of Fgf23−/− mice. Whereas H yp mice had survival rates indistinguishable from WT mice, combined Fgf23+/−/H yp mice also had high mortality rates indistinguishable from Fgf23−/− mice. Thus the complete loss of Fgf23 predominated over effects of Phex deficiency.

Effect of combined Fgf23 and Phex deficiency on serum biochemical parameters. Circulating Fgf23 levels in 6-wk-old WT (91.9 ± 7.0 pg/ml) and heterozygous Fgf23+/− mice (88.0 ± 19.5 pg/ml) were not significantly different, indicating that the loss of one Fgf23 allele is not sufficient to alter basal circulating levels of Fgf23 (Table 2). In contrast, homozygous Fgf23−/− and combined Fgf23−/−/H yp mice had undetectable serum Fgf23 levels, consistent with successful deletion of the Fgf23 gene. As expected, 6-wk-old H yp mice had an approximate 12-fold increase in Fgf23 levels (1,151.7 ± 159.2 pg/ml), consistent with the known effect of inactivating Phex mutations to increase circulating Fgf23 (15). Moreover, heterozygous Fgf23+/−/H yp mice displayed a 40% reduction in Fgf23 levels (724.6 ± 42.5 pg/ml) compared with H yp mice but remained eightfold higher in Fgf23+/−/H yp compared with WT mice (Table 2), indicating that Fgf23 gene dosage influences the level of Fgf23 when stimulated by Phex deficiency.

The absence of circulating Fgf23 levels in Fgf23−/− mice resulted in significantly higher mean serum phosphate levels compared with WT (Fgf23+/−) littermates, consistent with known phosphaturic activity of this hormone (9). Fgf23−/− mice also resulted in a nearly twofold increase in serum 1,25(OH)2D3 concentrations compared with WT controls (Table 2). In addition, serum calcium was slightly increased, and PTH levels were decreased by ~50% in Fgf23−/− mice, consistent with the increase in 1,25(OH)2D3 or possible effects Fgf23 on the parathyroid gland (see below). Heterozygous Fgf23+/− mice, consistent with their normal levels of Fgf23, had serum phosphate, calcium, 1,25(OH)2D3, and PTH levels that did not differ from WT mice (Table 2).

In H yp mice, the marked elevations in circulating Fgf23 levels were associated with a 40% reduction in mean serum

Table 1. Body weight and body length in various genotypes at 2, 3, and 6 wk of age

<table>
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<th>Age, wk</th>
<th>Fgf23+/−</th>
<th>Fgf23+/−</th>
<th>Fgf23−/−</th>
<th>Hyp</th>
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<th>Fgf23−/−/Hyp</th>
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<td>7.3 ± 0.2‡</td>
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<td>5.7 ± 0.2†</td>
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<tr>
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Data shown are means ± SE from 6-wk-old mice. Values sharing the same symbol with each row (across groups) are not significantly different at P < 0.05 by one-way ANOVA.

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Table 2. Serum biochemistries in the various genotypes

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<th>Serum Markers</th>
<th>Fgf23+/+</th>
<th>Fgf23+/-</th>
<th>Fgf23+/-/Hyp</th>
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<th>Fgf23+/-/Hyp</th>
<th>Fgf23+/-/Hyp</th>
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<td>724.6±42.5†</td>
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<td>14.1±0.6*</td>
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<td>14.2±0.5*</td>
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<td>8.7±0.1†</td>
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<td>1,25-(OH)2D3, pM</td>
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<td>189±43†</td>
<td>535±104*</td>
<td>88±14‡</td>
<td>252±21†</td>
<td>727±119*</td>
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<tr>
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<td>&lt;0.0001</td>
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Data shown are means ± SE from 6-wk-old mice. FGF23, fibroblastic growth factor-23; PTH, parathyroid hormone. Values sharing the same symbol with each row (across groups) are not significantly different at P < 0.05 by one-way ANOVA. Differences within groups at 3 and 6 wk of age are discussed in the text.

Phosphate concentrations and an ~63% reduction in 1,25(OH)2D3 levels compared with WT mice. Serum calcium levels were not different from WT controls, but serum PTH levels were significantly increased in Hyp mice (Table 2). In combined homozygous Fgf23+/-/Hyp mice, the serum phosphate levels were increased to levels indistinguishable from values in Fgf23+/- mice, and the 1,25(OH)2D3 concentrations were markedly elevated. Serum calcium and PTH levels in combined Fgf23+/-/Hyp mice were also identical to values found in Fgf23 null mice (Table 2). Indeed, the 10-fold reduction in PTH values in Hyp by superimposed Fgf23 deficiency could be due to increased 1,25(OH)2D3 or direct effects of Fgf23 on the parathyroid gland. Combined heterozygous Fgf23+/-/Hyp mice, which had an ~40% reduction in Fgf23 levels, remained hypophosphatemic with serum phosphate levels not significantly different from Hyp mice, but 1,25(OH)2D3 levels in 6-wk-old combined heterozygous Fgf23+/-/Hyp mice increased significantly from the suppressed 1,25(OH)2D3 levels in Hyp alone (Table 2), and this nearly threefold increase was not associated with a reduction in PTH.

Expression of eGFP in vivo under the control of the endogenous Fgf23 promoter. Next, we examined the cell distribution and expression levels of eGFP driven by endogenous Fgf23 promoter in heterozygous Fgf23+/- (1 copy of eGFP) and Fgf23+/- (2 copies of eGFP) mice and the effect of superimposed Phex deficiency in combined heterozygous and homozygous Fgf23-deficient Hyp mice of eGFP expression. We found evidence for eGFP driven by the endogenous Fgf23 promoter in bone cortex, venous sinusoids in bone marrow, and thymus as well as in ventrolateral thalamus. In addition to cortical bone, eGFP expression was also found in calvaria, trabecular bone in long bone, and vertebrae. Interestingly, eGFP was not observed in growth plate cartilage (data not shown), where Phex has been shown to be expressed in proliferating and hypertrophic chondrocytes (17). The presence of two copies of eGFP resulted in higher levels of eGFP expression in tissues of homozygous Fgf23+/- compared with heterozygous Fgf23+/- mice (Fig. 2B), indicating an eGFP gene dosage effect. We found no eGFP expression in heart, spleen, kidney, or liver (Fig. 2A). In particular, although eGFP was expressed in the venous sinusoids of the central bone marrow (Figs. 2, A and B, and 3B) and in venules of the thymus, it was not present in vascular beds of other organs (Fig. 2A and data not shown).

Superimposing Hyp onto Fgf23-deficient reporter mice resulted in marked upregulation of Fgf23 expression predominantly in mineralized bone, whereas levels of Fgf23 expression in bone marrow sinusoids were not altered by superimposed Phex deficiency (Fig. 2B), indicating cell type-specific differences in Fgf23 regulation. With regard to expression within the osteoblastic lineage, the upregulation of eGFP was limited to osteocytes embedded in bone, whereas surface osteoblasts did not express eGFP, although both osteoblasts and osteocytes are known to express Fgf (Figs. 2B and 3A) (17).

To define the cell types that express eGFP in the venules of bone marrow and thymus, we performed immunohistochemical staining with an antibody to the endothelial marker CD31. These studies colocalized eGFP and CD31 immunoreactivity in apparent endothelial cells in the venous sinusoids of both bone marrow (Fig. 3C) and thymus (data not shown). Confocal microscopic analysis confirmed the coexpression of eGFP and CD31 in the venous sinusoids (Fig. 3C, inset).

Expression of eGFP in bone marrow stromal cultures under control of the endogenous Fgf23 promoter. To further characterize the cell type expression of eGFP and its regulation by Phex and osteoblast development, we performed cultures of BMSCs derived from combined homozygous Fgf23+/-/Hyp for 14 days under conditions favoring the differentiation into osteoblasts. Similar to the in vivo findings, we observed eGFP expression in two cell populations (Fig. 4A). In early cultures, before the formation of mineralization nodules, we observed low levels of eGFP in CD31-positive fibroblast-like cells (data not shown). After the development of mineralization nodules in 14-day-old confluent cultures, we observed high levels of eGFP expression limited to cells within the mineralization nodules that were negative for CD31 and had dendritic processes resembling osteocytes. Fibroblast-like CD31-positive cells that were present in earlier cultures persisted in these late cultures as spindle-shaped cells interspersed with mesenchymal cells surrounding the mineralization nodules (Fig. 4A). In addition, we compared eGFP expression in BMSCs from Fgf23+/- and Fgf23+/-/Hyp mice to determine whether Phex deficiency regulates Fgf23 promoter activity in these different cell populations ex vivo. We found that the CD31-positive fibroblast-like cells located outside of the mineralization nodules continued to express low levels of eGFP (Fig. 4B, a and c) in the setting of superimposed Phex deficiency, whereas the osteocyte-like cells embedded in the mineralization nodules markedly upregulated eGFP expression (Fig. 4B, b and d) in the absence of Phex. Both the number of cells in mineralization...
nodules expressing eGFP and the intensity of eGFP expression per cell were increased in Hyp mice (Fig. 4B, d).

Effect of superimposed Fgf23 deficiency on rickets and osteomalacia in Hyp mice. The skeleton from heterozygous Fgf23<sup>+/−</sup> mice was indistinguishable from that of WT littersmates with regard to X-ray appearance (Fig. 5A), μCT analysis (Fig. 5B), BMD (Fig. 5C), and histological analysis (Fig. 6, A and B). In contrast, X-ray and μCT analysis of homozygous Fgf23<sup>−/−</sup> mice revealed miniaturized bones of normal shape, but with increased calcification of the subchondral region of the distal metaphysis by μCT (Fig. 5A). The absence of Fgf23 was associated with a reduction in trabecular bone volume, as measured by μCT (Fig. 5B) and an ~20% reduction in BMD (Fig. 5C). Histologically, the growth plate of 6-wk-old Fgf23<sup>−/−</sup> mice demonstrated an increase in the hypertrophic zone relative to the proliferative zone without appreciable widening of the growth plate, consistent with a dyschondroplasia, leading to impaired long bone growth (Fig. 6A). Compared with WT mice, Fgf23<sup>−/−</sup> mice also displayed increased osteoid volume and impaired mineralization, as evidenced by the reduced intensity and the lack of double-fluorescent labeling of bone (Fig. 6B), as previously reported (24).

Fig. 2. Expression of eGFP driven by endogenous Fgf23 promoter. A: tissue-specific expression of eGFP as a marker of Fgf23 promoter activity. Unstained frozen sections viewed under fluorescent light from various organs derived from combined Fgf23<sup>+/−</Hyp</sup> mouse line in which eGFP is driven by the Fgf23 promoter. Fgf23 is expressed in osteocytes in bone, venous sinusoids in bone marrow and thymus, and the ventrolateral thalamic nucleus of the brain (×200). B: Phex-dependent regulation of Fgf23 expression. Hematoxylin and eosin (H & E) stained section (top) viewed under transmitted light and unstained section (bottom) viewed with fluorescent light. Level of eGFP expression is gene-dose dependent (i.e., greater in homozygous Fgf23<sup>−/−</sup> mice with knock-in of 2 eGFP alleles than heterozygous Fgf23<sup>+/−</sup> mice) and is markedly increased in Phex-deficient Hyp mice in osteocytes embedded in bone but not cell surface osteoblasts (magnification ×100; also see Fig. 3).
Hyp mice displayed classical features of rickets, such as splaying of the ends of the long bones and widening of the growth plate by both X-ray and CT analysis (Fig. 5A). Histological analysis of growth plates revealed an increase in the zone of hypertrophic chondrocytes in Hyp mice (Fig. 5B). As previously reported, Hyp mice have a 54% reduction in BMD (Fig. 5C) that is a surrogate marker for osteomalacia and correlates with the degree of excess osteoid (14). Indeed, histological analysis revealed that Hyp mice displayed hyperosteoidosis and impaired mineralization as well as increased periosteocytic osteoid in cortical bone (Fig. 5C). Also, the fluorescent labeling was indistinct, consistent with impaired mineralization. The osteomalacia in Hyp, however, differs from that in Fgf23+/−/Hyp mice by the greater amounts of osteoid and relative greater intensity of diffuse alizarin red and calcine in bone in Hyp mice.

The radiographic appearance of bone in heterozygous Fgf23+/−/Hyp mice did not differ from Hyp mice (Fig. 5A), although, similarly to the slight increase in bone length (Table 1), these animals also had a small but significant increase in BMD (Fig. 5C). In contrast, combined Fgf23−/−/Hyp mice showed correction of both the radiographic and histological manifestations of rickets, which were replaced with growth plate abnormalities and overall bone appearance resembling those of Fgf23−/− mice (Figs. 5, A and B, and 6A). In addition, combined Fgf23−/−/Hyp mice developed a more pronounced increased density of subchondral bone in the distal metaphyseal bone just beneath the growth plate (Fig. 5, A and B, and 6A), suggesting that Phex deficiency results in changes distinct from FGF23 deficiency in this segment of bone. Overall, the BMD also increased in combined homozygous Fgf23−/−/Hyp mice to levels not significantly different from Fgf23 null mice but remained significantly lower than WT littermates (Fig. 5C). On histological examination, osteomalacia persisted in combined Fgf23−/−/Hyp mice but now resembled Fgf23−/− mice, resulting from a reduction in both the reduced amount of osteoid and diminished intensity of fluorescent labeling compared with Hyp mice (Fig. 5C).

DISCUSSION

We originally proposed that Phex and Fgf23, which are coexpressed in bone, are part of a bone-kidney axis regulating phosphate homeostasis and mineralization (21). The existence of the enzyme-hormone cascade was inferred from the fact that...
circulating concentrations of the phosphaturic hormone Fgf23 were increased in association with inactivating Phex mutations (34) and the observations that transcripts for Fgf23 were increased in bone and osteoblast cultures derived from Hyp mice that are deficient in Phex (15). In the present study, we have established an Fgf23 promoter/reporter and Fgf23 gene deletion model that permits us to directly test the Phex-Fgf23 bone-kidney hypothesis. The finding that the Fgf23 promoter drives expression of eGFP in bone in this model (Figs. 2 and 3) is consistent with prior reports (15) regarding the predominant expression of Fgf23 in bone. Also, Phex deficiency upregulates Fgf23 promoter activity in bone in combined Fgf23-deficient/Hyp mice (Fig. 2), validating this mouse model for investigating Phex regulation of Fgf23 gene transcription.

Our investigation of Fgf23-deficient mice expressing eGFP under the control of the endogenous Fgf23 promoter provides new insights into the cell type expression of Fgf23 in bone and clarifies the role of this hormone in the pathogenesis of renal and bone abnormalities in the Hyp homolog of XLH. The present studies show for the first time that Fgf23 is expressed in two distinct cell populations in the bone, namely osteocytes embedded in both cortical and trabecular bone and in cells lining the venous sinuses in bone marrow. Only the Fgf23 expression in osteocytes appears to be upregulated by Phex deficiency. In contrast, Fgf23 was expressed at similar levels in venous sinuses in both combined Fgf23-deficient/Hyp and Fgf23-deficient mice. The site-selective upregulation of Fgf23 by Phex deficiency in osteocytes, but not venous sinuses, parallels our prior observations that increased Fgf23 transcript in Hyp mice was limited to bone, because no increases of Fgf23 were observed in bone marrow by real-time PCR (15).

In addition, the present results further clarify the spatial-cell type relationship between Phex and Fgf23 expression. On the basis of prior studies showing that Phex and Fgf23 are coexpressed in differentiated osteoblasts (15, 23, 36) and the inability to demonstrate that Fgf23 is a substrate for Phex (5, 15), we propose that inactivating Phex mutations increase the biosynthesis of Fgf23 in osteoblasts, possibly as a result of
direct consequences of Phex deficiency or through autocrine/paracrine actions of putative Phex substrates, accumulating in the bone milieu as a result of inactivating Phex mutations (21). The present findings, however, show that the cell type expression of Fgf23 in bone is more limited than Phex. Whereas the expression of Phex has been shown to be limited to osteoblasts and osteocytes by in situ and immunohistochemical analysis (17, 22, 23), we show that Fgf23 is selectively upregulated in

Fig. 5. Effects of superimposed Fgf23 and Phex deficiency on bone. A: X-ray of femurs from 6-wk-old mice. B: microcomputed tomography coronal section images of femurs from 6-wk-old mice. Heterozygous Fgf23+/- mice were indistinguishable from wild-type mice, whereas homozygous Fgf23-/- mice have smaller bones with normal gross appearance. Hyp mice and combined Fgf23+/+Hyp mice have radiographic evidence of rickets, as evidenced by the widening and splaying of bone and increased distance between the metaphysis and ephiphysis. These rachitic features are not present in combined Fgf23-/-Hyp mice, which resemble homozygous Fgf23-/- mice, except for the increase in subchondral bone in the distal metaphysis. C: bone mineral density (BMD) of femurs from 6-wk-old mice. Both Fgf23-/- and Hyp mice have lower BMD. Lower BMD in Hyp mice is associated with a greater degree of hyperosteoosclerosis. BMD is increased in combined Fgf23-/-Hyp mice to levels not significantly different from Fgf23-/- mice.
osteocytes embedded in bone, but not in surface lining osteoblasts, in response to inactivating Phex mutations (Fig. 3A). Additional studies of BMSCs derived from Fgf23-deficient eGFP reporter mice confirm that Fgf23 gene transcription occurs when the cells within the osteoblast lineage become embedded in the extracellular matrix (Fig. 4). Thus additional factors related to terminal differentiation into osteocytes and/or matrix-derived factors are required to upregulate Fgf23 in the setting of Phex deficiency. The related observation that Phex immunostaining is pronounced in the cellular processes of osteocytes that transverse matrix favors indirect stimulation of Fgf23 resulting from altered Phex-dependent metabolism of extracellular matrix proteins. To date, only dietary phosphate, but not hyperphosphatemia per se, and 1,25(OH)₂D₃ have been shown to regulate FGF23 gene transcription, neither of which could account for the increased Fgf23 expression in Hyp. Additional information is needed to identify physiologically relevant Phex substrates and to define their role in the regulation of Fgf23 expression. Nevertheless, the present findings indicate that, although Phex deficiency is necessary, it is not sufficient to increase Fgf23 production in cells within the osteoblast lineage. Also, the upregulation of Fgf23 by inactivating Phex mutations in bone places Fgf23 downstream of Phex in what is likely to be a hormonal cascade involving yet to be discovered Phex substrates that are the proximate stimuli for increased Fgf23 expression.

The finding of Fgf23 in venous sinusoids of bone marrow is novel. Colocalized Fgf23 with CD31, a marker of endothelial cells in bone marrow, and the presence of eGFP and CD31-positive cells in BMSC cultures suggest that Fgf23 is produced by vascular endothelial cells, or possibly pericytes, that are in close proximity to vascular endothelial cells in venous sinusoids. The presence of Fgf23 in the bone marrow venules might explain the ability of bone marrow transplantation to partially rescue the hypophosphatemia in Hyp mice (18). The localization of Fgf23 to the venous circulation also raises interesting possibilities regarding the endocrine functions of Fgf23 as well as potential local function to regulate bone

Fig. 6. Effects of superimposed Fgf23 deficiency on cartilage and bone histology. A: growth plate of distal femur of 6-wk-old mice (Goldner stain, ×200). Fgf23⁻/⁻ mice have an increased zone of hypertrophic chondrocytes but an overall normal growth plate width. Hyp mice have a widened growth plate due to increased proliferative and hypertrophic zones. The growth plate in combined homozygous Fgf23⁻/⁻/Hyp mice resembles that of Fgf23⁻/⁻ mice, indicating that the loss of Fgf23 and consequent biochemical changes correct the growth plate abnormalities associated with Phex deficiency. B: cross section of radius (×100); top: Von Kossa stain; bottom: unstained serial section viewed under fluorescent light. Mineralized bone stains are black and unmineralized osteoid stains are red. There is impaired mineralization in homozygous Fgf23⁻/⁻ mice, as evidenced by the excess osteoid (red, transmitted light; top) and diffuse and indistinct fluorescent labeling compared with the narrow osteoid seams and distinct alizarin red and calcine green double labels in wild-type and heterozygous mice. Hyp mice have excess osteoid and diffuse fluorescent labels, but they differed from the osteomalacia of Fgf23⁻/⁻ mice by greater degree of hyperostosis and greater intensity of fluorescent label uptake. Combined homozygous Fgf23⁻/⁻/Hyp mice resemble Fgf23⁻/⁻ mice, as evidenced by a reduction both the amount of osteoid and fluorochrome labeling, indicating that superimposed Fgf23 deficiency also corrects the bone abnormalities associated with Phex deficiency.
marrow hematopoietic lineages. A local role is suggested by the related finding that Fgf23 is also localized to venous sinuses of the thymus, which undergoes involution in Fgf23 null mice (24). Expression of Fgf23 in the sinusoid does not appear to be regulated by Phex deficiency, making it less likely that Fgf23 production at this site is important in regulating systemic phosphate homeostasis or mineralization.

These results also provide insights into the biological effects of Fgf23 in Hyp. With regard to the kidney, the present studies are consistent with Fgf23 mediating the hypophosphatemia and abnormal vitamin D metabolism in Hyp. The absence of circulating Fgf23 levels results in increased serum phosphate and 1,25(OH)2D3 levels but fully corrects the hypophosphatemia and low 1,25(OH)2D3 levels in Hyp mice to levels identical to Fgf23 null mice, thereby showing the dominant role of Fgf23 in the regulating phosphate and 1,25(OH)2D3 levels in Hyp mice. If inactivating mutations of Phex were to result in the increased production of another phosphaturic factor, we might expect that the serum phosphate levels would remain lower in Fgf23−/− compared with combined Fgf23−/−/Hyp mice. Reduction of Fgf23 levels by ~50% in combined heterozygous Fgf23+/−/Hyp mice, however, failed to significantly increase serum phosphate or correct the skeletal abnormalities but did normalize 1,25(OH)2D3 levels, suggesting that the regulation of sodium phosphate transport and 1α-hydroxylase activity by Fgf23 in the kidney may have different sensitivities.

The potential interactions between Fgf23 and Phex on bone growth and mineralization remain to be fully elucidated. The presence of osteomalacia in Fgf23 null mice (i.e., excess osteoid and impaired fluorescent bone labeling), despite hyperphosphatemia, which should stimulate mineralization of extracellular matrix (19), the presence of growth plate abnormalities leading to growth retardation in Fgf23 null mice, and the expression of potential Fgf23 receptors in bone and cartilage (38) indicate a possible direct effect of Fgf23 on the skeleton. Preliminary reports (33) show that adenovirus-mediated overexpression of Fgf23 acts as a negative regulator of osteoblast development and matrix mineralization in fetal rat calvarial cultures. However, such actions would not account for the impaired mineralization observed in Fgf23-deficient mice. From our studies, it is difficult to distinguish possible direct osseous effects of Fgf23 from potential indirect effects resulting from hyperphosphatemia and elevated 1,25(OH)2D3. With regard to Phex, its inactivation causes both rickets and osteomalacia. The osteomalacia in Hyp differs from that observed in Fgf23 null mice in that Hyp has a greater degree of excess osteoid and greater accumulation of fluorescent labeling. Phex deficiency has also been purported to have indirect effects on bone mineralization through putative mineralization inhibitors, called mihhibin, that mediate an intrinsic defect in mineralization, although recent data indicate that phosphate loading can correct the defective mineralization in Hyp mice (19). The greater complexity is indicated by the finding that superimposing Fgf23 deficiency onto Hyp reversed the bone abnormalities typically associated with Phex deficiency, namely rickets, and replaced the Hyp bone phenotype with that of Fgf23 null mice, except for the selective increase in subchondral sclerosis in distal metaphyseal bone in Phex-deficient Hyp mice lacking Fgf23. The marked degree of subchondral sclerosis did not occur in Fgf23 null mice expressing wild-type Phex despite identical degrees of hyperphosphatemia and increased 1,25(OH)2D3. The presence of this Phex-dependent bone phenotype supports the presence of other Phex-dependent processes regulating mineralization.

On the basis of these studies, we conclude that inactivating mutations of Phex selectively increase Fgf23 transcription in osteocytes in bone, revealing that Phex deficiency is necessary, but not sufficient, for increased Fgf23 gene transcription in osteocytes. It is likely that the presence of other factors related to osteocyte differentiation and/or the presence of extracellular matrix factors are necessary for the selective upregulation of Fgf23 expression in Phex-deficient osteocytes. Insights into these additional factors and identification of physiologically relevant Phex substrates will be necessary to fill in the gap in our understanding of how inactivating Phex mutations increase Fgf23 production. Regardless, superimposing Fgf23 deficiency onto Hyp mice corrects the abnormalities in serum phosphate and 1,25(OH)2D3 associated with inactivation of Phex, thereby establishing Fgf23 as phosphatonin and placing this phosphaturic factor downstream of Phex. Finally, the presence of bone abnormalities in Phex-deficient mice not attributable to Fgf23 leaves open the possibility of additional Phex functions and/or substrates in bone that regulate mineralization.

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