Phosphate concentrations, inappropriately normal 1,25(OH)2D levels, severe reabsorption of phosphate and aberrant regulation of 1,25(OH)2D3 to rickets and osteomalacia, as well as impaired renal tubular phosphate reabsorption (39). These disorders have similar skeletal abnormalities, the small integrin-binding ligand N-linked glycoprotein (DMP1), an extracellular matrix phosphoprotein belonging to the phosphate-regulating gene with homologies to endopeptidases (PHEX) family (9, 38, 39). Mutations of PHEX that lead to increased production of FGF23 by osteocytes in bone. Circulating levels of FGF23 are increased in ARHR and its Dmp1-null mouse homologue. To determine the causal role of FGF23 in ARHR, we transferred Fgf23 deficient/enhanced green fluorescent protein (eGFP) reporter mice onto Dmp1-null mice to create mice lacking Fgf23 and Dmp1. Dmp1−/− mice displayed decreased serum phosphate concentrations, inappropriately normal 1,25(OH)2D3 levels, severe rickets, and a diffuse form of osteomalacia in association with elevated Fgf23 serum levels and expression in osteocytes. In contrast, Fgf23−/− mice had undetectable serum Fgf23 and elevated serum phosphate and 1,25(OH)2D3 levels along with severe growth retardation and focal form of osteomalacia. In combined Dmp1−/−/Fgf23−/−, circulating Fgf23 levels were also undetectable, and the serum levels of phosphate and 1,25(OH)2D3 levels were identical to Fgf23−/− mice. Rickets and diffuse osteomalacia in Dmp1-null mice were transformed to severe growth retardation and focal osteomalacia characteristic of Fgf23-null mice. These data suggest that the regulation of extracellular matrix mineralization by DMP1 is coupled to renal phosphate handling and vitamin D metabolism through a DMP1-dependent regulation of FGF23 production by osteocytes.

fibroblastic growth factor 23; dentin matrix protein 1; autosomal recessive hypophosphatemic rickets; hypophosphatemia; osteomalacia; phosphate homeostasis

X-LINKED HYPOPHOSPHATEMIA (XLH), autosomal recessive hypophosphatemic rickets (ARHR), and autosomal dominant hypophosphatemic rickets (ADHR) are hereditary hypophosphatemic disorders respectively caused by mutations of PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) (1, 37), dentin matrix protein 1 (DMP1), an extracellular matrix phosphoprotein belonging to the small integrin-binding ligand N-linked glycoprotein protein family (9, 24), and fibroblastic growth factor 23 (FGF23) (Fgf23) (1a, 5, 39). These disorders have similar skeletal abnormalities, including defective calcification of cartilage and bone, leading to rickets and osteomalacia, as well as 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 (1). In mice, inactivating mutations of Phex, deletion of the Dmp1 gene, and degradation-preventing mutations of Fgf23 result in phenotypic abnormalities homologous to XLH (6, 32), ARHR (9), and ADHR (1a, 5, 39).

It has been proposed that all three of these hereditary disorders share a common pathogenesis mediated by increased circulating FGF23 levels (21). Several findings support this possibility. First, FGF23 is produced predominantly by osteocytes in bone along with PHEX and DMP1. Second, FGF23 plays an essential role in regulating serum phosphate and 1,25(OH)2D3 levels, as evidenced by the fact that overexpression of FGF23 in mice causes hypophosphatemia and suppression of 1,25(OH)2D3 production by the kidney (4, 18, 29), whereas FGF23 deficiency (5, 28) or mutations increasing FGF23 degradation (17) result in hyperphosphatemia, increased serum 1,25(OH)2D3 levels, and soft tissue calcifications. Third, circulating FGF23 levels correlate with hypophosphatemia in ADHR, XLH, and ARHR (9, 38, 39). Mutations of a furin-like enzyme cleavage site, RXXR in FGF23, is the cause of ADHR (13). Recent data confirm that XLH is also caused by increased circulating levels of FGF23 (1b, 20, 40), since hypophosphatemia in Hyp mice is corrected by either the genetic ablation of Fgf23 (23, 30) or the administration of blocking antibodies for FGF23 (3). Interestingly, PHEX does not cleave FGF23 (7, 11, 20) but instead regulates FGF23 expression in osteocytes through unknown mechanisms involving intrinsic bone abnormalities (7, 20, 22, 23).

It remains to be established whether increased circulating FGF23 levels in ARHR and Dmp1-null mice are responsible for the hypophosphatemia and abnormalities in 1,25(OH)2D3 and bone mineralization (9). DMP1 is produced by osteoblasts/osteocytes and then localizes to the mineralization front and functions in bone as a nucleator for mineralization of extracellular matrix (10, 12, 33), suggesting that the rickets and osteomalacia in ARHR are directly mediated by the loss of DMP1. On the other hand, the colocalization of PHEX and DMP1 in osteocytes and the association of DMP1 mutations with increased FGF23 expression, hypophosphatemia, and reduced 1,25(OH)2D3 levels suggest that FGF23 may also contribute to the phenotype of ARHR.

In the current investigations, we examined the contribution of elevated Fgf23 levels to the systemic and skeletal abnormalities observed in Dmp1-null mice. To accomplish this, we crossed the Fgf23-null-eGFP reporter mouse (23) onto the Dmp1-null background. We found that Fgf23 expression in osteocytes was increased by Dmp1 deficiency and that the Fgf23-null phenotype was dominant to that of Dmp1 deficiency, resulting in conversion of hypophosphatemia and inappropriately low 1,25(OH)2D3 levels in Dmp1-null mice to normal levels.
hyperphosphatemia and elevated 1,25(OH)2D3 levels. With regard to the skeleton, superimposed Fgf23 deficiency and consequent increases in serum phosphate and 1,25(OH)2D3 led to rickets. With female double-heterozygous Fgf23- and Dmp1-deficient mice, we also collected long bone from Dmp1-WT and Dmp1-null mice carrying one Fgf23-eGFP reporter allele (Dmp1+/+/Fgf23-/- and Dmp1-/-/-/Fgf23-/-) at 5 wk of age.

**METHODS**

Mouse models. The Fgf23 promoter-eGFP reporter mouse model was created by knocking in an eGFP construct following the ATG in exon 1 of the Fgf23 gene, as described previously (23). Dmp1-deficient mice were created by replacing exon 6 with the lacZ and neo cassette, as described previously (8). Hyp mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in our vivarium. All mice were maintained and utilized in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals prepared by the Institute for Laboratory Animal Resources, National Research Council (Department of Health and Human Services Publication, National Institutes of Health 86-23, National Academy Press, 1996) and the guidelines established by the University of Kansas Medical Center Institutional Animal Care and Use Committee. All experiments reported here were approved by the Institutional Animal Care and Use Committee. Mice were maintained under standard 5K67 diet (PMI Nutrition International, Brentwood, MO), which contains 1.15% calcium, 0.85% phosphorus, and 4 IU/g vitamin D3.

Transfer of Fgf23 deficiency onto the Dmp1-deficient background. To generate double Fgf23- and Dmp1-null mice, we crossed male double-heterozygous Fgf23- and Dmp1-null mice (Fgf23+/+/Dmp1+/+) with female double-heterozygous Fgf23- and Dmp1-null mice (Fgf23+/-/Dmp1+/-). We examined male wild-type (WT) and mutant mice (Dmp1-/-, Fgf23+-/-, and Dmp1-/-/Fgf23-/-) at 5 wk of age. To assess Fgf23 promoter activity in bones of WT and Dmp1-null mice, we also collected long bone from Dmp1-WT and Dmp1-null mice carrying one Fgf23-eGFP reporter allele (Dmp1+/+/Fgf23-/- and Dmp1-/-/-/Fgf23-/-) at 5 wk of age.

**Genotyping.** Genomic DNA tissue was extracted from the tail or ear biopsy of each mouse using REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO). WT (Fgf23+/-), homozygous Fgf23-deficient (Fgf23-/-), and homozygous Dmp1-deficient (Dmp1-/-) and combined homozygous Fgf23- and Dmp1-deficient (Dmp1-/-/Fgf23-/-) mice were genotyped by PCR as, reported previously (Fig. 1) (8, 19). Mice with different genotypes were born at the expected frequencies.

**Serum biochemical measurements.** Serum Fgf23 levels were measured by using FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan), following the manufacturer's protocol. Serum calcium was measured using a Calcium CPC Liquicolor kit (Stanbio Laboratories, Boerne, TX), and serum phosphorus was measured using the Calcium CPC Liquicolor kit (Immunodiagnostic Systems, Fountain Hills, AZ).

**Histological analysis.** eGFP fluorescent imaging in tissues was performed using previously described methods (23). Briefly, mouse bone was quickly dissected and fixed in 4% paraformaldehyde in PBS (pH 7.4) and then embedded in frozen embedding medium. Cryosectioning was performed on a Leica CM1900 Cryostat (Leica, Nussloch, Germany) equipped with a CryoJane frozen sectioning kit (Instrumeditech, Hackensack, NJ). Five-micrometer sections were obtained from the nondecalcified bone samples. eGFP was examined with a Leica DM IRB inverted microscope equipped with a Leica DM 500 digital camera.

For the histological analysis of nondecalcified bone, mice were prelabeled at 6 days and 1 day before they were killed for tissue collection with alizarin complexone (Acros Organics, Fair Lawn, NJ).

**Table 1. Primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf23</td>
<td>GTGCAGATTTCGAGTACAG</td>
<td>GGATAGCTGTTAGGAGTGA</td>
</tr>
<tr>
<td>Phex</td>
<td>TGATGAGACAGGAAAGAC</td>
<td>CTTGGAACCTTGGAGGACC</td>
</tr>
<tr>
<td>Mepe</td>
<td>TCAAGAGAGCATTCAGAGGAC</td>
<td>GAAGGAGAGCAGCATAACC</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>GACCTTCAGAGTGCCAAAG</td>
<td>TCAGAAGAGGCTTAAACG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CGAAGGCTCAGGCACTGAAAG</td>
<td>ACTGCGGCTCAGCATTCC</td>
</tr>
</tbody>
</table>

Fgf23, fibroblast growth factor 23; Phex, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; Mepe, matrix extracellular phosphoglycoprotein.
and calcein (Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection. Tibias 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 10-µm unstained sections were evaluated under fluorescent light (23). Growth plate sections were evaluated under fluorescent light (23). Growth plate/Dmp1*, dentin matrix protein 1; PTH, parathyroid hormone; ND, not detectable. One-way ANOVA was used for statistical analysis. *Significantly different compared with WT (P < 0.05); †significantly different compared with Dmp1−/− mice (P < 0.05).

Bone marrow harvest and stromal cell culture. Bone marrow stromal cells (BMSCs) from long bones isolated from 6-wk-old male mice were cultured as described previously (23). Briefly, both femurs and tibias and bone marrow cells from those mice were flushed out from long bone collected from WT and Dmp1−/− mice at 5 wk of age. WT, wild type; Dmp1, dentin matrix protein 1; PTH, parathyroid hormone; ND, not detectable. One-way ANOVA was used for statistical analysis. *Significantly different compared with WT (P < 0.05); †significantly different compared with Dmp1−/− mice (P < 0.05).

RNA isolation and quantitative RT-PCR. Total RNAs were extracted from long bone collected from WT and Dmp1−/− null mice at 5 wk of age using TRI Reagent (Molecular Research Center, Cincinnati, OH) and then treated with RNase-free DNase (Qiagen, Valencia, CA). First-strand cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Total RNA (1 µg) was used in each 20-µl reverse transcriptase reaction. For real-time RT-PCR, 200 ng of total RNA was used in each PCR reaction. The iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad) were used for real-time quantitative PCR analysis. The relative gene expression was expressed as previously described using cycle threshold values of the gene of interest normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample and then divided by the values from WT (23). Sequences of primers used for real-time quantitative RT-PCR were listed in table 1.

Table 2. Serum biochemistry of WT, Dmp1−/−, Fgf23−/−, and Dmp1−/−/Fgf23−/− mice

<table>
<thead>
<tr>
<th>Serum Markers</th>
<th>WT</th>
<th>Dmp1−/−</th>
<th>Fgf23−/−</th>
<th>Dmp1−/−/Fgf23−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus, mg/dl</td>
<td>9.3±0.4</td>
<td>5.1±0.3*</td>
<td>14.3±0.5†</td>
<td>14.7±0.6†</td>
</tr>
<tr>
<td>Calcium, mg/dl</td>
<td>8.8±0.1</td>
<td>8.3±0.2</td>
<td>10.2±0.3†</td>
<td>10.1±0.4†</td>
</tr>
<tr>
<td>1.25(OH)2D3, pM</td>
<td>260±29</td>
<td>257±36</td>
<td>1,056±135†</td>
<td>1,121±163†</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>83±25</td>
<td>103±27</td>
<td>28±4†</td>
<td>26±4†</td>
</tr>
<tr>
<td>FGF23, pg/ml</td>
<td>77±7</td>
<td>140±108*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE from ≥9 mice at 5 wk of age. WT, wild type; Dmp1, dentin matrix protein 1; PTH, parathyroid hormone; ND, not detectable. One-way ANOVA was used for statistical analysis. *Significantly different compared with WT (P < 0.05); †significantly different compared with Dmp1−/− mice (P < 0.05).

Statistics. We evaluated differences among groups by one-way ANOVA followed by a Tukey posttest. The differences were considered to be statistically significant at P < 0.05. All values are expressed as means ± SE. Computations were performed using either GraphPad Prism (GraphPad Software, San Diego, CA) or Statgraphics statistical graphic system (STSC, Rockville, MD).
RESULTS

Gross appearance. Compared with WT mice, 5-wk-old Dmp1+/− mice displayed evidence of mild growth retardation and skeletal dysplasia (e.g., reduced body weight and shorter limbs and tails; Fig. 1, B and C). In contrast, by 5 wk of age, Fgf23−/− mice were noticeably smaller and had significantly shorter body length and body weight compared with WT littermates (Fig. 1, B and C), consistent with the previously reported effect of FGF23 ablation to cause severe growth retardation (28, 30). Transfer of homozygous Fgf23−/− onto Dmp1−/− mice resulted in a gross appearance identical to that of Fgf23−/− mice (Fig. 1, B and C). Dmp1-null mice had survival rates indistinguishable from WT mice, whereas combined Fgf23−/−/Dmp1−/− mice had mortality rates comparable with Fgf23−/− mice (i.e., mortality beginning 6 wk after birth, with the majority of mice dying by 10 wk of age; data not shown). Thus, the phenotype of the complete loss of Fgf23 predominated over the manifestations of Dmp1 deficiency. In contrast, heterozygous Fgf23+/− and Dmp1+/− mice were indistinguishable from WT mice, and superimposing heterozygous Fgf23+/− onto the Dmp1-null background resulted in mice that grossly resembled Dmp1−/− mice (data not shown).

Effect of combined Fgf23 and Dmp1 deficiency on serum biochemical parameters. The serum Fgf23 concentration in Dmp1-null mice was roughly 18-fold greater than in WT littermates, whereas serum Fgf23 levels in 5-wk-old homozygous Fgf23−/− and combined Fgf23−/−/Dmp1−/− mice were undetectable (Table 2). The increased Fgf23 levels in Dmp1−/− mice were associated with a significant reduction in serum phosphate and inappropriately normal 1,25(OH)2D levels for the degree of hypophosphatemia. In contrast, the absence of circulating Fgf23 levels in Fgf23−/− mice resulted in significantly higher serum phosphate and 1,25(OH)2D levels (Table 2). In combined homozygous Fgg23−/−/Dmp1−/− mice, the serum phosphate and 1,25(OH)2D levels were increased to levels indistinguishable from the values in Fgf23−/− mice. In addition, serum calcium was increased and PTH decreased in both Fgg23−/− mice and combined Fgg23−/−/Dmp1−/− mice, consistent with the in increase in 1,25(OH)2D.

Effects of Dmp1 ablation on Fgf23 promoter-eGFP reporter mice. Heterozygous Fgg23 promoter-eGFP reporter mice with intact Dmp1 displayed rare eGFP expression in osteocytes embedded in bone but no eGFP expression in osteoblast cells on the bone surfaces. Superimposing Dmp1-null onto Fgg23 promoter-eGFP reporter mice resulted in marked increases in expression of eGFP in the majority of osteocytes embedded in bone, whereas surface osteoblasts remained negative for eGFP expression (Fig. 2A). The faint fluorescence on bone surfaces observed in Dmp1+/−/Fgg23+/− and Dmp1−/−/Fgg23+/− is likely due to background autofluorescence, since Dmp1−/−/Fgg23+/− (WT) mice, which do not contain the eGFP cDNA cassette in their genome, had similar bone surface autofluorescence (Fig. 2A). We failed to detect increments in eGFP expression in combined Dmp1−/−/Fgg23+/− mice at any other site (data not shown). These findings are consistent with the published reports of increased expression of Fgg23 transcripts in bone of Dmp1-null mice by real-time PCR (9) and suggest that the increased circulating Fgg23 levels in Dmp1−/− mice is due to increased production by osteocytes. In addition, we examined the temporal pattern of eGFP expression in BMSCs from Dmp1+/−/Fgg23+/− and Dmp1−/−/Fgg23+/− mice grown for 16 days under conditions promoting osteoblast differentiation in vitro. We found that loss of Dmp1 resulted in a marked stimulation of eGFP expression only in cells embedded within mineralization nodules (Fig. 2B).

Effect of superimposed Fgf23 deficiency on rickets and osteomalacia in Dmp1-null mice. Dmp1-null mice displayed classical features of rickets, including splaying of the ends of the long bones and widening of the growth plate by radiographic and μCT analysis (Fig. 3, A and B). Homozygous Fgg23−/− mice were characterized by miniaturized bones of normal shape that lacked any features of rickets by radiographic and histological manifestations of rickets, including splaying of the ends of the long bones and widening of the growth plate by radiographic and μCT analysis (Fig. 3). Histological analysis revealed that the growth plates were wider in Dmp1−/− mice and narrower in Fgg23−/− mice compared with WT littermates (growth plate width of 151.0 ± 13.10 μm in WT, 240.4 ± 21.84 μm in Dmp1−/− mice, and 94.8 ± 24.99 μm in Fgg23−/− mice (means ± SE; n = 4)). The widened growth plate width in Dmp1−/−-null mice was due to an increase in the zone of hypertrophic chondrocytes (Fig. 4A). Interestingly, transfer of Dmp1-null onto Fgg23−/− mice resulted in correction of both the radiographic and histological manifestations of rickets, which were replaced with growth plate abnormalities and overall bone appearance resembling those of Fgg23−/− mice.
A. In this regard, the growth plates in combined homozygous Dmp1^{-/-}/Fgf23^{-/-} mice were not significantly different from Fgf23^{-/-} mice (growth plate width of 99.5 ± 10.73 μm in Dmp1^{-/-}/Fgf23^{-/-} and 94.8 ± 2.49 μm in Fgf23^{-/-}).

Dmp1^{-/-} mice also exhibited by both bone densitometry and μCT analysis significant reductions in bone mineral density (Table 3), a surrogate marker for osteomalacia in this setting (19). Indeed, histological analysis revealed that Dmp1^{-/-} mice displayed hyperosteoidosis and impaired mineralization (Fig. 4B), along with indistinct fluorescent labeling of bone, consistent with impaired mineralization (Fig. 4C). The absence of Fgf23 also results in impaired mineralization despite increased phosphate and 1,25(OH)2D3 levels, as reported previously.

Table 3. Bone densitometry and μCT analysis of femurs of WT and mutant mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Dmp1^{-/-}</th>
<th>Fgf23^{-/-}</th>
<th>Dmp1^{-/-}/Fgf23^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral BMD, g/cm²</td>
<td>0.050±0.001</td>
<td>0.028±0.001*</td>
<td>0.029±0.001*</td>
<td>0.029±0.001*</td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>5.3±0.4</td>
<td>1.3±0.2*</td>
<td>0.8±0.2*</td>
<td>1.2±0.2*</td>
</tr>
<tr>
<td>Tb. density/BV, mg HA/ccm</td>
<td>902.9±7.9</td>
<td>813.2±16.0*</td>
<td>737.0±14.5†</td>
<td>807.0±7.1‡</td>
</tr>
<tr>
<td>Ct. Th, mm</td>
<td>0.148±0.003</td>
<td>0.099±0.003*</td>
<td>0.090±0.005*</td>
<td>0.104±0.004‡</td>
</tr>
<tr>
<td>Ct. density/TV, mg HA/ccm</td>
<td>1.087±16</td>
<td>797±21*</td>
<td>839±17*</td>
<td>810±34*</td>
</tr>
<tr>
<td>Ct. bone density/BV, mg HA/ccm</td>
<td>1.256±15</td>
<td>1.054±18*</td>
<td>978±26*</td>
<td>988±32*</td>
</tr>
</tbody>
</table>

Values means ± SE from ≥9 mice at 5 wk of age. μCT, microcomputed tomography; BMD, bone mineral density; BV, bone volume; TV, total volume; HA, hydroxyapatite; Tb., trabecular bone; Ct., cortical bone; Ct. Th, cortical thickness. One-way ANOVA was used for statistical analysis. *Significantly different compared with WT (P < 0.05); †significantly different compared with Dmp1^{-/-} mice (P < 0.05); ‡significantly different compared with FGF23^{-/-} mice (P < 0.05).
ously (29). In this regard, Fgf23-null mice displayed a reduction in mineralized trabecular bone volume as measured by \( \mu \)CT (Table 3) and a reduced bone mineral density by bone densitometry and \( \mu \)CT (Table 3). Compared with WT mice, Fgf23\(^{-/-}\) 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. 4), as reported previously (28). In contrast to the diffuse osteomalacia observed in Dmp1\(^{-/-}\) mice, a patchy distribution of the widened osteoid seams (Fig. 4, B and C) and near absence of double-fluorescent labeling of bone in Fgf23-null mice (Fig. 4D) are consistent with a focal osteomalacia. Combined homozygous Dmp1\(^{-/-}\)/Fgf23\(^{-/-}\) mice resemble Fgf23\(^{-/-}\) mice, as evidenced by a reduction of the amount of osteoid and fluorochrome labeling, indicating that superimposed Fgf23 deficiency also corrects the diffuse hyperosteoïdosis observed in Dmp1\(^{-/-}\) mice. The focal osteomalacia consisting of bone surfaces with widened (Fig. 4B) and normal (Fig. 4C) osteoid seams and impaired fluorescent labeling (Fig. 4D) observed in Fgf23-null mice remained in Dmp1\(^{-/-}\)/Fgf23\(^{-/-}\) mice. Gene expression analysis in bone from WT and Dmp1-null mice. We compared Fgf23, Phex, matrix extracellular phosphoglycoprotein (Mepe), and osteocalcin expression in Dmp1-null mice and WT mice using quantitative real-time RT-PCR (Table 4). We observed an \( \sim \)150-fold increase in Fgf23 expression in Dmp1-null mice compared with WT mice, consistent with elevated promoter activity in Fgf23 promoter-eGFP reporter mice (Fig. 2). We also observed a minor increase in Phex and Mepe expression (1.6- and 1.5-fold, respectively) in Dmp1-null mice. However, the expression of the osteoblast marker osteocalcin was not changed in Dmp1-null mice.

**DISCUSSION**

ARHR, which is caused by inactivating mutations of DMP1 (9, 24), shares a common phenotype with two other hereditary hypophosphatemic disorders, ADHR and XLH, that are known to be caused by excess FGF23 (13, 38, 40). In this study, we confirm that Dmp1 deficiency in the mouse homologue of ARHR also results in the selective increase in osteocyte production of Fgf23 and elevated circulating levels of this phosphaturic hormone. In addition, we provide evidence that Fgf23 is the proximate cause of the hypophosphatemia and abnormal vitamin D metabolism in Dmp1-null mice by showing that ablation of Fgf23 results in increased serum phosphate and 1,25(OH)\(_2\)D levels in Dmp1-null mice. In fact, the phenotype of combined Fgf23 and Dmp1-null mice was identical to Fgf23-null mice, indicating the dominant role of Fgf23 in the regulating phosphate and 1,25(OH)\(_2\)D levels. The respective PTH and 1,25(OH)\(_2\)D values in the current study (83 pg/ml and 260 pM in WT and 28 pg/ml and 1,056 pM in Fgf23-null mice; Table 2) and in our previous study (21 pg/ml and 241 pM in WT and 10 pg/ml and 535 pM in Fgf23-null mice) (23) are directionally the same, but the absolute values differ, likely due to differences in the diet (1.15% calcium, 0.85% phosphorus in current study vs. 1.36% calcium and 1.01% phosphorus in the previous study), differences in the assay kits, and/or differences in the age of the animals. Regardless, we found no evidence to support the possibilities that Dmp1 deficiency might stimulate the production of another phosphaturic factor or directly regulate phosphate transport in the kidney, since either of these alternative mechanisms would have lowered serum phosphate levels in combined Dmp1\(^{-/-}\)/Fgf23\(^{-/-}\) mice compared with Fgf23\(^{-/-}\) mice.

The current studies do not address the mechanisms whereby Dmp1 deficiency stimulates transcription of Fgf23 in osteocytes. DMP1 is produced by osteoblasts/osteocytes and accumulates in the extracellular matrix, where it facilitates mineralization of collagen in bone and promotes osteoblast/odontoblast differentiation (1b, 26). In addition, DMP1 has the potential to regulate cell and extracellular matrix activity through its binding to integrin \( \alpha_\beta_1 \) and matrix metalloproteinase-9 (15, 16). Phex deficiency was not the cause of the observed phenotype in Dmp1-null mice, since Phex mRNA expression was increased in these mice. Moreover, since Fgf23 is not expressed in osteoblasts, which are precursors to osteocytes, it is unlikely that increased Fgf23 expression is due to a maturational defect in the osteoblast-to-osteocyte transition. Moreover, osteocalcin message expression, a marker of mature osteoblasts, was similar in Dmp1-null and WT mice. It is known that extracellular matrix contains a multitude of growth factors capable of targeting osteoblasts and osteocytes in the bone microenvironment (14, 34). Therefore, Dmp1 deficiency may stimulate Fgf23 gene transcription via direct effects on osteocyte function or indirectly through alterations in extracellular matrix-related factors. Further studies will be needed to determine the mechanisms whereby Dmp1 regulates FGF23 expression in osteocytes.

On the basis of the function of Dmp1 to act as a nucleator of mineralization, the a priori assumption is that defective bone mineralization in Dmp1-null mice would arise as a direct consequence of the lack of Dmp1 in bone; however, the current results indicate that hypophosphatemia and aberrant production of 1,25(OH)\(_2\)D\(_3\), consequent to elevated Fgf23, were principally responsible for rickets and diffuse osteomalacia in Dmp1-null mice. The possibility that increased phosphate is responsible for the lack of rachitic changes in combined Dmp1\(^{-/-}\)/Fgf23\(^{-/-}\) mice is consistent with the observation that defective cartilage mineralization in vitamin D-deficient states can be corrected by restoring phosphate (2). The contributions of Fgf23 and hypophosphatemia to Dmp1-associated defective mineralization of bone are more complex because of the separate effects of Fgf23 deficiency to cause a focal osteomalacia (23, 28, 30). Indeed, superimposing Fgf23 deficiency onto Dmp1-null mice and consequent increments in serum phosphate and 1,25(OH)\(_2\)D levels replaced the diffuse osteomalacia that characterized the Dmp1-null bone phenotype with the focal osteomalacia of Fgf23-null mice. The reduction in the extent of hyperosteoïdosis in combined Dmp1- and Fgf23-null mice is likely related to the effect of increased phosphate to

Table 4. Relative gene expression in bone measured by quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>Dmp1(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf23</td>
<td>1.0±0.40</td>
<td>154.4±27.17*</td>
</tr>
<tr>
<td>Phex</td>
<td>1.0±0.09</td>
<td>1.6±0.21*</td>
</tr>
<tr>
<td>Mepe</td>
<td>1.0±0.14</td>
<td>1.5±0.12*</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>1.1±0.2</td>
<td>1.1±0.19</td>
</tr>
</tbody>
</table>

Values are means ± SE from \( \geq 6 \) mice at 5 wk of age. Student’s \( t \)-test was used for statistical analysis. *Significantly different compared with WT (\( P < 0.05 \)).
promote bone mineralization (25), but the mechanism underlying the focal mineralization defect in Fgf23-null mice is unknown. Finally, 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 (41) also raise the alternative possibility of a direct effect of Fgf23 on the skeleton. In addition, high levels of 1.25(OH)2D, as observed in Fgf23-null mice, are paradoxically associated with defective mineralization in 24-hydroxylase-null mice (31), suggesting that the excessive levels of 1.25(OH)2D can lead to impaired mineralization.

We originally proposed that PHEX and FGF23, which are coexpressed in osteocytes in bone, are part of a bone-kidney axis regulating phosphate homeostasis and mineralization (27). The existence of this enzyme-hormone cascade was supported by the association between increased FGF23 expression and hypophosphatemia (22). In the current study, we have added another component to this putative bone-kidney axis by demonstrating that lack of Dmp1, a protein regulating mineralization, stimulates Fgf23 expression in osteocytes and ablation of Fgf23 corrects the hypophosphatemia in Dmp1-null mice. Remarkably, the phenotype of Dmp1-null mice is very similar to Hyp mice, suggesting the functional importance of elevated Fgf23 in both models. Whether PHEX and DMP1 are regulating FGF23 production through common or distinct pathways remains to be determined. Regardless, PHEX, DMP1, and FGF23 produced by osteocytes appear to coordinate mineralization and systemic phosphate homeostasis. Further studies are needed to understand the potential interdependent regulation of these three factors.

REFERENCES


1. 10.220.33.3 on April 7, 2017 http://ajpendo.physiology.org/ Downloaded from http://ajpendo.physiology.org/ by 10.220.33.3 on April 7, 2017

GRANTS

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