Role of Matrix Extracellular Phosphoglycoprotein in the Pathogenesis of X-Linked Hypophosphatemia

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X-linked hypophosphatemia (XLH), a disorder characterized by hypophosphatemia, impaired skeletal mineralization, and aberrant regulation of 1,25(OH)2D, is caused by inactivating mutations of Phex, which results in the accumulation of putative phosphaturic factors, called phosphatonins. Matrix extracellular phosphoglycoprotein (Mepe) is a proposed candidate for phosphatonin. The authors found that Hyp mice had increased expression of the MEPE and another phosphaturic factor, Fgf23. To establish MEPE’s role in the pathogenesis of the XLH, Mepe-deficient mice were back-crossed onto the Hyp mouse homologue of XLH and phenotypes of wild-type, Mepe−/−, Hyp, and Mepe−/−/Hyp mice were examined. Transfer of Mepe deficiency onto the Phex-deficient Hyp mouse background failed to correct hypophosphatemia and aberrant serum 1,25(OH)2D levels. Increased Fgf23 levels in Hyp mice were not affected by superimposed Mepe deficiency. In addition, Mepe-deficient Hyp mice retained bone mineralization defects in vivo, characterized by decreased bone mineral density, reduced mineralized trabecular bone volume, lower flexural strength, and histologic evidence of osteomalacia; however, cultures of Hyp-derived bone marrow stromal cells in the absence of Mepe showed improved mineralization and normalization of osteoblast gene expression profiles observed in cells derived from Mepe-null mice. These results demonstrate that MEPE elevation in Hyp mice does not contribute to the hypophosphatemia associated with inactivating Phex mutations and is therefore not phosphatonin.


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subjects with TIO and has phosphaturic activity when administered in vivo (30). However, elevated serum sFRP4 levels have not been reported in patients with TIO and expression of sFRP4 has not been assessed in XLH/Hyp.

Matrix extracellular phosphoglycoprotein (MEPE), also called OF45, a bone-specific extracellular matrix protein belonging to the SIBLING protein family (31), has also been implicated in the pathogenesis of XLH as a candidate for phosphatonin and minihbin. MEPE is expressed in osteoblasts and osteocytes (31–34), and its expression is temporally coordinated with PHEX (32–39). MEPE has phosphaturic activity when injected into mice (39), a function that appears to be mediated by C-terminal ASARM motif that localizes to the proximal renal tubule. The ASARM peptide also inhibits mineralization (39,40).

Mepe expression is increased in bone derived from Hyp mice (35), and MEPE C-terminal ASARM peptide is increased in serum of humans with XLH and Hyp (41). In addition, recent studies indicate that MEPE binds to PHEX (40), and the osteolysis of MEPE is inhibited by PHEX (36). Moreover, TIO patients have increased MEPE expression in tumors (32). Also, Mepe transcripts are positively correlated with Fgf23 expression in Hyp bone, suggesting a functional interrelationship between MEPE and FGF23 in the pathogenesis of XLH/Hyp phenotype (42).

In this study, we examine the role of Mepe in mediating the hypophosphatemia and impaired mineralization in Hyp mice by transferring Mepe deficiency onto the Hyp mice background.

Materials and Methods
Transfer of Mepe Deficiency onto the Phex-Deficient Hyp Background

We obtained male heterozygous Mepe-deficient mice (Mepe$^{+/−}$/XY) and female double heterozygous Mepe-deficient Hyp mice (Mepe$^{+/−}$/HypX) from the laboratory of Thomas A. Brown (Department of Genetic Technologies, Pfizer Global Research and Development, Groton, CT) (34). The Mepe-deficient mice had been back-crossed onto a C57BL/6 mouse background for more than nine generations and maintained on the Mepe deficient mice had been back-crossed onto a C57BL/6 background. The Mepe-deficient mice were used for more than nine generations and maintained on the C57BL/6 background. The Mepe-deficient mice had been back-crossed onto a C57BL/6 background for more than nine generations and maintained on the C57BL/6 background.

Genotyping

Genomic DNA tissue was extracted and purified from the tail of each mouse using a QIAGEN DNeasy Tissue Kit (QIAGEN Inc, Valencia, CA). To genotype Mepe-deficient mice, we performed PCR with the following primers specific for the native Mepe gene sequence and the inserted neomycin gene sequence: Mepe1016F (5’-CCCAAGAGCG-CAGAAAAGTAG-3’), Mepe1215R (5’-CCGTTGACATCCCTTTAT-3’), Neo50F (5’-AGAGGCTATTCCGGCTATGCTAG-3’), and Neo480R (5’-ATCCCTTATGCCCTTCGTACAGGATTC-3’). Amplification products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Because we can only genotype male Hyp mice, we only planned to use male mice in our original study design. After we found that Mepe-deficient Hyp females still had hypophosphatemia and skeletal phenotypes, we diagnosed female Hyp mice by hypophosphatemia, growth retardation, and characteristic on radiography (11).

Serum and Urine Assays

Serum calcium was measured using Calcium kit (Sigma-Aldrich, St. Louis, MO), and urine and serum phosphorus levels were measured by the phosphomolybdate–ascorbic acid method as described previously (11). Urine and serum creatinine levels were measured using the Stanbio Creatinine (Stanbio Laboratory, Boerne, TX). Serum parathyroid hormone (PTH) levels were measured by a mouse intact PTH ELISA kit (Immutopics, Carlsbad, CA). Serum 1,25-(OH)$_2$-vitamin D$_3$ levels were measured using Gamma-B 1,25-Dihydroxy Vitamin D kit (Immunodiagnostic Systems Limited, Boldon, UK). Serum Fgf23 levels were measured by using FGF-23 ELISA kit (Kainos Laboratories Inc, Tokyo, Japan) following the manufacturer’s protocol. Fractional excretion phosphate was calculated as described previously (39).

Bone Densitometry and Dry Ash Weight

Bone mineral density (BMD) of femurs was assessed at age 13 wk using a LUNARPIXIMUS bone densitometer (Lunar Corp, Madison, WI). Dry ash weight of femurs collected from 13-wk-old mice was measured as described previously (11).

Three-Dimensional Analysis of the Femurs by Microcomputed Tomography

High-resolution microcomputed tomograph (μCT) was used to evaluate trabecular volume fraction and microarchitecture in the distal femur (μCT40; Scanco Medical AG, Basserdorf, Switzerland). The femur was scanned in a 12.3-mm diameter sample holder at 45 kEv, with cone beam mode and a slice increment of 6 μm. Images from each group were generated at identical threshold. Morphometric parameters included the bone volume fraction (bone volume/trabecular volume, %), trabecular thickness (mm), trabecular number (mm$^{-1}$), and trabecular separation (mm). Trabecular thickness, trabecular number, and trabecular separation were computed using model-independent distance transformation algorithms (43). To determine cortical thickness, cortical bone was imaged over a distance of 0.4 mm at the mid-shaft of the femurs. The three-dimensional structure was generated and morphometric analysis was conducted on a length of 0.3 mm using the “midshaft” program built into the μCT system software.

Biomechanical Testing of the Femurs

Femurs were dissected and cleaned soft tissue attachments and stored at −20°C until testing. On the day of testing, the femurs were thawed and rehydrated with PBS then photographed in the anterior and medial views to measure bone length. Bending tests were performed using a three-point fixture on the ELF 3200 (EnduraTEC Inc, Minnetonka, MN). The femurs were flexed in the anterior–posterior plane by displacing the loading point at 5 mm/min to failure by modifications of previously described techniques (44).
Histomorphometric Analysis of Nondecalcified Bone

Skeletons of mice were prelabeled with tetracycline hydrochloride (Sigma-Aldrich, St. Louis, MO) and calcine (Sigma-Aldrich) by intraperitoneal injection at days 1 and 3 in 12-wk-old mice before collection of tibias. Tibias were removed from 13-wk-old mice, fixed in 70% ethanol, prestained in Villanueva stain, and processed for methyl methacrylate embedding. Five-micrometer sections were stained with Goldner’s stain and analyzed under transmitted light, and 10-μm Villanueva-prestained sections were evaluated under fluorescent light as previously reported (11).

Bone Marrow Harvest and Stromal Cell Culture

Bone marrow stromal cells (BMSC) were cultured as described previously from long bones isolated from 13-wk-old male animals from each group (45). Adherent cells were grown in the differentiation medium (α-MEM containing 10% FBS supplemented with 5 mmol/L β-glycerophosphate and 25 μg/ml of ascorbic acid) to induce osteoblastic differentiation. Mineralization was assessed by staining with Alizarin red-S, as described previously (11).

RNA extraction and Real-Time PCR

Total RNA was extracted from cultured BMSC with Trizol Reagent (Invitrogen, Carlsbad, CA). The real-time PCR were performed as described previously (42). Briefly, cDNA was synthesized from 2 μg of total RNA using TaqMan reverse-transcription reagent kit and random hexamers according to the manufacturer’s directions (Applied Biosystems). The reactions were incubated at 25°C for 10 min, at 42°C for 15 min, and at 99°C for 5 min, and then stored at −20°C until use. The primer sets for specific genes are shown in Table 1. The iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) were used for real-time PCR analysis. We optimized the real-time PCR conditions of all selected genes by testing efficiency of the reactions and the optimal annealing temperature. The relative differences in expression between groups were expressed using the formula of 2^(-ddCT). Statistical Analyses

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

Results

Effects of Superimposed Mepe Deficiency on Hypophosphatemia and Abnormal Vitamin D Metabolism in Hyp Mice

Hyp mice had significantly lower serum phosphate levels (40% reduction), a two-fold increase in fractional excretion of phosphate, and inappropriately normal 1,25-(OH)2-vitamin D3 levels for the degree of hypophosphatemia compared with WT mice (Table 2). Mepe knockout mice had serum phosphate and 1,25-(OH)2-vitamin D3 levels that were not statistically different from WT mice. Combined Mepe-null and Hyp mice had persistent hypophosphatemia and increased fractional excretion of phosphate, as well as inappropriately low 1,25-(OH)2-vitamin D3 for the degree of hypophosphatemia (Table 2).

Regarding other potential phosphaturic factors, we unexpectedly found that serum PTH levels were increased in Hyp mice compared with WT mice. We also found that serum Fgf23 was markedly elevated (18 fold) in Hyp mice (Table 2). Superimposed Mepe deficiency did not change the elevated Fgf23 or PTH levels in Hyp mice.

Effects of Mepe Deficiency Mice on the Skeletal Phenotype in Hyp Mice

Bone Histology. To assess the effect of superimposed Mepe deficiency on bone mineralization, we examined non-decalcified bone histology of tibial metaphyseal bone derived from WT, Hyp, Mepe−/−, and combined Mepe−/−/Hyp mice (Figure 1, A to H). Compared with normal amount of osteoid in WT littermates (Figure 1A), Hyp mouse bone exhibited a striking excess of osteoid (Figure 1B) that was caused by an increase in osteoid seam width as well as the extent of osteoid covered

### Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Cyclophilin A</td>
<td>NM_008907</td>
<td>CTGCAGCTGCAACAGACTGAAT</td>
<td>CCACAATGTTCATGCGCTTCT</td>
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<td>Fgf23</td>
<td>NM_022657</td>
<td>ACTTGTCGACAGACAGACTCA</td>
<td>GTGGGGAACAGACGGAGA</td>
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<tr>
<td>Sfrp 4</td>
<td>NM_016687</td>
<td>TGGCCATCGCAACAGATGATGAA</td>
<td>GCAGGCCACTCCAGGGTACAG</td>
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<tr>
<td>Mepe</td>
<td>AF314964</td>
<td>ATGCAGGGAGAGCTGGTACC</td>
<td>TGGTTCCCTTGGACACTTCT</td>
</tr>
<tr>
<td>Dmp1</td>
<td>NM_016779</td>
<td>AGTGAGGAGGAGAGCTGGTACC</td>
<td>GAGGGCTCTCGTGTTGACCTAC</td>
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<tr>
<td>Osteopontin</td>
<td>AF515708</td>
<td>TCTGATGAGACCCGTACCTG</td>
<td>CCTCATGATGAGACCCGTACCT</td>
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<td>Osteocalcin</td>
<td>NM_007541</td>
<td>AGCAGGAGGCGATAGGTTA</td>
<td>CAAGACGATGATGACTCGA</td>
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<td>AKP2</td>
<td>NM_007431</td>
<td>TCTCTGCTTCGTGCTTTT</td>
<td>GAGGAATGCTTGCAGTCTGT</td>
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<td>Cathepsin B</td>
<td>BC006766</td>
<td>GGAGATACTCCGAGGTTGCAA</td>
<td>CTGCCATGATCTCTCTTCACA</td>
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<td>Phex</td>
<td>NM_011077</td>
<td>GTGGTGAGTCTGTGAGATCC</td>
<td>AGCCGGTCTTCTCTCCACA</td>
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<tr>
<td>Osterix</td>
<td>AF184902</td>
<td>ACTGGCTAGTGTTGTCGCC</td>
<td>GGTAGGGACGGGTAGTAAA</td>
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<td>Runx2 type II</td>
<td>NM_009820</td>
<td>GCCCTCAACAAACACCAACACAAGA</td>
<td>TAAAAGGCGCAGACGGCTTCTT</td>
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<tr>
<td>RankL</td>
<td>NM011613</td>
<td>GCAGAAGGACAGCTCAGACACAC</td>
<td>TATGGTGAGGCTCAGATGGTGGT</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>MMU94331</td>
<td>GTTCTGCAGACAGCTCACAA</td>
<td>AAACAGGCTCAGATGGCCATT</td>
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Table 2. Serum and urine markers

<table>
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<tr>
<th>Serum Markers</th>
<th>WT (n=12)</th>
<th>Hyp (n=12)</th>
<th>Mepe−/− (n=24)</th>
<th>Mepe−/−/Hyp (n=11)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Phosphorus, mg/dl</td>
<td>8.9 ± 0.5a</td>
<td>5.3 ± 0.5b</td>
<td>9.4 ± 0.3a</td>
<td>5.8 ± 0.5b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Calcium, mg/dl</td>
<td>8.6 ± 0.2a</td>
<td>8.5 ± 0.2a</td>
<td>9.1 ± 0.1b</td>
<td>8.4 ± 0.2a</td>
<td>0.0050</td>
</tr>
<tr>
<td>1,25-(OH)2-VitD3, pM</td>
<td>217.3 ± 34.4</td>
<td>194.8 ± 43.5</td>
<td>262.2 ± 34.4</td>
<td>220.9 ± 43.5</td>
<td>0.6435</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>24.6 ± 12.3a</td>
<td>84.9 ± 14.0c</td>
<td>30.4 ± 10.2ab</td>
<td>60.5 ± 11.6bc</td>
<td>0.0035</td>
</tr>
<tr>
<td>Fgf23, pg/ml</td>
<td>123.1 ± 10.6a</td>
<td>2192.6 ± 159.2b</td>
<td>125.3 ± 10.0a</td>
<td>2287.2 ± 234.4b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FEP, %</td>
<td>9.3 ± 2.7a</td>
<td>20.7 ± 3.2b</td>
<td>7.6 ± 0.8a</td>
<td>19.3 ± 2.7b</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM from 12-wk-old mice, except for Fgf23, which are from 5-mo-old mice. Values sharing the same letter superscript with each row are not significantly different at P < 0.05 by one-way ANOVA analysis. FEP indicates fractional excretion of phosphate.

Figure 1. Representative nondecalcified bone sections of tibias from wild-type (WT) and mutant mice. (A to D) Goldner-stained sections are shown in the upper panel (×500). In Goldner-stained sections, mineralized bone is blue and unmineralized bone is red–brown in color. WT mice have small amounts of osteoid relative to mineralized bone (A). In contrast, Hyp mice have increased relative osteoid volume (B). Mepe-null mice did not alter the ratio of osteoid to mineralized bone (C). Superimposed Mepe deficiency in Hyp mice (D) failed to correct the hyperosteoiodosis, as evidenced by the persistent increase in relative osteoid volume in Mepe−/−/Hyp mice. (E to H) Villanueva-stained section viewed under fluorescent light in the lower panels (×500). Wild-type mice have two distinct double labels indicative of normal mineralization (E). Hyp bone has diffuse labels consistent with impaired mineralization (F). Trabecular bone from Mepe-null mice exhibit an apparent increase in mineral apposition, as evidenced by a greater distance between double labels (G). Similar to Hyp mice, combined Mepe−/−/Hyp mice have diminished fluorescent labeling of bone (H), indicating that Mepe deficiency failed to correct the mineralization defect in vivo.

Bone Mineral Density. We have previously shown that BMD of femurs measured by DEXA is highly correlated with dry ash weights and both serve as surrogate markers for the degree of impaired mineralization of bone in Hyp mice. Consistent with the presence of osteomalacia, Hyp mice exhibited significantly lower BMD (Figure 2A) and dry ash weight (Figure 2B) of the femur compared with WT littermates. We failed to observe an increase in BMD or dry ash weight of bone in Mepe-null mice. More importantly, superimposed Mepe deficiency failed to rescue the bone phenotype in Hyp mice.

The µCT analysis of femurs provided additional characterization of Hyp bone and the effects of superimposed Mepe deficiency (Figure 3, A through P and Table 3). Consistent with the presence of rickets and osteomalacia, the femurs of Hyp mice (Figure 3, B and F) were short, widened, and bowed compared with WT littermates (Figure 3, A and E). These changes were most pronounced in the distal femur. In addition, the growth plates were widened in Hyp compared with WT mice (Figure 3F, indicated by brackets). In addition, metaphyseal mineralized bone volume was markedly diminished in Hyp.
Hyp mechanical properties were more severe in combined failure compared with WT mice. The reduced bone biomechanical strength as evidenced by a significantly less force before failure.

In 13-wk-old mice, and superimposed Mepe deficiency failed to alter the trabecular and cortical parameters in Mepe-null mice (Figure 3, C and G), which was indistinguishable from WT. In addition, we did not observe any quantifiable differences in trabecular or cortical bone between Mepe-null and WT mice. Similar to the histologic and BMD results, the skeletal phenotype of combined Mepe<sup>−/−</sup>/Hyp mice (Figure 3, D and H) had the same appearance as Hyp mice, consisting of short and widened femurs width with diminished mineralized trabecular bone and increased cortical porosity consistent with persistent rickets and osteomalacia. Quantitative analysis of metaphyseal and cortical bone confirmed these impressions. In this regard, Hyp mice had significant reductions in all trabecular bone parameters and diminished cortical thickness compared with WT mice. We were unable to identify any effects of Mepe deficiency on trabecular and cortical bone measurements in 12-wk-old mice, and superimposed Mepe deficiency failed to alter the trabecular and cortical parameters in Hyp mice (Table 3).

Biomechanical Properties. Next, we investigated the biomechanical properties of WT and mutant mice. To accomplish this, we performed biomechanical testing on femurs using a three-point bending fixture (Table 4). Compared with WT mice, the bones of Hyp mice were more pliable and could tolerate significantly less force before failure. Mepe-null mice also exhibited a decreased biomechanical strength as evidenced by a significant reduction in the maximum force and deflection at failure compared with WT mice. The reduced bone biomechanical properties were more severe in combined Mepe-null and Hyp mice than in either Mepe-null or Hyp mice alone. Taken together, these findings indicate that Mepe deficiency worsened the mechanical properties of bone.

Effects of Mepe Deficiency on the BMSC in Hyp Mice

Although Mepe ablation did not rescue the bone phenotype in Hyp mice, we tested if Mepe deficiency altered the intrinsic mineralization defect observed in osteoblasts from Hyp mice (9). BMSC were grown in the presence of ascorbate and β-glycerophosphate to induce osteoblast differentiation and promote bone nodule formation. Consistent with an earlier report (10), BMSC derived from Hyp mice displayed impaired mineraliza-
tion compared with WT mice as assessed by alizarin red staining of mineralization nodules, suggesting an intrinsic defect in mineralization in the Hyp osteoblast cells (Figure 4). Also consistent with earlier report (34), BMSC derived from Mepe-null mice had an increased mineralization potential compared with WT BMSC ex vivo (Figure 4). More importantly, BMSC derived from combined Mepe-null and Hyp mice displayed greater mineralization potential after induction of osteoblast differentiation than BMSC derived from Hyp mice (Figure 4). The improvement of the mineralization defect, however, was limited, because mineralization remained significantly less in combined Mepe−/−/Hyp-derived BMSC than in WT cultures (Figure 4B).

To further explore the effects of combined Phex and Mepe deficiency on osteoblast function, we evaluated gene expression profiles of BMSC (Table 3). Except for impaired mineralization, Hyp-derived BMSC had normal levels of osteoblast transcripts, except for increased Mepe and Fgfr3 expression, and a small but significant decrease in the osteoblast transcription factor Runx2-II. In contrast, Mepe deficiency, consistent with the enhanced mineralization, resulted in significant alterations in the osteoblast phenotype ex vivo that was characterized by an increase in the osteoblastic transcription factors Runx2-II and osterix, as well as osteoblastic differentiation markers alkaline phosphatase, DMP-1, and osteocalcin, consistent with increased osteoblastogenesis and/or differentiation. Expression of the osteoclastic coupling factor Rank L was decreased in BMSC from Mepe-null mice. The presence of combined Phex and Mepe deficiency in the Mepe−/−/Hyp mouse-derived BMSC reversed all of the effects of Mepe deficiency on gene expression profiles, suggesting that the lack of Phex function somehow offset the effects of Mepe deficiency on osteoblast gene expression.

Table 3. Micro CT analysis of femurs in WT and mutant mice

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>Hyp (n = 5)</th>
<th>Mepe−/− (n = 14)</th>
<th>Mepe−/−/Hyp (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV, %</td>
<td>12.91 ± 0.73a</td>
<td>5.17 ± 0.81b</td>
<td>10.70 ± 0.48a</td>
<td>5.60 ± 0.96b</td>
<td>&lt;0.0001</td>
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<tr>
<td>Tb. N, mm−1</td>
<td>5.09 ± 0.14a</td>
<td>3.13 ± 0.20b</td>
<td>5.18 ± 0.10a</td>
<td>3.79 ± 0.13c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tb. Th, mm</td>
<td>0.042 ± 0.003ab</td>
<td>0.047 ± 0.005b</td>
<td>0.036 ± 0.001a</td>
<td>0.040 ± 0.006ab</td>
<td>0.0350</td>
</tr>
<tr>
<td>Tb. Sp, mm</td>
<td>0.193 ± 0.006a</td>
<td>0.337 ± 0.024b</td>
<td>0.189 ± 0.004a</td>
<td>0.267 ± 0.013c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ct.Th, mm</td>
<td>0.186 ± 0.004a</td>
<td>0.117 ± 0.006b</td>
<td>0.177 ± 0.002a</td>
<td>0.105 ± 0.012b</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM from 5 male 13-wk-old mice. Values sharing the same letter superscript with each row are not significantly different at P < 0.05 by one-way ANOVA analysis.

Table 4. Bone mechanical testing using three-point bending fixture

<table>
<thead>
<tr>
<th>Group</th>
<th>WT (n = 5)</th>
<th>Hyp (n = 5)</th>
<th>Mepe−/− (n = 14)</th>
<th>Mepe−/−/Hyp (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum force, N</td>
<td>25.8 ± 1.2a</td>
<td>8.0 ± 1.0 b</td>
<td>23.1 ± 0.7c</td>
<td>4.5 ± 0.9d</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maximum deflection, mm</td>
<td>0.71 ± 0.03a</td>
<td>0.96 ± 0.20a</td>
<td>0.62 ± 0.04a</td>
<td>1.89 ± 0.29b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Deflection at failure, mm</td>
<td>0.93 ± 0.14a</td>
<td>2.08 ± 0.08b</td>
<td>0.71 ± 0.06c</td>
<td>2.11 ± 0.22d</td>
<td>&lt;0.0001</td>
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<tr>
<td>Stiffness, N/mm</td>
<td>55.4 ± 4.3a</td>
<td>17.6 ± 2.5b</td>
<td>54.3 ± 2.5a</td>
<td>6.4 ± 1.4c</td>
<td>&lt;0.0001</td>
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</tbody>
</table>

Data shown are mean ± SEM from 13-wk-old male mice. Values sharing the same letter superscript with each row are not significantly different at P < 0.05 by one-way ANOVA analysis.

*Maximum deflection represents the deformation corresponding to maximum force.

Figure 4. Effects of Mepe deficiency on Hyp-derived bone marrow stromal cell maturation and mineralization. (A) Alizarin red-S staining of primary bone marrow stromal cells derived from WT, Hyp, Mepe-null, and combined Mepe-null Hyp mice. Compared with WT, bone marrow stromal cells derived from Hyp mice formed less mineralization nodules, whereas the cells from Mepe−/− mice formed much more mineralized nodules. Mepe−/−/Hyp mice formed more mineralized nodules compared with cells from Hyp. (B) Quantification of mineralization. The alizarin red-S stain was extracted with 10% cetylpyridinium chloride and quantified by absorbance measurement at 562 nm as described in Materials and Methods. Numeric values represent the mean ± SEM of six wells. Bone marrow-derived mesenchymal stem cells were culture from WT and mutant mice for 14 d in the presence of ascorbic acid and β-glycerophosphate to induce osteoblast differentiation. Values sharing the same letter superscript are not significantly different at P < 0.05 by one-way ANOVA analysis.
Discussion

We transferred Mepe deficiency onto the Hyp mouse background to examine the role of Mepe in the pathogenesis of hypophosphatemia and defective mineralization in XLH/Hyp. We found that Mepe-deficient mice had serum phosphate levels and fractional excretion of phosphate indistinguishable from WT littermates (Table 2), and Mepe-deficient Hyp mice remained hypophosphemic and phosphaturic, as well as had persistent inappropriately normal 1,25(OH)₂D₃ levels (Table 2). The inability of Mepe deficiency to rescue the renal abnormalities in Hyp mice indicates that Mepe is not the phosphaturic factor, phosphatonin.

Superimposing Mepe deficiency onto the Hyp background also did not correct the gross skeletal abnormalities associated with Phex mutations, indicating that hypophosphatemia and/or associated factors are more important than Mepe in the defective skeletal mineralization in Hyp mice. In vitro data, however, implicate a role for Mepe in regulating mineralization that may have been masked by persistent hypophosphatemia in vivo. We observed improved mineralization in Hyp-derived BMSC ex vivo in the absence of Mepe (Figure 4). In addition, Mepe deficiency was associated with increased osteoblastic markers, including DMP1, osteocalcin, Osterix, and Runx2-II, and inactivating Phex mutations counteracts the changes in osteoblastic gene expression profiles induced by Mepe deficiency (Table 5). Mepe deficiency also increases Phex expression in BMSC.

Our studies differ from previous studies by Gowen et al. (34) because we failed to observe an increase in bone mass by both DEXA and µCT or to demonstrate increased bone strength by biomechanical testing (Figures 1 to 3). The reasons for these discrepancies are not clear, although notable differences exist with regard to age, techniques for assessing the skeletal phenotype, and genetic background between the two studies. With regard to other potential phosphaturic factors, we found that sfrp-4 (30) was not different in BMSC from WT or Mepe-deficient mice (Table 5). Another interesting finding is the three-fold increased circulating levels of the phosphaturic hormone PTH in Hyp mice. PTH, however, is not the primary phosphaturic factor in Hyp, because parathyroidectomy does not correct the hypophosphatemia in Hyp mice (46). The mechanism of PTH elevation in Hyp remains an enigma, but Phex has been identified in the parathyroid gland (47), raising the possibility that a primary abnormality in parathyroid gland function may also exist in XLH.

Finally, we observed an 18-fold increase in serum Fgf23 levels in Hyp mice compared with WT littermates (Table 2) and an eight-fold increase in Fgf23 transcripts in BMSC from Hyp mice compared with WT mice (Table 5). Recent studies demonstrate that transfer of Fgf23 deficiency onto the Hyp background increases serum phosphorus in Hyp mice (23). Collectively, these findings implicate Fgf23, rather than Mepe, as the leading candidate for phosphatonin. The mechanisms whereby Phex deficiency results in increased Fgf23 expression are not certain, but the original notion that Fgf23 is a substrate of Phex (28) has not been confirmed (42,48). We interpret the concordant increase in serum levels and expression of Fgf23 to indicate that inactivating Phex mutations lead to the increased production of Fgf23 through intermediate steps that likely involve potential Phex substrates and/or other intermediate factors (13).

In conclusion, Mepe deficiency does not correct the hypophosphatemia in Hyp mice and therefore is not phosphatonin, but Mepe may play a role in regulating the local mineralization process in Hyp mice. It may be necessary to correct the hypophosphatemia in combined Mepe−/−/Hyp mice to uncover the in vivo function of Mepe in the pathogenesis of XLH. Regardless, the identification of the physiologically relevant Phex substrates will be necessary to fully understand the pathogenesis of XLH.

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Table 5. Real-time PCR of bone marrow stromal cells from WT and mutant mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>Hyp</th>
<th>Mepe−/−</th>
<th>Mepe−/−/Hyp</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phex</td>
<td>0.01 ± 0.001ᵃ</td>
<td>Not detectable</td>
<td>0.09 ± 0.017ᵇ</td>
<td>Not detectable</td>
<td>0.0003</td>
</tr>
<tr>
<td>Akp2</td>
<td>0.35 ± 0.043ᵃ</td>
<td>0.39 ± 0.115ᵃ</td>
<td>1.87 ± 0.593ᵇ</td>
<td>0.28 ± 0.073ᵃ</td>
<td>0.0165</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>4.89 ± 0.613ᵃ</td>
<td>3.60 ± 1.043ᵃ</td>
<td>3.15 ± 0.622ᵃ</td>
<td>3.20 ± 0.281ᵃ</td>
<td>0.3232</td>
</tr>
<tr>
<td>Mepe</td>
<td>0.002 ± 0.0005ᵃ</td>
<td>0.008 ± 0.0028ᵇ</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>0.0035</td>
</tr>
<tr>
<td>Dmp1</td>
<td>0.13 ± 0.028ᵃ</td>
<td>0.17 ± 0.023ᵃ</td>
<td>0.32 ± 0.055ᵇ</td>
<td>0.12 ± 0.017ᵃ</td>
<td>0.0120</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>0.05 ± 0.012ᵃ</td>
<td>0.06 ± 0.021ᵃ</td>
<td>0.53 ± 0.067ᵇ</td>
<td>0.06 ± 0.009ᵃ</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>0.04 ± 0.004ᵃ</td>
<td>0.04 ± 0.006ᵃ</td>
<td>0.02 ± 0.003ᵃ</td>
<td>0.03 ± 0.005ᵃ</td>
<td>0.1174</td>
</tr>
<tr>
<td>Fgf23</td>
<td>0.0006 ± 0.0001ᵃ</td>
<td>0.0046 ± 0.0012ᵇ</td>
<td>0.0001 ± 0.00001ᵃ</td>
<td>0.0064 ± 0.0020ᵇ</td>
<td>0.0022</td>
</tr>
<tr>
<td>sfrp4</td>
<td>0.11 ± 0.003ᵃ</td>
<td>0.08 ± 0.010ᵃ</td>
<td>0.09 ± 0.007ᵃ</td>
<td>0.08 ± 0.013ᵃ</td>
<td>0.0920</td>
</tr>
<tr>
<td>RankL</td>
<td>0.001 ± 0.00025ᵃ</td>
<td>0.001 ± 0.00017ᵃ</td>
<td>0.0001 ± 0.00002ᵇ</td>
<td>0.0012 ± 0.00025ᵃ</td>
<td>0.0147</td>
</tr>
<tr>
<td>Runx2 II</td>
<td>0.011 ± 0.0004ᵃ</td>
<td>0.007 ± 0.0012ᵇ</td>
<td>0.017 ± 0.0014ᶜ</td>
<td>0.008 ± 0.0007ᵃᵇ</td>
<td>0.0004</td>
</tr>
<tr>
<td>Osterix</td>
<td>0.004 ± 0.0007ᵃ</td>
<td>0.003 ± 0.0005ᵇ</td>
<td>0.014 ± 0.0037ᵇ</td>
<td>0.004 ± 0.0002ᵃ</td>
<td>0.0109</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM from bone marrow stromal cell cultures from WT and mutant mice at 14 d of culture duration with ascorbic acid and β-glycerophosphate (n ≥ 3). Values sharing the same letter superscript within each row are not significantly different at P < 0.05 by one-way ANOVA analysis.
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