Overexpression of Phex in Osteoblasts Fails to Rescue the Hyp Mouse Phenotype*

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Inactivating mutations of Phex, a phosphate-regulating endopeptidase, cause hypophosphatemia and impaired mineralization in X-linked hypophosphatemia (XLH) and its mouse homologue, Hyp. Because Phex is predominantly expressed in bone and cultured osteoblasts from Hyp mice display an apparent intrinsic mineralization defect, it is thought that reduced expression of Phex in mature osteoblasts is the primary cause of XLH. To test this hypothesis, we studied both targeted expression and/or additional factors are likely to be important in the pathogenesis of XLH.

Because the discovery that mutations of PHEX, or the Phosphate-regulating gene with homologies to Endopeptidases on the X chromosome, is the genetic defect underlying X-linked hypophosphatemia (XLH) (1–4), efforts have been underway to determine how this novel endopeptidase regulates phosphorus and mineral homeostasis. Phex is one of six members of the M13 family of zinc-dependent type II cell-surface membrane metalloproteases (5–7). The presence of renal phosphate wasting secondary to inactivating mutations of the Phex gene suggests that this endopeptidase degrades a novel phosphaturic hormone (referred to as phosphatonin) or inactivates a phosphate-conserving factor (8). Neutral endopeptidase substrates ZAAL-pNA and [Leu]enkephalin, as well as certain parathyroid hormone-related peptides (9–12), are cleaved by recombinant Phex in vitro, but the biological relevance of these substrates is not certain. Recent studies indicate that FGF-23 may be a physiologically important Phex substrate and a candidate for phosphatonin (14). Not only is FGF-23 cleaved by recombinant Phex in vitro (13), but mutations in the FGF-23 gene product cause the related disorder autosomal dominant hypophosphatemia (14), and FGF-23 induces hypophosphatemia and defective mineralization when administered to mice in vivo (15).

There is significant evidence indicating bone is a physiologically relevant site of Phex expression and is directly involved in the pathogenesis of XLH. Phex is expressed at high levels in osteoblasts and other mineralizing tissues such as teeth and growth plate cartilage (16–20) where its expression is temporally associated with the formation of mineralized extracellular matrix in cultured osteoblasts (18, 20). In addition, available data (21, 22) suggest that loss of Phex function in osteoblasts results in a nascent defect that leads to impaired mineralization of extracellular matrix, independent of the hypophosphatemia. Osteoblasts derived from Hyp mice, a murine homologue of XLH, display defective mineralization and other abnormalities in culture (21, 22), as well as fail to form mineralized bone after transplantation into normal mice (23). Finally, putative phosphate and mineralization inhibitory activities also have been identified in conditioned media of Hyp osteoblasts (21, 24), suggesting that Phex and its substrate are both produced in bone.

Although these data provide compelling evidence that the loss of Phex function in osteoblasts is causally related to the intrinsic abnormality of mineralization, several components of this model have not be substantiated. First, there is no direct evidence that Phex metabolizes endogenous phosphaturic or mineralization inhibitory factors synthesized by osteoblasts. Second, FGF-23, the current best candidate for phosphatonin (14, 15), has not been shown to be expressed in bone marrow (14), although its expression in osteoblasts has not been excluded. Third, several studies (25, 26) have failed to document nascent defects in Hyp-derived osteoblasts, indicating that the observed abnormalities of cultured osteoblasts may be secondary to the Hyp milieu. Fourth, the observation that parabiosis (27) and cross-kidney transplantation (28) between normal and Hyp mice lead to phosphaturia in the normal animal indicates the presence of extrinsic circulating factors (29) that modulate bone mineralization in the X-linked disorder, either directly or indirectly through the induction of hypophosphatemia. Finally, bone marrow transplantation via the intraperitoneal route,
which does not normalize Phex expression in bone, partially rescues the hypophosphatemia in Hyp mice (30). All of these studies indicate that factors extrinsic to the osteoblast might be responsible for the mineralization defect in XLI. No studies to date, however, have examined whether the apparent intrinsic abnormalities of mineralization in Hyp-derived osteoblasts are corrected by restoration of Phex expression and function in osteoblasts.

In the present investigation, we directly examined whether Phex deficiency is directly associated with impairment of osteoblast-mediated mineralization by two complementary approaches. We used a retroviral vector to overexpress a functional Phex cDNA in osteoblasts derived from Hyp mice and the osteocalcin (OG2) promoter to achieve targeted overexpression of Phex to Hyp osteoblasts in vivo. Neither of these approaches corrected the Hyp-related mineralization abnormalities, and the targeted expression of Phex to mature osteoblasts in bone did not rescue the hypophosphatemia. Thus, our findings fail to support the simple hypothesis that loss of Phex does not rescue the hypophosphatemia. Thus, our findings fail to support the simple hypothesis that loss of Phex expression in bone, partially rescues the hypophosphatemia in Hyp mice (30). All of these studies indicate that factors extrinsic to the osteoblast might be responsible for the mineralization defect in XLI. No studies to date, however, have examined whether the apparent intrinsic abnormalities of mineralization in Hyp-derived osteoblasts are corrected by restoration of Phex expression and function in osteoblasts.

In the present investigation, we directly examined whether Phex deficiency is directly associated with impairment of osteoblast-mediated mineralization by two complementary approaches. We used a retroviral vector to overexpress a functional Phex cDNA in osteoblasts derived from Hyp mice and the osteocalcin (OG2) promoter to achieve targeted overexpression of Phex to Hyp osteoblasts in vivo. Neither of these approaches corrected the Hyp-related mineralization abnormalities, and the targeted expression of Phex to mature osteoblasts in bone did not rescue the hypophosphatemia. Thus, our findings fail to support the simple hypothesis that loss of Phex in osteoblasts is primarily responsible for the Hyp phenotype.

**Experimental Procedures**

**Reagents—** α-Minimum essential medium, Dulbecco’s modified Eagle’s medium/Ham’s F-12, Dulbecco’s modified Eagle medium, penicillin/streptomycin solution, Hanks’ balanced salt solution, and Trizol Reagent for single-step isolation of total RNA from cells were obtained from Invitrogen. Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Pronase-E, ascorbic acid, β-glycophosphate, bovine serum albumin, and alkaline phosphatase kit were purchased from Sigma. Bio-Rad reagent for protein assay was obtained from Bio-Rad. OligoNucleotide primers were synthesized at the Duke University DNA Core Facility. We used Phex-specific rabbit antisera that recognize the C-terminal end of mouse Phex (17). Other antibodies used in this study included anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Recombinant Phex was produced in SF9 cells as described previously (17). The enhanced chemiluminescence detection kit (PerkinElmer Life Sciences) was used to detect horseradish peroxidase. Nitrocellulose membranes (0.45 μm) and other chemicals used for SDS-PAGE and Western blotting were purchased from Bio-Rad.

**Cell Culture—** Immortalized cells derived from normal and Phex transgenic mice (30) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum as described previously (9). TMOb-N1 and TMOb-Hyp immortalized cells derived from normal and Hyp mice calvaria were grown for periods of up to 14 days in TMOb-N1 and TMOb-Glyceraldehyde-3-phosphate dehydrogenase (glyceraldehyde-3-phosphate dehydrogenase) (17) were subjected to reverse transcription with either the empty retrovirus vector pLuvM, pLuvPhex-WT, or pLuvPhex-3’M. These constructs were cotransfected into 293 cells with either the empty retrovirus vector pLuvM, pLuvPhex-WT, pLuvPhex-3’M, or pLuvPhex-1’M, followed by monoclonal antibody (1:200 dilution) incubation, after washing the blots three times with TBST at room temperature for 40 min each, immunoreactivity was detected by a chemiluminescence system.

The expression of Phex in mice was confirmed by using specific rabbit polyclonal antisera (1:200 dilution). In addition, we used anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was performed with mouse monoclonal anti-β-actin antibody (1:2000 dilution) incubation, after washing the blots three times with TBST at room temperature for 40 min each, immunoreactivity was detected by a chemiluminescence system.

**Assessing Phex Enzyme Activity—** We assessed Phex activity by modifications of methods described previously (11). We used membrane fractions from the SF9 cells (50 μg) or TMOb-Hyp osteoblasts (100 μg) expressing vector or various Phex constructs as well as homogenized calvaria-derived protein (80 μg) from 6-week-old non-transgenic normal (+/+), Hyp (−/−), OG2-Phex transgenic, and OG2-Phex Hyp mice (see below). These protein samples were incubated with 50 μm Z-Ala-Ala-Leu-p-nitroanilide (ZAA-LpNA) (Bachem Biosciences, Inc., King of Prussia, PA) in 100 μL of 100 mM MES, pH 6.5, for 1 h at 37 °C. After completion of the initial incubation, the reaction mixture was further incubated with 0.4 milliunits of leucine aminopeptidase (Sigma) for 20 min at 37 °C. The reaction was stopped by the addition of 100 mM EDTA, and the absorbance was measured at 405 nm after centrifugation. In some studies membrane fractions were preincubated with 100 mM EDTA for 30 min before the addition of ZAA-LpNA. Phex endopeptidase activity was also assessed using [Leu]enkephalin (Bachem Biosciences, Inc., King of Prussia, PA) using modifications of methods described previously (9).

**Alkaline Phosphatase Activity—** We analyzed alkaline phosphatase in cell layers by calorimetric assay of enzyme activity using alkaline phosphatase kit (Sigma) as described previously (21). The activity was expressed as nanomoles of substrate transferred per min per mg of DNA.

**Mineralization Assays—** The formation of in vitro mineralization nodules was determined by alizarin red-S histochemical staining, and mineralization was quantified by modification of methods described previously (21). Briefly, the stained matrix was washed with water and phosphate-buffered saline, and the dye was destained with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate, pH 7.0, for 15 min at room temperature. The alizarin red-S was quantified at 562 nm.

**Generation of Transgenic Mice—** The transgene was constructed in the pW1 vector, which contains a multiple cloning site and an SV40 origin. This construct was cloned into the Blunt-ended Sall and SacI sites of the pLuvIRES-GFP construct. In addition, we used the pLuvIRES-GFP construct and pLuvPhex-WT, pLuvPhex-3’M, or pLuvPhex-3’M, which were cotransfected into 293 cells with either the empty retrovirus vector pLuvM, pLuvPhex-WT, pLuvPhex-3’M, or pLuvPhex-3’M, followed by monoclonal antibody (1:200 dilution) incubation, after washing the blots three times with TBST at room temperature for 40 min each, immunoreactivity was detected by a chemiluminescence system.
Persistent Hypophosphatemia in OG2 Phex Transgenic H yp Mice

ng/µl according to standard techniques (35). Mice that carried the transgene were identified by PCR using tail DNA, followed by Southern blot analysis. For Southern blots we used 10 µg of DNA from each mouse digested with *KpnI* and *Sphl* separated on 1% agarose gel and hybridized with a 28P-labeled Phex CDNA probe as described previously (21).

We obtained heterozygous female mice (Hyp +/−) with 3′ deletions of Phex gene from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council (Departments of Health and Human Services Publication NIH 86-23), and by guidelines established by the Institutional Animal Care and Use Committee of Duke University.

Male founder mice expressing Phex under the control of the OG2 promoter were bred with heterozygous female Hyp mice (X<sup>+/−</sup>) to create the following male and female offspring: Hyp mice; Phex transgenic male and female Hyp (OG2-Phex-Hyp); non-transgenic mice (designated +/+) Phex transgenic mice (designated OG2-Phex). Genotypes were determined by PCR using as primers: −67 OG2 forward 5′-TCCTGTCCATACAGAG-3′ and +930 Phex reverse 5′-GGGATCATAGGCGAGTTGCTAT-3′ for the OG2-Phex mice; and 19 forward 5′-CTTGGGCTAGTTTGCTATCT-3′ and 19 reverse 5′-TGATGATGCTATAACCAGGAG-3′ for Hyp mice.

**Serum Phosphorus**—Blood was obtained from 3-, 6-, and 12-week-old mice. Serum phosphorus was measured by a phosphomolybdate colorimetric assay (36).

**Dry Ash Weights of Femurs**—Femurs collected from 12-week-old mice were cleaned of muscle and dried to constant weight at 110 °C in a desiccator. The ashed bones were weighed in an electronic analytical balance at room temperature.

**Measurement of Bone Mineral Density**—We assessed the bone mineral density (BMD; g/cm<sup>2</sup>) of the whole skeleton and femur in 3-, 6-, and 12-week-old mice using a LUNAR PIXIMUS bone densitometer (Lunar Corp., Madison, WI). Slices of femurs were evaluated under fluorescent light as reported previously (38) by the Lunar Corporation (Rockville, MD).

**RESULTS**

**Restoration of Full-length Phex Expression in Hyp Mouse Osteoblasts**—First, we examined Phex transcript expression by RT-PCR analysis. Consistent with previous observations (21), the Phex mRNA levels were not detectable (<100 copies/mg) in 3- and 6-week-old mice; and 19-week-old mice. Moreover, consistent with the presence of other endopeptidases known to cleave ZAAL-NA (Fig. 1, 3rd lane), we detected Phex using this antibody in positive control Sf9 cells expressing high levels of recombinant wild-type Phex (Fig. 1b, 3rd lane). Moreover, consistent with attaining high levels of Phex expression in TMoB-Hyp cells transduced with pLuv-Phex-IRES-GFP, we observed in these cells the presence of the expected 100-kDa Phex protein representing the glycosylated monomer (Fig. 1b, 2nd lane). No endogenous Phex protein was detected in the TMoB-Hyp cells transfected with pLuv-IRES-GFP vector alone (Fig. 1b, 1st lane). In addition, we demonstrated that the level of the Phex transcripts remained elevated in retrovirally infected TMoB-Hyp cells throughout a 14-day culture period (Fig. 1c), a period when these cells undergo a temporal sequence of osteoblastic maturation (see below).

**Restoration of Phex Endopeptidase Activity to Membranes of Hyp Osteoblasts**—Recent studies (9, 11) indicate that Phex hydrolyzes several neutral endopeptidase substrates, including ZAAL-NA, and Leu-enkephalin, which contains a closely related Phe-proline peptidase activity. We initially confirmed that recombinant Phex produced in S9 cells cleaves ZAAL-NA (Fig. 2a), and we then used this substrate to demonstrate that the overexpression of Phex in Hyp mouse osteoblasts imparted endopeptidase activity to levels equal to that of normal osteoblasts (Fig. 2b). S9 membranes expressing recombinant Phex cleaved ZAAL-NA in an EDTA-dependent fashion, whereas the 3′-truncated Phex mutant lacking the catalytic domain resulted in cleavage not different from vector-transfected control cells (Fig. 2a). We found that the EDTA-dependent cleavage of ZAAL-NA was restored in Hyp mouse osteoblasts membranes by retrovirus expressing Phex to levels comparable with that of normal 14-day-old osteoblasts, whereas Hyp osteoblasts transduced with the empty retroviral vector displayed low activity, consistent with the absence of other endopeptidases known to

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be present in these cells (Fig. 2b). Similar results were obtained with [Leu]enkephalin as a substrate (Fig. 2c). [Leu]enkephalin alone migrated as a single peak (Fig. 2c, upper panel) and as a faster migrating peak after incubating [Leu]enkephalin with Hyp membranes overexpressing Phex (Fig. 2c, middle panel). Incubation with membrane preparations from vector-transfected Hyp membranes did not result in cleavage of [Leu]enkephalin (Fig. 2c, lower panel), similar to our prior reports with recombinant Phex (9). Thus, in addition to restoring high levels of Phex protein expression, we normalized Phex endopeptidase activity, a requirement for correcting this haplo-insufficient stage.

**Phenotype Characteristics of Hyp Osteoblasts Overexpressing Phex**—Next, we examined if retroviral mediated transduction of Phex-derived osteoblasts retained their capacity to undergo a temporal sequence of maturation characterized by an initial period of replication and subsequent expression of osteoblastic characteristics. Similar to our previous report, Phex-derived osteoblasts infected with the empty vector as well as non-transfected osteoblast derived from normal and Hyp mice (21) underwent a progressive period of cell proliferation that was characterized by increments in DNA content as an index of cell number (Fig. 3a). The overexpression of Phex did not affect the growth characteristics of Hyp-derived osteoblasts. During the period of rapid cell growth, both vector alone and Phex-expressing TMOb-Hyp cells expressed low levels of alkaline phosphatase (Fig. 3b), consistent with their immaturity. The slowing of cell replication was associated with a significant increase in the expression of alkaline phosphatase activity to similar degrees in both TMOb-Hyp vector alone and Phex-expressing cells (Fig. 3b). Similarly, the process of osteoblast maturation in both vector and Phex-expressing Hyp-derived cells was marked by similar levels of osteocalcin, osteopontin, and type 1 collagen transcripts in 14-day-old cultures (Fig. 3c).

**Failure to Correct the Impaired Mineralization in TMOb-Hyp Osteoblast Cultures by Overexpression of Phex**—In ensuing experiments, we assessed mineralization in normal and Hyp mouse osteoblasts using alizarin red-S histochemical staining. We confirmed our previous report (21) that TMOb-NI cells exhibited marked increments in alizarin red-S-stained mineralization nodules by day 14 of culture (Fig. 3, d and e). Similar to our previous report (21) of impaired mineralization in mature Hyp mouse derived osteoblasts, the Hyp osteoblasts infected with the vector also exhibited minimal alizarin red-S staining characterized by only ill-defined patches with limited dye uptake and the absence of discrete mineralization nodules (Fig. 3, d and e). Overexpression of Phex and restoration of Phex endopeptidase activity (Figs. 1 and 2) failed to correct the impaired mineralization in Hyp osteoblasts (Fig. 3d, middle panel). Indeed, the levels of alizarin red-S staining were not statistically different between Hyp osteoblasts infected with the vector alone and Hyp osteoblasts overexpressing Phex (Fig. 3e). In addition, we showed that the impaired mineralization in Hyp mouse derived osteoblasts was not attributable to differences in cellular composition of the cultures, as clonal cell lines obtained from the parent TMOb-Hyp cultures failed to mineralize after retroviral mediated transfection of Phex (data not shown).

As additional controls, we demonstrated that neither the overexpression of wild-type Phex nor its inactive mutant affect mineralization of normal osteoblasts. We achieved high levels of retroviral mediated Phex expression in 4-day-old normal TMOb cells (Fig. 4a, left panel, 2nd lane) as well as attained high levels of expression of the 3’-truncated Phex mutant in normal TMOb-NI cells (Fig. 4a, right panel, 2nd lane). As documented previously (18), normal osteoblasts display a maturation-dependent up-regulation of Phex, with little expression at 4 days and significant expression at 14 days of culture (Fig. 4a, middle panel, 1st and 2nd lanes). Regardless of whether they were transduced with vector alone, full-length Phex, or the 3’-truncated Phex constructs, normal TMOb osteoblast cultures exhibited indistinguishable maturation-dependent mineralization (Fig. 4, b and c). In addition, in separate studies we demonstrate that retroviral mediated overexpression of GFP did not affect osteoblast growth, differentiation, or mineralization (data not shown).

**Osteoblasts Do Not Express the Phex Substrate FGF-23**—Current data indicate that FGF-23 is a substrate for Phex (13, 15). Therefore, we examined whether FGF-23 might be present in osteoblasts and differentially expressed in osteoblasts derived from Hyp mice. By using RT-PCR analysis with FGF-23-specific primers, however, we failed to identify FGF-23 transcripts in either normal or Hyp-derived osteoblasts (Fig. 5).

**Generation of Hyp Transgenic Mice Expressing Phex in Osteoblasts**—Because we failed to correct the Hyp osteoblast phenotype in culture and failed to identify FGF-23 in osteoblasts,
we reasoned that factors required for Phex function may be lacking in the in vitro environment. Because Phex is predominantly expressed in mature osteoblasts and is temporally co-expressed with osteocalcin (20), we next determined whether restoring Phex expression to mature Hyp osteoblasts in vivo using the osteocalcin gene 2 (OG2) promoter corrected the mineralization defect as well as increased serum phosphorus levels in Hyp mice. We constructed transgenic mice expressing full-length Phex under the control of a 1.3-kb fragment of the mouse OG2 promoter (Fig. 6a). A total of five transgenic mouse lines were obtained (Fig. 6b). We selected two lines with the highest level of expression for further study (founder lines 1 and 2). Male founder mice expressing Phex, designated OG2-Phex, were bred with heterozygous female Hyp mice (X<sup>0</sup>X<sup>0</sup>) to create offspring that co-express the full-length Phex transgene and mutant Hyp allele, designated OG2-Phex-Hyp. The OG2-Phex-Hyp mice were identified and compared with non-transgenic and transgenic normal and non-transgenic Hyp littermates (Fig. 6c). To date we have analyzed results from 68 mice from line 2, including 19 nontransgenic normals, 21 OG2-Phex mice normals, 11 non-transgenic Hyp, and 17 OG2-Phex-Hyp and a more limited number from line 1. Expression of the Phex transgene was predominantly restricted to bone and bone marrow, with minimal expression in brain and no expression in other tissues tested by RT-PCR from 6-week-old animals (Fig. 6d).

FIG. 2. Proteolytic activity of the Phex expressed in the Sf9 and Hyp osteoblasts. a, membrane fractions (50 μg) derived from Sf9 cells after baculovirus-mediated transduction with vector alone, rPhex-WT, or rPhex-3 M were analyzed for peptidase activity using ZAAL-pNA as a substrate in the absence or presence of EDTA. Data are representative of at least three independent experiments and shown with mean ± S.E. b, membrane fractions (100 μg) from control Hyp osteoblasts transduced with the empty retroviral vector (vector) and Hyp osteoblasts infected with pLuv-Phex-IRES-GFP or normal osteoblasts were analyzed for peptidase activity using ZAAL-pNA as a substrate. Data are expressed as EDTA-dependent cleavage. Data are representative of four independent experiments and shown with mean ± S.E. Values sharing the same superscript are not significantly statistically different at p < 0.05. c, high pressure liquid chromatography analysis of hydrolysis of [Leu]enkephalin. [Leu]Enkephalin analyzed in the absence of membranes shows the migration of the uncleaved Try-Gly-Gly-Phe-Leu peptide (upper panel). Incubation of [Leu]enkephalin with 100 μg of Hyp osteoblast membrane infected with pLuv-Phex-IRES-GFP shows the degradation of the substrate and the shift of the peak (indicated by ← in the middle panel). Incubation with the empty vector-infected Hyp-derived osteoblast membranes shows no cleavage of the substrate (lower panel).
Experimental Procedures. The amount of mineralization. The alizarin red-S stain was extracted with 10% cetylpyridinium chloride and quantified as described under Hypogenic transgenic mice exhibited hypophosphatemia identical to non-transgenic normal osteoblasts with the capacity to form mineralized nodules (Fig. 6e). Overexpression of Phex-expressing osteoblasts. Mouse-specific primers were used to RT-PCR-amplify osteopontin, osteocalcin, and α1(I) procollagen from osteoblasts infected with empty vector or the Phex expression vector. The predicted size products for osteopontin (239 bp), osteocalcin (370 bp), and α1(I) procollagen (268 bp) were expressed at similar abundance in Hyp vector and Phex-expressing osteoblasts. Phex was detected only in Hyp osteoblasts infected with the pLuv-Phex-IRES-GFP expression construct. β-Actin served as a control for relative mRNA abundance. d, histochemical staining of mineralization nodules. Hyp osteoblasts (1st lane) failed to form mineralization nodules, whereas normal osteoblasts formed abundant mineralized nodules (3rd lane). Overexpression of Phex failed to impart Hyp osteoblasts with the capacity to form mineralized nodules (2nd lane) above that of vector-infected control cells (1st lane). e, measurement of the amount of mineralization. The alizarin red-S stain was extracted with 10% cetylpyridinium chloride and quantified as described under “Experimental Procedures.” Vector and Phex-expressing Hyp osteoblasts had significantly lower alizarin red-S accumulation at day 14 of culture compared with normal osteoblasts. Numeric values represent the mean ± S.E. of three separate determinations.

Fig. 3. Characterization of TMOb-Hyp osteoblasts after retroviral mediated overexpression of Phex. a, temporal changes in osteoblast growth during culture of Hyp mouse derived osteoblasts infected with the pLuv-IRES-GFP (vector) or the pLuv-Phex-IRES-GFP. Cell numbers as assessed by DNA content increased similarly during the initial period of culture corresponding to comparable rates of DNA synthesis in vector and Phex-expressing Hyp osteoblasts. b, alkaline phosphatase activity. Both the Hyp-vector and Hyp-Phex cell lines displayed a culture duration dependent increase in alkaline phosphatase activity. c, mRNA phenotype analysis of Hyp osteoblasts. Mouse-specific primers were used to RT-PCR-amplify osteopontin, osteocalcin, and α1(I) procollagen from osteoblasts infected with empty vector or the Phex expression vector. The predicted size products for osteopontin (239 bp), osteocalcin (370 bp), and α1(I) procollagen (268 bp) were expressed at similar abundance in Hyp vector and Phex-expressing osteoblasts. Phex was detected only in Hyp osteoblasts infected with the pLuv-Phex-IRES-GFP expression construct. β-Actin served as a control for relative mRNA abundance. d, histochemical staining of mineralization nodules. Hyp osteoblasts (1st lane) failed to form mineralization nodules, whereas normal osteoblasts formed abundant mineralized nodules (3rd lane). Overexpression of Phex failed to impart Hyp osteoblasts with the capacity to form mineralized nodules (2nd lane) above that of vector-infected control cells (1st lane). e, measurement of the amount of mineralization. The alizarin red-S stain was extracted with 10% cetylpyridinium chloride and quantified as described under “Experimental Procedures.” Vector and Phex-expressing Hyp osteoblasts had significantly lower alizarin red-S accumulation at day 14 of culture compared with normal osteoblasts. Numeric values represent the mean ± S.E. of three separate determinations.
Phex to osteoblasts, we performed in situ hybridization studies in 4-day-old normal (+/+), Hyp, and OG2-Phex-Hyp male mice to confirm the localization of the Phex transgene in bone and to compare the distribution of the Phex transgene to endogenous Phex (Fig. 8b). We found, similar to previous reports (20), that endogenous Phex is expressed at high levels in osteoblasts and osteocytes of trabecular bone (Fig. 8b, upper left panel) and in lower abundance in hypertrophic chondrocytes in the growth plate (data not shown). In contrast, Phex was absent in non-transgenic male Hyp mice at all sites (Fig. 8b, upper middle panel). The OG2-driven expression of Phex in transgenic Hyp mice was similar to that of endogenous Phex in trabecular bone, where it was expressed in osteoblasts and osteocytes (Fig. 8b, upper right panel). Transgenic Hyp mice, however, did not express Phex in chondrocytes (data not shown). Sections probed with the sense riboprobe gave only low level background staining in all groups (Fig. 8b, lower panels).

Non-decalcified bone sections from tibias of non-transgenic normals and OG2-Phex mice were indistinguishable by qualitative analysis, including normal appearing bone volume, osteoid thickness, and mineralization (Fig. 8c, compare 1st and 2nd column). In contrast, non-transgenic Hyp mice exhibited profound defects in mineralization characterized by hyperosteo- and defective mineralization as evidenced by the complete absence of double fluorescent bone labels (Fig. 8c, 3rd column). Only diffuse, non-quantifiable label was present beneath widened osteoid seams (Fig. 8c, 3rd column, 3rd panel). In addition, the growth plate and underlying metaphyseal bone of non-transgenic Hyp mice was disorganized and exhibited impaired mineralization. The bone histology of OG2-Phex-Hyp was indistinguishable from their non-transgenic Hyp mice littermates and exhibited histologic evidence of severe osteomalacia and rickets (Fig. 8c, 4th column). Thus, despite the successful overexpression of the Phex transgene in a pattern overlapping that of endogenous Phex, we failed to observe any effect of the Phex transgene on bone histology in either OG2-Phex transgenic or OG2-Phex Hyp mice.

**Discussion**

Because Phex is predominantly expressed in bone and osteoblasts derived from Hyp mice have an apparent intrinsic mineralization defect, we (8) and others (16, 17, 20) favored the hypothesis that diminished Phex expression in osteoblasts was primarily responsible for the pathogenesis of the XLH and the Hyp phenotype. Direct evidence that co-expression of Phex and
its putative substrates in the bone milieu participated in bone mineralization and regulation of systemic phosphate homeostasis, however, was lacking (21, 24). The results from the current study indicate that Phex expression in osteoblasts is not sufficient to explain the pathogenesis of XLH. Rather, we found that the targeted overexpression of Phex to osteoblasts in Hyp mice using the osteocalcin promoter failed to correct either the mineralization defect of bone or the systemic hypophosphatemia (Figs. 6–8). Despite attaining expression of Phex in mature osteoblasts to levels (Fig. 8b) and activity (Fig. 6e) comparable with endogenous Phex, OG2-Phex-Hyp mice exhibited hypophosphatemia (Fig. 7a), radiographic evidence of rickets (Fig. 8a), and histologic evidence of osteomalacia (Fig. 8c) identical to that of non-transgenic Hyp littermates. The overexpression of Phex also had no demonstrable effect in normal OG2-Phex mice, which displayed serum phosphate levels (Fig. 7a) and skeletal morphology (Fig. 8) indistinguishable from non-transgenic littermates, despite higher endopeptidase ac-
activity against a synthetic Phex substrate (Fig. 6e). Thus, our findings fail to support the hypothesis that abnormal Phex function in mature osteoblasts plays a primary role in the pathogenesis of hypophosphatemia and raises questions regarding whether Phex has a direct or indirect role in regulating osteoblast-mediated mineralization of bone.

Based on our findings, it is likely that expression of Phex at sites other than those controlled by the osteocalcin promoter is responsible for the persistent accumulation of Phex substrates, resulting in hypophosphatemia and the failure to rescue the Hyp phenotype. Although Phex and osteocalcin expression are concordant in mature osteoblasts and osteocytes (20), the osteocalcin promoter does not express Phex in teeth, cartilage, or other sites where Phex is normally present (20) and initiates expression later during embryogenesis at embryonic day 15.5 compared to day 11 for endogenous Phex. Consequently, persistent abnormalities in the spatial and temporal expression of Phex, and/or the failure to restore the full complement of Phex activities in the whole mouse (which may be the sum of Phex expression in many tissues), may account for the failure to rescue the Hyp phenotype in the current studies. In addition, other genes and environmental factors have been shown to affect the severity of hypophosphatemic rickets (44) and also could impact upon the inability of Phex to rescue the Hyp phenotype.

Other potential explanations for our in vivo findings seem unlikely. Even though recent reports indicate that mutant Phex proteins accumulate in the endoplasmic reticulum (45), we have shown that the 3′ deletion Phex mutant does not interfere with the function of the wild-type transfected Phex. For example, overexpression of the mutant Phex construct neither interferes with the mineralization of normal osteoblasts in vitro (Fig. 4) nor disrupts the enzymatic activity of wild-type Phex in vitro (9). In addition, our experimental design, using the 1.3-kb OG2 promoter fragment to drive expression of transgenes restricted to mature osteoblasts in vivo (34), achieved the successful restoration of Phex endopeptidase activity in bone (Fig. 6e). Demonstration of Phex activity in osteoblasts and calvaria also lessens the concern that endogenous Osteocalcin may inhibit the activity of co-expressed Phex, as suggested by a

**Fig. 7.** Serum phosphorus and bone density in non-transgenic and OG2 Phex transgenic normal and Hyp mice. a, serum phosphorus concentrations in 3-, 6-, and 12-week-old male and female non-transgenic normals, OG2 Phex transgenic normals, non-transgenic Hyp, and OG2-Phex transgenic Hyp littermates. BMD of the femur was assessed with the PIXImus™ mouse densitometer in 12-week-old male non-transgenic normals, OG2-Phex transgenic normals, non-transgenic Hyp, and OG2-Phex transgenic Hyp littermates. The decrease in mineralization of the skeleton in the Hyp compared with normal was confirmed by significantly reduced bone ash weight and BMD; however, OG2-Phex-Hyp exhibited a slight but significant increment in bone ash weight and BMD compared with non-transgenic Hyp mice. All numeric values represent the mean ± S.E. of at least five individual animals. Values sharing the same superscript are not significantly statistic different at p < 0.05.

*S. Liu, R. Guo, Q. Tu, and L. D. Quarles, unpublished observations.*
recent in vitro studies (12). Finally, because Phex is not expressed in osteoblastic precursors (20), it seems unlikely that targeting Phex to earlier states of the osteoblast lineage would have altered the results. We did observe a slight, but significant, increase in dry ashed weight and bone mineral density of femurs derived from OG2 Phex-Hyp mice (Fig. 7, b and c). These later findings, representing changes in bone mineralization not detected by conventional radiographic or histologic methods, raise the possibility that Phex may have a role in regulating bone mineralization that is masked by the persistent hypophosphatemia in the OG2 Phex-Hyp mice. It is possible that Phex directly or indirectly regulates the production of matrix proteins and/or local bone substrates, which in turn regulate the mineralization process and account for the small increase in bone mineral density in persistently hypophosphatemic OG2-Phex-Hyp mice. Proof of an effect independent of hypophosphatemia mediated by Phex direct regulation of osteoblast-mediated mineralization requires identification of additional physiologically important substrates in bone. Nevertheless, the observed increase in bone mass in OG2 Phex-Hyp mice is in keeping with the increased production of factors that regulate mineralization and phosphate transport by cultured osteoblasts derived from Hyp mice (21, 23). Recent studies have observed abnormalities of bone extracellular matrix proteins (46) and the accumulation of MEPE (47) in Hyp mice. The failure to detect FGF-23, the only known physiologic Phex substrate and the leading candidate for phosphatonin (13, 14, 35), in bone (Fig. 5) and bone marrow (15), however, indicates that this phosphaturic factor is not the putative Phex substrate in the local bone environment. Related members of the M13 family of metalloproteases have...
multiple substrates whose tissue-specific actions are derived from their co-localization (8). Therefore, Phex may separately metabolize distinct substrates that regulate phosphaturia and mineralization.

We confirmed previous reports (21) that osteoblasts derived from Hyp mice exhibit the inability to form mineralization nodules in culture (Figs. 3 and 4). However, restoration of Phex expression (Fig. 1) and enzymatic activity (Fig. 2) to Hyp osteoblasts did not restore their capacity to mineralize extracellular matrix in vitro (Fig. 3) under culture conditions supporting mineralization in normal osteoblasts (Fig. 4). The inability to rescue the Hyp osteoblastic phenotype by Phex overexpression in vitro is consistent with prior studies where co-culture of cells expressing Phex also failed to correct the mineralization defect in Hyp osteoblasts (21). The current negative findings are not due to the effects of the retroviral transduction or inadequate restoration of endopeptidase activity. Moreover, the presence of a truncated Phex in Hyp osteoblast does not interfere with the restoration of Phex function, because overexpression of a 3'-truncated mutant Phex failed to disrupt mineralization of normal osteoblasts (Fig. 4). It also is unlikely that aberrant temporal and/or the excessive amounts of retroviral mediated Phex expression or GFP could have influenced our results in vitro, because transduction of normal osteoblasts with the retroviral Phex construct with and without GFP did not affect their ability to mineralize (Fig. 4). We cannot exclude, however, potential variability resulting from differences in the cell culture models used to assess mineralization by alizarin red staining. Indeed, a preliminary report from another laboratory (48), unlike our in vitro studies, shows that overexpression of Phex in Hyp-derived osteoblasts, although not sufficient to fully normalize mineralization, results in partial rescue of their mineralization capacity.

Regardless, the in vitro studies of Hyp-derived osteoblasts indicate a more complex pathogenesis of the defective mineralization in cultured osteoblasts. There are differences in the gene expression profiles in osteoblastic cultures compared with bone in Hyp mice, indicating that these culture models do not fully mimic the in vivo state (21, 46). In addition, the exposure to hypophosphatemia or the Hyp milieu may somehow limit the expression of an accessory factor necessary for Phex function and/or lead to deficiencies in the full complement of genes necessary for mineralization. There is a precedent for hypophosphatemia to induce a similar intrinsic mineralization defect in osteoblasts derived from mice in which the renal sodium-dependent phosphate transporter has been ablated (49). Extracellular phosphate also may alter osteoblast gene expression through its actions to modulate nuclear export of the osteoblast transcriptional regulator Cbfal in bone cells (33). Identification of the potential Phex substrates in bone and these modulating factors will be necessary to unravel the relative contribution of local and systemic regulation of in osteoblast-mediated mineralization.

In conclusion, our current findings fail to support the simple hypothesis that the lack of Phex in Hyp osteoblasts is directly responsible for the impaired mineralization and abnormalities in systemic phosphate homeostasis. Rather, other sites and/or temporal aspects of Phex expression appear to be physiologically important in the metabolism of the phosphaturic factor phosphatonin. It is likely that the successful rescue of the Hyp phenotype will require restoration of normal Phex activity in one or more of these additional sites. In addition, we failed to establish a cause and effect relationship between Phex expression in osteoblasts and their ability to form a mineralized extracellular matrix in culture, indicating that hypophosphatemia has a predominant role in the defective mineralization. Additional studies that restore endogenous Phex expression in transgenic animals (possibly by using Phex promoter) and/or selectively disrupt Phex by tissue-specific targeted deletion strategies will be necessary to establish a cause and effect relationship between the site of Phex expression and the Hyp phenotype. In addition, confirming the identity of phosphatonin, as well as the identification of possible bone-derived substrates for Phex and/or phosphate-dependent accessory factors that regulate the mineralization process, will be important in unraveling the complex pathogenesis of XLH.

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REFERENCES

1. Anonymous (1995) Nat. Genet. 11, 130–136
2. Strom, T. M., Francis, F., Lorenz, B., Boddirch, A., Eoons, M. J., Lehrach, H., and Meitinger, T. (1997) Hum. Mol. Genet. 6, 165–171
3. Grieff, M., Mumm, S., Waelti, P., Mazzarella, R., Whyte, M. P., Thakkar, R. V., and Schlessinger, D. (1997) Biochem. Biophys. Res. Commun. 231, 635–639
4. Holm, I. A., Huang, X., and Kende, L. M. (1997) Am. J. Hum. Genet. 60, 579–587
5. Emoto, N., and Yanagisawa, M. (1995) J. Biol. Chem. 270, 15262–15268
6. Lee, S., Zambas, E. D., Marsh, W. L., and Redman, C. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6355–6357
7. Kiyu-Soo, S., Sasaki, M., Yokohama, H., Nakagami, S., Hirayama, T., Aoki, S., Wada, K., and Iyama, H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4545–4550
8. Quaresl L., and Drenzer, M. K. (2001) J. Clin. Endocrinol. & Metab. 86, 494–498
9. Guo, R., Liu, S., Sparney, R. F., and Quaresl, L. D. (2001) Am. J. Physiol. Endocrinol. & Metab. 281, 837–847
10. Lipman, M., Dhyendu, P., Hugh, P. J., Bennett, J. E., Henderson, E. S., Yingnian, S., Goltzman, D., and Karaplis, A. C. (1998) J. Biol. Chem. 273, 13729–13737
11. Shirotoni, K., Tsubuki, S., Iwata, N., Takagi, H., Hayigai, W., Maruyama, K., Kiyu-Soo, S., Kiyama, H., Iwata, H., and Tomita, T., Iwatsubo, T., and Suda, T. C. (2001) J. Biol. Chem. 276, 21885–21890
12. Bouleau, G., Tenenhouse, H. S., Desgroselliers, L., and Crine, P. (2001) Biochim. Biophys. Acta. 355, 707–713
13. Orange, A. E., Finnegan, R., Jan de Beur, S. M., Cho, J., Levine, M. A., Kumar, R., and Schaivi, S. C. (2003) Biochim. Biophys. Res. Commun. 294, 977–981
14. Strewler, G. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5945–5946
15. Shimada, T., Mitutani, S., Muto, T., Yoneya, T., Hino, R., Takeda, S., Takeuchi, Y., Fujita, T., Fukuzawa, M. S., and Yasamita, T. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6505–6505
16. Beck, L., Soumounou, Y., Marcel, J., Krishnamurthy, G., Gauthier, C., Goodyer, C. G., and Tenenhouse, H. S. (1997) J. Clin. Invest. 99, 1280–1299
17. Delvin, E. E., Richard, P., Desbarats, M., and Glorieux, F. H. (1990) Bone 7, 1775–1780
18. Guo, R., and Quaresl, L. D. (1997) J. Bone Miner. Res. 12, 1099–1101
19. Zoidis, E., Zapf, J., and Schmid, C. (2000) Mol. Cell. Endocrinol. 168, 41–51
20. Ruchon, A. F., Tenenhouse, H. S., Marcinkiewicz, M., Siegfried, G., Aubin, J. E., Desgroselliers, L., Crine, P., and Boileau, G. (2000) J. Bone Miner. Res. 15, 1440–1459
21. Delvin, E. E., Richard, P., Desbarats, M., Ecartor-Charrrier, B., and Glorieux, F. H. (1990) Bone 11, 87–98
22. Ecartor, B., Glorieux, F. H., Desbarats, M., Travers, R., and Labelle, L. (1992) J. Bone Miner. Res. 7, 523–530
23. Nishit, T., Fujita, T., Iwatsubo, T., Thomas, R., Xiao, Z. S., Quaresl, L. D., and Drenzer, M. K. (1999) J. Bone Miner. Res. 14, 2027–2035
24. Nishit, T., Coffman, T. M., Griffiths, R., and Drenzer, M. K. (1992) J. Clin. Invest. 89, 1435–1459
25. Miyamura, T., Tanaka, H., Inoue, M., Ichinoe, Y., and Seino, Y. (2000) J. Bone Miner. Res. 15, 1451–1458
26. Bonnier, C., and Nicolas, J. F. J. F. (1996) Methods Enzymol. 225, 451–469
27. Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756–1775
28. Halstead, L. R., Weinstein, R. S., Cheng, S. L., Riasl, F., and Avioli, L. V. (1996) Persistent Hypophosphatemia in OG2 Phex Transgenic Hyp Mice.
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Am. J. Physiol. 270, E141–E147
38. Garner, S. C., Pi, M., Tu, Q., and Quarles, L. D. (2001) Endocrinology 142, 3996–4005
39. Quarles, L. D., Siddhanti, S. R., and Medda, S. (1997) J. Cell. Biochem. 65, 11–24
40. Yamashita, T., Yoshioka, M., and Itoh, N. (2000) Biochem. Biophys. Res. Commun. 277, 494–498
41. Yang, H., Wanner, I. B., Roper, S. D., and Chaudhari, N. (1999) J. Histochem. Cytochem. 47, 431–446
42. Plummer, T. B., Sperry, A. C., Xu, H. S., and Lloyd, R. V. (1998) Diagn. Mol. Pathol. 7, 76–84
43. Sokal, R. R., and Rohol, F. J. (1981) Biometry: The Principals and Practice of Statistics in Biological Research, 2nd Ed, W. H. Freeman & Co., San Francisco, CA
44. Holm, I. A., Nelson, A. E., Robinson, B. G., Mason, R. S., Marsh, D. J., Cowell C. T., and Carpenter T. O. (2001) J. Clin. Endocrinol. & Metab. 86, 3889–3899
45. Sabbagh, Y., Boileau, G., DesGroseillers, L., and Tenenhouse, H. S. (2001) Hum. Mol. Genet. 10, 1539–1546
46. Miao, D., Bai, X., Panda, D., McKee, M., Karaplis, A., and Goltzman, D. (2001) Endocrinology 142, 926–939
47. Argiro, L., Desbarats, M., Glorieux, F. H., and Ecarot, B. (2001) Genomics 74, 342–351
48. Sabbagh, Y., Londowski, J. M., Mathiesen, D., Gauthier, C., Boileau, G., Tenenhouse, H. S., Poeschla, E. M., and Kumar, R. (2000) J. Am. Soc. Nephrol. 11, 413A
49. Thomas, R., Fujiwara, I., Tenenhouse, H. S., Quarles, L. D., and Drezner, M. K. (1999) J. Bone Miner. Res. 14, 189

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