Aberrant *Phex* function in osteoblasts and osteocytes alone underlies murine X-linked hypophosphatemia

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Patients with X-linked hypophosphatemia (XLH) and the *hyp*-mouse, a model of XLH characterized by a deletion in the *Phex* gene, manifest hypophosphatemia, renal phosphate wasting, and rickets/osteomalacia. Cloning of the *PHEX/Phex* gene and mutations in affected patients and *hyp*-mice established that alterations in *PHEX/Phex* expression underlie XLH. Although PHEX/Phex expression occurs primarily in osteoblast lineage cells, transgenic *Phex* expression in *hyp*-mouse osteoblasts fails to rescue the phenotype, suggesting that *Phex* expression at other sites underlies XLH. To establish whether abnormal *Phex* in osteoblasts and/or osteocytes alone generates the *HYP* phenotype, we created mice with a global *Phex* knockout (Cre-Phex<sup>Δflox/y</sup> mice) and conditional osteocalcin-promoted (OC-promoted) *Phex* inactivation in osteoblasts and osteocytes (OC-Cre-Phex<sup>Δflox/y</sup>). Serum phosphorus levels in Cre-Phex<sup>Δflox/y</sup>, OC-Cre-Phex<sup>Δflox/y</sup>, and *hyp*-mice were lower than those in normal mice. Kidney cell membrane phosphate transport in Cre-Phex<sup>Δflox/y</sup>, OC-Cre-Phex<sup>Δflox/y</sup>, and *hyp*-mice was likewise reduced compared with that in normal mice. Abnormal renal phosphate transport in Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice was associated with increased bone production and serum FGF-23 levels and decreased kidney membrane type IIa sodium phosphate cotransporter protein, as was the case in *hyp*-mice. In addition, Cre-Phex<sup>Δflox/y</sup>, OC-Cre-Phex<sup>Δflox/y</sup>, and *hyp*-mice manifested comparable osteomalacia. These data provide evidence that aberrant Phex function in osteoblasts and/or osteocytes alone is sufficient to underlie the *hyp*-mouse phenotype.

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Aberrant Phex function in osteoblasts and osteocytes alone underlies murine X-linked hypophosphatemia

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Patients with X-linked hypophosphatemia (XLH) and the hyp-mouse, a model of XLH characterized by a deletion in the Phex gene, manifest hypophosphatemia, renal phosphate wasting, and rickets/osteomalacia. Cloning of the PHEX/Phex gene and mutations in affected patients and hyp-mice established that alterations in PHEX/Phex expression underlie XLH. Although PHEx/Phex expression occurs primarily in osteoblast lineage cells, transgenic Phex expression in hyp-mouse osteoblasts fails to rescue the phenotype, suggesting that Phex expression at other sites underlies XLH. To establish whether abnormal Phex in osteoblasts and/or osteocytes alone generates the HYP phenotype, we created mice with a global Phex knockout (Cre-PhexΔflox/y mice) and conditional osteocalcin-promoted (OC-promoted) Phex inactivation in osteoblasts and osteocytes (OC-Cre-PhexΔflox/y). Serum phosphorus levels in Cre-PhexΔflox/y, OC-Cre-PhexΔflox/y, and hyp-mice were lower than those in normal mice. Kidney cell membrane phosphate transport in Cre-PhexΔflox/y, OC-Cre-PhexΔflox/y, and hyp-mice was likewise reduced compared with that in normal mice. Abnormal renal phosphate transport in Cre-PhexΔflox/y and OC-Cre-PhexΔflox/y mice was associated with increased bone production and serum FGF-23 levels and decreased kidney membrane type IIa sodium phosphate cotransporter protein, as was the case in hyp-mice. In addition, Cre-PhexΔflox/y, OC-Cre-PhexΔflox/y, and hyp-mice manifested comparable osteomalacia. These data provide evidence that aberrant Phex function in osteoblasts and/or osteocytes alone is sufficient to underlie the hyp-mouse phenotype.

Introduction
X-linked hypophosphatemia (XLH) is the archetypal vitamin D-resistant disease in humans and the most common form of inherited rickets, with an incidence of approximately 1 in 20,000 live births. The disease is characterized by renal phosphate (Pi) wasting with resulting hypophosphatemia, abnormal vitamin D metabolism, defective bone and cartilage mineralization, dentine defects, and stunted growth (1). Recently, the gene involved in the pathogenesis of XLH was identified by positional cloning (2–4) and designated as PHEX (the phosphate-regulating gene with homologies to endopeptidases on the X chromosome). The PHEX locus was mapped to Xp22.1, and more than 140 loss-of-function PHEX mutations have been reported to date in patients with XLH (2). The murine homolog of the human disease, the hyp-mouse, has a phenotype identical to that evident in patients with XLH and is due to a large deletion in the 3’ region of the Phex gene (5). These findings suggest that a mutation in the PHEX/Phex gene is responsible for the phenotypic changes in patients with XLH and the hyp-mouse.

The Phex gene has 22 exons and encodes for a 749-aa glycoprotein, which has homology with members of the M13 membrane-bound zinc metalloendopeptidase family. Investigation of murine tissues and cell cultures has revealed that Phex is predominantly expressed in bones and teeth (3, 6), although Phex mRNA and/or protein have been detected in lung, brain, muscle, gonads, skin, and parathyroid glands. The PHEX/Phex expression in bone is limited to cells of the osteoblast lineage (6, 7), osteoblasts, and osteocytes.

Despite the significant advances made in understanding the pathogenesis of XLH, further progress has been limited by unsuccessful efforts to completely rescue the HYP phenotype and determine the physiologically relevant site for the PHEX mutation. In attempts to determine whether abnormal PHEX/Phex expression in bone cells alone is the determining abnormality underlying the pathogenesis...
of XLH, several investigators have used osteoblast/osteocyte-targeted overexpression of Phex in order to normalize osteoblast mineralization, in vivo, and rescue the HYP phenotype in vivo (19, 20). Surprisingly, however, these studies documented that restoration of Phex expression and enzymatic activity to immortalized hyp-mouse osteoblasts, by retrovirus-mediated transduction, does not restore their capacity to mineralize extracellular matrix in vitro, under conditions supporting normal mineralization. Moreover, in complementary studies, Liu et al. (19) and Bai et al. (20) found that transgenic hyp-mice maintained characteristic hypophosphatemia and abnormal vitamin D metabolism, as well as histological evidence of osteomalacia, despite expressing abundant Phex mRNA and enzyme activity in mature osteoblasts and osteocytes, under the control of the bone-specific promoters osteocalcin (OC) and ColA1 (2.3 kb). These findings suggest that expression of Phex at sites other than bone is responsible for the HYP phenotype. However, similar studies performed with transgenic mice in which Phex overexpression was under the regulation of the ubiquitous human β-actin promoter likewise failed to normalize the Pi homeostasis (21).

To definitively explore this apparent paradox, we sought to determine whether conditional inactivation of Phex in osteoblasts and osteocytes generates a biochemical phenotype similar to that in mice with a global Phex knockout and comparable to that in hyp-mice. In order to accomplish this goal, we generated mouse lines with global and targeted deletion of exon 17 from the Phex gene, which codes a portion of the protein crucial for bioactivity. We compared biochemistries and bone histology in the global and targeted Phex–knockout mice with those in hyp- and normal mice.

**Results**

*Phex expression in knockout mice.* In order to determine the presence or absence of exon 17 in the Phex gene of the knockout models,
knockout mice. These mouse models, which carried the mutant Phex gene in a tissue-specific fashion, allowed investigation of the requisite abnormality necessary to express the HYP phenotype.

Biochemistries in the knockout mice. We examined the serum Pi and Ca²⁺ levels in Cre-PhexΔflox/y and OC-Cre-PhexΔflox/y mice, as well as in normal and hyp mice, at 8 weeks of age. Both Cre-PhexΔflox/y (3.29 ± 0.22 mg/dl) and OC-Cre-PhexΔflox/y (3.52 ± 0.17 mg/dl) mice had significantly decreased (P < 0.001) serum Pi levels compared with those of normal mice (7.05 ± 0.33 mg/dl). The decreased Pi levels were comparable to the significantly decreased (P < 0.001) concentration manifest in hyp mice (3.76 ± 0.17 mg/dl).

Similarly, the serum Ca²⁺ levels in Cre-PhexΔflox/y (8.51 ± 0.18 mg/dl) and OC-Cre-PhexΔflox/y (8.48 ± 0.16 mg/dl) mice were no different from those of hyp mice (8.35 ± 0.12 mg/dl). However, hyp mice and the knockout models maintained serum calcium levels that were not significantly different from those in normal mice (8.72 ± 0.25 mg/dl).

Renal Pi transport in the knockout mice. To determine whether the hypophosphatemia in the knockout mice was due to abnormal renal Pi handling, we examined brush border membrane Pi transport (Figure 2A). The Cre-PhexΔflox/y (146.7 ± 10.9 pmol/mg/10 s) and OC-Cre-PhexΔflox/y (134.3 ± 8.6 pmol/mg/10 s) mice exhibited significantly decreased (P < 0.01) Pi transport compared with that in normal mice (246.7 ± 13.8 pmol/mg/10 s) but similar to that characteristically observed in hyp mice (120.7 ± 10.9 pmol/mg/10 s).

Consistent with these observations, analysis of Western blots revealed that the aberrant Pi transport in Cre-PhexΔflox/y and OC-Cre-PhexΔflox/y mice, like that in the hyp mice, was due to a significant decrease (P < 0.05) in renal tubular type Ia sodium Pi cotransporter (Npt2) protein expression, compared with that in normal mice (Figure 2B). Indeed, in the knockout and hyp mice, a comparable approximately 20% decrement in the abundance of the Npt2 protein was observed.

Vitamin D metabolism in the knockout mice. In previous studies, we established that hyp mice manifest aberrant regulation of vitamin D metabolism, resulting from a unique abnormality in 25-hydroxyvitamin D-1α-hydroxylase [25(OH)D-1α-hydroxylase] translational activity (22). To ascertain whether Phex deletion resulted in a similar defect, we investigated Pi- and parathyroid hormone–mediated (PTH-mediated) vitamin D metabolism in both knockout mice. At the time of study, the knockout mice and hyp mice had comparable hypophosphatemia, as noted above. However, despite the stimulatory effects of hypophosphatemia on renal 25(OH)D-1α-hydroxylase activity, Cre-PhexΔflox/y and OC-Cre-PhexΔflox/y mice, similar to hyp mice, displayed no significant enhancement of enzyme function (Figure 3A). Nevertheless, each of these animal models displayed a 2.0-fold increment in 25(OH)D-1α-hydroxylase mRNA transcripts (Figure 3B). However, the Cre-PhexΔflox/y and OC-Cre-PhexΔflox/y mice, like hyp mice, manifested no stimulation of 25(OH)D-1α-hydroxylase protein content, consistent with a translational abnormality in vitamin D metabolism (Figure 3C).
To confirm the dissociation between mRNA expression and renal 25(OH)D-1α-hydroxylase activity in the knockout mice and further establish that the defect was similar to that in hyp-mice, we examined the effects of PTH stimulation on 25(OH)D-1α-hydroxylase activity in the various animal models. PTH stimulation of hyp-mice resulted in a 10-fold elevation of 25(OH)D-1α-hydroxylase mRNA, similar to the effects of stimulation in normal mice and to that evidenced in both Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice (Figure 3B). However, the increased mRNA in the hyp-mice and the knockout mice did not result in enhanced translation of the 25(OH)D-1α-hydroxylase protein, while normal mice, as expected, did manifest increased translational activity (Figure 3C). Consistent with these observations, PTH increased enzyme activity only in the normal mice and had no effect on 25(OH)D-1α-hydroxylase activity in hyp-mice or the Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice (Figure 3A).

Bone structure and histomorphology in the knockout mice. Radiographic evaluation of long bones from Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice revealed that length was decreased compared with that in normal mice but was similar to that in hyp-mice (Figure 4A). Indeed, measurements of femur length in normal and hyp-mice, as well as the Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice, confirmed that the femur length in the knockout models was significantly (*P < 0.001) less than that in normals, but no different from that in hyp-mice (Figure 4B).

In addition, both knockout mice, like hyp-mice, exhibited profound osteomalacia (Figure 5) characterized by hyperosteoendrosis, as evidenced by histological examination of Goldner-stained sections (Figure 5A), which revealed an apparent excess of unmineralized osteoid. The mineralization defect was confirmed in Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice and hyp-mice by the complete absence of distinct double-fluorescent labels in the respective bone sections from these animals (Figure 5B). Indeed, only diffuse, nonquantifiable label was present beneath widened osteoid seams. Most importantly, quantitative histomorphometric evaluation of the Goldner-stained bone sections revealed that the severity of the osteomalacia was equal in the knockout models and the hyp-mice (Figure 5C).

Further, confocal microscopic examination of bone sections from procorin red–treated mice revealed that in normal mice, the osteocyte lacunae were highly organized and regularly spaced in linear arrays, whereas the osteocyte lacunae in the bone from Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice, as well as those from hyp-mouse bone, were much larger and randomly oriented (Figure 6A). The marked abnormalities in the distribution and organization of the osteocyte-lacunocanalicular system in the knockout and hyp-mice were further documented with acid-etched scanning electron microscopic images (Figure 6B). Indeed, the inner lacunocanalicular wall was smooth in the bone sections from normal mice, but the wall was buckled and enlarged in the sections from Cre-Phex<sup>Δflox/y</sup>, OC-Cre-Phex<sup>Δflox/y</sup>, and hyp-mice (Figure 6C). These results established that loss of Phex function in all tissues or in bone (osteoblasts/osteocytes) alone created a biochemical and bone phenotype indistinguishable from that in hyp mice.

Serum levels and production of phosphatonin in the knockout mice. To establish whether the pathophysiological abnormalities in the knockout mice were due to a cascade of events similar to that observed in hyp-mice, we explored whether Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice, like the hyp-mice, exhibit a Phex-dependent increase in the circulating levels of the phosphatonin, as well as increased phosphatonin production. Serum FGF-23 levels were increased dramatically (*P < 0.001) in Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice to levels comparable to those in hyp-mice (Figure 7). Consistent with these findings, FGF-23 mRNA measurements in bone revealed a significant increase (*P < 0.01) in Cre-Phex<sup>Δflox/y</sup> and OC-Cre-Phex<sup>Δflox/y</sup> mice, equivalent to that observed in the hyp-mice (Figure 8).

In contrast, serum MEPE-ASARM-peptide (*P < 0.001) and sFRP-4 levels (*P < 0.05) were significantly elevated in Cre-Phex<sup>Δflox/y</sup> mice,
as in the hyp-mice, but not in OC-Cre-PhexΔflox/y mice (Figure 7). Not surprisingly, real-time PCR studies revealed a significant increase in MEPE \((P < 0.01)\) and sFRP-4 \((P < 0.05)\) expression in bone samples only of Cre-PhexΔflox/y mice to levels comparable to those in hyp-mice (Figure 8).

**Discussion**

Although many investigators suspect that mutated Phex and a lack of Phex activity in the hyp-mouse osteoblast is directly responsible for impaired mineralization and abnormal Pi homeostasis in the mutants, multiple strategies, including bone marrow transplantation (23) and targeted overexpression of Phex in osteoblasts (19, 20), have failed to substantiate this concept. However, it is uncertain whether such disparate observations resulted because: (a) physiologically relevant Phex expression occurs in cells other than osteoblasts; or (b) the experimental procedures employed had innate limitations, such as errant temporal or cellular expression of Phex in transgenic animals. Regardless, the need to discern with certainty the cell type(s) in which Phex expression is essential to regulate the phenotypic expression of XLH and the extent of the phenotype due directly and secondarily to a specific phosphatonin(s) produced by the osteoblast/osteocyte remains of substantial importance. Indeed, development of novel therapeutic strategies for the disease and identification of the putative phosphatonin(s) central to the pathophysiology of the disease have been hampered by the absence of such information. The Cre-loxP genetic strategy has been developed that enables inactivation of mouse genes in a tissue-specific (or cell-specific) fashion (24). The opportunity to employ the Cre-loxP strategy to knock out Phex in osteoblasts and osteocytes and determine with certainty the physiologically relevant site for gene expression has been facilitated by the recent development of FVB-N mice expressing Cre recombinase under the control of the osteoblast/osteocyte-specific promoter OC. Indeed, Zhang et al. (25) have found that in transgenic mice, OC-Cre mRNA is expressed at high levels in calvariae and vertebrae and is undetectable in other tissues, including brain, heart, kidney, liver, skeletal muscle, spleen, and stomach. Further, they demonstrated that the level of Cre expression in the transgenic FVB-N mice is sufficient for high-level recombination; indeed, crossing the OC-Cre+/– mice with a mouse carrying a floxed LacZ gene upstream of a human alkaline phosphatase gene revealed a greater than 90% recombination at the LacZ locus, as evidenced by the appearance of alkaline phosphatase–positive osteoblasts and osteocytes. More recently, the OC-Cre transgene has been backcrossed onto the C57BL/6J background for more than 7 generations, and subsequent crossing of the OC-Cre+/– mice, maintained on this background, again demonstrated that the level of Cre expression results in high-level recombination (T.L. Clemens, unpublished observations).

In the current studies, we used traditional techniques to successfully create C57BL/6J mice with a floxed Phex gene, in which exon 17 was targeted for deletion. Using Ella-Cre mice to remove the Neo cassette from floxed mice, we created global exon 17–knockout mice Cre-PhexΔflox/y for controls in our studies. Further, employing the C57BL/6J OC-Cre mice discussed above, we produced mice with

**Figure 4**

Femur length in normal, hyp-, and knockout mice. (A) High-resolution radiographics revealed that Cre-PhexΔflox/y and OC-Cre-PhexΔflox/y mice had shortened femurs comparable to those in hyp-mice. (B) The bar graph presents the average femur length in the normal, hyp-, Cre-PhexΔflox/y, and OC-Cre-PhexΔflox/y mice. Compared with normal mice, the hyp-, Cre-PhexΔflox/y, and OC-Cre-PhexΔflox/y mice had significantly decreased femur length. Measurements in at least 6 femurs in each group revealed a significant decrease (averaging 2.5 mm) in femur length in hyp-, Cre-PhexΔflox/y, and OC-Cre-PhexΔflox/y compared with normal mice. ***P < 0.001 compared with normal mice.
Figure 5
Bone histomorphology in normal, hyp-, Cre-Phex$^{Δflox/y}$, and OC-Cre-Phex$^{Δflox/y}$ mice. (A) Goldner-stained sections of cortical bone reveal at low magnification an apparent increase in unmineralized osteoid (red-brown colored) in the hyp-, Cre-Phex$^{Δflox/y}$, and OC-Cre-Phex$^{Δflox/y}$ mice, compared with that in normals. At higher magnification, the evident increased unmineralized osteoid in the cortical bone specimens from the hyp-, Cre-Phex$^{Δflox/y}$, and OC-Cre-Phex$^{Δflox/y}$ mice appears comparable in magnitude. (B) The double-labeled bone specimens, viewed under fluorescent light, show normal mineralization in the normal mice, manifested by distinct dual labels deposited beneath narrow osteoid seams. In contrast, the bone sections from the hyp-, Cre-Phex$^{Δflox/y}$, and OC-Cre-Phex$^{Δflox/y}$ mice have diffuse smudged fluorescent labels under widened osteoid seams, indicating a disorderly deposition of mineral characteristic of osteomalacia. The diffuse patchy double labels were too indistinct to permit quantitative assessment of the abnormal mineralization dynamics. (C) Quantitative histological exam of the Goldner-stained sections from a minimum of 6 animals in each group revealed significantly increased osteoid surface and osteoid volume in the hyp-, Cre-Phex$^{Δflox/y}$, and OC-Cre-Phex$^{Δflox/y}$ mice, as indicated by the asterisks denoting statistically significant values ($^{***}P < 0.001$). In contrast, there was no significant difference in these values in the knockout models and the hyp-mice (denoted by the black columns), again providing evidence that the osteomalacia was of comparable magnitude in these animal models.
targeted deletion of *Phex* exon 17 in osteoblasts and osteocytes, OC-Cre-*Phex*Δflx/y. Initial investigations of these animal models documented that we had achieved successful deletion of exon 17 in all tissues of the Cre-*Phex*Δflx/y knockout, while in the OC-Cre-*Phex*Δflx/y knockout, the truncated gene was confined to the osteoblasts and osteocytes in bone. Moreover, measurement of *Phex* mRNA indicated that the truncated gene resulted in absent *Phex* expression in multiple tissues of the Cre-*Phex*Δflx/y mice but only in the osteoblasts and osteocytes of OC-Cre-*Phex*Δflx/y mice (Figure 1).

The availability of these model systems enabled us to determine the physiologically relevant site of the *Phex* mutation in XLH. Indeed, the targeted knockout models we created established unequivocally that mutation of the *Phex* gene in osteoblasts and/or osteocytes alone is sufficient to generate the classical *HYP* phenotype. In this regard, we discovered that the OC-Cre-*Phex*Δflx/y mice exhibited a biochemical phenotype no different from that of the Cre-*Phex*Δflx/y mice and indistinguishable from that in the *hyp*-mouse. Thus, the OC-Cre-*Phex*Δflx/y mice manifest hypophosphatemia secondary to decreased renal Npt2 cotransporter protein and consequent decreased brush border membrane Pi transport, abnor-

osteoblast and/or osteocyte is a sufficient abnormality to produce the *HYP* phenotype. Bone biopsies from the OC-Cre-*Phex*Δflx/y mice displayed histomorphological evidence of osteomalacia, marked by abundant osteoid-covered bone surface, widened osteoid seams, and defective mineralization (Figure 5). These changes were indistinguishable from those observed in bone biopsies from the Cre-*Phex*Δflx/y mice, as well as those from *hyp*-mice. In addition, the canalicular organization of the bone sections from the OC-Cre-*Phex*Δflx/y mice was severely disrupted, similar to the disarray evident in the bone of Cre-*Phex*Δflx/y and *hyp*-mice (Figure 6).

Investigation of the bone pathology in the targeted knockout mice further established that mutation of the *Phex* gene in the tissues of the normal mice (Figure 3).

Moreover, the OC-Cre-*Phex*Δflx/y mice exhibited the unique defect in renal 25(OH)D-1α-hydroxylase activity previously reported in *hyp*-mice. Indeed, we confirmed that despite normal Pi and PTH-mediated control of 25(OH)D-1α-hydroxylase gene expression and consequent normal upregulation of 25(OH)D-1α-hydroxylase mRNA transcripts in the OC-Cre-*Phex*Δflx/y mice, as well as Cre-*Phex*Δflx/y and *hyp*-mice, these animals failed to substantially alter renal 25(OH)D-1α-hydroxylase activity in response to these stimuli. However, in accord with the dissociation between mRNA expression and renal 25(OH)D-1α-hydroxylase activity, decreased serum phosphorus levels in the hypophosphatemic animal models did not substantially increase renal 25(OH)D-1α-hydroxylase protein content. Similarly, PTH stimulation of the hypophosphatemia models likewise failed to alter the protein content. Hence aberrant translation of the 25(OH)D-1α-hydroxylase message, a unique defect in vitamin D metabolism, is present in the OC-Cre-*Phex*Δflx/y mice, as well as the Cre-*Phex*Δflx/y mice (Figure 2).

Although the present study establishes that *Phex* expression in osteoblasts and/or osteocytes initiates the cascade of events responsible for the pathogenesis of XLH, our data are seemingly inconsistent with previous reports that documented the unsuccessful efforts to completely rescue the bone mineralization defect and the complete failure to alter the biochemical phenotype in the *hyp*-mouse by targeted overexpression of *Phex* (19, 20). These data suggest that extraosseous *Phex* expression might play an important role in modulating the cascade of events underlying XLH. However, a subsequent study, using transgenic mice expressing *PHEX* under
the control of the human β-actin promoter, which allows more widespread tissue expression of the gene, likewise failed to rescue the bone mineralization defect and the biochemical phenotype in the hyp-mouse (21). Although these data seemingly excluded extraosseous Phex expression as a seminal factor in the genesis of XLH, it is possible that the β-actin–mediated Phex expression was below the level necessary to effect rescue of the phenotype, a possibility acknowledged by Erben et al. (21) in their report. Indeed, since in vivo β-actin may not be prominently expressed in cells that are mitotically quiescent, their conclusion seems plausible. That the level of expression might have so profound an effect is supported by the observations of Shih et al. (26), who demonstrated that in cultured human osteoblasts, moderate reduction of PHEX mRNA expression by antisense transfection impaired mineralization. Moreover, the conditioned culture medium from the antisense cells reduced Pi uptake in cultured kidney cells (OK cells) (26). In any case, the studies of Erben et al. (21) failed to resolve the issue regarding the possible role of extraosseous Phex expression in the genesis of the HYP phenotype.

Although the level of Phex expression may have influenced the data in the study by Erben et al. (21), the investigations of Phex overexpression in osteoblasts were not similarly impaired. Appropriate controls in these studies revealed that the osteoblasts from the transgenic animal models expressed abundant Phex mRNA and enzyme activity, levels, in fact, greater than those in normal osteoblasts. Thus, if extraosseous production of Phex is not pivotal to the expression of the HYP phenotype, another explanation for the failure to rescue the HYP phenotype by targeted overexpression of the gene in osteoblasts must have prevailed. In this regard, it is possible that the temporal and developmental expression of the osteocalcin- and pro-α1 (I) collagen promoter–driven Phex expression may not mimic the endogenous regulation of Phex. If this is the case, the transgenic animals may have experienced normal PHEX expression later than normal animals or in different osteoblast-related cell subpopulations. In fact, neither promoter is expressed in the preosteoblast, and the osteocalcin promoter appears at least 4 days later than PHEX in normally developing osteoblasts (19, 20). Thus, lack of Phex activity early in osteoblast development may result in propagation of immutable osteoblast dysfunction, which contributes to the impairment of mineralization and irreversible excess phosphatonin production. Consistent with this hypothesis, later expression of PHEX may not rescue the phenotype.

An alternative explanation for the failure of targeted Phex expression to rescue the HYP phenotype is the possibility that the mutant Phex protein in hyp-mice represents a dominant negative mutation that blocks the effects of the abundant normal Phex protein in the osteoblasts of the transgenic mice. In support of this possibility, a previous report revealed the accumulation of Phex mutant proteins in the endoplasmic reticulum of osteoblasts (7). However, Liu et al. (19) demonstrated that the 3’ deletion Phex mutant did not interfere with the mineralization of normal osteoblasts in vitro, nor did it disrupt the enzymatic activity of wild-type Phex in vitro. Therefore, the previously reported studies neither established whether extraosseous Phex expression is pivotal to the manifestation of the HYP phenotype nor excluded that mutation of Phex in the osteoblast is the requisite defect responsible for the abnormal mineralization and biochemical abnormalities characteristic of XLH. Hence, these studies provided no data that are inconsistent with our observations.
Several previous studies, however, are consistent with our documentation that the osteoblast and/or the osteocyte are the site of the physiologically relevant \textit{Phex} mutation in \textit{hyp}-mice. Since endopeptidases such as Phex have substrates that are coexpressed in an organ/cell type–specific fashion, physiologically relevant PHEX/Phex substrate is likely produced in osteoblasts or osteocytes, if these cells are the site of predominant \textit{PHEX/Phex} mutation. Hence, the osteoblast and osteocyte production of biological products with activity that can modulate such functions as bone mineralization and renal Pi transport would be anticipated. In this regard, several reports indicate that cultured \textit{hyp}-mouse osteoblasts secrete a factor(s) that not only inhibits renal Pi transport (27, 28) but impairs bone mineralization (9, 29). Thus, these data support the possibility that the osteoblast and/or the osteocyte are the relevant site(s) of \textit{Phex} mutation, a conclusion reaffirmed and established by our studies.

Although our observations provide compelling evidence that aberrant \textit{PHEX/Phex} function in osteoblasts and/or osteocytes underlies the characteristic biochemical and pathological phenotype in \textit{hyp}-mice and likely XLH, a surprising discordance between the biochemical phenotype in the targeted \textit{OC-Cre-Phex}^{Δflox/y} knockout mice and that in both the global \textit{Cre-Phex}^{Δflox/y} knockout and \textit{hyp}-mice became apparent with further study. Previous studies have implicated the phosphatins as critical factors in the pathogenesis of XLH. Such investigations have identified that the production rate and/or the circulating level of the phosphatins FGF-23, MEPE, and sFRP-4 are increased in affected humans and/or the \textit{hyp}-mouse. In our studies, however, we found that the \textit{OC-Cre-Phex}^{Δflox/y} mouse had only an increased osseous production rate and serum level of FGF-23, while the \textit{Cre-Phex}^{Δflox/y} and \textit{hyp}-mice exhibited increased production and serum levels of FGF-23, MEPE, and sFRP-4 (Figures 7 and 8). These observations provide the first successful attempt to our knowledge to discern whether integrated effects of these hormones or the activity of a single phosphatoin is essential for expression of the disease phenotype in \textit{hyp}-mice and affected patients with XLH. Indeed, the data suggest that increased bone production and serum levels of MEPE and sFRP-4 are not critical for development of the classical HYP phenotype (in the \textit{OC-Cre-Phex}^{Δflox/y} mouse), whereas increased osseous production and serum FGF-23 concentration appear requisite for this biological function. Therefore, FGF-23 is likely the phosphatonin pivotal to the pathogenesis of XLH, and the role of MEPE and sFRP-4, if any, remains uncertain.

The concept of an abnormality in the production and serum level of a single phosphatoin as the requisite abnormality in \textit{hyp}-mice and XLH seems virtually heretical. A variety of proteins, such as MEPE and sFRP-4, inhibit Pi transport, both in vitro and in vivo and influence bone and osteoblast mineralization. Moreover, in concert with a potential role as a phosphatoin, these proteins are produced by osteoblasts, markedly upregulated in the \textit{hyp}-mouse osteoblasts, and possibly activated directly or indirectly by Phex. Nevertheless, transgenic expression of FGF-23 (30) and a missense mutation of FGF-23 (31), which resists proteolytic cleavage and enhances biological activity, results in renal Pi wasting, abnormal vitamin D metabolism, rickets, and osteomalacia, identical to the phenotype observed in \textit{hyp}-mice and in patients with XLH. Moreover, Liu et al. (13) generated a combined FGF-23–deficient and
Phex-deficient hyp-mouse model, in which the characteristic hypophosphatemia, abnormal 1,25-dihydroxyvitamin D [1,25(OH)₂D] levels, and rickets and osteomalacia generally associated with Phex deficiency were reversed. Consistent with our data, these results suggest that a singular abnormality in the production and circulating levels of FGF-23 is sufficient to cause both the renal and bone phenotypes in hyp-mice.

In summary, therefore, use of Cre-loxP strategies enabled cre-lation of global and osteoblast/osteocyte-specific knockout mice that are suitable for investigating Phex gene function in the regulation of Pi and vitamin D homeostasis, as well as bone histology. In contrast to the suggestion raised by previous studies in which targeted overexpression of Phex was studied, our observations provide compelling evidence that aberrant Phex function in osteoblasts and/or osteocytes alone is sufficient to underlie the biochemical and bone phenotype in hyp-mice, establishing osteoblast lineage cells as the physiologically relevant gene mutation site in XLH. Moreover, our data undeniably indicate that the effects of a single phosphatonin, FGF-23, acting downstream of Phex, may cause all elements of the characteristic elements comprising the renal and bone phenotypes in hyp-mice.

Methods

Normal and hyp-mice

Six-week-old normal male C57BL/6J mice and C57BL/6J heterozygous hyp-mice purchased from The Jackson Laboratory and maintained at our laboratory were used in the present study.

Generation of mice with the floxed Phex gene [Phexfloxs/(Neo) and Phexfloxy/(Neo)]

We generated a mouse line with a floxed Phex gene (32–34) in which exon 17 was targeted for deletion in osteoblasts and osteocytes. We chose exon 17 for deletion, since previous studies documented that patients with XLH have missense mutations (2) and pseudoexon insertions (35) in this exon and it codes the zinc-binding site, a critical part of the protein catalytic site (2, 35, 36) and an area included in the 3′ deletion of the hyp-mice (5). This strategy assured that exon 17 excision, resulting in a frameshift, a premature termination codon, and truncated protein, would inactivate Phex.

Initially, we created a floxed targeting vector. Using primer pairs that specifically amplify exon 17, we PCR screened DNA pools from the CITB murine SV/129 BAC library and identified a BAC clone containing Phex exon 17. Thereafter, we digested the BAC DNA from the clone with EcoRI, BamHI, HindIII, and SalI and electrophoresed the DNA fragments on an agarose gel, transferred the separated products to a nylon membrane, and used a radiolabeled exon 17 PCR product for hybridization. We identified subclones containing gene fragments and prepared mini-libraries by digesting the BAC DNA with restriction enzymes and cloning appropriate fragments in the pBluescript KS vector. Subsequently, we restriction mapped

Figure 9

Generation of floxed Phex locus. Schematic structure of the targeting vector illustrates 3 loxP sites (triangles). Mutagenesis to the floxed Phex locus occurs upon 5′ and 3′ homologous recombination. Exon 17 is inserted between loxP sites.

Figure 10

Removal of Neo cassette and production of global (Cre-Phexfloxy) knockout mice and targeted Phexfloxyfloxy mice. (A) Pups from genotype F₀ heterozygous crossing with Elia-Cre mice were genotyped for the excision of floxed Phex. Female mosaics were mated with wild-type mice, producing variable excision of the 3 loxP sites and resulting in the Cre-Phexfloxy genotype (the global knockout mouse used in the study) and Phexfloxyfloxy and Phexfloxyfloxy genotypes (mice targeted for generation of Oc-Cre-Phexfloxy mice). (B) The PCR strategy for mouse identification is depicted.

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the exon 17–bearing clones to identify appropriate DNA fragments for subcloning into the flox vector (37), which contained a TK cassette and a loxP site upstream of a BamHI restriction site and 2 loxP sites surrounding a Neo cassette downstream of the restriction site (Figure 9). The DNA identified, representing 3.7- and 1.7-kb fragments adjacent to exon 17 (the 5’ and 3’ homology arms), was inserted upstream and downstream of the loxP sites, while a DNA fragment of Phex exon 17 was added at the BamHI site, surrounded by 2 loxP sites (Figure 9). We sequenced the inserted DNA to confirm identity and proper orientation. Using HindIII restriction sites, we linearized the floxed targeting vector (38), electroporated 25 μg into R1 mouse male ES cells, and selected homologous or randomly integrated transformed cells by growth on G418 and GANK. To identify correctly targeted clones, we replicated and expanded approximately 450 selected colonies and isolated DNA from ES clones prior to digestion with BamHI, electrophoresis on agarose gels, and transfer to charged nylon membranes. Hybridization to a radiolabeled 5’ probe, directed at DNA outside the targeting vector, identified potentially correctly targeted clones. We confirmed correct targeting by PCR and DNA sequence analysis, which revealed 3 loxP sites flanking exon 17 and the Neo cassette, and performed standard karyotype analysis to document haploidy. 

We selected cells from correctly targeted clones, carrying the intermediate targeted Phex allele with the Neo cassette and containing the normal complement of 40 chromosomes, and injected them into the blastocoeal cavities of E3.5 embryos from hyperovulated C57BL/6J/6J mice. Surviving blastocysts were transferred to the oviducts of pseudopregnant recipient females. At birth, Phex<sup>flo</sup> and Phex<sup>flo</sup> mice were selected by agouti spots, a marker of the ES genetic material, as well as by PCR selection using primers 1 (forward), 5’-CCACAGATGTTGAGACTGG-3’, and 2 (reverse), 5’-GCAATCAGGCACGCAATTCTCC-3’, to detect transgenic homology, and the wild-type Phex gene as a positive control. The targeted insertion of loxP sites was detected by using primers 1 and 3 (reverse), 5’-ATACGGCCATGCTGGGATG-3’, as well as by primer 4 (forward), 5’-GGCACCCTTGTTAGCCGCCC-3’, and primer 2 (Figure 10). The male chimeric founders identified were backcrossed with C57BL/6J males for 8 generations to ultimately obtain C57BL/6J floxed mice (Figure 10). Mating of female mosaic mice with normal male mice generated genetically mosaic mice (Figure 10). The male chimeric founders identified were backcrossed with C57BL/6J females for 8 generations to ultimately obtain C57BL/6J floxed mice (>7 generations). Since the osteocalcin-driven Cre recombinase enzyme was expressed in all tissues were used in all experiments. 

The mating of the mosaic mice with normal mice also produced targeted Phex<sup>flo</sup>-Neo and Phex<sup>flo</sup>-Neo mice with ES-Ilv-Cre mice purchased from the Jackson Laboratory (39), which generated genetically mosaic mice (Figure 10). Mating of female mosaic mice with normal male mice generated hemizygotic (Cre- Phex<sup>flo</sup>-Neo and heterozygotic (Cre-Phex<sup>flo</sup>-Neo) global Phex-knockout mice (Figure 10). The male hemizygotic mice with deletion of exon 17 in all tissues were used in all experiments. 

Production of targeted and global Phex-knockout mice

In subsequent breeding strategies, we removed the Neo cassette by crossing the Phex<sup>flo</sup>-Neo and Phex<sup>flo</sup>-Neo mice with Ilv-Cre mice purchased from the Jackson Laboratory (39), which generated genetically mosaic mice (Figure 10). Mating of female mosaic mice with normal male mice generated hemizygotic (Cre- Phex<sup>flo</sup>-Neo) and heterozygotic (Cre-Phex<sup>flo</sup>-Neo) global Phex-knockout mice (Figure 10). The male hemizygotic mice with deletion of exon 17 in all tissues were used in all experiments. 

The mating of the mosaic mice with normal mice also produced targeted Phex<sup>flo</sup>-Neo and Phex<sup>flo</sup>-Neo mice, and the osteoblast/osteocyte-specific (targeted) Phex-knockout mice (OC-Cre-Phex<sup>flo</sup>-Neo) were generated by crossing the Phex<sup>flo</sup>-Neo mice with C57BL/6J OC-Cre mice, produced by backcrossing the OC-Cre transgene from FVB-N mice (25) onto the C57BL/6J background for more than 7 generations. Since the osteocalcin-driven Cre recombinase enzyme is expressed only in osteoblasts and osteocytes (25), the floxed Phex gene deletion was confined to the osteoblasts and osteocytes. Except where noted, male hemizygotic mice with deletion of exon 17 in osteoblasts and osteocytes were used in the experiments. 

The normal and byp- mice noted above and the knockout mice received a diet containing 0.6% calcium and phosphorus (Teklad Co.) and deionized water ad libitum from the time of weaning until study. For biochemical and histological studies, we used hemizygotic male byp- and global and targeted knockout mice. Care of mice met or exceeded the standards set forth by the NIH in the Guide for the care and use of laboratory animals (NIH publication no. 85-23. Revised 1985). The University of Wisconsin Animal Care and Use Committee approved all procedures.

Biochemical measurements

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital, blood was collected through heart puncture, and serum samples were obtained by precipitation and centrifugation. Serum Pi levels were measured using a Phosphorus Liqui-UV kit from Stanbio Laboratory, as described previously (22). Serum calcium levels were assayed using a Calcium LiquiColor kit from Stanbio Laboratory following the protocol of the manufacturer. Serum intact FGF-23 levels were measured employing an FGF-23 ELISA kit obtained from Kainos Laboratories (40). As previously described, estimation of circulating MEPE levels was made by measurement of MEPE-ASARM (41) and serum sFRP-4 by a 2-antibody sandwich ELISA (15). 

Analytical methodology

In vitro assay of murine renal 25(OH)D-1-hydroxylase activity. We assayed the maximum velocity of renal 25(OH)D-1-hydroxylase activity in kidney homogenates by previously described methods (22, 42). Data are expressed as femtomoles per milligram (wt/wt) kidney per minute. 

Study of the renal 25(OH)D-1-hydroxylase activity, as well as mRNA and protein (see below) was accomplished in the various animal models in the baseline state and after PTH stimulation. The PTH was administered via surgically implantable Alzet osmotic minipumps (model 3001; Alza Corp.) as described previously (22). After 24 hours, the animals were sacrificed and their kidneys excised for measurement of enzyme activity, as well as 25(OH) D-1-hydroxylase mRNA and protein. 

Phosphate uptake in renal brush border membranes. Renal brush border membrane vesicles were prepared from kidney cortex by CaCl<sub>2</sub> precipitation as described previously (43) with modifications. Briefly, the kidneys were removed and washed free of blood with cold isotonic saline. The renal cortices were then isolated from the medulla and homogenized immediately in 30 volumes (vol/wt) of ice-cold 50 mM mannitol/2 mM Tris-HCl buffer, pH 7.0. A 1-M CaCl<sub>2</sub> solution was added to the homogenate to a final concentration of 10 mM, and the mixture was stirred for 10 minutes at 4 °C. The homogenate was centrifuged at 3,000 g for 15 minutes, and the supernatant was decanted and centrifuged again at 43,000 g for 20 minutes. The pellet was washed one more time with the same buffer and centrifuged again for another 20 minutes at 43,000 g. The final pellet was dissolved in incubation buffer containing 100 mM NaCl, 100 mM mannitol, and 20 mM Tris-HEPES, pH 7.4. 

The Pi uptake was assessed in quadruplicate using 40–60 μg of brush border membrane prepared as described above. The concentration of brush border membrane protein was measured and adjusted to 2–3 mg/ml using incubation buffer. A 20-μl aliquot of brush border membrane suspension solution was used for each measurement, and 0.01–10 mM KH<sub>2</sub>PO<sub>4</sub> (9,000 Ci/mmol; DuPont-NEW) was added for 1 minute followed by termination by 1 mL of iced-cold stop solution (100 mM mannitol/20 mM HEPES/Tris/0.1 mM KH<sub>2</sub>PO<sub>4</sub>/20 mM MgSO<sub>4</sub>/100 mM choline chloride). The final solution was separated by a premoistened filter (0.45-μm pore size) using the Millipore filtration method (44, 45). The filters were washed and associated 32P measured by liquid scintillation spectroscopy. 

Real-time PCR assay of mRNA. To isolated total RNA from decapsulated whole kidney mice using the TRizol protocol (Life Technologies Inc.), as described previously (22), and quantitated 25(OH)D-1-hydroxylase cytochrome P450 mRNA concentrations by real-time RT-PCR. Data were collected quantitatively, and the Ct number corrected by Ct readings of corresponding internal 18s rRNA controls. Data from a minimum of 6
determinations (mean ± SEM) are expressed in all experiments as fold changes compared with normal mice.

To isolate total RNA from bone, we euthanized mice and disarticulated the femurs. The femurs were trimmed of all fat, and the marrow was removed, frozen in liquid nitrogen, and stored at −80°C. To prepare the bone for RNA isolation, the samples were homogenized into fine powder in liquid nitrogen using a porcelain mortar and pestle. We extracted the total RNA from the resultant powder as described above and quantitated with a Bioanalyzer (Agilent Technologies). To test for any differences among the 4 groups of mice, followed, when a statistical significance was adjusted with a Bonferroni correction to compensate for multiple testing (48). Both cortical bone areas in the midshaft (2 mm proximal and 2 mm distal to the midshaft) were measured and the percent change compared with controls was expressed as fold changes compared with experimental controls.

Western blot analysis. We measured 25(OH)D-1α,25(OH)D, and PNP protein in the kidney samples from the various animal models. Half-kidneys were rinsed in PBS and homogenized in RIPA buffer (20 mM Tris, 1% SDS, 10 mM EDTA, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) (48). The homogenate was centrifuged at 1,500 × g for 10 minutes, and the supernatant was collected for the measurement of Npt2 protein. The supernatant was mixed with an equal volume of 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour in 5% nonfat dry milk and incubated with 1:5,000 Npt2 antibody (Alpha Diagnostic) and 1:4,000 goat anti–rabbit IgG conjugated with HRP. To quantify the immunoblots signal, the nitrocellulose membranes were analyzed using a standard chemiluminescence detection system (ECL Plus, Amersham). The density of bands was analyzed using ImageQuant 5.2 software (Molecular Dynamics). The antibody was then stripped off by incubation in reprobing solution (62.5 mM Tris-HCl, 2% SDS, 100 mM 2-mercaptoethanol, pH 6.7) for 30 minutes at 50°C. The membrane was then blocked and probed with specific anti–cytochrome c or β-actin antibody to verify the loading equivalence among samples.

Bone structure and histomorphology

High-resolution radiography (x-ray) of the femur. To obtain high-resolution radiographs of the long bones (48), mouse femurs were extracted and incubated in lysis buffer (2x SSC, 0.2% SDS, 10 mM EDTA, 10 mg/ml proteinase K) for 2 days. After the surrounding muscles were digested, the femurs were washed in PBS buffer and x-rayed on a Faxitron model MX-20 Specimen Radiography System with a digital camera attached (Faxitron X-ray Corp.).

Double fluorochrome labeling of the long bone. To assess mineralization dynamics of the bone, double fluorescence bone labeling was accomplished as described previously (48). Briefly, a calcine label (5 mg/kg i.p.; Sigma-Aldrich) was administered to 6-week-old mice. This was followed by injection of an Alizarin red label (20 mg/kg i.p.; Sigma-Aldrich) 5 days later. Mice were sacrificed 48 hours after injection of the second label, and the femurs were removed and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde at room temperature for 4 hours before serial dehydration. The specimens were then dehydrated through a graded series of ethanol (70%–100%) and embedded in MMA without prior decalcification. Sections of 50 μm were cut using a Leitz 1600 saw microtome. The unstained sections were viewed under epifluorescence illumination using a Nikon PCM-2000 confocal microscope, coupled to an Eclipse E800 upright microscope and interfaced with Osteomeasure histomorphometry software (version 4.1) to estimate mineralization dynamics.

Visualization of bone morphology by Goldner staining. Nondecalcified sagittal sections from femurs were stained using Goldner-Masson trichrome assay (48). Both cortical bone areas in the midshaft (2 mm proximal and 2 mm distal to the midshaft) were photographed using a Nikon microscope at 10x with Bioquant OSTEO v.7.20.10 (R&M Biometrics) software. Unmineralized osteoid stains red, and mineralized bone, green/blue. Osteoid surface (%) and osteoid volume (%) were measured using an interactive image analysis system, which ensures unbiased sampling and averaging of areas (Bioquant R&M Biometrics).

Visualization of bone morphology by procion red. This small molecule dye (0.8% procion red in sterile saline, 10 μl/g body weight) was injected 10 minutes prior to sacrifice through the tail vein. After sacrifice, the femurs were removed and fixed in 70% ETOH followed by dehydration and embedded using a standard epoxy method (48). The sections were sectioned at 180 nm using a Leitz 1600 saw microtome, viewed under a Nikon C100 confocal microscope, and photographed using an Optronics cooled CCD camera.

Resin-casted scanning electron microscopy. Femurs were dissected, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) at room temperature for 4 hours, and then transferred to 0.1 M cacodylate buffer solution. The specimens were dehydrated in ascending concentrations of ethanol, embedded in methyl-methacrylate, and then surface polished using 1 μm and 0.3 μm alumina alpha micropolish II solution (Buehler) in a soft cloth rotating wheel. The surface was acid etched with 37% phosphoric acid for 10 seconds, followed by 5% sodium hypochlorite for 5 minutes. The samples were then coated with gold and palladium as described previously (48) and examined using an FEI/Philips XL30 Field emission environmental scanning electron microscope.

Statistics. Data are expressed as the mean ± SEM of at least 6 individual determinations. We evaluated the data statistically employing an ANOVA test for any differences among the 4 groups of mice, followed, when a difference was determined, by 2-sample t tests in which all possible pairs of mice groups were evaluated. For the second-stage testing, the threshold for statistical significance was adjusted with a Bonferroni correction to compensate for multiple testing (49). A P value of less than 0.05 is considered statistically significant.

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