Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities

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ABSTRACT Npt2 encodes a renal-specific, brush-border membrane Na\(^+\)-phosphate (P\(_i\)) cotransporter that is expressed in the proximal tubule where the bulk of filtered P\(_i\) is reabsorbed. Mice deficient in the Npt2 gene were generated by targeted mutagenesis to define the role of Npt2 in the overall maintenance of P\(_i\) homeostasis, determine its impact on skeletal development, and clarify its relationship to autosomal disorders of renal P\(_i\) reabsorption in humans. Homozygous mutants (Npt2\(^{-/-}\)) exhibit increased urinary P\(_i\) excretion, hypophosphatemia, an appropriate elevation in the serum concentration of 1,25-dihydroxyvitamin D with attendant hypercalcemia, hypercalciuria and decreased serum parathyroid hormone levels, and increased serum alkaline phosphatase activity. These biochemical features are typical of patients with hereditary hypophosphatemic rickets with hypercalciuria (HHHR), a Mendelian disorder of renal P\(_i\) reabsorption. However, unlike HHHR patients, Npt2\(^{-/-}\) mice do not have rickets or osteomalacia. At weaning, Npt2\(^{-/-}\) mice have poorly developed trabecular bone and retarded secondary ossification, but, with increasing age, there is a dramatic reversal and eventual overcompensation of the skeletal phenotype. Our findings demonstrate that Npt2 is a major regulator of P\(_i\) homeostasis and necessary for normal skeletal development.

Inorganic phosphate (P\(_i\)) is essential for a variety of cellular processes including skeletal mineralization. Because a disturbance in P\(_i\) availability can affect the functional integrity of many organ systems, specialized tissues have evolved to maintain the extracellular P\(_i\) concentration. In mammals, the regulation of P\(_i\) homeostasis is largely determined by the kidney, with 60–70% of the filtered P\(_i\) load reclaimed in the proximal segment of the nephron (1). Transepithelial transport of P\(_i\) from the renal lumen to the blood compartment involves uptake across the brush-border membrane (BBM), translocation across the cell, and efflux across the basolateral membrane (2). Evidence suggests that P\(_i\) transport across the BBM is the rate-limiting step in the overall P\(_i\) reabsorptive process and the major site for its regulation (2, 3). Kinetics studies demonstrated that the transport is mediated by high capacity, low affinity, and low capacity, high affinity Na\(^+\)-P\(_i\) cotransport systems (4).

Recently, cDNAs encoding two distinct low capacity, high affinity, renal BBM Na\(^+\)-P\(_i\) cotransporters (NPT1 and NPT2) that share only 20% identity have been identified in several mammalian species by expression and homology cloning (5–13). That share only 20% identity have been identified in several mammalian species by expression and homology cloning (5–13).

NPT2 is expressed exclusively in the proximal convoluted tubule and is not subject to regulation by dietary P\(_i\) (10). X-chromosome-linked and autosomal disorders of renal P\(_i\) reabsorption have been described in humans (16). Both are characterized by growth retardation, rachitic and osteomalacic bone disease, and hypophosphatemia, secondary to a renal defect in P\(_i\) reabsorption (17). Although renal Npt2 gene expression is significantly reduced in the murine Hype (18) and G\(_y\) (19) homologs of X-chromosome-linked hypophosphatemia, localization of the Npt2 gene to human chromosome 5q35 (20, 21) and mouse chromosome 13B (22) excluded it as a candidate gene. However, it is not yet clear whether mutations in NPT2 are responsible for autosomal disorders of renal P\(_i\) wasting and mouse models for these disorders are not available.

To define the precise role of the NPT2 transporter in the overall maintenance of P\(_i\) homeostasis and to ascertain whether the phenotypic manifestations of autosomal hypophosphatemias can arise from mutations at the NPT2 locus, we have disrupted the murine Npt2 gene by homologous recombination in embryonic stem (ES) cells and introduced the mutation in the mouse germ line.

MATERIALS AND METHODS

Derivation of Mice Mutant for Npt2. A genomic clone encoding exons 2–12 of the Npt2 gene, obtained by screening a DASH II mouse (129/sv) strain genomic DNA library with a rat Npt2 cDNA probe (23), was used to generate a 2.2-kb EcoRI fragment encoding exons 2–5, and a 2.0-kb EcoRI–PstI fragment containing part of intron 10 (Fig. 1b). The Npt2 fragments were cloned into the pPNT plasmid (24), respectively, 5’ and 3’ of a neomycin resistance (neo\(^\gamma\)) gene (Fig. 1a). The pPNT plasmid also contained a herpes simplex virus thymidine kinase (hsv-tk) gene, permitting selection against random integration. The resulting targeting vector, pPNT-Npt2 (Fig. 1b), was CsCl purified and linearized at the unique NotI site before electroporation.

Exponentially growing D3 ES cells (4.2 × 10\(^6\)) were electroporated (Bio-Rad Gene Pulser at 240 V, 500 µF) with 20 µg of pPNT-Npt2 as described (25) and selected in a medium containing 350 µg/ml G418 and 0.2 µM FIAU [1-β-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil] for 5 days, and then for 3 more days with G418 alone. Genomic DNA was isolated from 170 doubly resistant ES cell clones, digested with EcoRI, transferred to supported nitrocellulose membranes [OptiTRAN BA-S(S)85, Schleicher & Shuell], and hybridized with probe A, located 3’ to the insertion point of the targeting

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Abbreviations: P\(_i\), phosphate; BBM, brush-border membrane; PTH, parathyroid hormone; ES, embryonic stem cell; ALPase, alkaline phosphatase; 1,25(OH)\(_2\)D, 1,25-dihydroxyvitamin D; FEI, fractional excretion index; HHRH, hereditary hypophosphatemic rickets with hypercalciuria; RT-PCR, reverse transcriptase–PCR.

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transmission of the mutation was determined by Southern blot analysis and PCR amplification of tail DNA (see below). To produce Npt2−/− mice, heterozygous offspring were intercrossed. The mice studied were of 129Sv and C57BL/6J mixed genetic background and maintained on Rodent Laboratory Chow (Ralston Purina, diet no. 5001) containing 1% calcium, 0.61% P2, and 4.5 units of vitamin D3/g. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

**Genotyping of Mice.** Mice were genotyped by Southern blot analysis and PCR. Genomic DNA was digested with EcoRI and analyzed by Southern blot hybridization using probe A (Fig. 1b). Expected sizes of fragments for the normal and disrupted alleles are 8 and 12 kb, respectively (Fig. 1d). PCR typing of tail DNA was performed by using Taq polymerase and 3 primers: sense primer 1 (5′-TGGCCAGGTGCGAC- GAAGC-3′) in exon 4, antisense primer 2 (5′-AGTCTCTGTCC- CCCTGCTGCA-3′) in exon 6, and antisense primer 3 (5′- TGATCATTCCATTTGTCAGTCC-3′) in the neo′ gene cassette (Fig. 1b). Expected sizes of amplified fragments are 1.8 kb for the normal allele (primers 1 and 2) and 1.4 kb for the disrupted allele (primers 1 and 3) (Fig. 1e).

**Analysis of RNA.** Total RNA (15 µg), isolated with TRIzol Reagent (GIBCO/BRL), was size fractionated on a 1.5% agarose/formaldehyde gel and transferred to Hybond-N nylon membranes (Amersham). The blots were probed as described (28) with a full-length rat Npt2 cDNA and an 18S rRNA oligonucleotide. Filters were washed to high stringency, exposed to Kodak Biomax MR1 film at −80°C, and RNA abundance quantitated by densitometric analysis using an LKB Ultrascan Laser Densitometer under conditions where linearity was observed. For reverse transcriptase–PCR (RT-PCR), total RNA (5 µg) was reverse transcribed by using random hexamers and Superscript reverse transcriptase (GIBCO/BRL), as recommended by the manufacturer. Primers 1 and 2 were used for detection of Npt2 sequences.

**BBM Vesicle Preparation, Western Blot Analysis, and Transport Studies.** Renal BBM vesicles were prepared from kidney cortex by the MgCl2 precipitation method as described (29) and used for both Western blot analysis and transport studies. BBM proteins were separated on 10% SDS/PAGE gels according to the method of Laemmli (30), transferred to supported nitrocellulose membranes (Hybond-C extra, Amersham), and probed sequentially with a rabbit polyclonal anti-rat Npt2 antibody (gift of H. Murcr) (18) and a mAb raised against the α-subunit of rat meprin (gift of P. Crine) (31). Primary antibodies were visualized by using an enhanced chemiluminescence kit (Amersham). Quantitation of the signal was achieved by densitometric analysis using an LKB Ultrascan Laser Densitometer under conditions where linearity was observed.

The uptakes of Pi (100 µM) and glucose (10 µM), each measured in quadruplicate, were measured at 6 s, 30 s, 60 s, and 90 min in medium containing either 100 mM NaCl or 100 mM KCl by the rapid filtration technique described (29).

**Serum and Urine Parameters.** Serum P, Ca, and alkaline phosphatase (ALPase) activity and urine P, Ca, and creatinine were assayed on a Hitachi 917 automatic analyzer (Hitachi, Tokyo). The fractional excretion indexes FEIPi and FEICa were calculated as follows: urine P or Ca/urine creatinine × serum P or Ca. The serum concentration of 1,25-dihydroxyvitamin D3 was measured by using a calf thymus radio-receptor assay kit (Incstar, Stillwater, MN). The serum concentration of PTH was measured by using a rat PTH immunoradiometric assay kit (Nichols Institute, San Juan Capistrano, CA). Urine amino acids were determined on a Beckman 6300 amino acid analyzer (Beckman) and urine glucose and protein with Aimes Multistix (Miles).

**Bone Histology.** Tibiae from 21-, 45-, 74-, 115-, and 184-day-old mice were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 12 h and then decalcified in 10% EDTA.

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**Fig. 1.** Targeted disruption of the murine Npt2 gene. (a) Production of the pPN1-Npt2 targeting vector. (Upper) Schematic representation of the murine Npt2 gene, with exons numbered and denoted as grey boxes. (Lower) pPN1 vector containing the neo′ and hsv-kb genes. The broken lines indicate sites where the Npt2 homologous arms were inserted. Relevant restriction enzyme sites are abbreviated as follows: E, EcoRI; H, HindIII; N, NotI; P, PstI; S, SstI; X, XhoI. (b) Targeting of Npt2 by homologous recombination. The top line represents the incoming pPN1-Npt2 targeting vector, the middle line the normal Npt2 allele, and the bottom line the targeted allele. The location of the probes used in Southern blot analysis is indicated. Probe A, 1.5-kb fragment external to the targeting vector; probe B, corresponding to the neo′ gene; probe C, a 0.8-kb fragment used as an internal probe. (c) Southern blot analysis of targeted ES cell clones. Genomic DNA (5 µg) derived from transfected ES cells (D3 wt) or from targeted clones (D3 +/−) was digested with EcoRI (E), HindIII (H), and SstI (S), Southern blotted, and hybridized with probes A, B, and C, as shown. The sizes of genomic DNA fragments expected from the normal and disrupted alleles are indicated. (d) Genotyping of 3-week-old offspring from heterozygous matings by Southern blot analysis. Probe A was hybridized to EcoRI-restricted tail genomic DNA. Wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) animals exhibited an 8-kb band that is absent in homozygous mutant (−/−) mice. Disruption of the Npt2 allele produced a 12-kb band. (e) PCR analysis of mouse tail DNA (see below). The expected sizes of amplified fragments are indicated above the corresponding alleles in B. M, size markers; Blk, negative control.
(pH 7.4) at 4°C for 10 days prior to dehydration with an increasing concentration of ethanol. Bones were then embedded in paraffin and sections were stained with haematoxylin/eosin. Number and length of trabeculae were determined in an area of 300 μm in length from the chondro-osseous junction to the diaphysis surrounded by cortical bone on both sides.

RESULTS

Targeted Disruption of the Npt2 Gene. To inactivate the Npt2 gene, we constructed a targeting vector in which Npt2 genomic fragments, encoding exons 2–5 and intron 10, respectively, were cloned into the XhoI and EcoRI sites of the pPNT plasmid (24), 5′ and 3′ to the neo gene (Fig. 1a). A double cross-over event would lead to the replacement of 7.7 kb of the Npt2 gene (corresponding to amino acids 178–389 of the Npt2 protein) with the neo gene (Fig. 1b). By using Southern blot analysis, 5 of 170 doubly resistant ES cell clones were found to have undergone homologous recombination, generating a 12-kb EcoRI fragment from the targeted allele and an 8-kb fragment from the normal allele (Fig. 1c).

Germ-line transmission of the inactivated Npt2 allele resulted in mice heterozygous for the mutation. Southern blot analysis (Fig. 1d) and PCR (Fig. 1e) were used to genotype the offspring of heterozygous matings. From a total of 404 mice, 110 were wild type (27%), 200 were heterozygotes (50%), and 94 were homozygous (23%) of heterozygous matings. From a total of 404 mice, 110 were wild type, 200 were heterozygous animals, and 94 were homozygous animals.

Phenotypic Features of Npt2-Deficient Mice. Npt2−/− mice were noticeably smaller than wild-type littermates and body weight was significantly reduced at birth (1.49 ± 0.08 g, n = 8 vs. 1.89 ± 0.12 g, n = 11, P < 0.05) and remained so for at least 4 months thereafter (Fig. 2). During the first 2 weeks of life, Npt2−/− mice showed apparent muscle weakness, some lethargy, and frequently had trouble feeding, suggesting that poor nutritional status likely contributed to their premature death. Npt2−/− mice that survived weaning appeared normal thereafter, despite smaller size and lower body weight (Fig. 2). Npt2−/− mice have a lower reproductive ability than wild-type and heterozygous animals.

Blood and urine parameters of Npt2−/− mice differed significantly from those of wild-type and heterozygous littermates (Table 1). The serum Pi concentration was markedly reduced at all ages examined and, in agreement with earlier work (32), decreased with age in all three genotypes (Table 1). The urine Pi/creatinine ratio and the fractional excretion of Pi (FEIPi) were strikingly elevated in Npt2−/− mice (Table 1). Although Npt2−/− mice had a normal serum Pi concentration, their urine Pi/creatinine ratio and FEIPi were significantly elevated compared with wild-type mice (Table 1). Npt2−/− mice also exhibited elevated serum 1.25(OH)2D levels (Table 1).

Npt2 Gene Expression. Northern blot analysis of total RNA isolated from bone, kidney, liver, and lung of animals from the three genotypes revealed the presence of a 2.6-kb transcript in wild-type mice and in mice with the homozygous mutant allele, but not in the kidney of homozgyous mutant mice (Fig. 3a). Quantitation of the 2.6-kb transcript, relative to 18S rRNA, indicated that Npt2−/− mice had <50% of the wild-type Npt2 mRNA, consistent with the presence of only one functional Npt2 allele. RT-PCR with Npt2 primers also confirmed the absence of detectable renal Npt2 mRNA in Npt2−/− mice (Fig. 3b). In agreement with previous work (8), Npt2 transcripts were only detectable in the kidney (Fig. 3a and b).

Western blot analysis of renal BBM proteins demonstrated the presence of an 86-kDa protein in Npt2−/− and Npt2−/− mice that was not detectable in homozygous mutant mice (Fig. 3c). Despite the reduction in Npt2 mRNA expression in Npt2−/− mice, the abundance of Npt2 protein, relative to that of meprin, was as prominent in Npt2−/− mice as in wild-type animals (Fig. 3c), consistent with the well-documented adaptive up-regulation of Npt2 protein in states of hypophosphatemia (14, 19, 33).

To assess the effect of the disrupted Npt2 allele on functional activity, we examined the time course of Na−Pi cotransport in renal BBM vesicles isolated from Npt2−/−, Npt2−/−, and Npt2−/− mice. The Na−dependent component of Pi uptake at 6, 30, and 60 s is similar in BBM vesicles from wild-type and heterozygous mice but is reduced by 79%, 71%, and 69%, respectively, in mutant homozygotes (Fig. 3d). However, at equilibrium (90 min), when the Na−gradient has dissipated, Pi uptake is comparable in all three groups of mice (Fig. 3d). Moreover, the Na−-independent component of Pi uptake (Pi uptake) is comparable in all three groups of mice (Fig. 3d). This indicates that the Na−-independent component of Pi uptake (Pi uptake) is comparable in all three groups of mice (Fig. 3d).

Skeletal Phenotype of Npt2-Deficient Mice. Histological analysis of tibial sections from 21-day-old animals revealed that Npt2−/− mice have poorly developed metaphyseal trabeculae and retarded secondary ossification in the epiphysis when compared with wild-type littermates (Fig. 4a). In Npt2−/− mice, these features are intermediate between wild-type and homozygous littermates (Fig. 4a). At 21 days of age, both the length (+/+ +/+ 163 ± 31; +/− 127 ± 33; −/− 98 ± 15; mean ± SD, μm; +/− vs. +/+ vs. −/−, P < 0.01; −/− vs. +/+ vs. −/−, P < 0.005) and number (+/+ 41 ± 3; +/− 37 ± 5; −/− 21 ± 3; mean ± SD; +/− vs. +/+ vs. −/−, not significant; −/− vs. +/+ vs. −/−, P < 0.005) of metaphyseal trabeculae are decreased in homozygous mutants and intermediate in heterozygotes when compared with wild-type littermates. In addition, differences in osteoclast (tartrate-resistant acid phosphatase positive cells) distribution and osteopontin-immunopositive bone surfaces as well as a
decrease in osteoclast number are apparent in tibiae of 21-day-old Npt2−/− mice and wild-type littermates, suggestive of a defect in bone remodeling in the homozygous mutants (data not shown).

In contrast, tibial sections from 45-day-old mice failed to demonstrate the genotype differences evident at 21 days of age (data not shown). At 115 days of age, however, the number of metaphyseal trabeculae in Npt2−/− mice is significantly greater than that of Npt2+/+ littermates (+/+; 16 ± 3; +/−, 21 ± 3; −/−, 25 ± 3; mean ± SD; +/+ vs. +/−, P < 0.05; −/− vs. +/−, P < 0.005) and the area of epiphyseal bone marrow in the homozygous mutants is partially occupied with calcified bone (Fig. 4b). Similar findings are observed at 74 and 184 days of age (data not shown).

**DISCUSSION**

We demonstrate that Npt2-deficient mice exhibit increased urinary Pi excretion, hypophosphatemia, and an appropriate adaptive increase in the circulating concentration of 1,25(OH)2D. Additional biochemical findings in Npt2−/− mice include hypercalcemia, hypercalciuria, decreased serum PTH levels, and elevated serum ALPase activity. These biochemical features are typical of patients with hereditary hypophosphatemic rickets with hypercalciuria (HHHRH), a Mendelian disorder of renal Pi reabsorption (17). In contrast to patients with HHRRH, Npt2−/− mice do not exhibit rickets and osteomalacia. Rather, the mutant homozygotes have a complex bone phenotype with retarded secondary ossification at weaning followed by a reversal and eventual overcompensation of the early bone phenotype with increasing age.

The phenotypic features that characterize Npt2−/− mouse are likely the direct consequence of impaired renal Pi reabsorption, secondary to the loss of Npt2 function in the kidney. This conclusion is based on the report that Npt2 is expressed exclusively in the kidney (8) and is confirmed by the present findings. We were unable to detect Npt2 mRNA expression in bone, liver, and lung of normal mice by either Northern blot analysis or RT-PCR. In addition, by using a RNase protection assay, we did not detect Npt2 transcripts in mouse intestine (data not shown), a tissue that has considerable morphological and functional similarity to the renal epithelium. Accordingly, the cellular entities that mediate Na+–Pi cotransport in tissues other than the kidney are distinct from Npt2 and, as such, would not contribute to the mutant phenotype.

We demonstrate that BBMs from Npt2−/− mice retain 20–30% of the Na+–Pi cotransport activity expressed in wild-type mice. Our data indicate that gene products other than Npt2 mediate Na+–Pi cotransport at the apical surface of the murine proximal neprhon. Likely candidates for this activity are Npt1 (7) Ram-1 and Glvr-1 (34). Indeed, Npt1 expression was previously localized to the renal BBM of proximal tubular cells (35, 36). In addition, it has been shown that Ram-1 and Glvr-1, ubiquitous cell surface viral receptors that mediate high affinity, Na+–dependent Pi transport, are expressed in kidney (34). At present, little is known about the relative contribution of Npt1, Ram-1, and Glvr-1 to overall renal BBM Na+–Pi cotransport. However, our data in homozygous mutant mice suggest that Npt2 accounts for ~70% of BBM Pi transport activity. Although these results are consistent with the relative distribution of Npt1, Npt2, Ram-1, and Glvr-1 mRNA/Ns in normal mouse kidney (37), it is not clear whether Npt1, Ram-1- or Glvr-1-mediated Na+–Pi cotransport is up-regulated in the absence of Npt2 activity.

Pi is an important determinant of 1,25(OH)2D synthesis in the kidney. Pi deprivation elicits an adaptive increase in the activity of the renal biosynthetic enzyme, 25-hydroxyvitamin D-1-

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**Table 1. Blood and urine profiles for Npt2+/+, Npt2+/−, and Npt2−/− mice**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Pi, mM</td>
<td>2.87 ± 0.07 (20)</td>
<td>2.78 ± 0.06 (37)</td>
<td>1.86 ± 0.08 (11)**</td>
</tr>
<tr>
<td>2</td>
<td>2.35 ± 0.09 (6)</td>
<td>2.41 ± 0.06 (6)</td>
<td>1.79 ± 0.06 (7)**</td>
</tr>
<tr>
<td>3</td>
<td>2.10 ± 0.08 (9)</td>
<td>2.30 ± 0.08 (9)</td>
<td>1.77 ± 0.10 (7)*</td>
</tr>
<tr>
<td>4</td>
<td>2.10 ± 0.07 (6)</td>
<td>2.08 ± 0.18 (6)</td>
<td>1.65 ± 0.14 (4)*</td>
</tr>
<tr>
<td>5</td>
<td>1.94 ± 0.14 (6)</td>
<td>1.97 ± 0.10 (7)</td>
<td>1.58 ± 0.03 (4)*</td>
</tr>
<tr>
<td>6</td>
<td>1.85 ± 0.09 (3)</td>
<td>2.02 ± 0.08 (7)</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>1.79 ± 0.08 (4)</td>
<td>1.88 ± 0.13 (5)</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Urinary Pn/creatinine†**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.83 ± 1.15 (26)</td>
<td>16.62 ± 1.61 (37)*</td>
<td>24.98 ± 2.02 (21)**</td>
</tr>
<tr>
<td>2</td>
<td>4.22 ± 0.63 (16)</td>
<td>6.79 ± 0.75 (28)*</td>
<td>13.24 ± 1.08 (9)**</td>
</tr>
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</table>

**Serum total Ca2+, mM**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.25 ± 0.03 (20)</td>
<td>2.21 ± 0.03 (34)</td>
<td>2.64 ± 0.05 (12)**</td>
</tr>
<tr>
<td>2</td>
<td>2.19 ± 0.04 (5)</td>
<td>2.21 ± 0.03 (6)</td>
<td>2.36 ± 0.05 (7)*</td>
</tr>
<tr>
<td>3</td>
<td>2.20 ± 0.05 (9)</td>
<td>2.13 ± 0.06 (8)</td>
<td>2.41 ± 0.08 (10)*</td>
</tr>
<tr>
<td>4</td>
<td>2.26 ± 0.03 (6)</td>
<td>2.24 ± 0.05 (6)</td>
<td>2.42 ± 0.04 (3)*</td>
</tr>
<tr>
<td>5</td>
<td>2.00 ± 0.12 (5)</td>
<td>2.13 ± 0.05 (7)</td>
<td>2.48 ± 0.02 (3)*</td>
</tr>
<tr>
<td>6</td>
<td>2.22 ± 0.09 (3)</td>
<td>2.32 ± 0.09 (8)</td>
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<tr>
<td>7</td>
<td>2.40 ± 0.06 (2)</td>
<td>2.23 ± 0.04 (3)</td>
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**Serum Ca2+/creatinine†**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.81 ± 0.07 (23)</td>
<td>1.00 ± 0.12 (36)</td>
<td>7.90 ± 1.07 (20)**</td>
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</tbody>
</table>

**FEI Pi**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.36 ± 0.04 (15)</td>
<td>0.52 ± 0.07 (27)</td>
<td>3.43 ± 0.46 (10)**</td>
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**Serum 1,25(OH)2D§ pg/ml**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>36.5 ± 9.5 (3)</td>
<td>73.0 ± 7.2 (3)*</td>
<td>107.3 ± 11.1 (3)**</td>
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**Serum PTH§ pg/ml**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>18.83 ± 1.14 (3)</td>
<td>19.79 ± 2.31 (3)</td>
<td>6.98 ± 0.60 (3)**</td>
</tr>
</tbody>
</table>

**Serum ALPase, units/liter**

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Npt2+/+ (n)</th>
<th>Npt2+/− (n)</th>
<th>Npt2−/− (n)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>425 ± 35 (19)</td>
<td>459 ± 22 (32)</td>
<td>869 ± 165 (12)**</td>
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<td>2</td>
<td>179 ± 28 (4)</td>
<td>208 ± 19 (6)</td>
<td>361 ± 17 (7)**</td>
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<tr>
<td>3</td>
<td>126 ± 9 (6)</td>
<td>99 ± 7 (9)</td>
<td>175 ± 15 (8)*</td>
</tr>
<tr>
<td>4</td>
<td>91 ± 6 (6)</td>
<td>82 ± 6 (6)</td>
<td>105 ± 10 (4)</td>
</tr>
<tr>
<td>5</td>
<td>71 ± 14 (5)</td>
<td>63 ± 3 (7)</td>
<td>98 ± 7 (3)</td>
</tr>
<tr>
<td>6</td>
<td>92 ± 5 (3)</td>
<td>64 ± 10 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>84 ± 0 (2)</td>
<td>61 ± 12 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Results are means ± SEM (n, number of mice analyzed). ND, test not performed. †, P = 0.05–0.01 for t test comparing mutant mice to Npt2+/+ mice. **, P < 0.05 for t test comparing mutant mice to Npt2+/+ mice.**

†Assays were done on urine collected from 1- to 3-month-old animals.

‡FEI indicates the ratio between urine Pi, or Ca (mM)/urine creatinine (mM) × serum Pi, or Ca (mM) (1-month-old animals).

§1,25(OH)2D assays were done in duplicate on three serum pools from six mice each (1- to 3-month-old animals).

§‡－PTH assays were done in duplicate on three serum pools from two or three mice each (2- to 3-month-old animals).
of renal Npt2 function, is also associated with a significant rise in the serum concentration of 1,25(OH)2D, thereby confirming the regulatory loop between extracellular Pi and renal vitamin D hormone synthesis. Previous studies have shown that the increase in 1,25(OH)2D elicits a marked stimulation in intestinal Pi (41), and calcium (42) absorption. Intestinal Pi hyperabsorption in Npt2 null mice would result in an increase in the filtered Pi load that, together with Npt2 ablation, is responsible for the substantial elevation in urinary Pi excretion. Intestinal calcium hyperabsorption in Npt2 null mice would lead to hypercalcemia, an increase in the filtered calcium load, and a suppression of PTH secretion, which contribute to the significant hypercalcuria observed in the homozygous mutant mice.

The phenotype of Npt2−/− mice is of considerable interest. Npt2−/− mice grow normally and are able to maintain normal serum Pi levels in the face of a modest increase in urinary Pi excretion. Moreover, the heterozygotes exhibit normal BBM Na−/−, Pi cotransport activity and an abundance of Npt2 protein that is comparable to that in wild-type mice in spite of a 50% reduction in Npt2 mRNA. These findings are likely the result of an adaptive response to the loss of one copy of the Npt2 gene. Previous studies have shown that renal BBM Na−/−, Pi cotransport and Npt2 cotransporter protein are both up-regulated by hypophosphatemia following low Pi challenge (14, 33). Although the renal Pi sensing mechanism that is involved in initiating the adaptive response to Pi deprivation has not yet been defined, our data suggest that kidneys of Npt2−/− mice are able to react appropriately to the increase in renal Pi loss and the resulting perturbation in Pi homeostasis.

Mice homozygous for the disrupted Npt2 gene have the biochemical features of patients with HHRH (43–47), yet the bone phenotype is markedly different in the human and mouse disorders. Children with HHRH present with radiological findings that are consistent with osteomalacia, such as irregular mineralization fronts, markedly elevated osteoid surface and seam width, increased number of osteoid lamellae, and prolonged mineralization time (48). In contrast, it does not appear from preliminary observations that the skeletal phenotype in Npt2−/− mice is similar to that in HHRH patients. There are several explanations for this discrepancy. (i) The bone response to hypophosphatemia and the Pi requirement for skeletal mineralization may differ substantially in humans and mice. Indeed, basal serum Pi levels are markedly different in the two species. (ii) Bone responses to elevated 1,25(OH)2D levels may differ in both species. The latter is suggested by a recent report showing differences in the regulation of human and murine osteocalcin gene expression by 1,25(OH)2D (49). (iii) A mutation in the NPT2 gene may not be the cause of HHRH.

The bone findings in mice homozygous for the disrupted Npt2 allele are clearly age dependent. With increasing age, Npt2−/− mice exhibit a dramatic reversal and eventual overcompensation of the skeletal changes apparent at 21 days of age. These differences may be attributed to age-related differences in the bone and intestinal response to 1,25(OH)2D. Recent studies in vitamin D receptor (VDR) null mice suggest that the phenotypic consequences of VDR ablation are not evident until after weaning, suggesting that 1,25(OH)2D may not play an important physiological role before 21 days of age (50, 51). Accordingly, osteoblast and osteoclast activity and intestinal Pi absorption may not be responsive to the elevated circulating levels of 1,25-(OH)2D in Npt2−/− mice before weaning, whereas after weaning, 1,25(OH)2D/VDR-dependent compensatory changes in intestinal Pi absorption and bone mineralization can occur. Further studies are necessary to determine whether this or other mechanisms contribute to the age-related differences in the bone phenotype of Npt2−/− mice.

We demonstrate that Npt2−/− mice have decreased circulating levels of PTH relative to wild-type littermates. Because PTH increases both osteocalcin number and activity, reduced

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**Fig. 3.** Npt2 gene expression. (a) Northern blot analysis of total RNA from bone, kidney, liver, and lung of Npt2+/+, Npt2+/−, and Npt2−/− mice. Total RNA (15 μg) was blotted to a nylon membrane and hybridized sequentially with 32P-labeled rat Npt2 cDNA (Upper) and 18S RNA oligonucleotide (Lower) probes as described. (b) RT-PCR of total RNA from Npt2+/+, Npt2+/−, and Npt2−/− mice. Total RNA (5 μg) from bone, kidney, liver, and lung was reverse transcribed and PCR amplified as described. An aliquot of each PCR was electrophoresed on 1.5% agarose gels and visualized with ethidium bromide. M, size markers, Blk, negative control. (c) Western blot analysis of renal BBM proteins from Npt2+/+, Npt2+/−, and Npt2−/− mice. The BBMs were solubilized and subjected to SDS/PAGE, transferred to nitrocellulose, and the blots probed sequentially with (Upper) a rabbit polyclonal anti-rat Npt2 antibody and (Lower) a mAb raised against the α-subunit of rat meprin. Molecular masses and quantity of protein (μg) applied to each lane are indicated. (d) Pi uptake in renal BBM vesicles from Npt2+/+, Npt2+/−, and Npt2−/− mice. Pi uptake (100 μM) was measured at times indicated in presence of KCl (filled symbols) and NaCl (open symbols) in BBM vesicles derived from Npt2+/+(circles), Npt2+/−(triangles), and Npt2−/−(diamond) mice as described. Each point represents mean ± SEM of quadruplicate determinations.

**Fig. 4.** Histological appearance of bone from Npt2+/+, Npt2+/−, and Npt2−/− mice. Longitudinal tibial sections were prepared from 21- (a) and 115-day-old (b) mice as described and stained with hematoxylin/eosin.
serum PTH levels in Npt2 null mice may contribute to compromised osteoclast function and a consequent bone remodeling defect. Preliminary data suggest that osteoclast number as well as tartrate-resistant acid phosphatase-positive and osteopontin-immunopositive cement lines are decreased in tibiae of 21-day Npt2−/− mice, relative to wild-type littermates, consistent with the notion that bone remodeling is impaired in young homozygous mutants.

In summary, we have demonstrated that mice homozygous for the disrupted Npt2 gene exhibit many of the features of HHRH and that the Npt2 gene plays an essential role in the regulation of Pᵢ homeostasis and in normal skeletal development and growth. We believe that Npt2−/− mice provide a unique animal model to define the effects of disturbed Pᵢ homeostasis not only on the skeleton but also on a variety of organ systems where Pᵢ plays a crucial role and in disease states where aberrant Pᵢ economy is manifest.

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