Mouse Model for Lowe Syndrome/Dent Disease 2 Renal Tubulopathy

Susan P. Bothwell,*† Emily Chan,*† Isa M. Bernardini,‡ Yien-Ming Kuo,*† William A. Gahl,‡ and Robert L. Nussbaum*†

*Department of Medicine and †Institute for Human Genetics, University of California, San Francisco, California; and ‡Medical Genetics Branch, National Human Genome Research Institute, Bethesda, Maryland

ABSTRACT
The Lowe oculocerebrorenal syndrome is an X-linked disorder characterized by congenital cataracts, cognitive disability, and proximal tubular dysfunction. Both this syndrome and Dent Disease 2 result from loss-of-function mutations in the OCRL gene, which encodes a type II phosphatidylinositol bisphosphate 5-phosphatase. Ocrl-deficient mice are unaffected, however, which we believe reflects a difference in how humans and mice cope with the enzyme deficiency. Inpp5b and INPP5B, paralogous autosomal genes that encode another type II phosphoinositide 5-phosphatase in mice and humans, respectively, might explain the distinct phenotype in the two species because they are the closest paralogs to Ocrl and OCRL in their respective genomes yet differ between the two species with regard to expression and splicing. Here, we generated Ocrl−/− mice that express INPP5B but not Inpp5b. Similar to the human syndromes, all showed reduced postnatal growth, low molecular weight proteinuria, and aminoaciduria. Thus, we created an animal model for OCRL and Dent Disease 2 tubulopathy by humanizing a modifier paralog in mice already carrying the mutant disease gene.


The Lowe Oculocerebrorenal syndrome (OCRL; MIM #309000) is a rare X-linked disorder of congenital cataracts, mental retardation, and proximal tubular dysfunction.1–5 OCRL is caused by loss of function of the OCRL gene,6–9 which encodes a type II phosphatidylinositol bisphosphate (PtdIns4,5P₂) 5-phosphatase,10,11 Ocrl also contains a noncatalytic Rho-GTPase activating protein (GAP) domain with affinity for small G-proteins,12,13 a clathrin binding domain,14 a pleckstrin-homology domain,16 and an ASPM, SPD-2, Hydin (ASH) domain17 and interacts with APPL1, a Rab5 effector protein,18,19 and the endosomal proteins Ses1 and Ses2.20 The renal tubular dysfunction in OCRL invariably includes low molecular weight (LMW) proteinuria, but may also include generalized aminoaciduria, hypercalciuria, and bicarbonaturia.1,5 Cataracts develop very early in gestation.21 Neurologic abnormalities include variable degrees of cognitive delay and stereotypic behavior.1–3

The renal complications of OCRL are similar to those of Dent disease, a renal disorder characterized by low molecular proteinuria, hypercalciuria, and nephrolithiasis.22,23 Dent disease is genetically heterogeneous.24 Over 50% of cases of Dent disease are caused by mutations in a chloride transporter gene, CLCN5, involved in endosomal acidification and are referred to as Dent Disease 1 (DD1), whereas approximately 20% of Dent disease patients have mutations in OCRL and are referred to as Dent Disease 2 (DD2).23,25–27 Our previous attempt to create a mouse model for OCRL failed when Ocrl−/− mice were shown to have no renal, ophthalmologic, or central nervous system abnormalities.28 We hypothesized that a difference between how humans and mice compensate for loss of Ocrl, rather than a difference between the species in Ocrl function itself, is responsible for the difference in phenotype. We turned our attention to Inpp5b, another type II PtdIns4,5P₂ 5-phosphatase, for three reasons. First, human INPP5B and mouse Inpp5b are the closest paralogs to OCRL and Ocrl in the human and mouse genomes, respectively, and share nearly all their functional domains.19 Second, Inpp5b and Ocrl overlap in function in vivo in mice; mice defective in Ocrl or Inpp5b have little or no phenotype, but mice deficient in both die before implantation.28,29 Finally, Inpp5b and INPP5B differ in transcription and splicing.30 We hypothesized that replacing mouse Inpp5b with human INPP5B in Ocrl-deficient mice would result in mice with a phenotype similar to OCRL. Here we show that such mice survive to term and...
have cardinal features of the renal tubulopathy seen in OCRL and DD2.

Seven founder mice expressing and transmitting INPP5B were generated by oocyte injection of a bacterial artificial chromosome (BAC) containing INPP5B. Three of these lines of mice (lines C, F, and G) were bred with Inpp5b−/− mice and subsequently with Ocr−/− mice to generate live-born Ocr−/−;Inpp5b−/− females and Ocr−/−;Inpp5b−/− males that were either heterozygous or homozygous for human INPP5B (referred to subsequently as 1H or 2H mice, respectively). The live birth of 1H and 2H mice demonstrated that human INPP5B complements the embryonic lethality in Ocr−/−;Inpp5b−/− mice, and insertion of the BAC into the genomes of the mice was not deleterious. Controls were Ocr−/− mice that were either Inpp5b+/+ or Inpp5b+/− (referred to as 2M or 1M mice, respectively).31

We measured the expression of INPP5B in the transgenic mouse line C relative to endogenous Inpp5b with quantitative reverse transcriptase PCR (Table 1). The amount of INPP5B message in 1H mice was 4.4-fold greater and the amount in 2H mice was 9.4-fold greater than the amount of Inpp5b message in 2M mice. These data suggest that the BAC in this line inserted in multiple tandem copies. Whether this difference in mRNA levels translated into greater amounts of enzyme could not be determined because of the lack of suitable antibody.

Patients with Lowe syndrome have a normal birth weight but are below the 3rd percentile for height by 3 years of age.32 At birth, mice of each genotype were largely indistinguishable in size, although occasionally a few of the neonatal 1H mice, but not the 2H or 1M mice, were runted and died by 6 weeks of age. The majority of the 1H and 2H mice of line C showed significantly reduced postnatal growth compared with 1M mice by 4 weeks of age (Figure 1). This growth defect was replicated in lines F and G (data not shown).

The single most consistent renal abnormality in Lowe syndrome is LMW proteinuria.3 INPP5B mice from line C had substantial LMW proteinuria detectable by Coomassie (Figure 2) and silver (data not shown) stain of a sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the region between albumin and the major urinary protein (Mup1). By comparison, 2H mice from line C showed no LMW proteinuria. For comparison, 1M and various control mice did not have LMW proteinuria. 1H mice made from lines F and G mice replicated the LMW proteinuria pattern found in line C mice (data not shown).

We confirmed and quantified the generalized LMW proteinuria by Western blot analysis of secretoglobin (CC16) and vitamin D binding protein (DBP), which are typically seen in the urine in low molecular weight proteinuria. 1H mice from line C had obvious vitamin DBP and CC16 in their urine (Figure 3, A and B). The CC16 proteinuria was quantified by densitometry of the Western blot of urine from the C line (in arbitrary units), with 2M arbitrarily set to 1. Urine from 1H mice measured 19 as compared with 3 for 2H and 1 for 1M. The 2H line C and F mice had no DBP, whereas 2 of 8 2H mice from line G had trace amounts of DBP (data not shown). 1M and 2M mice from all three lines (C, F, and G) showed no DBP or CC16.

Generalized aminoaciduria is seen in both OCRL and DD2, but is significantly more frequent in OCRL (82%) than in DD2 (52%) patients.23 There is increased fractional excretion of arginine, lysine, and cysteine, less so for branched chain amino acids.1 DD2 patients also have generalized aminoaciduria with prominent glycinuria.23 1H mice from line C had significant increases in urine amino acids compared with 2M mice in a pattern similar, but not identical, to what is seen in OCRL and DD2 patients (Figure 4, Table 1). Quantitative PCR of INPP5B, Inpp5b, and Hprt transcripts in kidney tissue of 2M, 1H, and 2H mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Ct INPP5B ± SEM</th>
<th>Ct Inpp5b ± SEM</th>
<th>Ct Hprt ± SEM</th>
<th>INPP5B or Inpp5b expression as % of Hprt expression ± 2 SEM</th>
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<tbody>
<tr>
<td>2M</td>
<td>5</td>
<td>28.06 ± 0.24</td>
<td>21.99 ± 0.05</td>
<td>21.21 ± 0.11</td>
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<tr>
<td>1H</td>
<td>4</td>
<td>25.53 ± 0.15</td>
<td>22.11 ± 0.11</td>
<td>22.29 ± 0.08</td>
<td>7.5 ± 0.6%</td>
</tr>
<tr>
<td>2H</td>
<td>5</td>
<td>24.50 ± 0.13</td>
<td>22.29 ± 0.08</td>
<td>22.29 ± 0.08</td>
<td>16 ± 2%</td>
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</table>

Figure 1. Early growth retardation in 1H mice. Mouse weights (mean ± SEM) for 1H (n = 14), 2H (n = 10), and 1M (n = 10) mice established from transgenic line C. Mice were weighed weekly from 4 to 16 weeks. Growth curves were fitted empirically with cubic equations centered at 10 weeks and the null hypothesis that the curve for 1M could be described with the same four coefficients as either 1H or 2H could be rejected at P < 0.0001, whereas the null hypothesis that 1H and 2H could be fitted with a curve with the same four coefficients could not be rejected (P = 0.1335). 2M mice were published previously and had no growth deficit compared with wild-type mice.
A through C). Nine amino acids were significantly elevated in the urine of 1H versus 2M mice (Table 2). For 16 of 19 amino acids (panels A and B), urinary amino acids correlated significantly with genotype, increasing from 2M to 1M to 2H to 1H mice.

There were no histologic differences between 1H and 2M mice in H&E stained sections of the kidney examined by light microscopy (data not shown), similar to what is seen in young OCRL patients.33 There were also no cataracts by light microscopy. This may be either because overexpression of INPP5B in our mouse lines was adequate to allow normal lens development or lens development is different enough between humans and mice to make mouse lens development independent of both Ocrl and Inpp5b.

Low molecular weight proteins are absorbed from the proximal tubule by clathrin-mediated endocytosis by two receptors, megalin and cubilin, present at the apical surface of renal proximal tubules.34–36 Megalin and cubilin recycle between the apical plasma membrane and early/recycling endosomes. Reduced amounts of megalin at the brush border were observed in a kidney sample from one of two patients with DD1.37 DD1 and OCRL patients both have reduced urine levels of the proteolytic cleavage products of megalin that are normally generated in the apical brush border, suggesting they may have reduced amounts of megalin at the apical surface.38 The Clcn5 knockout mouse model of DD1 suggested decreased megalin at the apical surface was due to defective megalin recycling, caused by a failure to acidify endosomes.39 Given the intracel-
Table 2. Statistical significance of comparisons of urine amino acid concentration relative to creatinine in 2M, 1M, 2H, and 1H mice

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>ANOVA</th>
<th>Tukey Multiple Comparison Test for 1H versus 2M</th>
<th>Correlation*</th>
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<tr>
<td>Ala</td>
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<td>&lt;0.001</td>
<td>&lt;0.0001</td>
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<tr>
<td>Gly</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Tyr</td>
<td>0.130</td>
<td>NS</td>
<td>0.0289</td>
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</table>

NS = P ≥ 0.05.

* Spearman correlation of urine amino acid concentration relative to creatinine versus arbitrary numerical genotype ranking, with 2M = 1, 1M = 2, 2H = 3, and 1H = 4.

Tables

Table 2. Statistical significance of comparisons of urine amino acid concentration relative to creatinine in 2M, 1M, 2H, and 1H mice.

Clinical manifestations of Lowe syndrome, created by ablating the mouse ortholog of the disease gene in combination with humanizing a compensatory mouse paralog of the disease-causing gene. These mice are a model system in which to study how a defect in the Ocrl phosphatase leads to the tubular defects seen in OCRL and DD2 and provides an animal model to test potential therapies for these disorders.

CONCISE METHODS

Mouse Strains

Mice with disruption of Inpp5b are a 129/Sv and FVB/N mixture. Ocrl-deficient mice are in a 129/Sv and C57BL/6 mixed background. Both strains have been previously described. Animals were housed and studied according to NIH Guidelines for the Care and Use of Laboratory Animals under UC SF Protocols AN076327 and AN18551.

Analysis of BAC RP11–431J04

BAC RP11–431J04 was introduced into fertilized FVB/N oocytes by pronuclear injection as described previously. For INPP5B expression studies in offspring of the various founder mouse lines, brain and kidneys were dissected from mice and stored at 4°C overnight in RNAlater (Ambion). RNA was isolated from tissues using Trizol (Invitrogen) and converted to cDNA using a First Strand cDNA Synthesis kit with random primers (GE Healthcare) according to the manufacturer’s instructions and then subjected to PCR.

Quantitative Reverse-Transcriptase PCR

Quantitative PCR of INPP5B, Inpp5b, and Hprt were performed by TaqMan assay (Applied Biosystems) according to the manufacturer’s instructions using Mm01182224_m1 for Inpp5b, Hs00413235_m1 for INPP5B, and as control Mm01318743_m1 for Hprt. Equivalent amplification efficiencies for INPP5B, Inpp5b, and Hprt were confirmed by the serial dilution method.

Urine Protein Analysis

Spot urine samples were collected for protein stain, Western blot analysis, and amino acid analysis. Protein concentration was measured by BCA Protein Assay (Pierce) according to the manufacturer’s instructions. Twenty micrograms of protein were boiled in loading buffer and separated by electrophoresis on a 4 to 20% (Coomassie blue or silver stain), 12% (DBP Western blot), or 10 to 20% (CC16 Western blot) polyacrylamide gel. Silver and Coomassie stains were performed according to the manufacturer’s instructions (Invitrogen). Gels for Western blots were transferred to a polyvinylidene fluoride membrane (Bio-Rad) and probed with rabbit polyclonal antibodies against DBP (Dako, 1:5000) or CC16 (Abcam, 1:1000). A horseradish per-
oxidase–linked sheep anti-mouse antibody (GE Healthcare, 1:2000) was used as the secondary antibody. Bands were visualized using chemiluminescence (GE Healthcare).

**Amino Acid Measurements**
Spot urine was pooled from collections over 3 consecutive days for amino acid measurements in 4-month-old M2 mice (n = 8), M1 mice (n = 6), H2 mice (n = 5), and H1 mice (n = 10). Amino acids were measured on a Biochrom 30 amino acid analyzer (Cambridge, U.K.), which uses an ion-exchange column with postcolumn ninhydrin derivatization. Creatinine was measured by the Jaffe script.

**Histologic Analysis**
Tissues were dissected from mice and fixed in fresh 4% paraformaldehyde overnight. They were dehydrated and embedded for sectioning followed by staining with hematoxylin and eosin.

**Statistical Analysis**
Statistical analysis was performed in Prism (Prism 4, GraphPad Software). Growth curves (weight over time) were fitted by a third-order polynomial. The null hypothesis that one third-order polynomial curve fit the 1H, 2H, and 1M growth curves was tested by the extra sum-of-squares F test. Differences in amino acid concentration were analyzed by a one-way ANOVA followed by Tukey’s post hoc test. Trends in aminoaciduria over mouse versus human gene and copy number were analyzed by Spearman correlation.

**ACKNOWLEDGMENTS**
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**DISCLOSURES**
None.

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