In vivo *Drosophila* genetic model for calcium oxalate nephrolithiasis

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1Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota; 2Mayo Clinic O’Brien Urology Research Center, Mayo Clinic College of Medicine, Rochester, Minnesota; and 3Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

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Hirata T, Cabrero P, Berkholz DS, Bondeson DP, Ritman EL, Thompson JR, Dow JA, Romero MF. In vivo *Drosophila* genetic model for calcium oxalate nephrolithiasis. *Am J Physiol Renal Physiol* 303: F1555–F1562, 2012. First published September 19, 2012; doi:10.1152/ajprenal.00074.2012.—Nephrolithiasis is a major public health problem with a complex and varied etiology. Most stones are composed of calcium oxalate (CaOx), with dietary excess a risk factor. Because of complexity of mammalian system, the details of stone formation remain to be understood. Here we have developed a nephrolithiasis model using the genetic model *Drosophila melanogaster*, which has a simple, transparent kidney tubule. *Drosophila* reliably develops CaOx stones upon dietary oxalate supplementation, and the nucleation and growth of microliths can be viewed in real time. The Slc26 anion transporter dPrestin (Slc26a5/6) is strongly expressed in *Drosophila* kidney, and biophysical analysis shows that it is a potent oxalate transporter. When dPrestin is knocked down by RNAi in fly kidney, formation of microliths is reduced, identifying it is a potent oxalate transporter.

**MATERIAL AND METHODS**

*Drosophila*. Flies were kept on standard medium or dietary salt substitution in vials at 22°C, 12:12-h photoperiod, and 40% relative humidity. CantonS was used as wild-type and line c825, which expresses GAL4 in the principal cells of the initial and main segments of the Malpighian tubule (50). *Cell-type specific knockdown of dPrestin*. An elegant transgenic system, GAL4/UAS (upstream activation sequence), allows tissue- or cell-type specific genetic intervention in *Drosophila* (4). The “GAL4 driver” lines contain the gene for the yeast transcription factor GAL4 under control of a *Drosophila* promoter of choice. When crossed to a second transgenic fly line in which a genetic construct of choice is placed downstream of the UAS promoter, then the transgene is expressed only in those cells where GAL4 is being expressed. In this case, we used the Uro-GAL4 driver (53), in which the promoter of the uterine tubule principal-cell specific gene urate oxidase drives GAL4 expression, and crossed it to NIGFly line 5845R3 (National Institute of Genetics), which contains a hairpin dsRNA sequence directed against dPrestin.

**Dietary salt substitution.** This was performed as described previously (51). Na-oxalate was dissolved in 100 ml of standard growth media (0.1% low; 1% high) just after its preparation and mixed, and the diet was left to set. Diet was freshly prepared to avoid any changes in the concentration of the salts due to evaporation.

**Birefringence experiments.** Adult flies (7 days) were allowed to feed in normal food or dietary substitution for 24 h. Malpighian tubules were dissected in Schneider’s medium and transferred immediately to poly-l-lysine-coated slides with PBS for visualization using a Zeiss Axiophot microscope. For in vitro experiments, PBS + 0.75 mM Na-oxalate was used.

**X-ray diffraction.** *Drosophila* Malpighian tubules were drawn into a quartz capillary and dried, and diffraction data were collected on an in-house X-ray source (2θ = 180° at 1°/min.). Images were background corrected using either pixel-by-pixel subtraction or radial averaging.

**Real-time RT-PCR.** *Drosophila* Malpighian tubule cDNA was generated as described (5). The cDNA was used as a template for the generation of the complete prestin open reading frame and the PCR fragment used for RNAi.

**Quantitative RT-PCR.** Quantitative RT-PCR validation was performed as described elsewhere (49). The mRNA was prepared from 7-day-old CantonS or experimental tubules using Qiagen RNAeasy column. Superscript II and an oligo-dT were used for reverse transcription. For each sample, 500 ng of cDNA were added to 12.5 μl of 2 μM SYBR green reaction mix (Finnzymes, GRI, Essex, UK) and 1 μl of 6.6 μM forward and reverse primers. An Opticon 2 thermocycler was set as follows: 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The ribosomal protein 49 (rp49) gene was used as a standard in all experiments. For each
condition, we used four independent samples; each PCR was repeated three independent times to verify results.

Animal health and welfare. *Xenopus laevis* were housed and cared for in accordance and approv of the Institutional Care and Use Committees of the Mayo Clinic.

*Drosophila* Slc26a5 constructs. The sequence of *Drosophila* prestin (Slc26a5; CG5485) has been previously reported (59). We designed PCR primers to amplify the open reading frame plus restriction sites. The *Drosophila* prestin sequence was verified.

Oocyte experiments. *Drosophila* prestin was subcloned into the pGEMHE *Xenopus* expression vector, capped cRNA synthesized; oocytes injected with 50 nl cRNA (0.2 μg/μl, 10 ng/oocyte) or water as previously for other transporters (22, 30, 39, 41); and incubated at 16°C in OR3 media. Oocytes were studied 3–10 days after injection.

Electrophysiology. Electrophysiology protocols were performed as we previously reported for Slc26a6 (30, 32, 62). All solutions were either ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.5) or iso-osmotic ion replacements (47). Cl− was replaced by gluconate. For HCO3− solutions, we used 5% CO2/33 mM HCO3− (pH 7.5).

Two electrode voltage clamp. For these experiments, membrane currents were recorded with an OC-725C voltage clamp (Warner Instruments, Hamden, CT), filtered at 2–5 kHz, digitized at 10 kHz. I-V protocols consisted of 40-ms steps from Vh (−60 mV) to −140 mV and +60 mV in 20-mV steps (30, 47).

Ion-selective microelectrodes. Ion-selective microelectrodes were used to monitor pH3 and intracellular Cl− ([Cl−]) of oocytes (41, 42). Intracellular pH and Cl− microelectrodes had slopes of at least −54 mV/pH unit or decade, respectively.

RESULTS

Dietary loading recapitulates oxalate nephrolithiasis in *Drosophila*. In humans, high dietary oxalate levels are associated with increased incidence of CaOx stones. Experimental food for flies does not normally contain oxalate. To induce increased oxalate elimination via Malpighian tubules, *Drosophila* larvae were fed on diet supplemented with sodium oxalate (see MATERIALS AND METHODS). Remarkably, CaOx microliths were observed within 2 days (Fig. 1, A–C); CaOx crystals show characteristic birefringence (Fig. 1, A and D–F), allowing a rapid screen for their existence, even in live larvae.

To confirm that monolith formation was a direct product of oxalate transport by the renal tubules, dissected tubules were incubated in vitro in culture medium supplemented with oxalate. Rapid nucleation of microliths was observed within minutes (Fig. 1, D–F) and could be followed over several hours (see Supplemental Movie S1; Supplemental Material for this article is available online at the *Am J Physiol Renal Physiol* website). The *Drosophila* tubule is a site of rapid calcium excretion (15), so transport of oxalate into the lumen could result in supersaturation with respect to CaOx (which has a solubility product $\sim 2.4 \times 10^{-9}$ at 25°C), exactly mimicking conditions conducive to stone formation in humans.

Likewise feeding adult *Drosophila* with oxalate supplementation results in rapid (6–12 h) CaOx-microlith formation. These crystals can be observed by tubule microdissection and birefringence (Fig. 1) or alternatively in the intact animal using micro-computed tomography (micro-CT; Fig. 2). As with a human patient, CT allows an in situ assessment of CaOx microlith location and abundance. Figure 2 illustrates that oxalate fed-*Drosophila* have CT dense material (CaOx) in the Malpighian tubules (MTs) while control *Drosophila* do not.

*Drosophila* prestin (Slc26a5/α6) is an electrogenic Cl−/ox2− exchanger. Next, we sought to determine the protein responsible for the CaOx accumulation. Recently, Schaechner and Oliver (46) showed that nonmammalian prestin (Slc26a5) could function as an electrogenic Cl−/ox2− exchanger. Therefore, we cloned and tested the function of *Drosophila* prestin (dPrestin) in *Xenopus* oocyte expression...
system. Figure 3 shows an experiment in which oxalate is exchanged for Cl\(^-\) when dPrestin or mammalian Slc26a6 are expressed in Xenopus oocytes and monitored intracellular Cl\(^-\) with a Cl\(^-\) electrode (Fig. 3A). In oocytes expressing dPrestin and mSlc26a6, Cl\(^-\) removal caused marked reduction of [Cl\(^-\)], and a marked hyperpolarization. Readdition of Cl\(^-\) elicited depolarization and recovery of [Cl\(^-\)]. Control (water-injected) oocytes did not show these responses. When a similar solution protocol is repeated while voltage clamping oocytes, voltage- and oxalate-dependent currents were observed (Fig. 3B). Since Cl\(^-\) removal in the absence of oxalate caused only slight currents in dPrestin and mSlc26a6 expressing oocytes (Fig. 3D), almost all of their transport currents are due to Cl\(^-\)/ox\(^{2-}\) exchange. These data illustrate that dPrestin mediates electrogenic Cl\(^-\)/ox\(^{2-}\) exchange (Fig. 3A, right). This activity is similar to the activity that we and others have reported for Slc26a6 (9, 19, 26, 30, 32, 37, 58, 62).

dPrestin knockdown decelerates crystal accumulation. As dPrestin is expressed in epithelia, and functions as a Cl\(^-\)/ox\(^{2-}\) exchanger, it could mediate tubule oxalate transport. As mammalian Slc26a6, dPrestin mRNA is enriched in the tubule and the gut (Fig. 4A). Within the tubule, it is possible to titrate expression of genes cell specifically using the GAL4/UAS system with appropriate GAL4 drivers (51), so dPrestin was taken down in the initial and main segments of the tubule with the Drosophila GAL4 line c825, which drives expression in tubule principal cells. The different dsRNA lines were found to reduce dPrestin mRNA levels by 50–70% (Supplemental Fig. S1), and this was sufficient to produce a marked phenotype (Fig. 4B). Birefringent luminal concretions were markedly reduced in the tubule (Fig. 4B), confirming that dPrestin mediates rapid oxalate transport. This effect is more pronounced in the anterior Malpighian tubules (Fig. 4C), which have higher overall transport (15).

**DISCUSSION**

**Drosophila model for Ox\(^{2-}\) transport and regulation.** Mutations in Slc26 anion transporter-channel proteins cause a variety of human diseases, e.g., diarrhea, deafness, goiter, and
**Diastrophic Dysplasia** (1, 18, 23, 25, 33–35, 38, 43, 64). Mouse studies have revealed additional roles for these Slc26 proteins in mammalian physiology: deafness, goiter, and acidosis (Slc26a4 and pendrin; Refs. 17, 44); cochlear motor protein (Slc26a5 and prestin; Refs. 10, 14); proximal tubule NaCl absorption, mouse urolithiasis, and intestinal HCO3 secretion (Slc26a4 and pendrin; Refs. 26, 48, 57, 58); sperm motility (Slc26a8 and Tat-1; Ref. 55); and gastric acid secretion (Slc26a9; Ref. 63). Additionally, several Slc26 proteins participate in the process (Fig. 7).

**Table 1.** *Km* oxalate transport kinetics in oocytes

<table>
<thead>
<tr>
<th>Vm (mV)</th>
<th>dPrestin (µA)</th>
<th>mSlc26a6 (µA)</th>
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<tr>
<td>−40</td>
<td>1.93 ± 0.58*</td>
<td>1.38 ± 0.09</td>
</tr>
<tr>
<td>−20</td>
<td>1.42 ± 0.09*</td>
<td>3.45 ± 2.0</td>
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<tr>
<td>0</td>
<td>0.64 ± 0.09*</td>
<td>3.51 ± 1.10</td>
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<tr>
<td>+20</td>
<td>0.60 ± 0.12t</td>
<td>2.45 ± 0.26</td>
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<tr>
<td>+40</td>
<td>0.37 ± 0.05t</td>
<td>1.74 ± 0.20</td>
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<tr>
<td>+60</td>
<td>0.23 ± 0.04t</td>
<td>1.44 ± 0.24</td>
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</tbody>
</table>

Values are means ± SE; ns, not significant; *P < 0.02 for dPrestin vs. mSlc26a6 at the same Vm; †P < 0.005 for dPrestin vs. mSlc26a6 at the same Vm (n = 3 for each data point of each clone).

The present work shows that a major renal pathophysiology (CaOx kidney stones) can be recapitulated rather precisely in the potent genetic model, *Drosophila*, and identify a key transporter (dPrestin) that participates in the process (Fig. 7). Recently, ethylene glycol intoxication associated with oxalate
stones was reported in *Drosophila* (6) and we did further examination including genetic association with oxalate stones for establishing fly stone model. The studies in this report show that *Drosophila* can specifically mimic 1) CaOx crystal formation (Figs. 1 and 5), 2) tubule oxalate secretion (Figs. 1 and 4), 3) gut and renal tubule Cl⁻/HCO₃⁻ exchange (Fig. 3), and finally 4) whole animal gut oxalate absorption to CaOx crystal formation (Figs. 1 and 2). These studies also illustrate that dPrestin (Slc26a5/a6) is key to this physiological process and mimics the pathophysiology of CaOx renal stone formation.

Using Slc26a6 knockout mice, the group of Aronson (31) reported that oxalate secretion into intestinal lumen is important for protecting against stone formation, which means that oxalate transporter (Slc26a6) in gut contributes to keep out oxalate from body fluid by mediating oxalate secretion into the intestinal lumen. This is a first defense system to eliminate

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**Fig. 4.***Drosophila* prestin: localization and knockdown. A: dPrestin mRNA expression across different *Drosophila* tissues and life stages. dPrestin is broadly expressed at low level but conspicuously abundant in the epithelia of the alimentary canal (midgut, tubules, and hindgut). Data from FlyAtlas.org (7). B: impact of selective dPrestin knockdown in tubule on oxalate formation in vivo. dPrestin RNAi mutants [dPrestin RNAi (5834R3xUro-Gal4)] show remarkably reduced deposition of oxalate, compared with corresponding parental controls (Uro-Gal4). C: birefringence quantification of pixel intensity for anterior (A; black) and posterior (P; orange) tubules. Open boxes are tubule pixel background; solid boxes are control +oxalate; hatched boxes are RNAi-dPrestin + oxalate. *P < 0.05. Quantitative PCR of dPrestin mRNA in Malpighian tubules is available as Supplemental Fig. 1.

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**Fig. 5.** Crystal identity by X-ray diffraction of tubules. A: negative image and peak profile of X-ray diffraction pattern from *Drosophila* Malpighian tubules after exposure to Na-oxalate (see MATERIALS AND METHODS). Diffraction rings characteristic of crystal powders are annotated (1–18). B: single Malpighian tubule of 48-h high oxalate fed flies was placed in a quartz pipette for X-ray beam shooting (in blue line circle). Quartz does not disturb X-ray diffraction.
oxalate at gut. Once oxalate goes into body fluid, it should be excreted from renal tubules but direct evidence of oxalate secretion at renal tubules was reported in few studies (3). In this study, we used c42- and Uro-drivers for specific expression of dPrestin at Malpighian tubules but not at gut and showed that renal secretion is also a quite important defense factor for oxalate secretion. For preventing oxalate accumulation in body fluid, the transporter-depending oxalate secretion system in both gut and renal tubules is important. This study showed the direct evidence that oxalate transporter (dPrestin) at renal tubules (MT) excrete oxalate to the lumen.

Given that nucleation and growth of CaOx crystals can be followed in real time in an intact renal tubule, and the ease of genetic and physiological intervention in this model, these results confirm that Drosophila can be used to rapidly study factors that modulate ion transport and crystallization within the tubules. This approach is particularly valuable, because there are presently very limited clinical therapies for this very common kidney disorder. Nonetheless, we have found the pharmacology of transport in Drosophila to be very similar to that of humans. For example, well-known drugs like ouabain (54), amiloride (12, 20), or the antidiabetic sulfonylureas (16) work well on Drosophila renal tubule and with similar IC50s to human. Furthermore, the low solubility product of CaOx implies that once formed, stones are unlikely to redissolve rapidly. Thus the ability to view the nucleation event, and screen for therapies that inhibit it, is uniquely valuable.

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GRANTS

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Table 2. X-ray diffraction ring peaks

<table>
<thead>
<tr>
<th>Identification/Ring No.</th>
<th>Peak</th>
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<tr>
<td>CaOx dihydrate*</td>
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</tr>
<tr>
<td>1</td>
<td>6.19</td>
</tr>
<tr>
<td>7</td>
<td>3.17</td>
</tr>
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<td>9</td>
<td>2.96</td>
</tr>
<tr>
<td>11</td>
<td>2.78</td>
</tr>
<tr>
<td>CaOx monohydrate*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.93</td>
</tr>
<tr>
<td>5</td>
<td>3.64</td>
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<td>Lipid acyl chains</td>
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*x-ray diffraction reflections (rings) shown in Fig. 5A. CaOx, calcium oxalate.* and * identify specific rings annotated in Fig. 5A.


