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 dPrestin as a key player in oxalate excretion. CaOx stone formation is an ancient conserved process across >400 My of divergent evolution (fly and human), and from this study we can conclude that the fly is a good genetic model of nephrolithiasis.

oxalate; Cl− transport; prestit; Malpighian tubules; gene knockdown; Slc26

Kidney stones are a major healthcare burden (>US$5.3 billion/yr in the US alone), with a complex and varied etiology that is poorly understood at the genetic level (36, 45, 61). Up to 75% of human kidney stones are made of calcium oxalate (CaOx). Some of these CaOx stones are associated with rare genetic disorders (primary hyperoxaluria) or intestinal disorders that cause fat malabsorption and enteric hyperoxaluria. In the remainder, the pathogenesis seems more complex, and multiple environmental and genetic factors that ultimately influence the urinary composition appear to play a role. Urinary oxalate excretion is not the only determinant of CaOx stone disease. Since oxalate is required for the formation of CaOx stones, factors that determine oxalate absorption from the diet, metabolic production, and elimination in the urine are all likely to play some role in stone risk.

Recent work has shown *Drosophila* to provide a surprisingly good model for renal function (13), including calcium and now oxalate handling. Although human SLC26A6 is known to transport oxalate, only one study to date has evaluated it in oxalate nephrolithiasis (37). Here we describe the characterization of a Slc26 homolog (prestin, Slc26a5/6) in *Drosophila* 1 which mediates tubule oxalate crystal formation, 2) which is an electrogenic Cl−/Ox2− exchanger, and 3) which when knocked down decreases CaOx crystal formation.

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.

Bioirreerenceg 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 (20°C at 0.3× magnification). Images were back-ground 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). 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 approval 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 V0 (−60 mV) to −140 mV and +60 mV in 20-mV steps (30, 47).

Ion-selective microelectrodes. Ion-selective microelectrodes were used to monitor pH 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 \(2.4 \times 10^{-9}\) 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, Schaechinger 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

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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, 27, 30, 32, 37, 58, 62).

**dPrestin is functionally analogous to mammalian Slc26a6.**

The above experiment can be repeated with sulfate or HCO\(_3\) and Cl\(^-\) removal to illustrate electrogenic Cl\(^-\)/SO\(_4\)\(^2-\) (Fig. 3C) or electrogenic Cl\(^-\)/nHCO\(_3\) exchange, respectively (24). These activities have also been reported and characterized for Slc26a6 (Fig. 3C; Refs. 9, 19, 26, 27, 30, 32, 58, 62). Interestingly, the absolute magnitude of Cl\(^-\)/ox\(^2-\) exchange is similar in Fig. 3. These new data reveal that dPrestin-mediated Cl\(^-\)/SO\(_4\)\(^2-\) exchange is faster than Cl\(^-\)/ox\(^2-\) exchange in insects (Fig. 3C, left), while the reverse is true for mouse Slc26a6 (Fig. 3C, right). This difference in substrate preference likely represents a *Drosophila* (perhaps even Diptera) adaptation not needed in mammals. Varying [ox\(^2-\)] and performing similar experiments reveals that dPrestin is close to saturation at 1 mM (K\(_m\) = 0.64 ± 0.09 mM at 0 mV; V\(_{max}\) = 0.85 ± 0.06 μA at 0 mV) while mouse Slc26a6 is not (K\(_m\) = 3.5 ± 1.1 mM at 0 mV; V\(_{max}\) = 6.00 ± 1.49 μA at 0 mV). Table 1 details the voltage dependence of the oxalate K\(_m\) for both dPrestin and mSlc26a6. For V\(_m\) > 0 mV, the K\(_m\) for dPrestin compared with mSlc26a6 are significantly different (P < 0.02). In contrast for V\(_m\) close to resting V\(_m\) of epithelial cells (−60 mV), there is no statistical difference.

**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).

**Tubule crystals are CaOx.** To make sure that crystals formed in *Drosophila* Malpighian tubules were indeed CaOx (2), we used X-ray diffraction to definitively identify the crystals that formed in the tubules (Fig. 5). Figure 5B shows a Malpighian tube with crystals, which was placed in the beam path of an X-ray diffractometer. The resulting diffraction pattern (Fig. 5A, 1–18) was identified by comparison to standards (21, 52, 56). Peaks assigned to CaOx monohydrate and dihydrate were identified (Table 2; Supplemental Fig. S2) as well as NaCl and hexagonal lipid from the partly dried tubule preparation.

**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...
Additional roles for Slc26 proteins in mammalian physiology: deafness, goiter, and acidosis (Slc26a5 and prestin; Refs. 10, 14); proximal tubule NaCl absorption, mouse urolithiasis, and intestinal HCO3 secretion (Slc26a4 and pendrin; Refs. 17, 44); cochlear motor protein (Slc26a6, Pat-1, and CFEX; Refs. 26, 48, 57, 58); sperm motility (Slc26a8 and Tat-1; Ref. 55); and gastric acid secretion (Slc26a9; Ref. 63). Furthermore, Slc26 genes, these appear to be an adaptive divergence into vertebrate subfamilies. Although there are other Drosophila homologue transporters, perhaps representing ancestral gene members with poorly understood transport function. dPrestin mediates the transports of not only oxalate and chloride but also bicarbonate, sulfate; and formate and thus likely contributes to the metabolism of these compounds in gut and Malpighian tubules.

The present work shows that a major renal pathophysiology (CaOx kidney stones) can be recapitulated rather precisely in flies, Drosophila prestin (Slc26a5) seems to function as a Slc26a5-homologue transporter, perhaps representing ancestral gene function. Figure 6 shows that insect and worm Slc26a5a6 proteins sit between vertebrate Slc26a5 and Slc26a6 clades, confirming their ancestral origin apparently predating the divergence into vertebrate subfamilies. Although there are other Drosophila Slc26 genes, these appear to be an adaptive radiation of a Slc26a11 ancestor (Supplemental Fig. S3), a homologue transporter, perhaps representing ancestral gene members with poorly understood transport function. dPrestin mediates the transports of not only oxalate and chloride but also bicarbonate, sulfate; and formate and thus likely contributes to the metabolism of these compounds in gut and Malpighian tubules.

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 metabolism is well known for producing kidney stones in humans and mice. A model for oxalate kidney stones can be recapitulated rather precisely in flies, Drosophila, and identify a key transporter (dPrestin) that participates in the process (Fig. 7).

### Table 1. $K_m$ oxalate transport kinetics in oocytes

<table>
<thead>
<tr>
<th>$V_m$ (mV)</th>
<th>dPrestin (mM)</th>
<th>mSlc26a6 (mM)</th>
</tr>
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<tbody>
<tr>
<td>-40</td>
<td>1.93 ± 0.58**</td>
<td>3.18 ± 0.09</td>
</tr>
<tr>
<td>-20</td>
<td>1.42 ± 0.09</td>
<td>3.45 ± 2.0</td>
</tr>
<tr>
<td>0</td>
<td>0.64 ± 0.09*</td>
<td>3.51 ± 1.10</td>
</tr>
<tr>
<td>+20</td>
<td>0.60 ± 0.12†</td>
<td>2.45 ± 0.26</td>
</tr>
<tr>
<td>+40</td>
<td>0.37 ± 0.05†</td>
<td>1.74 ± 0.20</td>
</tr>
<tr>
<td>+60</td>
<td>0.23 ± 0.04†</td>
<td>1.44 ± 0.24</td>
</tr>
</tbody>
</table>

Values are means ± SE; ns, not significant; *P < 0.02 for dPrestin vs. mSlc26a6 at the same $V_m$; †P < 0.005 for dPrestin vs. mSlc26a6 at the same $V_m$ (n ≥ 3 for each data point of each clone).
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−/oxalate 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...
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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Table 2. X-ray diffraction ring peaks

<table>
<thead>
<tr>
<th>Identification/Ring No.</th>
<th>Peak</th>
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<tbody>
<tr>
<td>CaOx dihydrate^</td>
<td>6.19</td>
</tr>
<tr>
<td>1</td>
<td>3.17</td>
</tr>
<tr>
<td>7</td>
<td>2.96</td>
</tr>
<tr>
<td>9</td>
<td>2.78</td>
</tr>
<tr>
<td>CaOx monohydrate*</td>
<td>5.93</td>
</tr>
<tr>
<td>2</td>
<td>3.64</td>
</tr>
<tr>
<td>5</td>
<td>2.59</td>
</tr>
<tr>
<td>13</td>
<td>2.49</td>
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<td>14</td>
<td>2.35</td>
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<td>16</td>
<td>2.21</td>
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<td>17</td>
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<td>6</td>
<td>2.82</td>
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<tr>
<td>10</td>
<td>1.99</td>
</tr>
<tr>
<td>Lipid acyl chains</td>
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</tbody>
</table>

*X-ray diffraction reflections (rings) shown in Fig. 5A. CaOx, calcium oxalate. ^ and * identify specific rings annotated in Fig. 5A.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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