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Graphical abstract

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Claudin-2 deficiency associates with hypercalciuria in mice and human kidney stone disease

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Introduction

Kidney stone disease is common, with a lifetime risk of the United States of about 19% in men and 9% in women.1 Studies of families (2), twin concordance (3), and genome-wide association (4–6) have revealed a significant role for polygenic inheritance in the pathogenesis of the disease. The vast majority of stones are composed of calcium crystals, primarily calcium phosphate or calcium oxalate (CaOx) (7, 8), and the major risk factor is an elevation in urine calcium, which is termed idiopathic hypercalciuria (9). Additional risk factors for kidney stone formation include low urine volume, hyperoxaluria, and hypocitraturia (9). The common pathogenic factor in these metabolic abnormalities is supersaturation of calcium salts leading to precipitation and crystal growth (9).

The pathogenesis of stone formation in patients with idiopathic hypercalciuria varies depending on the composition of the stone, but the majority are believed to begin with deposition of calcium within the renal papilla, or papillary nephrocalcinosis (8).

The major risk factor for kidney stone disease is idiopathic hypercalciuria. Recent evidence implicates a role for defective calcium reabsorption in the renal proximal tubule. We hypothesized that claudin-2, a paracellular cation channel protein, mediates proximal tubule calcium reabsorption. We found that claudin-2–null mice have hypercalciuria due to a primary defect in renal tubule calcium transport and papillary nephrocalcinosis that resembles the intratubular plugs in kidney stone formers. Our findings suggest that a proximal tubule defect in calcium reabsorption predisposes to papillary calcification, providing support for the vas washdown hypothesis. Claudin-2–null mice were also found to have increased net intestinal calcium absorption, but reduced paracellular calcium permeability in the colon, suggesting that this was due to reduced intestinal calcium secretion. Common genetic variants in the claudin-2 gene were associated with decreased tissue expression of claudin-2 and increased risk of kidney stones in 2 large population-based studies. Finally, we describe a family in which males with a rare missense variant in claudin-2 have marked hypercalciuria and kidney stone disease. Our findings indicate that claudin-2 is a key regulator of calcium excretion and a potential target for therapies to prevent kidney stones.

For patients with urinary stones composed of primarily CaOx, the precursor lesions are known as Randall’s plaques and are characterized by deposition of calcium phosphate within the interstitial space surrounding the basement membranes of the thin limbs of loops of Henle (10, 11). By contrast, patients with predominantly calcium phosphate urinary stones often have early intratubular calcium phosphate deposits in inner medullary collecting ducts, as well as interstitial aggregates of calcium phosphate, termed novel interstitial plaque structures, that have microscopic features distinct from Randall’s plaques (8). All of these forms of intrarenal deposits are believed to eventually rupture through the papillary surface and thereby form a nidus for the growth of urinary stones.

The etiology of idiopathic hypercalciuria is incompletely understood, but can generally be ascribed to increased bone resorption, intestinal hyperabsorption, and/or reduced renal reabsorption of calcium (12). Studies of diuretic response and lithium clearance in patients with idiopathic hypercalciuria suggest that they have a specific defect in calcium reabsorption in the proximal renal tubule (PT) (13, 14). The PT reabsorbs approximately 60% of calcium filtered by the glomerulus (15). It is highly permeable to calcium, and the tubule fluid/plasma concentration ratio is about 1.1, suggesting that transport of calcium follows that of sodium and water. (15). In isolated perfused PTs from the pars convoluta, net calcium trans-

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We now show that expression of calbindin-D28k, the apical calcium entry channel TRPV5, the Na-Ca exchanger NCX1, and Claudin-14 was also not different between Cldn2–/– and WT mice (Supplemental Figure 1).

Increased net intestinal absorption of calcium in Cldn2–/– mice. If the hypercalcuria in Cldn2–/– mice is solely due to primary renal calcium wasting, we would expect them to be in negative calcium balance and hence to exhibit a compensatory increase in the calciotropic hormones parathyroid hormone (PTH) and 1,25(OH)2-vitamin D3 (24). However, there were no significant differences in the levels of these hormones (Table 1). We would also expect Cldn2–/– mice to have net calcium loss from bone. However, we found no detectable difference in either total or lumbar bone mineral density in male Cldn2–/– mice, on either a normal or a low-calcium diet (Figure 1). Micro-CT analysis of femurs from 10-week-old animals confirmed normal bone volume and quality (Figure 1C and Supplemental Table 2). The finding that there was no change in the levels of PTH, 1,25(OH)2-vitamin D3, and bone mineral density in Cldn2–/– mice despite marked hypercalcuria suggests that they may have a concurrent primary increase in net intestinal absorption of calcium. Indeed, concurrent renal calcium wasting and intestinal hyperabsorption of calcium is typical of idiopathic hypercalciuria in patients with kidney stones and in hypercalciuric rats (12, 25). Moreover, Claudin-2 is known to be expressed in intestinal epithelial cells.

To test the hypothesis that there is also a primary increase in intestinal absorption of calcium in Cldn2–/– mice, we determined whether the hypercalcuria was attenuated by a reduction in dietary calcium content. We placed animals in metabolic cages on a control diet (0.6% calcium), and after 5 days switched half of them to a calcium-deficient (<0.01% calcium) diet (Figure 2A). We found that urinary calcium excretion in Cldn2–/– mice is highly sensitive to dietary calcium intake (Figure 2A). On the control diet, FECa2+ in Cldn2–/– mice was approximately 5 times higher than in WT mice (1.37% vs. 0.28%) (Figure 2B). In contrast, the FECa2+ of Cldn2–/– mice on the calcium-deficient diet was only twice that of WT mice (0.44% vs. 0.23%) (Figure 2B). No differences in serum calcium, phosphorus, or PTH were found between the groups (Figure 2, C–E).

These results suggest that hypercalcuria in Cldn2–/– mice is due to increased net intestinal absorption of calcium, in addition to a primary renal calcium leak. To confirm this, we performed metabolic balance studies over a 3-day period on the control diet (0.6% calcium) (Figure 3, A–C). As expected, renal calcium excretion was increased in Cldn2–/– mice. Intestinal calcium absorption, determined from the difference between dietary calcium intake

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Table 1. Serum and urinary parameters of male mice on standard lab chow

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>Cldn2–/–</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Calcium (mg/dL)</td>
<td>10.24</td>
<td>10.74</td>
<td>0.27</td>
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<tr>
<td>Phosphorus/Cr (mg/mg)</td>
<td>7.73</td>
<td>6.65</td>
<td>0.67</td>
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<tr>
<td>OXalate/Cr (mg/mg)</td>
<td>0.09</td>
<td>0.12</td>
<td>0.22</td>
</tr>
<tr>
<td>Citrate/Cr (mg/mg)</td>
<td>6.79</td>
<td>7.11</td>
<td>0.76</td>
</tr>
<tr>
<td>Mg/Cr (mg/mg)</td>
<td>3.57</td>
<td>3.11</td>
<td>0.18</td>
</tr>
</tbody>
</table>

**Parameter Wild type Cldn2–/– P value**

**Mean SEM n Mean SEM n**

**Serum**

**Calcium (mg/dL)** 10.24 0.17 9 10.74 0.43 8 0.27

**Phosphorus/Cr (mg/mg)** 7.73 1.64 8 6.65 1.76 5 0.67

**Oxalate/Cr (mg/mg)** 0.09 0.01 5 0.12 0.01 4 0.22

**Citrate/Cr (mg/mg)** 6.79 0.58 8 7.11 0.95 5 0.76

**Mg/Cr (mg/mg)** 3.57 0.20 8 3.11 0.25 5 0.18

**Urine**

**FECa (%)** 0.23 0.02 8 0.40 0.02 7 0.0001

**Phosphorus/Cr (mg/mg)** 7.73 1.64 8 6.65 1.76 5 0.67

**Oxalate/Cr (mg/mg)** 0.09 0.01 5 0.12 0.01 4 0.22

**Citrate/Cr (mg/mg)** 6.79 0.58 8 7.11 0.95 5 0.76

**Mg/Cr (mg/mg)** 3.57 0.20 8 3.11 0.25 5 0.18

**PTH, parathyroid hormone; FECA, fractional excretion of calcium; Cr, creatinine.**

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port is zero in the absence of osmotic or potential differences, and reabsorption can be induced by application of a lumen-positive potential difference (16). This suggests that calcium reabsorption in the PT is passive and likely occurs by paracellular diffusion.

Paracellular transport is mediated by a family of tetraspanning membrane proteins known as claudins that are found at the tight junction between epithelial cells. These proteins act as charge-selective channels or barriers regulating the movement of solutes across epithelial layers (17). Claudin-2 is a cation-selective isoform (18, 19) that is permeable to both sodium and calcium (19, 20) and is highly expressed in the PT and the descending thin limb of the loop of Henle (21). Isolated PTs from claudin-2-knockout mice have increased transepithelial resistance and reduced sodium permeability, and exhibit increased fractional excretion of calcium (FECa2+) (22), suggesting that claudin-2 mediates paracellular calcium reabsorption in the PT.

In this study we tested the hypothesis that loss of claudin-2 predisposes mice to nephrocalcinosis and nephrolithiasis, mimicking the pathogenesis of human kidney stone disease. We explored the underlying mechanism and tested whether variants in the claudin-2 gene are associated with human kidney stone disease.

**Results**

**Deletion of Cldn2 in mice causes hypercalcemia due to defective renal tubular calcium reabsorption.** We first confirmed that claudin-2-knockout mice (Cldn2–/–) are hypercalcicuric. FECA2+ was increased in Cldn2–/– mice compared with their WT littermates (Table 1). There was no significant difference in serum calcium levels (Table 1), and we previously showed that glomerular filtration rate is unchanged in Cldn2–/– mice (23). Hence, the filtered load of calcium is unchanged. This suggests that renal tubule calcium reabsorption is decreased in Cldn2–/– mice. As claudin-2 is predominantly expressed in the PT and is permeable to calcium (20), these results suggest that Cldn2–/– mice are hypercalcicuric as a result of impaired PT paracellular calcium transport. Our urine calcium studies were replicated in a separate experiment that showed that both male Cldn2–/– and female Cldn2–/– mice were similarly hypercalcicuric (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI127750DS1). We found no evidence for compen-
sumably paracellular transepithelial permeability. There was no difference in the serosal-to-mucosal flux and permeability of the duodenum and ileum. However, the colonic serosal-to-mucosal flux and permeability were significantly reduced in Cldn2–/y mice. Moreover, mucosal-to-serosal flux in the colon was very similar to serosal-to-mucosal flux (i.e., net flux was negligible) (Figure 4B), indicating that the measured fluxes represent passive transport, and was reduced similarly in Cldn2–/y mice, as would be expected for an effect on the paracellular pathway.

To confirm these findings, we mounted full-thickness segments of proximal colon in Ussing chambers and determined their ionic permeability from equilibrium potential measurements. As expected, colons from Cldn2–/y mice had significantly lower permeability to Na+ (PNa) and Ca2+ (PCa) and permeability ratio of Na+ to Cl– (PNa/PCl) than colons of WT male littermates (Figure 5). In experiments comparing full-thickness preparations with colon stripped of its seromuscular layer, we found no differences in ion permeability (Supplemental Table 3). Additionally, we confirmed that stripped proximal colon segments from female Cldn2–/– mice and fecal calcium content, was greater in Cldn2–/y mice (5.3% ± 4.2% of intake) than in WT mice (–2.3% ± 7.5% of intake; P < 0.05). As a consequence, net calcium balance was more positive in Cldn2–/y mice than in WT mice. On the calcium-deficient diet (Figure 3, D–F), fecal calcium exceeded dietary intake, so that there was net intestinal secretion of calcium, which was decreased in magnitude in the Cldn2–/y mice. Net calcium balance was markedly negative. Nevertheless, Cldn2–/y mice were still hypercalciuric compared with WT mice, indicating that a primary renal calcium leak had been unmasked.

Cldn2–/y mice have decreased colonic permeability to calcium. To elucidate the mechanism for increased net intestinal absorption of calcium in Cldn2–/y mice, the calcium permeability of different intestinal segments was determined from unilateral, serosal-to-mucosal 45Ca tracer flux assays in ex vivo everted gut sacs (Figure 4). Because there is no known active secretory mechanism for calcium in intestinal epithelium, the serosal-to-mucosal flux should represent passive and pre-

Figure 2. Hypercalciuria in Cldn2–/y mice is sensitive to dietary calcium intake. (A) Metabolic cage experiments show 24-hour urine calcium (Ca2+) excretion on control diet until day 5, after which half of the animals (n = 5–7 per group) are switched to a calcium-deficient diet (<0.01% calcium). At day 10, the experiment was terminated and serum was collected. (B) Fractional excretion of calcium (FECa) at day 10. (C–E) Serum Ca2+ (C), serum inorganic phosphorus (D), and serum intact parathyroid hormone (1–84) (E) at day 10. Bars are mean ± SEM. *P < 0.05, ****P < 0.0001 using 2-way ANOVA with Bonferroni’s correction for multiple comparisons.
had reduced permeability to calcium in comparison with WT animals (Supplemental Table 4).

The expression levels of intestinal epithelial transcellular transport proteins, including TRPV6, calbindin-D9k, and plasma membrane calcium ATPase-1, and of the intestinal paracellular cation channel proteins claudin-12 and -15, were no different between the genotypes (Supplemental Figure 2). Taken together with the calcium balance studies, these findings suggest that loss of claudin-2 leads to a reduction in paracellular permeability to calcium in the colon, and hence to a reduction in passive secretion of calcium in the distal intestine.

Cldn2−/− mice exhibit marked papillary nephrocalcinosis. Hypercalciuria is a major risk factor for nephrocalcinosis, the precipitation of calcium deposits within the kidney parenchyma and tubules, and the formation of kidney stones (8). We observed abundant mineral deposits in the renal papilla of 6-month-old Cldn2−/− mice that stained with von Kossa stain and alizarin red S at pH 4.2 (26), and alizarin red–stained deposits were birefringent under polarized light. These findings are suggestive of the presence of calcium phosphate (Figure 6, A–C). Micro–Fourier transform infrared spectroscopy (micro-FTIR) confirmed the composition as primarily calcium phosphate in the form of hydroxyapatite, with a small amount of calcium carbonate (Figure 6D). Transmission electron microscopy (TEM) revealed large, circular, laminated mineral deposits typical of hydroxyapatite (Figure 6E). These deposits appeared to be surrounded by a basement membrane, although no intact epithelium could be identified in the sections. There was scant mineral in WT kidneys at all age groups examined. None of the Cldn2 −/− mice had papillary deposits at 4.5 months, but all had developed papillary deposition at 6 months and 1 year (Figure 6, F and G). Additionally, Cldn2−/− females developed similar papillary deposits (Supplemental Figure 3).

We used a combination of von Kossa and immunofluorescence staining to determine the localization of these deposits. No deposits were found within inner medullary collecting ducts or the vasa recta (Figure 7, A and C). Occasional deposits were observed within the lumen of aquaporin-1–positive (AQP1-positive) descending thin limbs or kidney-specific chloride channel–positive (CLC-K–positive) ascending thin limbs of the loops of Henle (Figure 7, A and B, arrow). However, the vast majority of deposits did not associate with any epithelial or endothelial markers, suggesting either that they formed in the interstitium or that they originated within tubules that subsequently degenerated. The diameter of the calcium deposits on histological cross sections and micro-CT reconstructions was approximately 20 μm (Supplemental Figure 4), similar to the diameter of thin limbs of the loops of Henle (27). To detect earlier deposits, we examined the papillae of 5-month-old Cldn2−/− mice by TEM. Early calcium deposits in these mice were detected within lumina of intact tubules with type 4 cells of ascending thin limbs of the loops of Henle, identifiable by their extensive lateral interdigitations (Figure 7D), as well as type 3 cells of descending thin limbs of the loops of Henle, characterized by

**Figure 3. Calcium balance studies in Cldn2−/− mice and WT controls.** Mice were housed in metabolic cages for 3 days, during which urine and feces were collected for measurement of calcium, and dietary consumption determined by weighing of the food. (A–C) Normal-calcium (0.6%) diet (n = 11 per group). (D–F) Calcium-deficient (<0.01%) diet (n = 6–7 per group). (A and D) Total 3-day urinary calcium excretion. (B and E) Total 3-day intestinal calcium absorption (calcium consumed minus fecal calcium content). (C and F) Net calcium balance (intestinal absorption minus urinary excretion). Bars are mean ± SEM. *P < 0.05, ****P < 0.0001 using unpaired 2-tailed t test.
infrequent tight junction strands (ref. 27 and Figure 7E). This suggests that the deposits originate from tubular plugs in the descending and ascending thin limbs of the loops of Henle.

Common variants in the CLDN2 gene are associated with nephrolithiasis in the general population. The phenotype of claudin-2–knockout mice, with hypercalciuria due to both proximal tubular calcium wasting and increased intestinal calcium absorption, in conjunction with papillary deposition of hydroxyapatite in thin limbs of the loops of Henle, resembles the phenotype of patients with idiopathic hypercalciuria and kidney stone disease. We therefore hypothesized that genetic variants in CLDN2 might be associated with the risk of kidney stones in humans. To date, several genome-wide association studies (GWAS) of nephrolithiasis have been performed in the general population (4, 5, 28), but none of these studies have included the X chromosome in their analyses. To evaluate the role of CLDN2 in human kidney stones, we examined the association of 12 single-nucleotide polymorphisms (SNPs) in the CLDN2 locus with disease risk (29). In this analysis, a total of 11,130 kidney stone cases and 187,639 controls were analyzed (Supplemental Table 5). Nine SNPs showed significant association with chronic pancreatitis (30).

Our hypothesis predicts that causal variants for nephrolithiasis in CLDN2 should decrease claudin-2 function or tissue expression. All 9 positive SNPs were in the noncoding region. We therefore performed an analysis for cis-acting expression quantitative trait loci (eQTLs) in CLDN2 using the data set from the Genotype-Tissue Expression (GTEx) project. Seven of the SNPs associated with nephrolithiasis were genotyped in GTEx, and in all 7 cases, the nephrolithiasis risk allele was strongly associated with decreased claudin-2 expression in pancreatic tissue (to a highly statistically significant degree in 6 of the 7; Table 2). The number of kidney cortex samples in GTEx was insufficient to perform eQTL analysis of kidney expression. However, we know that CLDN2 has 3 alternative first exons and hence 3 alternative promoters. In the GTEx expression data, human kidney cortex and pancreas both exclusively express the same transcript (ENST00000540876.1) and therefore use the same promoter. Thus, it is highly likely that transcriptional regulation by cis-acting eQTLs in these 2 tissues is identical. Interestingly, the alleles in CLDN2 that are associated with increased risk of pancreatitis (30) are all associated with increased claudin-2 expression in the pancreas, and with decreased risk for nephrolithiasis (Supplemental Figure 5).

Rare missense mutation in CLDN2 causes hypercalciuria and kidney stones. We recently reported a family of Iranian origin with 9 infertile men over 3 generations (31). Five of these males were diagnosed with obstructive azoospermia due to a rare missense variant in CLDN2 (p.Gly161Arg) (Supplemental Figure 6). All 5 patients had a history of kidney stones (one of which, I.7, was known to have passed a calcium stone), whereas none of the 9 unaffected adult males who participated in the study had ever had kidney stones (P = 0.0002 by chi-squared test). All 5 patients were markedly hypercalciuric (Table 3), with 24-hour urine calcium excretion of 390 ± 18 mg (mean ± SD; normal male <300 mg). Daily urine calcium in a female carrier was 274 mg (normal <250 mg). By contrast, urine calcium excretion was normal in 2 unaffected male siblings (223 and 270 mg).

Discussion

In this study, we tested the hypothesis that loss of claudin-2 leads to defective PT calcium reabsorption, thereby increasing the distal delivery of calcium to the loops of Henle and predisposing mammals to nephrocalcinosis, and hence kidney stone disease. We showed that constitutive loss of claudin-2 in mice leads to hypercalciuria attributable to both a renal leak of calcium and increased net intestinal calcium absorption, the latter being due to impaired colonic calcium secretion. We identified multiple common CLDN2 SNPs that associate with modestly increased risk of kidney stone disease and are significant cis-acting eQTLs in human tissue. Finally, we identified one family with a rare missense mutation in CLDN2 that is strongly associated with both hypercalciuria and kidney stones. Our results suggest that claudin-2 plays a pathogenic role in idiopathic hypercalciuria and kidney stone disease.

PT calcium reabsorption is passive and follows sodium and water reabsorption. We and others have shown that claudin-2 is highly expressed in the PT (21, 32), that it functions as a paracel-
Calcium reabsorption, claudin-2 deletion also causes a primary renal calcium leak and that claudin-2 is the mediator of paracellular calcium reabsorption in the PT. Micro-puncture experiments will be needed to confirm this.

In addition, we found that Cldn2–/− mice have a more positive calcium balance and an exaggerated reduction in urinary calcium excretion with dietary calcium restriction. These findings were unexpected and suggest that, in addition to a reduction in renal calcium reabsorption, claudin-2 deletion also causes a primary increase in net calcium absorption in the gastrointestinal tract. The phenotype of Cldn2–/− mice resembles that of patients with idiopathic hypercalciuria that are kidney stone formers, who often exhibit both intestinal hyperabsorption of calcium and reduced renal calcium reabsorption (14).

To determine the mechanism for increased net intestinal absorption of calcium, we measured unidirectional $^{45}$Ca flux in the presence of symmetrical solutions. We found no difference in fluxes in the duodenum and ileum, but a marked decrease in both serosal-mucosal and mucosal-serosal flux in the colon. This indicates that the colon has decreased passive permeability to calcium. Our findings were confirmed by measurement of permeability from equilibrium potentials in Ussing chambers, and are partially consistent with the observation by Tamura et al. that the small intestines of claudin-2–knockout animals have reduced $P_{\text{ab}}$ (34). Furthermore, we found no difference in active calcium absorption in the duodenum (not shown) and no difference in the expression of transcellular calcium transport proteins.

It is known that calcium transport exhibits a proximal-to-distal gradient. In the proximal small intestine, calcium is absorbed both actively (via vitamin D–dependent transcellular mechanisms) and passively, whereas in more distal segments, and particularly in the colon on a normal calcium diet, transport is predominantly passive and the net direction of flux becomes increasingly secretory. This has been demonstrated both in vitro Ussing chamber experiments (35), and by measurements in intestinal segments perfused in vivo (36). That the electrochemical driving force for passive calcium diffusion favors secretion is explained by 2 observations: (a) The soluble fraction of total calcium in intestinal contents is surprisingly low (averaging ~3%) and decreases in distal segments because of the high luminal pH (37); and (b) the transepithelial voltage is lumen-negative throughout the intestine and greatest in magnitude in the colon, with measured values ranging from ~20 mV to ~60 mV (38, 39).

Thus, the preponderance of evidence suggests that normal intestinal absorption of calcium is accompanied by a continuous passive backleak of calcium into the lumen in the colon. Our findings indicate that this backleak occurs paracellularly through claudin-2 channels in the colon. In Cldn2–/− mice, the colonic calcium permeability is decreased, and so there is greater net calcium absorption overall by the gut.

Another important similarity between Cldn2–/− mice and recurrent kidney stone formers is the presence of calcium deposits in the renal papilla. We show that claudin-2 deletion leads to papillary nephrocalcinosis in mice. In humans, nephrocalcinosis is appreciable on CT scan in approximately one-sixth of CaOx stone formers and three-quarters of hydroxyapatite stone formers (40). Randall’s plaques, which are papillary hydroxyapatite deposits found in the basement membranes of thin ascending limbs and the interstitium, are found in 65%–75% of stone formers and are a precursor to the most common stones, composed of CaOx (41). Twenty-five percent to 35% of stone formers have intratubular plugs of calcium, typically in the inner medullary collecting ducts and the ducts of Bellini, and these patients typically form brushite and hydroxyapatite stones (42). Plugs in the thin limb have also been observed in stone formers, most notably in patients with ileostomy, primary hyperoxaluria type I, and cystinuria (42). Interestingly, a recent study of kidneys removed for cancer suggested that tubular plugs are common even in individuals without kidney stones (43). These intratu-
bular plugs were composed of calcium phosphate and located within AQP1-negative loops of Henle in the papilla (43), consistent with both the lowermost portions of descending thin limbs and the ascending thin limbs (44). It has been hypothesized that medullary tubule plugging might even precede and precipitate plaque formation and subsequent stone growth (45–47), though this remains unproven. The nephrocalcinosis that we observe in Cldn2–/y mice resembles the medullary plugging within the loops of Henle found in some human kidney stone patients. Because Cldn2–/y mice and kidney stone formers with idiopathic hypercalciuria (14) have in common a defect in PT calcium reabsorption, we propose that the site of the defect uniquely predisposes to papillary nephrocalcinosis by increasing the delivery of calcium to Henle’s loop (44). It has been hypothesized that medullary tubule plugging might even precede and precipitate plaque formation and subsequent stone growth (45–47), though this remains unproven. The nephrocalcinosis that we observe in Cldn2–/y mice resembles the medullary plugging within the loops of Henle found in some human kidney stone patients.

Because Cldn2–/y mice and kidney stone formers with idiopathic hypercalciuria (14) have in common a defect in PT calcium reabsorption, we propose that the site of the defect uniquely predisposes to papillary nephrocalcinosis by increasing the delivery of calcium to Henle’s loop. In a similar scenario, reabsorption of water in the thin descending limb is predicted to increase tubular calcium and phosphate levels to the point of supersaturation by the bend of the loop (48). In addition, the medullary thick ascending limb is proposed to compensate for the PT defect by increasing reabsorption of luminal calcium, which could then enter the descending vasa recta by passive diffusion and be returned to the inner medulla and papilla (dubbed “vas washdown”), thereby generating an axial calcium gradient in the medullary interstitium (49, 50). This mechanism is consistent with the recent finding of preferential deposition of plaques in the basement membrane of thin ascending limbs (10), and with the predictions of a recent mathematical model (51), lending it further credence. Overall, these 2 processes could explain how defective PT calcium reabsorption causes both intratubular plugging and interstitial plaques in the papilla, and suggest that Cldn2–/y mice may be a useful model for studying the complex pathogenesis of human kidney stone disease.

The similarities between Cldn2–/y mice and kidney stone formers led us to test the hypothesis that genetic variants in the CLDN2 locus might predispose to kidney stone disease. In our population-based study, we identified 9 SNPs that are associated with nephrolithiasis, all of which are common variants. Cis-acting eQTL analysis revealed that 6 of the 7 risk variants available for analysis share a strong association with reduced claudin-2 tissue expression. Our findings provide strong evidence that genetic variants in the claudin-2 gene that decrease its tissue expression are causal in kidney stone disease. Unexpectedly, the non-risk alleles of several of these SNPs have been identified previously as risk alleles for alcohol-related pancreatitis and increased pancreatic claudin-2 expression. The strong reciprocal relationship between risk variants for these 2 diseases and tissue expression levels increases the likelihood that these represent true causal disease associations.

The pathogenic link between claudin-2, calcium excretion, and kidney stone disease is further corroborated by our finding of a family with a very rare missense mutation in CLDN2, in which
the affected males are afflicted with both hypercalciuria and kidney stone disease.

Currently, treatments for the prevention of idiopathic hypercalciuria and nephrolithiasis are limited. These patients are often treated with thiazide diuretics, which induce hypocalciuria in part by stimulating PT calcium reabsorption (9, 52). While affordable and relatively safe, thiazide diuretics are often contraindicated in patients because of off-target side effects. Identification of claudin-2 regulatory pathways may help to identify potential pharmacologic strategies to increase renal claudin-2 expression. We speculate that such a strategy will be effective in the prevention of kidney stones.

Methods

Animal and metabolic balance studies. Mice with global, constitutive knockout of the claudin-2 gene (Cldn2<sup>m1Lex/Mmcd</sup>) were originally generated by targeted deletion of the coding exon of Cldn2 and backcrossed onto the C57BL/6J background, as described previously (23). Male mice were used for all experiments, except where indicated otherwise. This is due to the location of the Cldn2 gene on the X chromosome, which precludes generation of WT and knockout female littermates. Baseline measurements of urine and serum parameters were performed on 15- to 17-week-old mice fed standard lab chow (Teklad Rodent Diet 8604, Envigo). Urine was collected by spontaneous voiding onto a Parafilm mat. Mice were then anesthetized with xylazine (15 mg/kg i.p.) and ketamine (150 mg/kg), and blood was collected by cardiac puncture. To avoid possible interference of ketamine with PTH measurements, blood for this assay was collected from the submandibular vein of unanesthetized 16-week-old mice into EDTA plasma tubes. For urine and serum studies listed in Supplemental Figure 3, 17- to 26-week-old mice were used.

Metabolic balance studies were performed using mice between 14 and 18 weeks of age. Control (0.6% calcium, TD.97191) and calcium-deficient (≤0.01% calcium<sup>+</sup>, TD.95027) diet was purchased in powdered form (Envigo) and prepared as a soft agar diet using 1% Difco Noble agar (BD Biosciences) in a ratio of 5:8 wt/vol diet to water. Mice were first acclimated in metabolic cages (MMC100, Hatteras Instruments) for 48 hours with free access to food and water. Control diet was started at this time (day –4). At day –2, mice were returned to conventional housing for 2 days of recovery. At day 0, mice were returned to metabolic cages for collection of urine and feces. Food weight, water weight, and urine volume were recorded each day, and mice were removed for approximately 10 minutes each day during collection of urine, feces, and cleaning of cages. At day 5, a subset of mice was switched to the low-calcium diet. At the completion of the study, serum was collected by cardiac exsanguination.

Serum, urine, and fecal analysis. Urine was collected in 2 separate tubes. HCl (final 0.3 N) was added to 1 aliquot for calcium determination. Both aliquots were then centrifuged at 5000 g for 1 minute and the supernatant collected. Feces were collected in glass vials, and calcium was extracted by wet ashing. Briefly, 2:1 nitric/perchloric acid was added to each sample (3 mL total) and heated at 95°C until dissolved completely (~7 hours), then serially diluted to a total volume of 400 mL. Serum, urine, and fecal calcium was measured by a colorimetric assay (Quantichrom, BioAssay Systems). Urine creatinine (Cr) was measured by the Jaffe reaction (Cayman Chemical). Serum creatinine (Cr) was measured by the University of Texas Southwestern Medical...
Table 2. CLDN2 gene variants associated with nephrolithiasis

<table>
<thead>
<tr>
<th>Reference SNP ID</th>
<th>Position</th>
<th>Major allele</th>
<th>Minor allele</th>
<th>MAF</th>
<th>OR</th>
<th>P value</th>
<th>NES</th>
<th>GTEx</th>
<th>P value</th>
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<td>Intron</td>
<td>T</td>
<td>C</td>
<td>0.4848</td>
<td>1.029</td>
<td>0.0152</td>
<td>−0.48</td>
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<td>T</td>
<td>0.1099</td>
<td>0.917</td>
<td>0.0113</td>
<td>0.13</td>
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<td>T</td>
<td>0.231</td>
<td>1.0303</td>
<td>0.0200</td>
<td>−0.45</td>
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<td>A</td>
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<td>A</td>
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<td>0.915</td>
<td>0.0085</td>
<td>0.15</td>
<td>5.7 × 10⁻¹</td>
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<td>T</td>
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<tr>
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<td>G</td>
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<td>rs14782672</td>
<td>3′-UTR</td>
<td>C</td>
<td>A</td>
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<td>0.927</td>
<td>0.0080</td>
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</table>

MAF, minor allele frequency (1000 Genomes project). OR, odds ratio for nephrolithiasis with minor allele as the reference allele. When major allele is associated with disease risk, OR is >1. METAL P value, P value for association of variants with nephrolithiasis by meta-analysis of 2 independent GWAS cohorts. NES, normalized effect size of the eQTL in GTEx, defined as the slope of the linear regression between genotype and expression (effect of the risk allele relative to the alternative allele). Negative values indicate that the major allele is associated with decreased tissue expression in pancreas. GTEx P value, P value for the test of the hypothesis that the slope of the linear regression between genotype and expression deviates from 0. Blue text indicates loci where the major allele is associated with increased risk of nephrolithiasis (OR > 1.0) and with decreased tissue expression (NES < 0); red text indicates loci where the major allele is associated with decreased risk of nephrolithiasis and with increased tissue expression.

Center O’Brien Center Kidney Physiology Core by capillary electrophoresis. FECa²⁺ was calculated as

\[
(\text{urine Ca}^2+ \times \text{serum Cr}) / (0.5 \times \text{serum Ca}^2+ \times \text{urine Cr})
\]

(Equation 1)

A correction factor of 0.5 was used to estimate the ionized fraction of serum calcium (53). Plasma intact PTH (Immunotubes Mouse Intact PTH 1-84) and 1,25-dihydroxyvitamin D (Immunodiagnostic Systems) were measured by ELISA. Urine was also assayed for oxalate (EnzyChrom, BioAssay Systems), citrate (EnzyChrom, BioAssay Systems), magnesium (Quantichrom, BioAssay Systems), and inorganic phosphorus (Pointe Scientific).

Quantitative reverse transcription PCR. Whole tissue RNA was extracted with TRI Reagent (Sigma-Aldrich). First-strand cDNA iScript Reverse Transcription Supermix for quantitative reverse transcription PCR (RT-qPCR) (Bio-Rad) was used for first-strand cDNA synthesis. For kidney and duodenal tissue, a CFX96 Touch Real-Time PCR Detection System and iTag Universal SYBR Green SuperMix (Bio-Rad) were used for quantitative PCR using the primers listed in Supplemental Table 7. mRNA quantitation of proximal colon, ileum, and jejunum was performed by TaqMan assay using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) and primers sequences previously reported (54). Expression levels were normalized to 1 of 3 housekeeping genes, β-actin, ezrin, or GAPDH.

Immunoblotting. Kidney tissue was minced with a razor blade and collected in protein isolation buffer composed of 20 mM HEPES (pH 7.4), 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA with protease inhibitors (cOmplete Mini, Roche Diagnostics). Each sample was homogenized for 15-second increments every minute for 5 minutes at a low-speed setting with an Ultra-Turrax T25 (IKA-Labortechnik) and then centrifuged at 1800 g for 10 minutes. Lysates were heated at 95°C for 10 minutes in reducing SDS-PAGE buffer and loaded into a polyacrylamide gel. Protein was then transferred to a PVDF membrane and blocked using 5% nonfat dry milk. Primary antibody incubation was performed overnight at 4°C using the following antibodies: mouse anti-calbindin-D28k (1:1000; Swant 300), mouse anti-β-claudin-2 (1:500; Thermo Fisher Scientific 32-5600), and mouse anti-β-actin (1:1000; Sigma-Aldrich A2066). HRP-conjugated secondary antibodies were incubated at a concentration of 1:5000 (GE Healthcare Bio-Sciences) and bands detected using chemiluminescence (Pierce).

Quantitation of densitometry was achieved using ImageJ (NIH) and normalized to β-actin.

Histological analysis. Six-month-old mice fed standard lab chow were anesthetized using ketamine and xylazine, and cardiac perfusion fixation was performed using 4% paraformaldehyde (PFA) in PBS. Kidneys were postfixed in 4% PFA for 4 hours at room temperature. Initial calcium staining was performed on paraffin-embedded sections that were deparaffinized and rehydrated. For von Kossa staining, sections were incubated in 5% aqueous silver nitrate for 1 hour under UV light, and 5% sodium thiosulfate for 1 minute. For alizarin red S staining, tissues were placed in 2% alizarin red S (pH 4.3) for 1–3 minutes.

Immunofluorescence was performed on frozen sections. Fixed kidneys were cryoprotected in sucrose and embedded in OCT compound, and 5-µm sections were cut. Slides were first stained using the von Kossa technique. Antigen retrieval was then performed with 10 mM sodium citrate in a steamer for 6 minutes. Aldehyde quenching was accomplished by incubating in 0.3 M glycine in PBS; then slides were blocked for 1 hour in PBS with 5% normal goat serum, 1% BSA, and 0.3% Triton X-100. The primary antibodies used were CLEK-K (1:200; Alomone Labs ALC-0044), AQP1 (1:500; Abcam ab9566), AQP2 (1:500; a gift from Mark Knepper, National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA), MEGA32 (1:100; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, USA). Lipofuscin was quenched with 0.1% Sudan Black B in 70% ethanol for 15 minutes, before application of secondary antibodies (Alexa Fluor 488– and 555–conjugated goat anti-rabbit and anti-mouse IgG, 1:1000; Thermo Fisher Scientific). Confocal fluorescent images were captured along with bright-field images for von Kossa stain (Leica TCS...
SPE microscope). Von Kossa images were pseudocolored, then overlaid onto the fluorescent images using ImageJ (NIH).

**Micro-CT analysis of kidneys.** Kidneys from 6-month-old mice were fixed and ethanol-dehydrated and then scanned with a high-resolution micro-CT scanner (μCT40, Scanco Medical) as previously described (55). Data were acquired at 55 keV and 6 μm cubic resolutions. Renal calculations were assessed with a threshold of 220.

**Bone measurements.** Dual-energy x-ray absorptiometry (DEXA; Lunar PIXimus, GE Medical Systems) was used to measure bone mineral density in anesthetized mice (0.25× ketamine/xylazine cocktail) at 4.7, 6, 8, and 10 weeks of age. Two different sites were determined by adjustment of the region of interest: femur and lumbar vertebrae. Some animals were fed a pelleted calcium-deficient diet (TD.95027, Envigo) at 12 weeks of age for 4 weeks. During the low-calcium diet, measurements were taken at 12, 13, 14, 15, and 16 weeks of age. Micro-CT analysis was performed on femurs from 10-week-old animals as described previously (55).

**Transmission electron microscopy.** Renal papillae were dissected from fresh kidneys, fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour. They were then dehydrated in ethanol and propylene oxide and embedded in EMbed 812 resin (Electron Microscopy Sciences). Cross sections were cut through the papilla block at 80 nm thickness and picked up on 250-mesh copper grids. Next, blocks were sectioned 10 μm deeper, and the same sectioning process was repeated twice. These thin sections were then contrasted with 3% uranyl acetate aqueous and Sato’s lead stain. Grids were viewed at 100 kV in a JEOL JEM 1400 transmission electron microscope, and images were captured as TIFF files with an AMT camera.

**Micro–Fourier transform infrared spectroscopy.** Kidney sections (~5 μm) were mounted on low-E glass slides (Kevley Technologies) for attenuated total internal reflection (ATR) imaging analysis. A serial section stained with Yasue silver replacement was used as a control section. Before infrared analysis, the control was visually examined with an Olympus white light microscope (×20 objective) to determine the areas of interest. Sections for ATR-FTIR imaging were not stained. ATR infrared images were collected with a PerkinElmer Spectrum Spotlight 400 infrared imaging microscope interfaced to a PerkinElmer FTIR spectrometer, as described previously (11). Each image (400 × 400 μm area) had a spatial resolution of 1.56 μm/pixel and contained 65,746 infrared spectra collected at a spectral resolution of 8 wavenumbers. Each spectrum in the image is the average of 4 individual scans. The images were further processed using Spectrum Image software (PerkinElmer).

**Everted gut sac assays.** Ex vivo everted intestinal sac assays were performed by a modification of previously published methods (56, 57). Intestinal segments from the duodenum (defined as the first 4 cm of small intestine), ileum, and colon were excised, perfused with PBS to remove any fecal matter, and everted over a wetted glass rod. They were then filled with 0.4 mL of solution (serosal side) and ligated at both ends so that the final length of the sealed sac was approximately 6 cm. Sacs were placed in individual Erlenmeyer flasks and bathed with 5 mL of solution (mucosal side) continuously bubbled with 100% O2, and stirred by vigorous shaking in a 37°C water bath. The composition of both serosal and mucosal solutions was identical and consisted of 125 mM NaCl, 10 mM fructose, 30 mM Tris-Cl (pH 7.4), and either 0.25 mM CaCl2 (low calcium) or 5.0 mM CaCl2 (high calcium). For unidirectional tracer flux studies, 45CaCl2 was added to a final activity of 16.7 μCi/L to the solution on one side (cis). For serosal-to-mucosal flux assays, 45CaCl2 was included in the serosal solution at a final activity of 16.7 μCi/L. Samples were collected from the mucosal solution at 30-minute intervals up to 90 minutes, for liquid scintillation counting. For mucosal-to-serosal flux assays, 45CaCl2 was included in the mucosal solution at the same final activity as above. After 60 minutes, the

### Table 3. Urine calcium and kidney stone disease in a family with X-linked obstructive azoospermia due to CLDN2 mutations

<table>
<thead>
<tr>
<th>Subject IDa</th>
<th>Age when studied</th>
<th>Sex</th>
<th>CLDN2 mutation</th>
<th>Male infertility</th>
<th>Urine total volume (mL/24 h)</th>
<th>Urine calcium (mg/24 h)</th>
<th>Kidney stones</th>
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<tr>
<td>I.4</td>
<td>48</td>
<td>M</td>
<td>Unknown</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>I.5</td>
<td>60</td>
<td>M</td>
<td>G161R</td>
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<td>1300</td>
<td>364</td>
<td>Y</td>
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<tr>
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<tr>
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<tr>
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*aIncludes all affected patients and unaffected family members who had ascertainment of kidney stone history. Subject ID is generation-individual number (please refer to pedigree chart in Supplemental Figure 6).
and apical buffer (3.6 mM K⁺, 146 mM Cl⁻, 1 mM Mg²⁺, 1.3 mM Ca gluconate, 2 mM PO₄³⁻, pH 7.4) at 37°C and bubbled with 5% CO₂ (balance O₂). After 15 minutes, a 90-μA current was pulsed across the tissue 3 times for 4 seconds, and the resulting voltage was used to determine transepithelial resistance by Ohm’s law. The apical solution was then changed to a low-NaCl solution (30 mM Na⁺, 3.6 mM K⁺, 32 mM Cl⁻, 1 mM Mg²⁺, 1.3 mM Ca gluconate, 2 mM PO₄³⁻, pH 7.4). The resulting peak change in transepithelial voltage was used to determine the permeability ratio of Na⁺ to Cl⁻ (PNa/PCl) and absorbance to Na⁺ using the Goldman-Hodgkin-Katz and simplified Kimizuka-Koketsu equations (58, 59). The apical buffer was then exchanged for the control buffer. When the voltage stabilized, transepithelial resistance was determined as above. Bi-ionic diffusion potential was then used to assess permeability to calcium (Pcₐ) by exchanging the basolateral buffer (140 mM Na⁺, 3.6 mM K⁺, 146 mM Cl⁻, 1 mM Mg²⁺, 1.3 mM Ca gluconate, 5 mM mannitol, pH 7.4) and apical buffer (3.6 mM K⁺, 146 mM Cl⁻, 1 mM Mg²⁺, 70 mM Ca²⁺, 3 mM HEPES, pH 7.4) simultaneously and recording peak change in transepithelial voltage. Both buffers were then exchanged for the control buffers, and transepithelial resistance was determined as above when voltage stabilized. A change in resistance of no more than 25% was used to determine tissue viability (54). To further test tissue viability, forskolin (0.1 μM) was applied to both chambers and a potential difference measured. All samples included for analysis demonstrated a decrease of at least 50% of all changes in potential were corrected for voltage stabilization. A decrease of at least 50% was used to determine tissue viability. Studying intestinal epithelial cells in a normalized space, so its magnitude has no direct biological interpretation.

Family-based genetic study. A family of Iranian origin with obstructive azoospermia, which has previously been reported (31), was enrolled at the Infertility Clinic & Reproductive Biomedicine Research Center of Royan Institute, Tehran, Iran. A history was obtained from all participants to ascertain prior kidney stone events, and 24-hour urine collections were obtained from selected individuals to determine urinary calcium excretion. The genotypes of family members for CLDN2 loci associated with kidney stone risk were assessed by logistic regression. Covariates used in each study were age and principal components 1-10 (71).

cis eQTL analysis. SNPs associated with kidney stone risk were queried to identify cis-acting eQTLs. Analysis was performed with FastQTL using Release V7 of the Genotype-Tissue Expression (GTEx) project data set (74) (https://gtexportal.org/; accessed February 16, 2018). Nominal P values were generated for each CLDN2 gene variant by testing of the alternative hypothesis that the slope of a linear regression model between genotype and claudin-2 expression, in pancreas samples from 220 individuals, deviates from 0. The normalized effect size of the eQTLs, defined as the slope of the linear regression, was computed in a normalized space, so its magnitude has no direct biological interpretation.
the University of Kansas Medical Center or the University of Alber-
ta animal ethics committee, Health Sciences Section. For the genetic
studies, the study protocols conformed to the Declaration of Helsinki,
and all human subjects provided written informed consent. The pop-
ulation-based genome association study was approved by the ethical
committee at the University of Tokyo. The family-based genetic study
was approved by the Institutional Review Board of the Royan Institute
Research Center and the Royan Ethics Committee, Tehran, Iran.

Author contributions
JNC and ASLY designed the overall study and drafted the manu-
script. JNC, MS, LP, and MBF conducted the main set of mouse
experiments. MRB and RTA conducted the Ussing chamber stud-
ies. AJ’S conducted the micro-FTIR analysis. PSNR assisted with
micro-CT and DEXA experimental design and analysis. TF assisted
with interpretation of the histological studies. APE assisted in
the analysis and interpretation of nephrocalcinosis data. KM, YK,
and CT designed and conducted the genome association data anal-
ysis. MA and MT performed the family genetic study. All authors
reviewed and edited the manuscript and approved the final draft.

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ing Research Council of Canada Discovery Grant RGPin 05842 (to
RTA). The Genotype-Tissue Expression (GTEx) project is suppor-
ted by the NIH Common Fund and by NCI, NHGRI, NHLBI, NIDA,
NIMH, and NINDS. The University of Kansas Medical Center Elec-
tron Microscope Research Laboratory and Confocal Imaging Core
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of Texas Southwestern O’Brien Kidney Center Research Core is
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Rainbow Boulevard, Kansas City, Kansas 66103, USA. Email:
ayu@kumc.edu.

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