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

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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.

Introduction

Kidney stone disease is common, with a lifetime risk in 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). 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-
and hence to exhibit a compensatory increase in the calcitropic 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 hypercalciuria 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 hypercalciuria 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, FECA⁺ in Cldn2⁻/⁻ mice was approximately 5 times higher than in WT mice (1.37% vs. 0.28%) (Figure 2B). In contrast, the FECA⁺ 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 hypercalciuria 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 and fecal calcium, was increased by a factor of 2.5 in Cldn2⁻/⁻ mice compared with WT mice (0.65% vs. 0.26%, respectively) (Figure 3C). This suggests that the increased net intestinal calcium absorption in Cldn2⁻/⁻ mice is in part due to a reduction in the renal calcium leak. We found no evidence for compensatory changes in expression of calcium transporters in more distal segments of the nephron. We previously showed that expression of claudin-16, claudin-19, and the Na-K-2Cl cotransporter (NKCC2) is unchanged (23).

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).

### Results

**Deletion of Cldn2 in mice causes hypercalciuria due to defective renal tubular calcium reabsorption.** We first confirmed that claudin-2-knockout mice (Cldn2⁻/⁻) are hypercalciuric. FECA⁺ 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 hypercalciuric 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 hypercalciuric (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI127750DS1). We found no evidence for compensatory changes in expression of calcium transporters in more distal segments of the nephron. We previously showed that expression of claudin-16, claudin-19, and the Na-K-2Cl cotransporter (NKCC2) is unchanged (23).

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).
and fecal calcium content, was greater in Cldn2<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice. Net calcium balance was markedly negative. Nevertheless, Cldn2<sup>−/−</sup> mice were still hypercalciuric compared with WT mice, indicating that a primary renal calcium leak had been unmasked.

Cldn2<sup>−/−</sup> mice have decreased colonic permeability to calcium. To elucidate the mechanism for increased net intestinal absorption of calcium in Cldn2<sup>−/−</sup> 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 1. Bone mineral metabolism is normal in Cldn2<sup>−/−</sup> mice. Bone analysis of WT (blue) and Cldn2<sup>−/−</sup> (red) animals on a standard chow diet. (A and B) Total (A) and lumbar (B) bone mineral density (BMD) was measured using DEXA at 4.7, 6, 8, and 10 weeks (n = 14–18 per group). A group of these animals was started on a low-calcium diet for 4 weeks and BMD measured weekly (n = 4 per group). (C) Representative micro-CT reconstructions of femurs showing normal cortical and trabecular bone in Cldn2<sup>−/−</sup> mice. (D and E) Total BMD (D) and total bone mineral content (BMC) (E) were measured in 1-year-old animals by DEXA (n = 5 per group). There were no significant differences between groups using unpaired 2-tailed t test. Bars are mean ± SEM.

Figure 2. Hypercalciuria in Cldn2<sup>−/−</sup> mice is sensitive to dietary calcium intake. (A) Metabolic cage experiments show 24-hour urine calcium (Ca<sup>2+</sup>) 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–E) Serum Ca<sup>2+</sup> (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–/y_ 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–/y_ 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–/y_ 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).
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 hypercalcuria 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 hypercalcuria 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 disease risk \( \chi^2 = 0.0002 \) by \( \chi^2 \) test. All 5 patients were marked-

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A – B PNa/PCl (Cldn2–/y), diet, despite being in negative calcium balance. This suggests that ciuric, relative to WT littermates, after 5 days on a calcium-deficient that deletion of claudin-2 causes a reduction in P Na in PTs and an um as well as sodium (20). It was previously shown by Muto et al. lular cation channel (19, 20, 33), and that it is permeable to calci-Figure 5. Ussing chamber measurements of intestinal ion permeability. (A) Transepithelial resistance (TER). (B–D) Pm/Pm, (B), Pm/Pm, (E), and Pm/Pm, (D) as determined from dilution potential measurement. (E and F) Pm/Pm, (E) and Pm, (F) as determined from bi-ionic potentials (n = 7 per group). Individual ion permeabilities were estimated by the method of Kimizuka and Koket-su. Bars are mean ± SEM. *P < 0.05, **P = 0.005 using unpaired 2-tailed t test. Pm, transepithelial permeability to X.
lular cation channel (19, 20, 33), and that it is permeable to calcium as well as sodium (20). It was previously shown by Muto et al. that deletion of claudin-2 causes a reduction in Pm in PTs and an increase in FECa2+, indicating that renal tubule calcium reabsorption is decreased (22). Our work confirms that Cldn2–/y mice have a large decrease in renal calcium reabsorption and indicates that this cannot be explained by reduced expression of calcium transporters in the distal nephron. We found that Cldn2–/y mice remain hypercalciuric, relative to WT littermates, after 5 days on a calcium-deficient diet, despite being in negative calcium balance. This suggests that Cldn2–/y mice have 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–/y 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–/y 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 45Ca 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 Pm, (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 by 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–/y mice, the colonic calcium permeability is decreased, and so there is greater net calcium absorption overall by the gut.

Another important similarity between Cldn2–/y 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 intra-
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. In such a 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 expression in human tissue. 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...
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+, 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 was measured by the Jaffe reaction (Cayman Chemical). Serum creatinine (Cr) was measured by the University of Texas Southwestern Medical 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>tm1Lex/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+, 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.

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Table 2. CLDN2 gene variants associated with nephrolithiasis

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<td>C</td>
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<td>3′-UTR</td>
<td>C</td>
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<td>0.927</td>
<td>0.0080</td>
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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 those 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; 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 (ImmunoTec Mouse Intact PTH 1–84 and 1,25-dihydroxyvitamin D (ImmunoDiagnostics 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 iQ 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 primer 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–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 CLC-K (1:200; Alomone Labs ACL-004), AQP1 (1:500; Abcam ab9566), AQP2 (1:500; 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.

Reference SNP ID: Reference SNP identifier.
Position: Position of the SNP in the gene.
Major allele: The major allele of the SNP.
Minor allele: The minor allele of the SNP.
MAF: Minor allele frequency (1000 Genomes project).
METAL: METAL P value, P value for association of variants with nephrolithiasis by meta-analysis of 2 independent GWAS cohorts.
P value: P value for association of variants with nephrolithiasis.
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 those 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; red text indicates loci where the major allele is associated with decreased risk of nephrolithiasis and with increased tissue expression.

**The Journal of Clinical Investigation**
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 ID</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>
</tr>
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<tr>
<td>I.4</td>
<td>48</td>
<td>M</td>
<td>Unknown</td>
<td>Y</td>
<td>–</td>
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<td>Y</td>
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<tr>
<td>I.5</td>
<td>60</td>
<td>M</td>
<td>G161R</td>
<td>Y</td>
<td>1300</td>
<td>364</td>
<td>Y</td>
</tr>
<tr>
<td>I.7</td>
<td>51</td>
<td>M</td>
<td>G161R</td>
<td>Y</td>
<td>1000</td>
<td>390</td>
<td>Y</td>
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<td>N</td>
</tr>
<tr>
<td>I.10</td>
<td>65</td>
<td>F</td>
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<td>700</td>
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<tr>
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<tr>
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<td>52</td>
<td>M</td>
<td>G161R</td>
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<td>415</td>
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<tr>
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<tr>
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<tr>
<td>III.19</td>
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<tr>
<td>III.29</td>
<td>36</td>
<td>M</td>
<td>G161R</td>
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<tr>
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<td>M</td>
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<td>N</td>
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<td>–</td>
<td>N</td>
</tr>
</tbody>
</table>

*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)."
sac was taken out, washed briefly by submerging twice in 150 mL of ice-cold solution, and blotted dry, and its contents emptied and sampled for scintillation counting. In each case, the transepithelial flux and permeability were determined from the initial rate of ⁴⁰Ca accumulation in the trans compartment.

**Ussing chamber permeability assays.** Proximal colon (1.2 cm distal to the ileocecal junction) was excised from claudin-2-knockout mice and WT littermates (9–13 weeks old) and linearized. Whole-thickness preparations were initially used. To test the contribution of the seromuscular layer, unstripped preparations were compared with preparations in which the seromusculature was visualized with a dissecting microscope (Olympus SZ60, Olympus America) and gently stripped away with a pair of fine forceps. The colon segments were then mounted in P2407B sliders (1.2-mm-diameter circular aperture) and placed into P2400 Ussing chambers connected to a VCC Multichannel Voltmeter (Physiologic Instruments). Both hemichambers were filled with 4 mL modified Krebs-Ringer buffer (“control” buffer) (144 mM Na⁺, 3.6 mM K⁺, 146 mM Cl⁻, 1 mM Mg²⁺, 1.3 mM Ca gluconate, 2 mM PO₄⁻, 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 PO₄⁻, pH 7.4). The resulting peak change in transepithelial voltage was used to determine the permeability ratio of Na⁺ to Cl⁻ (PNa/PCl) and absolute permeability 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 increase 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.

**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.

**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 the CLDN2 missense variant, c.481G>C (p.Gly161Arg), determined by Sanger sequencing, were previously reported (31). For each study, we stratified samples into male and female groups, and analyzed the association of SNPs with kidney stone risk separately, then conducted a meta-analysis of the combined male and female data using METAL (72). The effect of allelic dosage was modeled as 0 or 2 in males and 0, 1, or 2 in females (i.e., dosage compensation for X inactivation). A meta-analysis of study 1 and study 2 was then conducted. Heterogeneity across each pair of studies was examined using Cochran’s Q test (73).

**Biological interpretation.** SNPs were excluded if they met the following criteria: MAF < 0.005; Hardy-Weinberg equilibrium P value < 1 × 10⁻⁶; call rate = 0 (study 1) or < 0.01 (study 2). Imputation of the ungenotyped SNPs was conducted by MaCH (68) and minimac (69) using the data from the Japanese in Tokyo (JPT), Han Chinese in South, China (CHS), and Chinese in Denver, Colorado, USA (CHD) and using the 1000 Genomes project phase 1 (release 16, March 2012) as a reference. We excluded SNPs with a large allele frequency difference between the reference panel and the GWAS (>0.16) as described previously (70). We also excluded SNPs with low imputation quality score (Rsq < 0.3) and insertion/deletion polymorphisms. Finally, we selected 12 SNPs within CLDN2 loci for association analysis. Association of these SNPs with kidney stone risk was assessed by logistic regression. Covariates used in each study were age and principal components 1-10 (71).

For each study, we stratified samples into male and female groups, and analyzed the association of SNPs with kidney stone risk separately, then conducted a meta-analysis of the combined male and female data using METAL (72). The effect of allelic dosage was modeled as 0 or 2 in males and 0, 1, or 2 in females (i.e., dosage compensation for X inactivation). A meta-analysis of study 1 and study 2 was then conducted. Heterogeneity across each pair of studies was examined using Cochran’s Q test (73).
the University of Kansas Medical Center or the University of Alberta 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 population-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 manuscript. JNC, MS, LP, and MBF conducted the main set of mouse experiments. MRB and RTA conducted the Ussing chamber studies. AJS conducted the micro-FTR 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 analysis. MA and MT performed the family genetic study. All authors reviewed and edited the manuscript and approved the final draft.

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