Transgenic RNAi Depletion of Claudin-16 and the Renal Handling of Magnesium

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Tight junctions play a key role in mediating paracellular ion reabsorption in the kidney. Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is a human disorder caused by mutations in the tight junction protein claudin-16. However, the molecular mechanisms underlying the renal handling of magnesium and its dysfunction causing FHHNC are unknown. Here we show that claudin-16 plays a key role in maintaining the paracellular cation selectivity of the thick ascending limbs of the nephron. Using RNA interference, we have generated claudin-16-deficient mouse models. Claudin-16 knock-down (KD) mice exhibit chronic renal wasting of magnesium and calcium and develop renal nephrocalcinosis. Our data suggest that claudin-16 forms a non-selective paracellular cation channel, rather than a selective Mg²⁺/Ca²⁺ channel as previously proposed. Our study highlights the pivotal importance of the tight junction in renal control of ion homeostasis and provides answers to the pathogenesis of FHHNC. We anticipate our study to be a starting point for more sophisticated in vivo analysis of tight junction proteins in renal functions. Furthermore, tight junction proteins could be major targets of drug development for electrolyte disorders.

The human renal disorder, familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), is characterized by progressive renal Mg²⁺ and Ca²⁺ wasting, leading to impaired renal function and chronic renal failure. FHHNC is genetically linked to mutations in the gene of claudin-16 (CLDN16, also known as paracellin-1; Ref. 1), which is expressed exclusively in the kidney (2). The claudins comprise a 22 gene family that encodes essential structural components of the tight junction, the principal regulator of paracellular permeability. In vitro studies have shown that ion selectivity of the paracellular conductance (see review: Ref. 3) is a complex function of claudin subtype and cellular context (4, 5). Thus, in vivo models of FNNHC are essential to our understanding of its pathogenesis.

To develop an in vivo model of FNNHC, we have employed transgenic RNA interference (RNAi), which is in theory more rapid and flexible than a conventional knock-out approach. While the use of transgenic RNAi has been limited thus far, it has been shown that an RNAi knockdown of Ras1 recapitulates a null phenotype in mice (6). In addition, transgenic RNAi has been used to establish a role for Ryk in axon guidance (7) and a role for Nramp1 in controlling susceptibility to Type 1 diabetes (8). We used lentiviral transgenesis because it is more resistant than onco-retroviral transgenesis to epigenetic silencing during embryonic development (9–11).

In this study, we report the generation of CLDN16-deficient transgenic mouse lines using RNAi and have established physiological functions of CLDN16. We observed homeostatic changes of Mg²⁺, Ca²⁺, Na⁺, and K⁺ resulting from RNAi-mediated knockdown. The lumen-positive transepithelial potential in the thick ascending limb (TAL) of the nephron drives the reabsorption of Mg²⁺ and Ca²⁺ (see review, Ref. 12). It is generated by the electrogenic NaCl reabsorption and, as a direct consequence of the tubular fluid dilution, it is mainly a diffusion potential between luminal and basolateral extracellular spaces if they are separated by cation-selective tight junctions. We show here that the loss of CLDN16 caused tight junctions in TAL to lose the cation selectivity, leading to the dissipation of the lumen-positive potential with concomitant loss of the driving force for Mg²⁺ reabsorption. This model is consistent with our in vitro analysis (5) but strongly contrasts with previous models of CLDN16 function (1, 13). Furthermore, our study supports the utility of RNAi knockdown (KD) of gene expression in vivo as a complement to traditional gene knock-out approaches.

EXPERIMENTAL PROCEDURES

Antibodies, Cell Lines, and Animals—The following antibodies were used in this study: rabbit polyclonal anti-CLDN16 (Zymed Laboratories); fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (Chemicon). 293T cells (a kind gift from Dr Joan Brugge, Harvard Medical School) were cultured in Dulbecco’s modified Eagle’s medium supplemented...
with 10% fetal bovine serum, penicillin/streptomycin, and 1 mm sodium pyruvate. Animals (strain: B6D2F1, CD-1 female foster mice, and CD-1 vasectomized male stud mice) were from Charles River Laboratory.

Molecular Cloning and Lentivirus Production—For siRNA studies, the siRNA hairpin oligonucleotides (complementary to the mouse CLDN16 mRNA sequence (GenBankTM AF323748)) were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and annealed and cloned into the pFUGW lentivirus backbone downstream of the human snRNA U6 or H1 promoter to create the CLDN16 siRNA constructs (4). A set of twelve short hairpin oligonucleotides (shRNAs) were tested in vitro for the ability to efficiently deplete CLDN16 mRNA in cultured primary tubular cells isolated from the renal segment of the thick ascending limb. VSV-G pseudotyped lentivirus were produced in 293T cells and used to inject the single cell mouse embryos at a titer of 1 × 10^6 units/μl, as described before (11).

Animal Protocols—All mice were bred and maintained according to the Harvard Medical School animal research requirements, and all procedures were approved by the Institutional Animal Research and Care committee. To increase the yield of embryo collection following superovulation in female mice and to facilitate the rapid generation of transgenic animals, we utilized a common hybrid mouse strain (B6D2F1: F1 cross between DBA/2 male and C57BL/6 female) as the donor strain (14). The transgenic founders were crossed to B6D2F1 wild-type mice, and progeny were analyzed. All mice were fed ad libitum and housed under a 12-h light cycle.

Generation of Transgenic Mice Using Lentivirus—Transgenic mice using lentivirus were generated as described (11). Female donor mice (B6D2F1 hybrid strain) were superovulated with a combination of pregnant mare serum (5 units) and human chorionic gonadotropin (5 units). On average, around 20–30 embryos were collected per female. Approximately 10–100 pl of concentrated lentivirus at 10^6 units/μl were injected into the perivitelline space of single cell mouse embryos and allowed to develop to 2-cell embryo stage. Around 20 embryos were implanted into each pseudopregnant female (CD-1 strain; 0.5 dpc) and carried to term. The transgenic founder mice and their progeny were identified by direct observation of GFP fluorescence using a hand-held GFP flashlight (Nightsea, MA).

Surgical Protocols and Renal Clearance—The methods for preparing mice for renal clearance measurements, for monitoring intra-arterial mean arterial blood pressure, and for collecting urine samples have been previously described (15). Mice were anesthetized by intraperitoneal injection of (5-ethyl-5-(L-methylpropyl)-2-thiobarbituric acid (Inactin, 100 mg kg^-1; Sigma)). A tracheotomy was performed, and the jugular vein and carotid artery were catheterized for intravenous infusion and blood sampling. Following surgery, each animal received an intravenous infusion of 140 mm NaCl and 5 mm KHCO3 at 0.5 ml h^-1, with [3H]inulin included in the infusate (10 μCi ml^-1; 10 μCi of primer). After an equilibration period of 60 min, renal clearance measurements were initiated for a 60-min period. Urine was collected under mineral oil, and a 30-μl blood sample was taken at hourly intervals. Blood pressure was measured at the beginning, middle, and end of each clearance period. Urine and plasma Na+, K+, Mg2+, and Ca2+ concentrations were measured by flame photometry (type 480 Flame Photometer, Corning Medical and Scientific, Corning, NY).

Renal Tubule Perfusion—The methods for perfusion and transepithelial measurements in freshly isolated mouse TAL segments were performed as described previously (16). The tubule was held and perfused by a concentric glass pipette system. The perfusion pipette was double-barreled with an outer diameter of 10–12 μm. One barrel was used for perfusion, fluid exchange, and voltage measurement. The second barrel was used for constant current injection (13 nA). The collection side consisted of a glass pipette with an inner diameter of 45 μm. Cable equations as described (16) were used appropriately to calculate transepithelial resistance Rte. Equivalent short circuit current Isc was calculated from Rte and Vte according to Ohms law. The length constant for a tubular length of at least three times the length constant was calculated with λ = ΔV / ρ × (I_inj / Rte); V_o = voltage deflection at the perfusion side; r = tubular radius, I_inj = injection current; ρ = resistivity of the perfusion solution. The rates of perfusion were 10–20 nl/min. The bath was thermostated at 38 °C. Continuous bath perfusion at 3–5 ml/min was obtained by gravity perfusion. Relative permeabilities were calculated from the observed transepithelial diffusion potentials according to the Goldman equation.

Bone Analysis—Femurs were evaluated using a desktop microtomographic imaging system (μCT40, Scanco Medical AG, Bassersdorf, Switzerland) equipped with a 10-mm focal spot microfocus x-ray tube. Transverse CT slices of the distal femoral metaphysis were acquired using 12-μm isotropic voxel size. To assess the trabecular bone parameters for the distal femur, 200 CT slices were acquired, and trabecular bone properties were evaluated in a region starting 0.36 mm proximal to the growth plate and extending 1.8 mm proximally. Images were reconstructed, filtered, and thresholded using a specimen-specific threshold. Morphometric parameters were computed using a direct three-dimensional approach that does not rely on any assumptions about the underlying structure. Measured parameters were expressed according to bone histomorphometry nomenclature (17). For trabecular morphology, we assessed the following variables: trabecular bone volume (BV), total bone marrow volume including trabeculae (TV), trabecular thickness (TbTh, μm), trabecular separation (TbSp, μm), trabecular number (TbN, 1/mm) (18), connectivity density (ConnD 1/mm²; measurement of the interconnectivity of the trabecular network; Ref. 19) and structure model index (SMI); predominance of the shape of the trabeculae; 0 = plate-like; 3 = rod-like; Ref. 20). Transverse CT slices were also acquired at the femoral mid-shaft (diaphysis) using 12-μm slice increment (50 μCT slices per specimen). For this cortical region, we assessed the total cross-sectional area, cortical bone area and medullary area (TA, BA, and MA, respectively, mm²), bone area fraction (BA/TA, %), and cortical thickness (μm) (18). Furthermore, bone mineral density (BMD, g/cm²) and bone mineral content (BMC, g) of the whole body was measured using dual-energy x-ray absorptiometry (PIXImus GE Lunar, Madison, WI).
mix (Bio-Rad) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Total RNA was extracted from the kidney using the TRizol reagent (Invitrogen). The amount of kidney total RNA for each reaction was adjusted within the range 0.05 to 0.2 μg depending on the gene to ensure that gene expression was within the range of linear correlation between the log (amount of total RNA) and threshold cycle number. The housekeeping gene β-actin was used as an endogenous control, and the expression levels of genes of interest were presented as ratios relative to the expression level of β-actin. The primers annealed to two adjacent exons to avoid amplifying genomic DNA and the primer sequences (5’-end to 3’-end) are summarized as follows: claudin-16, CAAACGCTTTTGATGGGATC and TT-TGGGTCTACAGGTAAGG; β-actin, CTGCTTGACGCCC-AAGT and CAAGAGGAAAGCTGGAAAGA.

**Hormonal Assays**—Mouse serum PTH and 1,25-(OH)2D3 levels were measured using ELISA kits (Alpco Diagnostics) according to the manufacturer’s instruction; mouse serum aldosterone was measured using ELISA (Alpha Diagnostic). To provide enough serum for separation of 1,25-(OH)2D3 from 25-(OH)D3 by immunochromatography (Alpco Diagnostics), serum from two animals within each group were pooled at 1:1 ratio. In this assay, six mice within each group were sacrificed, and the N is counted as three for statistics.

**Histology**—The Von Kossa staining method was used to demonstrate nephrocalcinosis in kidney (21). Kidneys from both wild-type and transgenic mice were freshly dissected, fixed with 10% formalin, dehydrated with ethanol and xylene, and embedded into paraffin at 60 °C. 5-μm sections were deparaffinized using xylene and ethanol and rehydrated. Sections were incubated with 5% silver nitrate, followed by an exposure to UV light and a wash with 5% sodium thiosulfate. Finally, sections were stained with Nuclear Fast Red, dehydrated, and mounted with Permount (Fisher Scientific). Light micrographs were captured using a Spot RT camera mounted on a Nikon E800 photomicroscope.

**Immunolabeling and Confocal Microscopy**—For viewing GFP expression in tissues, mice were anesthetized with ketamine (100 mg/ml) and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Tissues were isolated and fixed in 4% PFA at 4 °C overnight, washed three times in PBS, cryoprotected for 24 h in 30% sucrose in PBS, and embedded in OCT prior to cryostat sectioning. Cryostat sections (10 μm) were fixed with cold methanol at −20 °C, followed by blocking with PBS containing 10% fetal bovine serum, incubation with primary antibodies (CLDN16, Zymed Laboratories Inc.; 1:300) and fluorescein isothiocyanate (FITC)-labeled secondary antibodies (1:200). After washing with PBS, slides were mounted with Mowiol. Confocal analyses were performed using the Nikon TE2000 confocal microscopy system equipped with Plan-Neofluar ×40 (NA 1.3 oil) and ×63 (NA 1.4 oil) objectives and krypton-argon laser (488 and 543 lines). For the imaging of FITC, fluorescent images were collected by exciting the fluorophores at 488 nm (FITC) with argon laser. Emissions from FITC were detected with the band-pass FITC filter set of 500–550 nm. All images were converted to JPEG format and arranged using Photoshop 6.0 (Adobe).

**Protein Electrophoresis and Immunoblotting**—Both kidneys from each mouse were homogenized using a Dounce homogenizer in ice cold water containing 250 mM sucrose and 50 mM Tris (pH 8.0), rupturing the membrane of cells. The homogenate was centrifuged in conical tubes at 5,000 × g for 10 min to remove cytosolic proteins. The sediment was resuspended in CSK buffer (150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 1X protease inhibitor mixture (Pierce)) to extract membrane proteins. 50 μg of membrane protein were subjected to SDS-PAGE under denaturing conditions and transferred to a nitrocellulose membrane followed by blocking with 3% milk, incubation with claudin-16 antibodies (Zymed Laboratories Inc.; 1:1,000) and the horseradish peroxidase-labeled secondary antibody (1:5000), and exposure to an ECL Hyperfilm (Amersham Biosciences). Molecular mass was determined relative to protein markers (Bio-Rad).

**Statistical Analyses**—The significance of differences between groups was tested by ANOVA (Statistica 6.0, Statsoft 2003). When the all-effects F value was significant (p < 0.05), post-hoc analysis of differences between individual groups was made with the Neuman–Keuls test. Values were expressed as mean ± S.E. unless otherwise stated.

**RESULTS**

**Lentivirus-directed Transgenesis**—To characterize the efficiency of lentiviral transgenesis, we used lentivirus based on the pFUGW vector in which the ubiquitin-C promoter drives expression of GFP (Fig. S1A of supplemental information; Ref. 11). Lentivirus was injected into the perivitelline space of single cell mouse embryos. Nearly all injected embryos developed to the two-cell stage, at which time they were implanted into pseudopregnant females (0.5 dpc). On average, 50% of the implanted embryos developed to full term. The rate of transgene expres-
sion was very high, with >70% of the pups expressing the marker protein GFP (Table 1), consistent with the original study by Lois et al. (11).

To express short hairpin RNAs (shRNA) in vivo, we modified pFUGW by inserting a U6 or H1 RNA polymerase III promoter to drive the expression of shRNA hairpins (pUG-U6 or pUG-H1, shown in Fig. S1A). The rate of shRNA transgenesis (assayed by expression of GFP, shown in supplemental Fig. S2) was 25–53% (Table 1). 70% of the pUG-U6 transgenic animals grew normally and did not show any gross differences compared with the wild types in body weight (see Table 2) and longevity (vide infra), while 30% of the transgenic pups (n = 20) were runted or died shortly after birth. Our founder animals exhibited multiple-copy integration (data not shown), consistent with previous studies (11).

The analysis of pFUGW expression described by Lois et al. (11) was limited to a small number of tissue and cell types. Thus, it was important to rigorously establish the transgene expression profile and possible gene silencing effects. Because the signal from cytoplasmic GFP was diffuse, it was difficult to distinguish from backgrounds variably present in cryostat sections. To allow unambiguous identification of GFP-positive cells, we modified pUG-U6 by fusing a nuclear localization sequence (NLS) onto the C terminus of GFP (pUG-nlsGFP-U6). A sample of nuclear GFP expression in 10 μm cryostat sections from pUGnls-U6 transgenic mice is presented in Fig. 1 and a comprehensive set in supplemental Fig. S4. GFP was detected in most cells in stomach, duodenum, jeju-

### TABLE 2

Body weight, mean blood pressure, and plasma electrolytes in WT and CLDN16 KD mice

| Genotype | N<sup>a</sup> | BW<sup>b</sup> | BP | P<sub>Na</sub><sup>c</sup> | P<sub>K</sub><sup>c</sup> | P<sub>Mg</sub><sup>c</sup> | P<sub>Ca</sub><sup>c</sup> |
|----------|---------------|---------------|----|-----------------|-----------------|-----------------|-----------------|
| WT       | 9             | 25.78 ± 1.27  | 91.91 ± 0.94 | 138.76 ± 2.24  | 4.45 ± 0.20     | 0.71 ± 0.06     | 2.01 ± 0.08     |
| KD       | 9             | 24.00 ± 1.00  | 71.56 ± 2.24 | 141.59 ± 2.60  | 3.68 ± 0.20     | 0.51 ± 0.03     | 2.16 ± 0.04     |

<sup>a</sup> N, number of animals.
<sup>b</sup> BW, body weight.
<sup>c</sup> P<sub>Na</sub>, P<sub>K</sub>, P<sub>Mg</sub>, P<sub>Ca</sub>: plasma Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> concentrations measured on anesthetized animals during renal clearance study.
<sup>d</sup> Significant difference between WT and KD, p < 0.05.

![FIGURE 1. Silencing of lentivirus.](image) 1) The silencing of a type of cells: the fibroblasts and the endothelial cells in the connective tissue of the pancreas (A, arrow) showed gene silencing; and the endothelial cells and the Kupffer cells of the liver (B, arrowhead) showed gene silencing. 2) The silencing within a type of cells: a cell (D, arrow) within a distal convoluted tubule (D, circle) of the kidney (C and D) showed gene silencing, whereas the rest of cells of the same tubule were active in the gene expression. 3) The silencing of the germ cells: the oocytes of the ovary (E) and the germ cells of the testis (F) showed gene silencing. Bar, 20 μm (A–C, E and F); 10 μm (D).
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num, ileum, colon, pancreas, liver, kidney, lung, cerebellum, spinal cord, myocardium, skeleton muscle, skin, lens, cornea, retina, and ciliary epithelium. Typically, GFP expression was observed in all or nearly all cells of a given type. However, expression was silenced in pancreatic fibroblasts and endothelial cells (Fig. 1A), hepatic endothelial and Kupffer cells (Fig. 1B) and germ cells from ovary and testis (Fig. 1, E and F). More selective silencing was observed in the kidney; for example, a single cell within a distal convoluted tubule showed gene silencing, whereas the rest of cells of the same tubule were active in the gene expression (Fig. 1, C and D).

To determine the stability of lentiviral expression with age, we examined the patterns of expression in animals at postnatal day 1 as well as 3 weeks, 8 weeks, and 4 months of age. Expression patterns were similar or identical in each case (data not shown). To determine heritability, a similar analysis was performed on founders and 3 subsequent generations (for pUG-U6 and pUG-nlsGFP-U6). No significant differences were observed (data not shown).

In Vivo RNA Interference Generated by Expression of shRNA or miRNA—A set of twelve short hairpin oligonucleotides (shRNAs) were tested in vitro for the ability to efficiently deplete CLDN16 mRNA (see “Experimental Procedures”). Each set contained a unique, complementary 19-nt sequence within the coding region of mouse CLDN16 (for diagram of vector topology see supplemental Fig. S1B; Refs. 4 and 22). ShRNA oligonucleotides were cloned into pUG-U6 or pUG-H1 for lentiviral expression. After infection, transcribed shRNAs are processed into siRNAs by Dicer, and siRNAs are incorporated into an RNA-induced silencing complex which cleaves homologous mRNAs (see review, Ref. 23). Sequence 551 was highly effective in vitro (>100-fold knockdown, see supplemental Fig. S3B). Sequence 453 was equally effective, but sequence 756 was completely ineffective and was used subsequently as the negative control (data not shown).

After transgenesis, the analysis of total RNA (supplemental Fig. S3C) and total membrane protein (supplemental Fig. S3D) from mouse kidneys showed that shRNA-mediated depletion of CLDN16 mRNA and protein was highly effective in vivo (>100 fold knockdown). Real-time quantitative RT-PCR was used to determine in vivo expression levels of CLDN16 relative to β-actin (WT: 0.113 ± 0.004, KD: 0.0010 ± 0.0001; n = 3, p < 0.05). Animals transgenic for pUG-U6-551 (supplemental Fig. S3), pUG-U6-453 and pUG-H1-453 (data not shown) showed >100-fold reduction in CLDN16 mRNA levels. Control animals transgenic for pFUGW and pUG-U6-756 showed no effects on CLDN16 expression (data not shown).

In WT mouse kidney, CLDN16 was immunolocalized to a subset of tubules extending from outer medulla through cortex and in bundles within cortico-medullary rays (Fig. 2, A and B). This is consistent with previous findings that CLDN16 is expressed exclusively in the thick ascending limbs (TAL) of the loops of Henle (2). In pUG-U6-551 transgenic mouse kidneys, CLDN16 staining was completely eliminated (Fig. 2, C and D). Similar results were obtained using pUG-U6-453, pUG-H1-453, and constructs incorporating both shRNA sequences and NLS-eGFP (data not shown). On the other hand, control animals transgenic for pFUGW and pUG-U6-756 showed normal patterns of CLDN16 staining (data not shown).

We also tested the efficacy of microRNAs (miRNAs) to suppress CLDN16 expression. MiRNAs are single-stranded RNA molecules of 19–25 nt in length that are generated from endogenous hairpin-shaped transcripts (24) which base pair with their target mRNAs inducing either translational repression or mRNA destabilization. Unlike shRNAs, the expression of miRNAs can be driven by RNA polymerase II (25), making tissue-specific or inducible RNAi knockdowns feasible. Studies to establish the effectiveness of miRNAs in generating human disease models are lacking, though a recent study by Xia et al. (26) has demonstrated its effectiveness in knocking down gene expression in vivo. Therefore, we generated transgenic animals expressing the miR30 minigene driven by the ubiquitin-C promoter, where the endogenous miR30 hairpin sequence was replaced with the CLDN16 shRNA sequence 453 (supplemental Fig. S1C). In these animals, CLDN16 immunostaining was reduced to background levels (data not shown).

As with GFP expression, the loss of CLDN16 mRNA and protein in shRNA and miRNA transgenic animals was not affected by the age of the animal over a range of at least 4 months or by propagation over at least three generations, which argues against the safety concerns over RNAi-induced liver failure and fatality in mice recently reported by Grimm et al. (27). Together, these data illustrate the utility of shRNA and miRNA for long-term modulation of endogenous gene expression.

Loss of CLDN16 Resulted in Hypomagnesemia, Hypercalcemia, and Nephrocalcinosis—The founder mice generated with pFUGW, the siRNA control [756] and the two effective siRNAs [453 and 551] were crossed to wild-type mice, and F1 offspring were analyzed for changes in plasma and urine electrolyte levels. Initial characterization showed that CLDN16 KD mouse lines transgenic for pUG-U6-551, pUG-U6-453, pUG-H1-453, and pUG-miR-453 all had significantly suppressed plasma Mg²⁺ levels and their 24-h urinary excretions of Mg²⁺ and Ca²⁺ were significantly increased (see table in supplemental
TABLE 3
Urine volume, GFR, Ca\(^{2+}\), and Mg\(^{2+}\) excretion in WT and CLDN16 KD mice

Data are mean \pm S.E.

| Genotype   | N\(^a\) | UV\(^b\) | GFR\(^c\) | \(E_{Ca}d\) | \(E_{Mg}d\) | \(FECa\(^e\)\) | \(FEMg\(^e\)\) |
|------------|---------|----------|-----------|-------------|-------------|-------------|-------------|
| Wild-type  | 9       | 2.11 ± 0.04 | 0.89 ± 0.09 | 12.99 ± 2.34 | 0.106 ± 0.025 | 0.75 ± 0.09 | 18.01 ± 3.22 |
| KD         | 9       | 2.29 ± 0.03 | 0.65 ± 0.09 | 38.62 ± 8.44 | 0.214 ± 0.022 | 2.80 ± 0.54 | 73.28 ± 10.34 |

\(^a\) N, number of animals.

\(^b\) UV, urine volume.

\(^c\) GFR, glomerular filtration rate.

\(^d\) \(E_{Ca}\), absolute Ca\(^{2+}\) excretion.

\(^e\) \(FECa\) and \(FEMg\), fractional excretion of Ca\(^{2+}\) and Mg\(^{2+}\).

\(^f\) Significant difference between WT and KD mice (\(p < 0.05\)).

Fig. S5), compared with WT animals. These phenotypes are summarized in Table 1. In contrast, control mouse lines transgenic for pFUGW and pUG-U6-756 had normal phenotypes.

To rigorously analyze the renal handling of Mg\(^{2+}\), Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\), we performed renal clearance measurements on WT and CLDN16 KD animals (pUG-U6-551) using 14–16 week old animals (see Tables 2–4). The plasma Mg\(^{2+}\) levels in CLDN16 KD mice was 28% lower than in WT while the Ca\(^{2+}\) level was not significantly altered (Table 2). However, analysis of urinary electrolytes showed that CLDN16 KD mice were losing both Ca\(^{2+}\) and Mg\(^{2+}\) more rapidly than WT mice. The absolute excretion of Ca\(^{2+}\) and Mg\(^{2+}\) in KD mouse (Table 3, \(E_{Ca}\) and \(E_{Mg}\)) was 2.97- and 2.02-fold higher, respectively, than in WT, while the fractional excretion (\(FECa\) and \(FEMg\)) was increased 3.73- and 4.07-fold. Von Kossa staining of renal sections from 6-month-old animals revealed calcium deposits in the basement membranes of medullary tubules and the surrounding interstitium of the kidney in CLDN16 KD but not WT mice (Fig. 3). This was qualitatively similar to FHHNC patients (28). The delivery of calcium to the medullary interstitium in FHHNC most likely arises from transcellular transport mediated by TRPV5 channels in the distal convoluted tubules and collecting ducts (29–30). These physiological changes in the KD mice are very similar to those reported in FHHNC patients, providing an accurate animal model of the human disease.

The KD mice are hypokalemic with plasma K\(^{+}\) levels 17% lower than WT (Table 2). The fractional excretion of K\(^{+}\) was increased 2-fold over WT (Table 4). Though the absolute Na\(^{+}\) excretion was not significantly increased, CLDN16 KD mice were hypotensive (Table 2) and had significantly elevated plasma aldosterone levels (KD: 662.4 \pm 110.0 \text{ pg/ml}; WT: 402.0 \pm 94.1 \text{ pg/ml}; \(p < 0.05\), \(n = 5\)), suggesting increased Na\(^{+}\) reabsorption in the distal nephron (see “Discussion”). There was no significant difference in urinary volume and osmolality between WT and KD (Table 4); thus, loss of CLDN16 did not result in a urine-concentrating defect.

Phenotypes of plasma and urine electrolyte abnormalities of the transgenic line pUG-U6-453 were identical to those of pUG-U6-551. In addition, hypotension and hyperaldosteronism were confirmed independently in the transgenic line pUG-U6-453.

Disturbances in Bone Structure—Although KD animals displayed abnormally high urinary excretion of calcium, circulating calcium levels were maintained normally. Extracellular Ca\(^{2+}\) concentration is controlled by the kidney, intestine, and bone through the action of the calciotropic hormones, including PTH and 1,25-dihydroxycholecalciferol (1,25-(OH)\(_2\)D\(_3\)). While serum PTH levels were not significantly changed (data not shown), 1,25-(OH)\(_2\)D\(_3\) levels were >3-fold higher in CLDN16 KD mice compared with WT (KD, 120.3 \pm 23.3 \text{ pg/ml}; WT, 35.8 \pm 12 \text{ pg/ml}; \(p < 0.001\), \(n = 3\); see “Experimental Procedures”), suggesting that increased urinary excretion of Ca\(^{2+}\) was being compensated by increased intestinal absorption.

We tested this hypothesis using dietary restriction. On a normal diet (0.8% Ca\(^{2+}\)), the overall bone mineral density...
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TABLE 4
Na⁺, K⁺ excretion and osmolality in WT and CLDN16 KD mice

Data are mean ± S.E.

| Genotype | N  | E₅₀ᵇ | E₇₀ᵇ | FENₐᶜ | FEN₇₀ᶜ | Osmᶜ  |
|----------|----|------|------|-------|--------|-------|
| WT       | 9  | 0.77 ± 0.24 | 1.02 ± 0.23 | 0.74 ± 0.22 | 26.05 ± 4.24 | 1504.2 ± 123.0 |
| KD       | 9  | 0.85 ± 0.24 | 1.11 ± 0.20 | 0.98 ± 0.25 | 53.49 ± 11.81 | 1334.0 ± 125.9 |

ᵃ N, number of animals.
ᵇ E₅₀, E₇₀: absolute Na⁺ and K⁺ excretion.
ᶜ FENₐ, FEN₇₀: fractional excretion of Na⁺ and K⁺.
ᵈ Osm, urine osmolality.
ᵉ Significant difference between WT and KD mice (p < 0.05).

TABLE 5
Bone analysis of WT and CLDN16 KD mice

Microcomputed tomography of the femur showed that cortical thickness (CtTh) of the diaphysis was significantly reduced in KD animals. The cross-sectional cortical bone area (BA) decreased while the medullary area (MA, marrow cavity area) increased. Reduction in the thickness (CtTh) and the volume (BA/TA) of the cortical compact bone showed no significant changes. Instead, the total bone marrow volume (TV) of the femoral head significantly decreased, leading to decreased trabecular separation (TbSp) and increased trabecular shape, was altered significantly. The trabecular bone volume (BA), trabecular number (TbN) and trabecular thickness (TbTh) showed no significant changes.

![Image](https://example.com/image.png)

**Figure 4.** Representative cross-sectional x-ray images of the distal femur (femoral head) and the diaphysis in WT (A and C) and KD (B and D) mice. Note the decreased cortical bone width in both the distal femur (arrows; comparing B with A) and the diaphysis (arrows; comparing D with C) of the KD animals. Also note the decrease in the signal intensity of the trabeculae of the distal femur in KD animals (comparing B with A), reflecting increased x-ray transparency and demineralized bones.

(BMD) and bone mineral content (BMC) were not significantly affected in KD mice (data not shown). Dietary restriction of Ca²⁺ (0.02% Ca₅⁺) for 20 days overwhelmed the compensatory effects of elevated 1,25-(OH)₂D₃, resulting in a dramatic reduction in BMD and BMC values (Table 5). Microcomputed tomography of the femur revealed a variety of structural changes consistent with the overall loss of mineral content from the skeleton of whole body (data summarized in Table 5 and Fig. 4).

**Ion Selectivity of TAL**—Using electrophysiological measurements in isolated, perfused renal tubules, we tested the ion selectivity of the thick ascending limbs (TAL) of CLDN16 KD mice. The mean active transepithelial potential difference (Vte, lumen positive), the mean length constant and the mean transepithelial resistance (Rte) were not significantly different comparing WT with CLDN16 KD mice (see Table 6). The effects of furosemide (0.1 mM, inhibiting active transcellular transport) were evident, reducing Vte to zero. To determine the ion selectivity of the tight junction, changes in the junctional diffusion potential (PD) produced by a NaCl gradient of 145 vs 30 mM were recorded in the absence of active transport (inhibited by furosemide). When the NaCl concentration of the bath was lower than that of the lumen, the PD decreased, indicating a cation selective pathway. Corresponding increases in PD were observed when the NaCl gradient was reversed. The magnitude of the junctional diffusion potential was independent on the direction of the NaCl gradient. Table 6 shows that for an outward gradient, mean PD was −18.0 ± 1.4 mV, and the mean
In this study, we have generated CLDN16-deficient mice using shRNA and miRNA to induce RNA interference in vivo and produce animal models of human FHHNC. The clinical presentation of FHHNC invariably includes hypomagnesemia, hypercalcemia, and nephrocalcinosis, and most patients also display impaired bone homeostasis (32). These phenotypes are invariably observed in CLDN16 KD animals. Most FHHNC patients show elevated parathyroid hormone (PTH) levels, although occasional increases in 1,25-dihydroxycholecalciferol levels have been reported. The CLDN16 KD animals compensate for severe renal wasting of Ca\(^{2+}\) primarily by increasing the production of 1,25-dihydroxycholecalciferol and stimulating the intestinal absorption of Ca\(^{2+}\), similar to the TRPV5 knockout mice which provide another Ca\(^{2+}\)-wasting animal model (29). Polypuria and polydipsia, chronic renal failure, nephro lithiasis and urinary tract infections are noted in many FHHNC patients, which are possibly associated with nephrocalcinosis and which complicate the diagnosis and management of the disease. In particular, the progression rate of renal failure correlates well with the severity of nephrocalcinosis (33). The CLDN16 KD mice do not display these secondary complications, which may actually improve their utility as models for the development of therapeutic treatments. Inconsistently, patients present with convulsions, muscular tetany, ocular abnormalities, and hearing impairment. We have not yet explored possible extrarenal effects in the KD mice. Recently, Konrad et al. (34) identified mutations in a second family member, claudin-19, in a population of FHHNC patients. Intriguingly, CLDN19 is also exclusively expressed in the TAL, colocalizing with CLDN16. Together, these data highlight the pivotal role of paracellular pathways in the pathogenesis of FHHNC.

The reabsorption of Mg\(^{2+}\) in TAL is a passive process via the paracellular pathway and highly dependent on the transepithelial potential as a driving force (35–37). When TAL is perfused with isotonic NaCl solutions, the lumen voltage is positive at 3–10 mV (Vte) owing to apical membrane K\(^{+}\) secretion (38). The active reabsorption of NaCl via NKCC2 in TAL results in diluted tubule fluid in the lumen and a higher concentration of NaCl in the peritubular space. A transcellular NaCl concentration gradient (from peritubular space to lumen) develops and becomes pronounced. Because the paracellular pathway in TAL is cation selective (with a mean PNa/PCl value of 3.1), the transepithelial potential can increase to as much as 30 mV with the lumen positive, as a result of the diffusion potential generated by the backflow of NaCl from peritubular space to tubule lumen down its concentration gradient via the paracellular pathway. This large voltage drives the bulk of reabsorption of Mg\(^{2+}\). The loss of CLDN16 function leads to a decrease in PNa/PCl and therefore the dissipation of the lumen-positive potential and the loss of driving force for Mg\(^{2+}\) reabsorption. Without CLDN16 and the cation-selective tight junction in TAL, more Cl\(^{−}\) and less Na\(^{+}\) will flow back to the lumen side but the total conductance of Na\(^{+}\) and Cl\(^{−}\) is likely to be the same (indicated by the lack of changes in Rte). Increases in luminal Cl\(^{−}\) will be sensed by the macula densa and result in decreases in tubuloglomerular feedback (TGF). More luminal Cl\(^{−}\) will lead to increases in apical K\(^{+}\) secretion, to reach electric neutrality. Thus, renal wasting of K\(^{+}\) in CLDN16 KD mice could be explained. It is also possible that the stimulation of K\(^{+}\) secretion occurs in the distal nephron, induced by high aldosterone levels. Renal handling of Na\(^{+}\) in CLDN16 KD mice is more complex. Though the precise site of Na\(^{+}\) loss is under investigation, our preliminary data have shown that the amiloride sensitive currents in isolated, perfused cortical collecting ducts (CCD) of CLDN16 KD animals were significantly increased compared with WT animals, indicating up-regulated Na\(^{+}\) reabsorption in the distal nephron. Na\(^{+}\) homeostasis is intricately linked to the extracellular fluid volume (ECFV) and the mean arterial blood pressure (MABP). ECFV is primarily determined by the total body Na\(^{+}\) content (39–40). ECFV and MABP are highly interdependent. Therefore, the reduced MABP in CLDN16 KD animals likely reflected decreases in total body Na\(^{+}\) content and ECFV. Reduced ECFV likely leads to decrease in glomerular filtration rate (GFR).

The efficiency of lentiviral transgenesis compared with pronuclear DNA injection is reflected by the higher rate of embryo survival and higher percentages of transgene expression. This efficiency results in part from the ability of the lentivirus to integrate into sites of active gene transcription (41). Retroviral

### DISCUSSION

In this study, we have generated CLDN16-deficient mice using shRNA and miRNA to induce RNA interference in vivo and produce animal models of human FHHNC. The clinical presentation of FHHNC invariably includes hypomagnesemia, hypercalcemia, and nephrocalcinosis, and most patients also display impaired bone homeostasis (32). These phenotypes are invariably observed in CLDN16 KD animals. Most FHHNC patients show elevated parathyroid hormone (PTH) levels, although occasional increases in 1,25-dihydroxycholecalciferol levels have been reported. The CLDN16 KD animals compensate for severe renal wasting of Ca\(^{2+}\) primarily by increasing the production of 1,25-dihydroxycholecalciferol and stimulating the intestinal absorption of Ca\(^{2+}\), similar to the TRPV5 knockout mice which provide another Ca\(^{2+}\)-wasting animal model (29). Polypuria and polydipsia, chronic renal failure, nephrolithiasis and urinary tract infections are noted in many FHHNC patients, which are possibly associated with nephrocalcinosis and which complicate the diagnosis and management of the disease. In particular, the progression rate of renal failure correlates well with the severity of nephrocalcinosis (33). The CLDN16 KD mice do not display these secondary complications, which may actually improve their utility as models for the development of therapeutic treatments. Inconsistently, patients present with convulsions, muscular tetany, ocular abnormalities, and hearing impairment. We have not yet

### TABLE 6

Properties of the thick ascending limbs of loops of Henle in WT and CLDN16 KD mice

|          | WT | KD | p  |
|----------|----|----|----|
| Length constant (µm) | 85.6 ± 5.1 | 80.3 ± 4.3 | NS |
| Vte (mV)     | 9.0 ± 0.7 | 7.4 ± 0.8 | NS |
| Rte (Ω/cm²)  | 13.6 ± 1.3 | 12.3 ± 1.2 | NS |
| Isc (µA/cm²) | 703 ± 72 | 645 ± 96 | NS |
| PD (mV)      | -18.0 ± 1.4 | -6.6 ± 0.9 | <0.01 |
| PNa/PNa⁺      | 3.1 ± 0.3 | 1.5 ± 0.1 | <0.01 |
| PNa/PNa⁺      | 1.6 ± 0.1 | 1.7 ± 0.1 | NS  |
| PNa/PNa⁺      | 1.9 ± 0.1 | 2.3 ± 0.2 | NS  |

* n = 9 for WT, n = 8 for KD.
* NS, not significant.
* Vte, transepithelial potential difference.
* Rte, transepithelial resistance.
* Isc, equivalent short circuit current.
* PD, diffusion potential.
* PNa, Na⁺ permeability; PCl, Cl⁻ permeability.
* Rte, Li⁺ permeability.
* PNa/PNa⁺, Na⁺ permeability; PCl, Cl⁻ permeability.
* PNa/PNa⁺, Na⁺ permeability.

The table above provides data on properties of the thick ascending limbs of loops of Henle in WT and CLDN16 KD mice. It shows that the length constant, transepithelial potential, resistance, and other parameters differ between WT and KD mice, with significant differences noted in the transepithelial potential and resistance.
expression is often subject to transcriptional silencing in mouse embryonic stem (ES) cells and in transgenic animals (Refs. 9–10, 42). Similar effects have been reported using lentivirus in cultured ES cells (43) and in vivo (8). However, using pFUGW and U6, H1 and miRNA minigene variants, we found minimal epigenetic silencing and expression variation in somatic cells. The most prominent silencing was observed in germ cells, although hepatic endothelia, Kupffer cells, and pancreatic endothelia were also affected. Relevant to our studies on CLDN16 function in the kidney, occasional variegation was observed in the epithelium of the distal nephron. In our physiological measurements, however, expression variation in a low number of cells would not likely affect interpretation, because the gross phenotypes reflect the collective properties of the all the distal tubule cells. Consistent with the results of Lois et al. (11), we found transgene expression and physiological phenotypes stably inherited by through germline transmission for at least three generations.

The major advantage of using RNAi for functional genomic analyses is efficiency of animal line generation compared with conventional homologous recombination. It is feasible to observe physiological changes in the F0 offspring, and phenotypically interesting animals then outbred to establish lines. Two different lines with complementary genes can be crossed with direct production of animals carrying both siRNAs for a “double knock-down” study, avoiding the time and expense of breeding multiple generations to capture both alleles of knocked out genes. An additional advantage is that RNAi-silenced animals can be rescued by crossing with mice carrying transgenes expressing either wild-type or mutant human isoforms (e.g. as found in human FHHNC of CLDN16), allowing generation of humanized mouse models to recapitulate human FHHNC disease. These mouse models will be particularly useful in screening for new treatments (see review, Ref. 44).

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