Mice With a Targeted Disruption of Slc4a11 Model the Progressive Corneal Changes of Congenital Hereditary Endothelial Dystrophy

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PURPOSE. To establish an animal model of congenital hereditary endothelial dystrophy (CHED) using Slc4a11 knockout (KO) mice and evaluate the abnormalities in the cornea and kidney.

METHODS. The Slc4a11 KO mouse model was generated by gene deletion. Corneal abnormalities were evaluated using slit-lamp photography, anterior segment optical coherence tomography (AS-OCT), immunohistochemistry, RT-PCR, corneal endothelial cell staining, and electron microscopy. The temporal corneal changes were also monitored. Histological and functional changes of the kidney were also evaluated.

RESULTS. Successful knockout of the Slc4a11 gene was confirmed by immunohistochemistry and RT-PCR. Slit-lamp photography and AS-OCT showed progressive corneal edema. Increased corneal endothelial cell size with decreased corneal endothelial cell density was observed with increased age. Scanning electron microscopy also revealed progressive cell swelling and distortion of the hexagonal cell morphology with time. Transmission electron microscopy showed characteristic ultrastructural findings of CHED, including endothelial vacuolization, thickening of the Descemet membrane, disorganization of collagen fibrils, deposition of amorphous material, and progression of these changes with age. Decreased urine osmolality and electrolyte concentrations suggesting abnormality in water resorption were also detected.

CONCLUSIONS. Our Slc4a11 KO mouse model successfully represents clinical manifestations of human CHED. We were able to show chronological corneal progression for the first time in a knockout mouse model as well as renal abnormalities.

Keywords: animal model, congenital hereditary endothelial dystrophy, corneal endothelial dystrophy, Slc4a11

Congenital hereditary endothelial dystrophy (CHED) is a rare, inheritable corneal dystrophy characterized by bilateral diffuse corneal opacification.1,2 Excessive fluid collection in the stroma leads to disruption of the collagen fibrils, light scattering, and opacification that causes the “ground glass” appearance.3,4 Histological features include diffuse stromal edema, dystrophic endothelial cells, and a thickened Descemet membrane (DM).2,5

Based on the differences in the mode of inheritance, CHED is classified into two subtypes: autosomal dominant (CHED1; OMIM 121700) and autosomal recessive (CHED2; OMIM 217700).6 CHED2 is generally the more severe phenotype and presents at birth or in early infancy, whereas CHED1 often presents later in childhood.3 The gene for CHED2 was identified as SLC4A11 (OMIM 610206) via a positional candidate approach.7 Mutations in SLC4A11 have also been described in Harbayan syndrome, which is characterized phenotypically as CHED2 with sensorineural hearing loss.8 Heterozygous SLC4A11 mutations have also been identified in patients with Fuchs dystrophy, although these may only be responsible for approximately 5% of late onset cases.9,10

SLC4A11 is related to the SLC4 (solute carrier family 4) family of genes that consists of a functionally diverse group of 10 members that play an essential role in the transport of HCO3−.11–13 The protein encoded by SLC4A11 was initially called BTR1 (bicarbonate transporter-related protein-1) on the basis of its sequence homology with other bicarbonate transporter proteins.14 Subsequently, motivated by its homology with the plant borate transporter BOR1,15 its function as an electrogenic sodium-borate cotransporter was suggested and the protein was renamed NaBCT.16

The nature of the identified SLC4A11 mutations in CHED2 patients thus far has indicated loss of function of the protein as the underlying cause.7 However, the exact functional role of
NaBC1 in the corneal endothelium or the associated disease mechanism are not clear, although abnormalities of borate transport, disturbance of ion homeostasis and fluid transport, or dysregulation of cell proliferation have been suggested. Animal models of disease can be used to provide key insights into disease pathology. We therefore generated a Slea11 knockout (KO) mouse model and describe in detail the corneal and renal abnormalities associated with the functional loss of NaBC1 in this mouse model.

**Materials and Methods**

**Animals**

All animal experiments were performed according to the care of experimental animal guidelines and all protocols were approved by the Institutional Animal Care and Use Committee of SingHealth, Singapore. All animals were treated according to the tenets of the Association for Research in Vision and Ophthalmology’s statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice were bred and maintained at the SingHealth Experimental Medical Centre (Singapore General Hospital, Singapore).

**Generation of the Slea11 KO Mouse**

Heterozygous Slea11 KO C57BL/6 mice with a targeted deletion of exons 9 to 13 of the murine Slea11 gene were generated by Ozgene (Bentley, Australia), the details of which have been described previously. Briefly, mice deficient for Slea11 were generated using the Cre-Lox system, whereby the gene locus was modified by lox P elements flanking exons 9 to 13 of the Slea11 gene. Upon Cre-mediated deletion, a frameshift was introduced in exon 16, with a premature stop. The altered mRNA transcript, lacking the sequences of the transmembrane helices of the wild type (WT) protein, was a target for degradation by nonsense-mediated decay. Initially, heterozygous Slea11 (WT/lox) were generated and then crossed with strain OzCre, which are homozygous for a Cre transgene in the ROSA26 locus. The resulting mice were screened for the presence of the Cre gene and the deletion of Slea11 exons 9 to 13. These mice (WT/KO-WT/Cre) were backcrossed to C57BL/6 (WT) to eliminate the Cre gene and the deletion of exons 9 to 13. These mice (WT/KO–WT/Cre) were bred to homozygosity and used for experiments.

**Anterior Segment Examination**

To evaluate changes of the anterior segment, slit-lamp photography was taken in three mice at each time point (10, 30, and 50 weeks) with a zoom photo slit lamp (Nikon FS-5V; Nikon, Tokyo, Japan). Anterior segment optical coherent tomography (AS-OCT) using commercial OCT equipment (RTVue; Optovue, Fremont, CA) was also done (three mice for each group at 10, 20, 30, 40, and 50 weeks). The measurement was performed three times in each eye, and the average of the three readings was taken.

**Immunohistochemistry**

Under deep anesthesia, mice were euthanized at 10 to 12 weeks and 40 to 48 weeks of age by intraperitoneal overdose anesthesia with sodium pentobarbital. For immunohistochemistry, whole globes were embedded in optimal cutting temperature compound (Leica Microsystems, Nussloch, Germany). Frozen tissue blocks were stored at −80°C until sectioning. Serial sagittal corneal 6-μm sections were cut using a cryostat (Microm HM550; Microm, Walldorf, Germany). Sections were placed on polylsine-coated glass slides (Thermo Scientific, Waltham, MA) and dried for 15 minutes.

Corneas from 12- and 48-week-old Slea11+/− (WT) and Slea11−/− (KO) mice, respectively, were used for immunohistochemistry. Triplicate sections (single sections from three mice) were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 20 minutes, washed with 1× PBS, blocked with 4% bovine serum albumin (BSA; Sigma-Aldrich) in 1× PBS, 0.15% Triton X-100 (Sigma-Aldrich) for 1 hour, and incubated with rabbit polyclonal antibodies listed in Table 1 at room temperature for 2 hours. After washing with 1× PBS, the sections were incubated with goat anti-rabbit AlexaFluor 488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) at room temperature for 2 hours. Slides were then mounted with mounting medium containing DAPI (UltraCruz; Santa Cruz Biotechnology, Santa Cruz, CA). For negative controls, nonimmune serum was used instead of the specific primary antibody. Sections were observed and imaged with a fluorescence microscope (Zeiss Axioplan 2; Carl Zeiss, Oberkochen, Germany).

**RT-PCR**

To detect the deletion of Slea11, RT-PCR was performed using five specific primer pairs designed using the PrimerBlast website (in the public domain at http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The five primer pairs were designed to specifically amplify regions of Slea11 genes flanking part of the deleted sequence exons 9 to 13 (primer 1); within the deleted region (primers 2 and 3); or encompassing the whole deleted region (primers 4 and 5). The following primer pairs were used: primer 1 (product size 421 base pair [bp]): F1-5′ ATG CTT CCA CAC CTT CAG C 3′ and R1-5′ GCACAGTCAGAGGCTCTC3′; primer 2 (product size 547 bp): F1-5′ ATTCCAGGAGGCCTCGGTG3′ and R1-5′ GCTGAGGTTGGAGAAAGCAT3′; primer 3 (product size 579 bp): F1-5′ AAGCTCTGAGACCTGCCAC3′ and R1-5′ CAGGCTGAGGTTGGAAGGAG3′; primer 4 (product size 967 bp): F1-5′ GGTCGACTGAGCAGCTCCT3′ and R1-5′ CAGGCTCCCAAGGAGGAG3′; and primer 5 (product size 965 bp): F1-5′ TCGAGTCGAGGCAGCTCTGG3′ and R1-5′

### Table 1. Details of the Antibodies

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<th>Type</th>
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<th>Dilution Titer</th>
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<tbody>
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<td>Rabbit polyclonal</td>
<td>Joseph R. Casey (Alberta, Canada)</td>
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<tr>
<td>Aquaporin-1</td>
<td>Rabbit polyclonal</td>
<td>Abcam (Cambridge, MA)</td>
<td>1:400</td>
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<tr>
<td>Fibronectin</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>Laminin</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>1:150</td>
</tr>
<tr>
<td>Collagen VIII</td>
<td>Rabbit polyclonal</td>
<td>Albert Jun (Baltimore, MD)</td>
<td>1:3000</td>
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</tbody>
</table>

* Generated against the murine NaBC1 N-terminal sequence: 292-SSLHTHRHPQPPKC-305.
GGCTCACAAGGAGGAGTC3 (Fig. A1). Briefly, two mice aged 60 to 80 weeks from each group were killed, and the corneal tissue was excised. The corneal tissue was then disrupted and homogenized using commercial equipment (Tissuelyser LT; Qiagen, Hilden, Germany). RNA was extracted using an extraction kit (RNeasy Mini Kit; Qiagen) according to the manufacturer's instructions. RT-PCR was done using a reverse transcriptase kit, in accordance with the manufacturer's protocol (Superscript III; Invitrogen). The RT-PCR products were visualized on 1.5% agarose gel electrophoresis.

**Endothelial Cell Staining**

In 10-week-old and 40-week-old WT and KO mice, four eyes in each group were used for the evaluation of the morphology and density of endothelial cells. Corneal buttons excised from the enucleated eyes were placed endothelial-side up and stained with alizarin red S (0.20%; pH 4.2) for 90 seconds, and then were immersed in the glutaraldehyde fixative solution (2.48%; osmolality 301 mosm/kg; pH 7.2; Sigma-Aldrich) for 10 minutes. The corneas were mounted on a glass slide endothelial-side up under a coverslip, and were observed using an inverted light microscope (model Eclipse Ti; Nikon).

In each cornea, five randomly selected, nonoverlapping 0.1-mm × 0.1-mm squares (×400) were selected, and the density and the size of the endothelial cells were evaluated using Java-based image processing software (ImageJ, version 1.46; National Institutes of Health, Bethesda, MD), as shown in the user guide.19

**Electron Microscopy**

To evaluate the ultrastructural changes, the corneas from both groups at the ages of 10, 30, 70, and 90 weeks were prepared for electron microscopy. For scanning electron microscopy (SEM), the globes were immersed in a fixative solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4; Electron Microscopy Sciences [EMS], Hatfield, PA) overnight at 4°C. The corneas excised from the globes were washed three times in distilled water for 10 minutes each, and were kept in 1% osmium tetroxide (FMB Trading and Engineering Pte Ltd., Singapore) at 22°C for 2 hours for final fixation. The corneas were then dehydrated through serial dilutions of ethanol (25%, 50%, 75%, 95%, and 100% each for 10 minutes, with the 100% being performed twice). The samples were then dried in a critical point dryer (Bal-Tec AG, Balzers, Liechtenstein) and mounted on SEM stubs using carbon adhesive tabs. Samples were then sputter-coated with a 10-nm thick layer of gold (Bal-Tec AG) and examined with a scanning electron microscope (JSM-5600; JEOL, Tokyo, Japan) at 15 W.

For transmission electron microscopy (TEM), corneal specimens were prefixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C, and were secondarily fixed in 1% osmium tetroxide and potassium ferrocyanide (EMS). After rinsing with sodium cacodylate buffer, they were dehydrated in a graded series of ethanol and embedded in araldite (EMS). All semithin sections of 0.5- to 1-μm thickness were cut with a ultramicrotome (Reichert-Jung Ultracut E; C. Reichert Optische Werke AG, Vienna, Austria), counterstained with toluidine blue/basic fuchsin stain, and examined under a light microscope (Zeiss DxyC800 and Synchron LX20; Beckman Coulter Inc., Brea, CA), as described in the literature.21,22

**RESULTS**

**Immunohistochemistry**

Expression of NaBC1 was examined in WT and Slc4a11 KO mouse corneas using immunohistochemistry. The endothelial cells stained positive for NaBC1 in corneas of 12-week-old and 48-week-old WT mice. In KO mice, no immunoreactivity for NaBC1 was detected in cornea from mice of either age, indicating the successful deletion of the gene (Fig. 1A).

The endothelial cells of WT and KO mice also reacted positively to aquaporin-1 (Fig. 1B). In the stroma, immunoreactivity was detected for fibronectin, collagen VI, and collagen I in both groups. Collagen I did not stain the DM or endothelial cells of either the KO or the WT mice. Immunoreactivity to collagen VIIIα2 and laminin was also found in both genotypes with the DM visibly thicker in the KO mice at 48 weeks (Figs. 1C–G). Both collagen VIIIα2 and laminin immunoreactivity was detected in the anterior banded zone of the DM.

In kidney tissue, expression of NaBC1 was observed close to the proximal tubule, identified by staining of brush border with phalloidin-TRITC in WT mice, whereas no NaBC1 immunofluorescence was found in KO mice. In both groups, immunoreactivity to aquaporin-1 was noted (Fig. 2).

**RT-PCR**

RT-PCR of the corneal tissue of wild-type mice showed amplified products of 421 bp, 547 bp, 579 bp, 967 bp, and 965 bp, respectively, corresponding to the expected sizes amplified by the five primer pairs. As expected, no product was detected for primer pairs 1, 2, or 3, and truncated products were amplified by primer sequence 4 and 5 in corneal tissue samples of KO mice, confirming the deletion of the Slc4a11 gene between exons 9 to 13 in the KO mice (Fig. 3).

**Anterior Segment Examination and AS-OCT**

Anterior segment photography demonstrated no substantial change in the corneal thickness of WT mice throughout the
Immunoreactivity in the stroma and DM was noted for collagen VIII (Table 2). There was no significant difference in the corneal thickness of the WT mice among the five time points (93.9 ± 8.6 μm, 94.1 ± 8.0 μm, 94.7 ± 10.1 μm, 101.8 ± 4.7 μm, and 99.4 ± 3.6 μm at 10, 20, 30, 40, and 50 weeks, respectively; P = 0.259, F = 1.413, one-way ANOVA), whereas there was a significant difference in the corneal thickness of the KO mice chronologically among the five time points (132.7 ± 8.2 μm, 151.0 ± 7.7 μm, 169.3 ± 29.1 μm, 198.4 ± 35.4 μm, and 215.8 ± 21.1 μm at 10, 20, 30, 40, and 50 weeks, respectively; P = 0.001, F = 12.902, one-way ANOVA). Post hoc pairwise multiple comparisons adjusted by the Bonferroni–Dunn method are shown in Table 2. Although the corneal thickness was not different between any time points in WT mice, significant differences in the corneal thickness were found between 10- and 40-week (P = 0.001); 10 and 50-week (P = 0.001); 20- and 40-week (P = 0.016); 20- and 50-week (P = 0.001); and between 30- and 50-week-old (P = 0.018) KO mice.

**Endothelial Cell Staining**

Endothelial cell density (ECD) was not significantly different between WT and KO mice aged 10 weeks (3168 ± 292 cells/mm² [WT] vs. 3027 ± 296 cells/mm² [KO], P = 0.137). However, at 40 weeks, the ECD was significantly lower in KO mice compared with WT mice (2717 ± 235 cells/mm² [WT] vs. 2036 ± 236 cells/mm² [KO], P < 0.001). Although there was no significant difference in the mean endothelial cell area between 10-week-old WT and KO mice (190.9 ± 38.3 μm² [WT] vs. 193.9 ± 43.4 μm² [KO], P = 0.196), the mean cell area was significantly larger in 40-week-old KO mice compared with 40-week-old WT mice (196.4 ± 43.7 μm² [WT] vs. 293.1 ± 64.5 μm² [KO], P < 0.001). A significantly larger cell area and lower ECD were noted in 40-week-old KO mice compared with 10-week-old KO mice (P < 0.001 for both parameters).

Increased cell area and decreased ECD were remarkable in 40-week-old KO mice, although these findings are not prominent in 10-week-old KO mice (Fig. 6).

**Electron Microscopy**

SEM of the WT mouse endothelial cells showed regular size and hexagonal morphology throughout all the time points. In the KO mouse, mild endothelial cell swelling was observed at 10 weeks, although the hexagonal morphology and clear cell border was maintained. The cell swelling progressed with increasing age, and led to increasing distortion of the hexagonal morphology. At 90 weeks, destruction of the cell membrane was also noted (Fig. 7).

TEM of the KO mice showed the characteristic findings of CHED2, such as intracellular vacuolization of the endothelial cell, thickening of DM, deposition of amorphous material, and disorganization of collagen fibrils in the stroma (Figs. 8A, 8B). TEM also demonstrated that thickening of DM and endothelial vacuolization worsen with the increasing age (Fig. 8C).

**Urinalysis**

Urine osmolality was significantly decreased in KO mice compared with WT mice. Concentrations of all electrolytes measured were also significantly lower in KO mice than in WT mice, suggesting a dysfunction in water resorption in kidney. The concentration of urea was unable to be measured because the concentration was over the upper range that our urine analyzer was able to measure in all mice except four KO mice (Table 3).
DISCUSSION

In this study, we provide a detailed description of the corneal phenotype observed in the *Slc4a11* KO mice, generated by the targeted deletion of exons 9 to 13 of the murine *Slc4a11* gene using the Cre-Lox system. Previously, this model was used to demonstrate an edematous cornea due to the absence of *Slc4a11* gene function, while the corneal endothelium remained intact with no evidence of cell loss. In the present study, a more detailed characterization of this mouse is presented and includes histological and imaging data gathered at time points ranging from 10 to 90 weeks. We were able to show the development of the characteristic morphological changes of CHED2, such as corneal edema, disorganization of collagen fibril, and deposition of amorphous material in corneal stroma, swelling of endothelial cells, and thickening of DM and vacuolization of endothelial cells. Moreover, we have also demonstrated the progression of these corneal changes for the first time in a mouse model similar to that seen in human patients. Our immunohistochemical analyses of proteins present normally in basement membranes also support what has been suggested previously in human studies. In recessive CHED, it has been shown that the DM is thickened not by a nonspecific fibrillary post collagenous layer, but by accumulation of normal, posterior non-banded zone (PNBZ) material. The absence of collagen I

**Figure 2.** Immunohistochemistry of NaBC1 and aquaporin-1 in 48-week-old WT and KO mouse kidney. **Upper:** NaBC1 immunofluorescence (green) is present close to proximal tubules marked by phalloidin staining in red (upper left) in WT mice. However, NaBC1 immunofluorescence is undetectable in KO mice. **Lower:** Expression of aquaporin-1 (green) is detectable in both groups. Scale bars: 50 μm.

**Figure 3.** RT-PCR showing the deletion of the *Slc4a11* gene in the KO mice. Tissue sample from WT mice displayed products of all the five primers, while samples from KO mice failed to display any product for primer pairs 1, 2, or 3 and produced truncated products for primer pairs 4 and 5. Neg, negative control with no DNA lysate.
staining in DM and similar staining patterns for collagens IV, VIIIα2, fibronectin, and laminin seen between WT and KO mice, despite the obvious thickening of DM seen in the KO, also indicate that the deranged murine endothelial cells of the KO have an accelerated rate of deposition of normal PNBZ material.2,5,23

Recently, there has been much interest in the development of \textit{Slc4a11} KO mouse models.25,26 The \textit{Slc4a11} knockout model by Lopez et al.25 was generated using a retroviral gene trap vector and had a more pronounced phenotype in the ear (i.e., sensorineural deafness) similar to Harboyan syndrome while there was no phenotypic changes in the cornea. Gröger et al.26 generated \textit{Slc4a11} KO mice by inserting a beta-galactosidase coding sequence in frame into the tenth exon of \textit{Slc4a11}. This mutation led to the truncation of the SLC4A11 protein before the first predicted transmembrane domain resulting in a cytoplasmic localization of the beta-Gal-fusion protein in vivo. Morphological analyses of the cornea of this model revealed morphological alterations in all layers of the cornea of 12-month-old mice. The authors did not report an earlier phenotype nor did they show if these phenotypic features progressed with age or remained stationary.

Although CHED2 was initially believed to be a stationary disease, studies in patients have demonstrated evidence of its progressive nature.5,25,27 A case report in a patient revealed that a corneal button taken at the time of transplantation of the right eye at 2.5 years of age showed only mild changes including mild corneal edema, slightly irregular epithelium, disorganization of collagen fibrils of only superficial anterior stroma, and early selective endothelial dystrophy while DM remained intact.25 However, a corneal button of the left eye taken at 14 years of age revealed more severe changes, including corneal thickening of 2.5-fold compared with the right cornea, DM thickening by 5-fold, and severe change of endothelial cells with numerous vacuoles, debris, and disrupted mitochondria.25 McCartney and Kirkness24 also described the progressive DM thickening with age in human patients. The results of the present study in mice demonstrate that the corneal thickening and structural distortions are mild at an early age and progress with time, and replicate the reports from patients.

NaBC1 was previously postulated to stimulate cell growth and proliferation by increasing intracellular borate and activating the mitogen activated protein kinase pathway.16 However, a role for borate has not been clearly demonstrated in humans.10 As borate can diffuse across biological membranes as an uncharged molecule at physiological pH, the requirement for an active transporter for borate is also not evident.10,16,28 Moreover, recent in vitro studies have shown that the human NaBC1 does not have borate transporter activity. Ogando et al. (Ogando DG, et al. \textit{IOVS} 2012;53:ARVO E-Abstract 6005) demonstrated that NaBC1 is a transporter with \textit{Na}⁺:\textit{OH}⁻/\textit{C}⁰⁻ cotransport activity and not a bicarbonate transporter or a borate-linked cotransporter. Accumulation of \textit{NaCl} in the corneal stroma of the \textit{Slc4a11} KO mice generated by Gröger et al.26 was attributed to the failure of fluid transport from the corneal endothelium that is coupled to transcellular solute movement.8,26,29 \textit{Slc4a11} is also expressed in the inner ear and kidney, and studies have demonstrated defects of these organs in other mouse models (i.e., sensorineural hearing loss and urinary concentrating defects).25,26,30 The results of urine analyses showed significantly decreased urine osmolarity and electrolyte concentrations in the KO mice, suggesting impaired water resorption in the kidney. However, although the \textit{SLC4A11} gene is expressed in human kidney,13 the phenotypic change in human kidney has never been extensively studied. Vlas et al.18 showed that NaBC1 mediates water movement across the basolateral surface of endothelium, working in concert with apical aquaporin proteins.18 However, they suggested that the only driving force for water flux is osmotic gradient, and that it was not coupled to solute movement.

**Figure 4.** Representative slit-lamp photography of the cornea of WT (upper) and KO (lower) mice. Whereas the corneal thickness of WT mice remained stationary, the corneal thickness of KO mice increased with age.
Our mouse model also showed that aquaporin expression remained intact, indicating that the functional role provided by NaBC1 in the corneal endothelium is essential for a homeostatic cornea and therefore nonredundant, and the water transport mediated by NaBC1 may be independent of aquaporin.

The corneal endothelium is derived from neural crest cells, and it is possible that NaBC1, given its postulated role in cell growth and proliferation, is required for the growth, proliferation, and migration of neural crest cells.31,32 However, none of the previous Slc4a11 KO models indicated reduced proliferation or accelerated cell death of corneal endothelial cells.18,25,26 Our results show that the corneal endothelial cell density of young KO mice at 10 weeks of age was comparable with WT mice, suggesting that cell proliferation is unaffected during development and the population of the endothelium (Fig. 5). The results that 40-week-old KO mice had significantly increased endothelial cell size and lower cell number compared with 10-week-old KO mice suggest that the depletion of the Slc4a11 gene caused progressive cell damage and resultant CEC apoptosis. A recent in vitro study revealed...
that Slc4a11 knockdown in human CEC led to suppressed cell growth and reduced cell viability by activating the apoptotic pathway. However, the exact mechanism of the early CEC death is still unclear, and further studies are necessary to elucidate the mechanism.

The current study has limitations as follows:

1. Due to lack of available expertise, we were unable to explore the abnormalities of the inner ear of the KO model.
2. We were not able to determine daily urine output; therefore, we cannot demonstrate an increase of urine volume that may further support a dysfunction of water resorption in the kidney. However, significant decrease in the urine osmolarity and concentrations of all electrolytes suggests an abnormality in the urine concentration mechanism in the kidney.

3. Although mice are used in widespread models of human genetic disorders, they often fail to completely mimic the human phenotype. Previous mouse models of CHED also demonstrated successful knockout of the causative gene, but only minimal or mild corneal changes. Sultana et al. demonstrated the reduction of ECD by 10-fold in CHED2 patients compared with age-matched controls. By contrast, the results of this study show that the ECD of the KO mouse is not significantly decreased at 10 weeks and decreased only by one-fourth at 40 weeks. Lopez et al. also reported that their Slc4a11 KO mouse model did not show any significant difference in endothelial cell density, size, and shape compared with the WT mouse of the same age (3–10 months). These findings suggest that the phenotypic changes in

![Figure 8](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932986/)

**Figure 8.** TEM. (A) A TEM photo of a 10-week-old KO mouse showed deposition of amorphous material in the stroma (black arrows) and endothelial vacuolization (white arrows) and thickening of DM (black arrowheads). (B) A TEM photo of a 90-week-old mouse revealed disorganization of collagen fibril (black arrowheads) and thickening of DM (white arrowheads). (C) Sequential TEM photos revealed that the thickening of DM and endothelial vacuolization is mild at 10 weeks, and worsened with the increase of age. DM thickness of each sample was as follows: 1.47 μm at 10 weeks, 1.59 μm at 20 weeks, 2.87 μm at 30 weeks, and 2.98 μm at 40 weeks for WT mice; and 1.78 μm at 10 weeks, 3.89 μm at 20 weeks, 4.85 at 30 weeks and 5.72 μm at 40 weeks for KO mice. Scale bars: 1 μm.

### Table 3. Comparison of Urine Osmolarities and Concentrations of Urine Electrolytes Between Slc4a11+/+ (WT) and Slc4a11−/− (KO) Mouse

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<th>WT, Mean ± SD</th>
<th>KO, Mean ± SD</th>
<th>P Value*</th>
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<tr>
<td>Osmolarity, mOsm/L, n = 11 for WT and 12 for KO</td>
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<td>Potassium</td>
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</table>

* Mann-Whitney U test.
† The concentration of urea was beyond the upper range that our machine can measure in all mice except four KO mice.
this model are possibly milder than those in human patients. However, this phenomenon might prove advantageous as the prolonged course endothelial deterioration may enable the serial monitoring of the cellular changes and the study of disease mechanisms.

In conclusion, the Slc4a11 mouse model described here demonstrates the progressive changes of the corneal abnormalities as seen in human CHED2. This model will therefore be useful for future studies on the pathophysiology of CHED2 and for the evaluation of treatment modalities such as gene replacement therapy for CHED2.

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References

APPENDIX

FIGURE A1. PCR primers. For the detection of the deletion of the Slc4a11 gene, five pairs of primers were used. Primer 1 amplified part of the deleted sequence and part of the nondeleted sequence. Primers 2 and 3 amplified a sequence that is within the deleted sequence. Both primers 4 and 5 amplified a region encompassing exon 9 to exon 13. The details of the primers were as follows: primer 1 (product size 421 bp): F1-5’ATGCCTTCCTCAACCAGC3’ and R1-5’GCACAGTCAGACGGGTCTC3’; primer 2 (product size 547 bp): F1-5’ATTCAAGAGGCTGTGGGTGC3’ and R1-5’GCTGAGGTTGAGGAAGCAT3’; primer 3 (product size 579 bp): F1-5’AAGCTCTGAGCAGACTGCAC3’ and R1-5’CAGGCTAGGTTGAGGAAGG3’; primer 4 (product size 967 bp): F1-5’GGTCAGTACGAGTCTG3’ and R1-5’GGCTCACCAAGGGAAGT3’; and primer 5 (product size 963 bp): F1-5’TGCAGTACGAGTCCTG3’ and R1-5’GGCTCACCAAGGGAAGTC3’.