Depletion of *SLC4A11* Causes Cell Death by Apoptosis in an Immortalized Human Corneal Endothelial Cell Line

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PURPOSE. To investigate the effects of *SLC4A11* gene depletion in human corneal endothelial cells.

METHODS. To achieve stable downregulation of *SLC4A11* gene expression in immortalized human corneal endothelial cells (HCECs), short-hairpin RNA (shRNA) targeted against *SLC4A11* was used. Cell growth and viability were determined using the real-time cell analyzer and trypan blue staining respectively. Apoptosis was investigated by Annexin V and TUNEL assays. Alterations in apoptotic gene expression following *SLC4A11* silencing were determined using the RT²Profiler PCR array for human apoptosis while activation of the apoptotic pathway was ascertained by western analysis.

RESULTS. *SLC4A11* silencing in HCECs could be achieved by stable expression of shRNA targeted against *SLC4A11*. *SLC4A11* knockdown suppressed HCEC growth and reduced HCEC viability compared to the control. This reduction in cell growth is associated with increased apoptosis in *SLC4A11*-silenced cells.

CONCLUSIONS. Our data suggest that the reduction of cell number with time in *SLC4A11*-depleted HCECs is due to an increase in cell death by apoptosis. This suggests that *SLC4A11* is necessary for cell survival and may explain the pathologic corneal endothelial cell loss in endotheliopathies due to *SLC4A11* mutations. (*Invest Ophthalmol Vis Sci.* 2012; 53:3270–3279) DOI:10.1167/iops.11-8724

The *SLC4A11* gene encodes an 891 amino acid membrane protein that was phylogenetically identified as a member of the Solute Carrier 4 (SLC4) protein family.¹ This family is composed of integral membrane proteins that mediate Cl[−]/

HCO₃[−] exchange or Na⁺-coupled HCO₃[−] cotransport across the plasma membrane.^{1–3} *SLC4A11* is the most divergent member of the family and reported to function as an electrogenic Na⁺-coupled borate cotransporter.⁴

SLC4A11 plays an important role in cornea functions as mutations in *SLC4A11* are associated with recessive congenital hereditary endothelial dystrophy (CHED), corneal dystrophy and perceptive deafness (Harboyan syndrome, HS) as well as late onset Fuchs endothelial corneal dystrophy (FECD).^{5–8} CHED MIM #121700 and MIM #217700 is an inherited bilateral disorder of the corneal endothelium characterized by corneal opacification which ranges from a diffuse haze to a ground glass, milk appearance.^{9,10} The Descemet's membrane in CHED consists of a normal anterior banded zone (ABZ) but the posterior nonbanded zone (PNBZ) is thickened, implying alterations in growth regulation during the terminal differentiation and reorganization of the endothelium.¹⁰ The endothelium in CHED also shows a reduction in cell number and a loss of the typical hexagonal cellular structure with many cells appearing vacuolated and dystrophic.^{10,11} FECD is a late onset disease characterized by the progressive degeneration of corneal endothelial cells, resulting in corneal decompensation, a thickened Descemet's membrane, and a collagen-rich basal lamina secreted by the endothelium. The gradual impairment of endothelial cell function and cell loss in FECD commonly lead to stromal edema and impaired vision.¹²

Although *SLC4A11* involvement in these corneal endothelial dystrophies has been known for a few years, the associated disease mechanisms are just beginning to be unraveled. There are considerable gaps in knowledge as little is known yet of the exact physiological role played by *SLC4A11* in the endothelium. Our previous studies indicated haploinsufficiency as the underlying disease mechanism for FECD-associated mutations, based on the observed failure of the mutant SLC4A11 protein to translocate to its normal position in the plasma membrane, presumably due to improper posttranslational modification.⁸ Based on these findings and clinical features, we further hypothesized that reduced levels of *SLC4A11* influence the long-term viability of the neural crest derived corneal endothelial cells.⁸ Other studies in HeLa cells suggested that endothelial dystrophy might result from improper proliferation during fetal development, possibly caused by borate-dependent effects on cell proliferation mediated via the mitogen-activated protein kinase (MAPK) pathway.⁴ Studies in *Slc4a11* knockout mice did not, however, report reduced proliferation in the murine corneal endothelium, in apparent contrast to what had been observed in gene-depleted HeLa cells.^{4,13,14} Moreover, these mice did not show any endothelial cell loss unlike in CHED and FECD patient corneas although cornea function was obviously compromised with apparent corneal edema in at least one of the mouse models.^{10,11,14}

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To carry out long-term gene knockdown studies in cells with relevance to CHED and FECD and better understand the cellular and molecular phenotype associated with the loss of the *SLC4A11* activity, we used small hairpin RNAs (shRNAs) to deplete *SLC4A11* in immortalized human corneal endothelial cells (HCECs). In agreement with the reduced cell proliferation observed in *SLC4A11*-depleted HeLa cells,⁴ we also observed inhibition of proliferation in the *SLC4A11*-depleted HCECs compared to control cells. We further demonstrate that the reduced proliferation in the *SLC4A11*-depleted HCECs was associated with increased cell death by apoptosis.

MATERIALS AND METHODS

Antibodies and Reagents

Mouse monoclonal anti-human ZO-1 was obtained from Invitrogen (Carlsbad, CA). Anti-collagen I, Anti-Collagen IV, and anti-Fibronectin were from Abcam (Cambridge, UK). The mouse monoclonal 9.3E antibody,¹⁵ polyclonal anti-rabbit *SLC4A11*/NaBC1,¹⁶ and polyclonal anti-rabbit COL8A2¹⁷ were gifted by Monika Valtink, Jeppe Praetorius, and Albert June respectively. Rabbit monoclonal anti-human Caspase-3, cleaved Caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), cleaved Caspase-7, and cleaved Caspase-9 antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For immunofluorescence analysis, the secondary antibodies used were Alexa Fluor-488 conjugated anti-mouse or anti-rabbit antibodies (Invitrogen). For Western analysis, the secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Inc.). Puromycin and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). All cell culture reagents were purchased from Invitrogen.

Generation, Characterization and Culture of Immortalized HCECs

Primary human corneal endothelial cultures were generated from two donor human corneas procured from the Heartland Eye Bank (St. Louis, MO). These corneas were considered to be unsuitable for transplantation, due to lack of a blood sample from the donor to conduct serology tests. The left and the right corneas from a 62 year old Caucasian female donor, whose primary cause of death was pneumonia, were used and corneal endothelial cells grown using reported culture conditions.¹⁸ To immortalize the HCECs, a retroviral vector (pLXSN Retroviral Vector; Clontech Laboratories, Mountain View, CA) expressing human papilloma virus (HPV) 16-E6/E7 was transduced into the human corneal endothelial cultures as described previously.¹⁹ The resulting clones were characterized by morphology, population doublings (PD), PD time, as well as HPV16-E6/E7 RNA and HPV16-E7 protein expression following methods described previously.¹⁹ The clones showing polygonal morphology similar to human corneal endothelium and active proliferation (PD 43.2) were selected and pooled, and used for the current study. The immortalized HCEC line was cultured in Earl's minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS), essential amino acids, non-essential amino acids, 200 mM L-Glutamine, and antibiotic-antimycotic solutions at 37°C with 5% CO₂.

qRT-PCR and Quantitative Real-time PCR (Reverse Transcription PCR)

Total RNA from HCECs was isolated using an RNA purification kit (RNeasy Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol and treated with DNase digestion. First-strand cDNA was

synthesized from total RNA (500 ng) by reverse transcription using random primer (Invitrogen) and reverse transcriptase enzyme (Superscript III; Invitrogen). Table 1 lists the gene specific primers used in the reverse transcription PCR and qRT-PCR. qRT-PCR was performed in a 10 µL mixture containing 1 µL of the cDNA preparation diluted five times, 5 µL master mix (SYBR Green PCR Master Mix; Applied Biosystems, Carlsbad, CA) and 500 nM of each primer in the real-time PCR system (LightCycler 480; Roche, Basel, Switzerland) using the following PCR parameters: 95°C for 5 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. The fluorescence threshold value (Ct) was calculated using the thermocycler system software. The absence of the nonspecific products was confirmed by both the analysis of the melt curves and by electrophoresis in 2% agarose gels. To find the stable housekeeping genes from a set of tested genes (*ACTB*, *GAPDH*, *HPRT*, *TBP*, *HMBS*, *B2M*) in the HCECs treated by shRNA, the Ct values of these genes in the samples 7 days or 10 days after treatment with control shRNA or shRNA1 were analyzed by data analysis software (geNorm; PrimerDesign Ltd., Southampton, UK) according to the manufacturer's manual.²⁰ We found that human β-actin (*ACTB*) gene was the most stable and henceforth used as the normalizing standard for mRNA expression analysis in this report. Values were expressed as fold change over the corresponding values for the control by the 2^{-ΔΔCt} method.

Immunofluorescence Staining

Cells were seeded on coverslips until approximately 80% confluent. The cells were fixed for 10 minutes in ice-cold methanol, incubated with primary antibody for 2 hours at room temperature, washed three times with 1× PBS, and then incubated with Alexa Fluor 488-conjugated secondary antibody for 1 hour. Nuclear DNA was visualized by staining with DAPI. The cells were mounted in mounting media (VECTASHIELD; Vector Laboratories, Burlingame, CA) and the fluorescent images were observed using a fluorescent microscope with ApoTome attachment (Axio Imager Z1; Zeiss, Stuttgart, Germany).

Knockdown of *SLC4A11* with shRNA

Two shRNA plasmids targeted against different regions of *SLC4A11* were constructed using the piGENE U6 Rep vector (iGene Therapeutics Inc., Tsukuba, Ibaraki, Japan):

shRNA1: 5'-GCCTGAAAGAGAAACCATT-3'

shRNA2: 5'-GCACAGAGGAGGAATTCAA-3'

The piGENE U6 Rep vector containing seven tandem repeats of thymidine (T7) served as the negative control vector. The shRNAs were transfected into cells by Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfected cells were selected with 500 ng/mL puromycin 24 hours after transfection and changed to fresh selection medium 5 days after transfection. ShRNA1-transfected HCECs used in all experiments were confirmed to be knocked down for *SLC4A11* expression by Western blotting.

Western Blot Analysis

Cells were washed twice with ice-cold 1× PBS and resuspended in ice-cold lysis buffer. The lysis buffer comprised 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol (DTT), and was supplemented with proteinase inhibitors and phosphatase inhibitors cocktail tablets (Roche, Basel, Switzerland). Cell lysates were centrifuged at 11,000g for 30 minutes at 4°C. Protein concentrations of the cell lysates were determined with a protein assay kit (Bradford Protein Assay Kit; Bio-Rad, Philadelphia, PA). The protein samples (20 µg) were resolved by 10% to 15% SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk in 1× Tris-buffered saline with Tween 20 (TBST) and then incubated with primary and HRP-tagged secondary antibody. Immunostaining with antibodies was detected using the enhanced chemiluminescence substrate (Western

TABLE 1. The Sequences of Primers Used for Reverse Transcription PCR and qRT-PCR

Name	Sequences	Amplicon Size (bp)	Annealing Temp (°C)
<i>SLC4A11</i> -F	5' CTGCTTCCCTTGCAGAAAAC 3'	165	58
<i>SLC4A11</i> -R	5' TACTCTCGCCAGACACGATG 3'		
<i>ZO-1</i> -F	5' GAATGATGGTTGGTATGGTGCG 3'	196	58
<i>ZO-1</i> -R	5' TCAGAAGTGTGTCTACTGTCCG 3'		
<i>FN1</i> -F	5' GCTCATCATCTGGCCATTTT 3'	230	58
<i>FN1</i> -R	5' ACCAACCTACGGATGACTCG 3'		
<i>COL8A2</i> -F	5' TGTCTTCGCTGCTGCTGCT 3'	209	58
<i>COL8A2</i> -R	5' TCCCTTCAGGTCCATCGGCA 3'		
<i>COL1A1</i> -F	5' GTGCTAAAGGTGCCAATGGT 3'	128	58
<i>COL1A1</i> -R	5' ACCAGGTTACCGCTGTTAC 3'		
<i>ATP1A1</i> -F	5' TGCCTAGTACACGGCAGTGATCTAA 3'	163	58
<i>ATP1A1</i> -R	5' CACCAGTCACAGCCACGA 3'		
<i>COL4A1</i> -F	5' ACTCTTTTGTGATGCACACCA 3'	151	58
<i>COL4A1</i> -R	5' AAGCTGTAAGCGTTTGCCTA 3'		
<i>ACTB</i> -F	5' CCAACCGCGAGAAGATGA 3'	234	58
<i>ACTB</i> -R	5' CCAGAGGCGTACAGGGATAG 3'		
<i>GAPDH</i> -F	5' GAAGGTGAAGGTCGGAGTCA 3'	109	58
<i>GAPDH</i> -R	5' AATGAAGGGTCATTGATGG 3'		

Lightning-ECL; Perkin-Elmer, Waltham, MA) followed by exposure to X-ray film (Fujifilm, Tokyo, Japan).

Real-time Cell Growth Measurement

A real time cell analyzer (RTCA) (xCelligence RTCA SP; Roche Diagnostics GmbH, Penzberg, Germany) was used to assess cell proliferation according to manufacturer's instructions.²¹ HCECs were seeded onto the RTCA's E-96 plate 5 days after transfection in quadruplicates. The plated cells were allowed to equilibrate for at least 30 minutes in the tissue culture incubator before electrode resistance was recorded. Cell growth was monitored continuously for up to 7 days.

Viable Cell Counting

Viable HCEC cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA) after staining with trypan blue (Sigma-Aldrich). Trypan blue-stained HCECs were counted at 5, 7, and 10 days after shRNA transfection.

Detection of Apoptosis

Apoptosis was measured using two methods. Early apoptosis was assessed by flow cytometry using the Guava Nexin Reagent (Guava Technologies, Hayward, CA). Briefly, cells were harvested using dispersal reagent (Guava ViaCount; EMD Millipore, Billerica, MA) according to the manufacturer's instructions. After washing twice with 1× PBS and being treated with Guava ViaCount dispersal reagent, the cells were resuspended in cell culture medium and centrifuged at 300g for 7 minutes. The cell pellet was then stained with the Guava Nexin reagent (Guava Technologies) according to the manufacturer's instructions. Five thousand cells from each sample were analyzed. Cell populations were quantified using a flow cytometry system (Guava EasyCyte Plus; Guava Technologies).

To detect cells in the late phase of apoptosis, a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-FITC nick-end labeling (TUNEL) assay was performed using the TUNEL assay kit (EMD Millipore) and quantified using the Guava EasyCyte Plus flow cytometry system (Guava Technologies) according to the manufacturers' instructions. The data were analyzed using the easyCyte software module (Guava Express Pro; Guava Technologies). As for TUNEL staining, HCEC cells were transfected, selected with puromycin, and then fixed in 4% paraformaldehyde for 15 minutes at room

temperature. The fixed cells were then stained with DeadEnd TUNEL assay kit (Promega, Madison, WI) according to manufacturer's instruction and mounted in mounting media (VECTASHIELD; Vector Laboratories). Fluorescent images were observed under the fluorescent microscope with ApoTome attachment (Axio Imager Z1; Zeiss).

Human Apoptotic PCR Array

The RT² Profiler apoptotic PCR array (PAHS-012A, SA Biosciences, Qiagen) was used to analyze the apoptotic gene expression profile of *SLC4A11* knockdown cells. The cDNA sample and SYBR Green master mix were added to the PCR array plate, and the PCR reactions were performed in the real-time PCR system (LightCycler 480; Roche) according to the manufacturer's instructions. The data were analyzed by the online RT² Profiler PCR array data analysis software (Qiagen).

Statistical Analysis

All the data used for statistical analysis were obtained from three independent experiments. The significance of difference between groups was determined by the 2-tailed Student's *t*-test using spreadsheet software (Excel 5.0; Microsoft, Redmond, WA), with significance at $P \leq 0.05$.

RESULTS

Characterization of the Immortalized HCEC Line

Immortalization of HCECs was achieved by transfecting primary HCECs with the HPV E6 and E7 genes. To ascertain that the HCECs retained the phenotypic properties of primary corneal endothelial cells, we examined the expression of several genes normally present in endothelial cells by reverse transcription PCR and immunofluorescent analyses (Fig. 1). We observed that the HCECs expressed mRNAs for *SLC4A11*, *ZO-1*, fibronectin, collagen type VIII alpha 2, collagen type I, collagen type IV, and the alpha-1 subunit of sodium/potassium-transporting ATPase (Fig. 1A). Immunofluorescent analyses revealed the expression of the corneal endothelial-specific 9.3E antigen in a pattern similar to that previously described in primary HCECs (Fig. 1B).¹⁵ Also observed was the expression of collagen I, collagen IV, collagen type VIII alpha 2, the junctional protein ZO-1, and fibronectin (Fig. 1B). Hence, the

FIGURE 1. Characterization of immortalized HCECs. (A) mRNA expression of *SLC4A11*, *ZO-1*, *FN1*, *COL8A2*, *COL1A1*, *ATP1A1*, and *COL4A1* in HCECs. Cultured HCECs were harvested and analyzed by reverse transcription PCR. Size markers are shown in the first lane from the left. (B) Cellular localization of collagen I (*COL1*), collagen IV (*COL4*), and collagen VIII alpha 2 (*COL8A2*), 9.3E, *ZO-1*, and fibronectin in HCECs. Cells were immunostained with the indicated antibodies and visualized by fluorescent microscopy. Scale bar: 10 μ m.

HCECs retained essential features of primary corneal endothelial cells.

Depletion of *SLC4A11* Using shRNA

To achieve stable and long-term knockdown of *SLC4A11*, HCECs were transfected with either of two shRNAs targeted against two independent regions of *SLC4A11* (shRNA1 and shRNA2). The T7 shRNA-transfected HCECs served as the negative control. Gene depletion was evaluated by both quantitative RT-PCR (qPCR) and Western analysis (Fig. 2). Seven days after shRNA transfection, the transcription level of *SLC4A11* was reduced to 60% (40% knockdown) or 40% (60% knockdown) in cells transfected with shRNA1 or shRNA2 respectively compared to cells transfected with control (Fig. 2A). Ten days after shRNA transfection, the transcription level of *SLC4A11* was reduced to 48% or 35% in cells transfected with shRNA1 or shRNA2, respectively, compared to cells transfected with control (Fig. 2A). The knockdown effects of shRNA1 on *SLC4A11* mRNA expression on both day 7 and day 10 were not significantly different from that due to shRNA2.

Western analysis using the SLC4A11 antibody revealed endogenous SLC4A11 as a protein of approximately 100 kDa, as seen previously.¹⁶ In corroboration with the mRNA data, the protein level of SLC4A11 was also reduced by 40% or 30% in

FIGURE 2. Knockdown of *SLC4A11* in HCECs by shRNA. (A) mRNA expression of *SLC4A11* after shRNA transfection. Two shRNAs targeting different regions of *SLC4A11* (shRNA1, 2) or control T7 shRNA were transfected into HCECs. The cell lysates were harvested 7 days or 10 days after transfection and reverse transcribed into cDNA, which were then used as templates for quantitative RT-PCR. Data are expressed as mean fold change $\pm$ SD in mRNA expression relative to control of three independent experiments. * $P < 0.05$. (B) Protein expression of *SLC4A11* after shRNA transfection. Two shRNAs targeting *SLC4A11* genes (shRNA1, 2) and the control shRNA were transfected into HCECs, respectively. *Upper panel*: the cell lysates were harvested 7 days or 10 days after transfection and subjected to Western blot analysis with the indicated antibodies. Immunoblotting with GAPDH antibody was used as loading control. *Lower panel*: densitometric analysis of the immunoblot represented in the upper panel. Values are normalized against GAPDH. Data shown are the mean $\pm$ SD from three independent experiments. * $P < 0.05$.

cells transfected with shRNA1 or shRNA2 respectively compared to cells transfected with the control shRNA after 7 days (Fig. 2B). At day 10 posttransfection, expression of endogenous SLC4A11 in cells treated with either shRNA1 or shRNA2 was approximately 40% of that in the negative control (Fig. 2B). Again, the knockdown effects of shRNA1 on *SLC4A11* protein expression on both day 7 and day 10 were not significantly different from that due to shRNA2. These results indicate that sustained knockdown of endogenous *SLC4A11* expression in HCECs is achievable with shRNAs at both the RNA and protein levels. As shRNA1 and shRNA2 were equally effective in silencing *SLC4A11* expression, we decided to use shRNA1 for subsequent experiments to study the effect of *SLC4A11*-silencing in HCECs.

Depletion of *SLC4A11* Reduced HCEC Proliferation

Next, we investigated the effect of *SLC4A11* knockdown on HCEC cell growth. The RTCA SP instrument was used to analyze cell growth.²¹ Cell index values produced by the shRNA1-transfected cells indicated the lack of proliferation while the cell index values of the control transfected cells

FIGURE 3. Proliferative capacity of HCEC after *SLC4A11* silencing. (A) The real-time growth rate of HCECs transfected with either control (T7 shRNA) or shRNA1 was analyzed using the xCelligence RTCA SP instrument in quadruplicates. (B) Cell viability was analyzed by trypan blue staining. Cell viability is expressed as % viable cells [(number of viable cells / total number of cells) $\times$ 100%]. Data shown are the mean $\pm$ SD from three independent experiments.

increased progressively with time suggesting normal growth proliferation (Fig. 3A). Moreover, when the trypan blue-stained cells were counted, we observed dramatic decreases in viable cell numbers in shRNA1-transfected HCECs 7 and 10 days after transfection compared to control transfected HCECs (Fig. 3B). At day 5 following shRNA transfection, the cell viability of HCECs with control shRNA and those with shRNA1 was near 80%. However at day 7, the cell viability of HCECs transfected with shRNA1 had reduced significantly compared to the control (27.6% vs. 77.1%; $P = 0.01$). At day 10, viability was further significantly reduced to 16.4% in shRNA1-transfected HCECs compared to 56.4% in control ($P = 0.01$). Hence, knockdown of *SLC4A11* suppressed cell proliferation and decreased cell viability suggesting that *SLC4A11* is essential for normal HCEC growth and survival.

Knockdown of *SLC4A11* Induced HCEC Apoptosis

To determine if the suppression of HCEC cell proliferation was associated with the induction of apoptosis, we analyzed Annexin V expression on the cell surface of shRNA1-transfected HCECs using flow cytometry and compared that against control transfected cells. We observed that approximately 12.7% more cells in shRNA1-transfected HCECs exhibited early apoptosis when compared to control transfected cells 7 days after transfection ($P = 0.01$; Fig. 4A, upper panels). At day 10 following transfection, we observed a similar profile although the gap between control and *SLC4A11* knockdown cells was smaller in terms of apoptotic cells but the 7.6% difference remained significant ($P = 0.02$, Fig. 4A, lower panels). To determine if *SLC4A11* knockdown also affects late apoptosis, we examined the cells by TUNEL assay via flow cytometry and immunofluorescence analysis. At day 7 following transfection with shRNA1, the *SLC4A11*-depleted cells undergoing late apoptosis were approximately 7.7% more than those cells with control shRNA ($P = 0.008$). At day 10 following transfection with shRNA1, there were 14.1% more cells in the

late apoptotic stage compared to control transfected cells ($P = 0.01$). Immunofluorescent analysis confirmed the increased number of TUNEL-positive cells upon transfection with shRNA1 compared to cells transfected with control shRNA (Lower panel of Fig. 4B). Hence, *SLC4A11* silencing increases the apoptosis of HCECs.

Expression of Apoptotic Genes in *SLC4A11* Knockdown Cells

Apoptosis is a tightly regulated and highly efficient cell death program which requires the interplay of a multitude of factors. To better understand the molecular apoptotic signature induced by the silencing of *SLC4A11* expression, we analyzed the knocked down cells using the RT² Profiler PCR array for human apoptosis. For this assay, HCECs were transfected with either control or shRNA1 for 7 or 10 days before being harvested for the array analysis. Compared with the samples transfected with control shRNA, we observed that several prominent proapoptotic genes were upregulated in the shRNA1-transfected cells at day 7, although a few antiapoptotic genes were also upregulated (Table 2). Notably, caspases-1, -2, -3, -6, -7, and -9 were significantly upregulated in the shRNA1-transfected cells. It is evident that by day 10 post-transfection, expression of the majority of the early stage proapoptotic genes was no longer significant; suggesting that apoptosis at this time point may be entering the late stage. Analysis of the fold change values of the apoptotic genes between day 7 and day 10 (Table 2) also suggests that a handful of proapoptotic genes, namely *BNIP3*, *BNIP3L*, *FAS*, and *TNFRSF11B*, are significantly different between day 7 and day 10, suggesting that these genes play significantly more important roles in day 7 compared to day 10.

To validate the qPCR array data, we performed Western blot analysis for activated caspase expression in shRNA1 or shRNA2 transfected cells compared to control transfected cells (Fig. 5). As observed before (Fig. 2B), both shRNA1 and shRNA2 effected knockdown of *SLC4A11* protein expression at 7 days post-transfection with greater knockdown after 10 days (Fig. 5B). Cleaved caspase-9, -7, and -3 were observed to be increased in the *SLC4A11*-depleted cells at both 7 and 10 days after transfection compared to cells transfected with control shRNA (Fig. 5B). The level of cleaved PARP, a substrate for caspase-3 and -7, was correspondingly increased in *SLC4A11*-depleted cells compared to control cells (Fig. 5B). Taken together, the data from the PCR array and Western blot analyses strongly suggest that reduced levels of *SLC4A11* potentiate HCECs to undergo cell death via apoptosis.

DISCUSSION

Mutations in *SLC4A11* have been identified in two different human corneal endothelial dystrophies, CHED and FECD, where the disease mechanisms are still unclear.^{5,22,23} Previous studies of *SLC4A11* knockdown in HeLa cells by short interfering RNAs (siRNAs) showed reduced cell proliferation and growth.^{4,24} In the postnatal human cornea, however, the corneal endothelial cells do not proliferate because they are arrested in the G1 phase of the cell cycle.²⁵ Hence, *SLC4A11* is unlikely to be involved in the proliferation of these cells in vivo. Rather, we suspect that this protein would be more critical for cell survival and cell viability. In this scenario and considering the delayed onset of symptoms in FECD harboring heterozygous *SLC4A11* mutations, long-term inhibition of *SLC4A11* expression would be a more appropriate strategy to study the effects of this gene loss on corneal endothelial cell property. In culture, corneal endothelial cells are released from

FIGURE 4. Depletion of *SLC4A11* induces apoptosis in HCECs. **(A)** Quantitative detection of early apoptosis by Annexin-V assay of cells. Seven days or 10 days after transfection with either control or shRNA1, the HCECs were harvested, stained with the Nexin reagent, and analyzed by the Guava flow cytometry system. Representative dot plots are shown. Early apoptotic cells are shown within the black box. The indicated values are the mean $\pm$ SD from three independent experiments. $*P < 0.05$. **(B)** Quantitative detection of late apoptosis by TUNEL staining and flow cytometry. Cells were transfected and harvested as indicated in **(A)** and then processed using the TUNEL assay kit followed by analysis via the Guava flow cytometry system. Representative plots are shown. Values shown are the mean $\pm$ SD from three independent experiments. $*P < 0.05$.

(C) Qualitative detection of late apoptosis by TUNEL staining. Ten days after transfection with either control or shRNA1, the HCECs were fixed and immunostained using the TUNEL assay kit. Cells positive for TUNEL staining are visualized as green fluorescence while nuclei were visualized as blue fluorescence by DAPI staining. Scale bar: 50 μ m.

contact inhibition and are able to enter the cell cycle but only for a limited number of passages. Hence, to effect long-term depletion of *SLC4A11* expression, we enlisted the help of a transformed corneal endothelial cell line and used shRNAs to deplete *SLC4A11* in these cells. The knockdown of *SLC4A11* in HCECs at the transcriptional and protein levels could be observed 7 days after shRNA transfection and maintained for at least 10 days. Cell proliferation was suppressed in cells with reduced levels of the *SLC4A11* and in addition, we have demonstrated in this study that this is associated with decreased cell viability and increased cell death via apoptosis.

The precise physiological role of the sodium borate cotransporter SLC4A11 in the corneal endothelium is not well understood, nor is the biological relevance of borate to the

cornea. Nevertheless, the present study suggests that the failure of *SLC4A11* function will lead to apoptosis as has been shown previously for knockdown of SLC4A7, a sodium bicarbonate cotransporter, in coronary endothelial cells.²⁶ Failure to elicit the transport function of SLC4A11 may lead to a DNA damage signal culminating in the assembly of the apoptosome comprised of cytochrome c and apoptotic protease-activating factor (Apaf)-1, which is induced in the *SLC4A11* knockdown cells. According to the caspase cascade, the recruitment and activation of initiator caspases such as caspases-2 and -9 follow, which then activate the effector caspases such as caspase-3, -6, and -7.²⁷ All these mentioned caspases demonstrated increased expression in the *SLC4A11* knockdown cells, which we further showed to be activated

TABLE 2. Expression of Apoptotic Genes in *SLC4A11* Knockdown Cells

Gene Name	Proapoptotic or Antiapoptotic	Fold Change in mRNA Relative to Control (Day 7)	Fold Change in mRNA Relative to Control (Day 10)	P Value
Caspases				
<i>CASP1</i>	Pro	5.46 $\pm$ 1.85*	2.44 $\pm$ 0.34*	0.249
<i>CASP2</i>	Pro	2.14 $\pm$ 0.50*	1.04 $\pm$ 0.14	0.168
<i>CASP3</i>	Pro	2.07 $\pm$ 0.70*	0.90 $\pm$ 0.12	0.241
<i>CASP6</i>	Pro	2.22 $\pm$ 0.29*	1.14 $\pm$ 0.10	0.074
<i>CASP7</i>	Pro	2.65 $\pm$ 0.75*	0.87 $\pm$ 0.07	0.142
<i>CASP9</i>	Pro	4.04 $\pm$ 1.24*	2.08 $\pm$ 0.26*	0.261
Bcl2 family				
<i>Bak1</i>	Pro	2.17 $\pm$ 0.28*	1.67 $\pm$ 0.24*	0.248
<i>Bax</i>	Pro	1.91 $\pm$ 0.39*	1.12 $\pm$ 0.11	0.193
<i>BCL2L1</i>	Anti	2.58 $\pm$ 0.67*	1.08 $\pm$ 0.12	0.158
BCL2-interacting				
<i>BAG4</i>	Anti	2.42 $\pm$ 0.59*	0.73 $\pm$ 0.15	0.109
<i>BNIP3</i>	Pro	1.87 $\pm$ 0.12*	1.25 $\pm$ 0.03	0.039*
<i>BNIP3L</i>	Pro	1.77 $\pm$ 0.10*	1.01 $\pm$ 0.06	0.008*
IAP family				
<i>XIAP</i>	Anti	1.92 $\pm$ 0.39*	1.40 $\pm$ 0.41	0.351
<i>BIRC2</i>	Anti	1.79 $\pm$ 0.14*	1.56 $\pm$ 0.29*	0.512
CARD-containing				
<i>APAF1</i>	Pro	2.73 $\pm$ 0.57*	1.06 $\pm$ 0.20	0.070
<i>NOD1</i>	Pro	1.94 $\pm$ 0.41*	1.16 $\pm$ 0.24	0.202
Death receptors				
<i>FAS</i>	Pro	1.98 $\pm$ 0.27*	0.80 $\pm$ 0.11	0.027*
<i>TNFRSF9</i>	Pro	3.37 $\pm$ 0.63*	0.98 $\pm$ 0.13	0.065
<i>TNFRSF11B</i>	Pro	2.67 $\pm$ 0.31*	1.30 $\pm$ 0.34	0.041*
<i>LTBR</i>	Pro	2.37 $\pm$ 0.71*	1.40 $\pm$ 0.05	0.304
Adaptors				
<i>TRADD</i>	Pro	2.47 $\pm$ 0.75*	1.66 $\pm$ 0.36	0.405
<i>TRAF2</i>	Anti	1.71 $\pm$ 0.38*	0.79 $\pm$ 0.04	0.140
<i>TRAF3</i>	Pro	2.44 $\pm$ 0.67*	1.38 $\pm$ 0.21	0.271
Miscellaneous				
<i>CIDEB</i>	Pro	2.21 $\pm$ 0.45*	1.26 $\pm$ 0.25	0.160

HCECs were harvested at 7 or 10 days after shRNA treatment, and analyzed using the RT² Profiler PCR array for human apoptosis. Cells transfected with the T7 shRNA vector were used as control. Data were analyzed by the RT² Profiler PCR Array Analysis Software. Values shown are the mean $\pm$ SE of three independent experiments ($n = 3$). Only genes that are significantly changed on day 7 are presented and the corresponding values on day 10 are shown for comparison. *P* values in the last column indicate the significance between the Day 7 and Day 10 fold change values from three independent experiments.

**P* < 0.05.

FIGURE 5. Activation of apoptosis in *SLC4A11*-silenced HCECs. (A) Activation of caspases in *SLC4A11* knocked down HCECs. Cell lysates were harvested at the indicated time points following shRNA transfection (day 7 or day 10) and subjected to Western blot analysis with the indicated antibodies. Immunoblotting with the GAPDH antibody was used as loading control. Representative immunoblots are shown. (B) Densitometric analysis of the immunoblot in (A). Values shown are normalized against GAPDH and expressed as the fold change relative to control. Data shown are the mean $\pm$ SD from three independent experiments.

and presumably participated in the cleavage of the downstream target PARP. The upregulation of some antiapoptotic genes may be due to the stochastic behavior of cells, each harboring different amounts of the knockdown vector. It could even be an attempt by transfected cells to 'rescue/evade from' the death phenotype. What is important to note is that the majority of genes that are upregulated are proapoptotic at this

stage. Hence, the function of *SLC4A11* is imperative, essential, and necessary for the survival of HCECs.

There seems to be a correlation between cell viability and the level of *SLC4A11* expression in corneal endothelial cells. The development of CHED2 at birth or early infancy is associated with almost negligible amounts of mutant proteins at the cell surface.²⁸ In FECD patients with heterozygous

SLC4A11 mutations there appears to be sufficient protein expression to delay onset of symptoms until late in life.²⁹ Specular microscopy in these FECD patients had indeed shown reduced corneal endothelial densities (indicating endothelial cell loss) in advanced stages of the disease.⁸ As the shRNAs utilized in this study did not completely deplete the endogenous *SLC4A11* in the HCECs, we suggest that this scenario more closely mimics the situation in FECD corneal endothelial cells bearing heterozygous *SLC4A11* mutations. Based on this study's findings, the endothelial cell loss in corneal endotheliopathies due to *SLC4A11* mutations may now be attributed to the increase in apoptotic cell death instigated by diminished or lack of *SLC4A11* function. Indeed, the importance of apoptosis in endothelial cell degeneration in FECD has been demonstrated in two previous studies. One analyzed 47 corneal buttons from 41 patients with FECD by nucleus labeling, transmission electron microscopy (TEM), and TUNEL.³⁰ Another revealed DNA damage as well as increased expression of TNF receptor superfamily member 6 (Fas), Fas ligand (FasL), and Bcl-2-associated X protein (Bax) in the FECD versus normal corneal endothelia.³¹ However, both of these studies were limited by not being able to attribute the observed apoptotic death to any primary molecular defect/cause within the analyzed FECD cases. Immunohistochemical and TUNEL analysis of corneal buttons of FECD or CHED patients with *SLC4A11* mutations will add further evidence for apoptotic cell death in corneal endotheliopathies due to *SLC4A11*.

In seeming contradiction to the present study and what is generally observed in human corneal endotheliopathies, the *Slc4a11* knockout mouse did not show any apparent corneal endothelial cell loss.¹⁴ However, the rodent corneal endothelium has the capacity to regenerate itself upon injury, unlike the human counterpart.³² It is therefore possible that any cell loss due to lack of *SLC4A11* may be compensated by new proliferating cells which will explain the rather mild corneal phenotype in these animals compared to humans. We speculate that the turnover rate of corneal endothelial cells in the *Slc4a11* knockout mice would be much higher than normal mice, and other compensatory pathways may be induced. Understanding the difference between the human versus mouse condition may help us find new ways to prevent corneal endothelial cell loss due to failure of *SLC4A11* function.

In summary, the present study suggests that the endothelial cell loss observed in FECD and recessive CHED cases with *SLC4A11* mutations may be attributed to the increase in apoptotic cell death initiated by diminished or complete loss of *SLC4A11* function. We propose that further studies be undertaken to determine the exact role of *SLC4A11* in the human corneal endothelium given the importance of this protein in the development and homeostasis of the corneal endothelium.

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