Transcription Factor 4 Regulates the Regeneration of Corneal Endothelial Cells

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RESULTS. Cell proliferation rates and TCF4 levels were reduced in senescent cells. TCF4 CRISPR activation enhanced corneal endothelial wound healing. TCF4 regulated mitochondrial functions including mitochondrial membrane potential, mitochondrial superoxide levels, and energy production. The percentage of cells in the S-phase was reduced with si-TCF4 and increased with pl-TCF4. Cell proliferation and cell cycle-associated proteins were regulated by TCF4. Autophagy was induced by si-TCF4. In vivo transfection of CRISPR/dCas9 activation systems (a-TCF4) induced regeneration of corneal endothelium.

CONCLUSIONS. Corneal endothelial diseases are associated with TCF4 reduction; TCF4 may be a potential target for hCEC diseases. Gene therapy using TCF4 CRISPR/dCas9 may be an effective treatment for hCEC diseases.

Keywords: transcriptional factor 4, mitochondrial functions, energy production, cell proliferation, human corneal endothelial cells

Human corneal endothelial cells (hCECs) have limited regenerative capacity in vivo. Reduced hCEC density results in bullous keratopathy requiring corneal transplantation. This study reveals the role of transcription factor 4 (TCF4) in hCEC diseases and suggests that TCF4 may be a molecular target for hCEC regeneration.

METHODS. Cell shape, cell proliferation rates, and proliferation-associated proteins were evaluated in normal or senescent hCECs. TCF4 was blocked by siRNA (si-TCF4) or activated using clustered regularly interspaced short palindromic repeats (CRISPR)/dCas9 activation systems (pl-TCF4). The corneal endothelium of six-week-old Sprague-Dawley (SD) rats was transfected by electroporation followed by cryoinjury.

RESULTS. Cell proliferation rates and TCF4 levels were reduced in senescent cells. TCF4 CRISPR activation enhanced corneal endothelial wound healing. TCF4 regulated mitochondrial functions including mitochondrial membrane potential, mitochondrial superoxide levels, and energy production. The percentage of cells in the S-phase was reduced with si-TCF4 and increased with pl-TCF4. Cell proliferation and cell cycle-associated proteins were regulated by TCF4. Autophagy was induced by si-TCF4. In vivo transfection of CRISPR/dCas9 activation systems (a-TCF4) induced regeneration of corneal endothelium.

CONCLUSIONS. Corneal endothelial diseases are associated with TCF4 reduction; TCF4 may be a potential target for hCEC diseases. Gene therapy using TCF4 CRISPR/dCas9 may be an effective treatment for hCEC diseases.

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limited regenerative capacity,\textsuperscript{18} whereas rat CECs proliferate throughout life.\textsuperscript{19} In this study, we used cryoinjury to damage the corneal endothelium of rats. This model has long been used to replicate in vivo corneal endothelial failure.\textsuperscript{20,21} Although studies using corneal endothelium in rats may have limitations in explaining the function of TCF4 in hCECs.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9) have been developed into a robust RNA-guided gene editing system.\textsuperscript{22} Deactivated Cas9 (dCas9) is used for a CRISPR/dCas9 activation system, which increases the transcription expression.\textsuperscript{23} Small-interference RNA (siRNA) has been commonly used for gene silencing. In this study, we investigated the regulatory role of TCF4 in CECs using a CRISPR/dCas9 activation system to activate TCF4 and siRNA to repress TCF4 in vitro and in vivo.

**Materials and Methods**

**Role of TCF4 in Cultured Human Corneal Endothelial Cells**

**Isolation and Culture of Human Corneal Endothelial Cells.** This study was performed in accordance with the tenets of the Declaration of Helsinki and was reviewed and approved by the institutional review board and ethics committee of Hallym University Medical Center. Cells were cultured in accordance with previously published methods.\textsuperscript{23,24} Corneas were obtained from the Eversight (Ann Arbor, MI, USA), which obtained informed consent for the use of all tissue samples collected and cultured for the study. Corneas from a total of six donors (56-year-old man, 33-year-old women, 45-year-old man, 62-year-old man, 60-year-old woman, and 55-year-old woman) were used.\textsuperscript{25} All cells remained attached to the Descemet's membrane. The endothelial cell's Descemet's membrane complex was incubated for 10 minutes in 0.25% trypsin, 0.02% EDTA solution. Cells were then plated in six-well plates coated with a fibronectin–collagen combination (FNC) coating mix (Athena Environmental Sciences, Inc., Baltimore, MD, USA). Cells were cultured for 14 to 21 days until they attained confluency and were then passaged as a ratio of 1:3 using 0.25% trypsin, 0.02% EDTA solution.

**RNA Interference.** To silence TCF4 expression, we used siRNA. The siRNA for TCF4 was purchased from sc-43525, Santa Cruz, Dallas, TX, USA. The siRNA for TCF4 (sc-43525) includes 3 different siRNA duplexes: sc-43525A (sense: 5'- CUGAGUGCCAGGUUGAAGA-3'; antisense: 5'- UCUUCCAGUUGCAUCAG-3'); sc-43525B (sense: 5'- GAAAGACCAAGGCAAUAUC-3'; antisense: 5'- AGAUUUCGGCUUGCCUCUC-3'; and sc-43525C (sense: 5'- CCAACCCUGAGCCCUAA-3'; antisense: 5'- UGAAGGGCAAGGAUUGAG-3'). Nonspecific control siRNA (sc-36869) used as a negative control were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). In brief, primary human corneal endothelial cells (hCECs) at a density of 5 × 10\textsuperscript{4} cells/cm\textsuperscript{2} were transfected with siRNA specific for TCF4 at 10 nM concentrations, with a non-coding sequence siRNA as a negative control, using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfections were performed at 70% confluency. After incubation for 48 hours, the cells were collected for experiments. The cells were separated into two groups, an siRNA group targeting TCF4 (si-TCF4) and a control group (si-control). The effect of TCF4 silencing was confirmed by Western blot analysis 48 hours after transfection.

**TCF4 Activation Plasmid and Transfection.** The CRISPR/dCas9 system using an activation plasmid for TCF4 was used to evaluate the effect of TCF4 activation. CRISPR/dCas9 activation plasmid for TCF4 was purchased from Santa Cruz Biotechnology (sc-400607-ACT, guide RNA sequence: 5'-ACAATGTACCTTTGCCCGGC-3'). TCF4 CRISPR/dCas9 activation plasmid (h) is a synergistic activation mediator (SAM) transcription activation system designed to specifically upregulate gene expression. It consists of three plasmids at a 1:1:1 mass ratio: 1) a plasmid encoding the dCas9 nuclease (D10A and N863A) fused to the transactivation domain VP64 and a blasticidin resistance gene; 2) a plasmid encoding the MS2-p65-HSF1 fusion protein and a hygromycin resistance gene; and 3) a plasmid encoding a target-specific 20 nt guide RNA and a puromycin resistance gene. The resulting SAM complex binds to a site-specific region approximately 200 to 250 nt upstream of the transcriptional start site and provides robust recruitment of transcription factors for highly efficient gene activation. Transfections of cells were performed using Lipofectamine 3000 according to the manufacturer’s protocols. Briefly, cells were plated in six-well plates before transfection to reach 70% confluency. Five μg of plasmid or 20 nM siRNA was diluted in 125 μl Opti-MEM (Invitrogen), and 7.5 μL Lipofectamine and 10 μl P3000 were diluted in 125 μl Opti-MEM. The Lipofectamine solution was added to the plasmid or siRNA solution, briefly vortexed and left for 5 minutes to allow complex formation. Plasmid-lipid complex was added to cells. The cells were separated into two groups: an activation plasmid group targeting TCF4 (pl-TCF4) as well as an activation plasmid control group (pl-control). The effect of TCF4 overexpression was tested by Western blot analysis 48 hours after transfection.

**Cell Viability Using WST-8.** Cells (1 × 10\textsuperscript{4}) were cultured in a 96-well plate. Cell viability was measured using a cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) based on the water-soluble monosodium tetrazolium salt, WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium). The plates were incubated with CCK-8 solution for 1 to 2 hours. Cell viability was determined by measuring the absorbance at 450 nm using a microplate spectrophotometer. Cell viability was expressed as the mean ± standard deviation, as a percentage of the control (100%). Experiments were repeated three times, and a representative experiment is shown.

**Cell Proliferation Rate Assay.** Cell proliferation rate was measured using a commercial bromodeoxyuridine (BrdU) proliferation assay kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells (5 × 10\textsuperscript{4} cells/well) were placed in 96-well plates and incubated for 48 hours in a humidified atmosphere containing 5% CO\textsubscript{2}. Cells were transfected and then labeled with BrdU at 37°C under 5% CO\textsubscript{2}. After incubating the plate in the FixDenat solution for 30 minutes at room temperature, the cells were incubated with anti-BrdU-POD solution for approximately 90 minutes at room temperature. Then, the substrate solution was added to each well, and the plate was incubated for 20 minutes at room temperature. Thereafter, 1 M H\textsubscript{2}SO\textsubscript{4} was added to each well to stop the reaction. The optical density was measured at 450 nm using an enzyme-linked immunoassay (ELISA) plate reader. Proliferation rates were expressed as the
percentage of controls after subtraction of the corresponding blanks.

**Cell Cycle Analysis.** Cell cycle analysis was performed using the NucleoCounter (NC-3000; ChemoMetec, Allerod, Denmark). Briefly, 1 × 10^5 cells were used. After the floating and adherent cells were collected and washed twice with phosphate-buffered saline (PBS), the cells were fixed with 70% ethanol and then incubated with a staining solution containing 0.1% Triton X-100, 0.1% EDTA (pH 7.4), RNase A (30 μg/ml) and propidium iodide (50 μg/ml) in phosphate-citrate buffer (pH 7.4). Cellular DNA content was analyzed using Muse cell analyzer or NC-3000. The results were displayed as histograms.

**Mitochondrial Viability Staining.** Mitochondrial viability stain (ab129732, Abcam, Cambridge, England) was used for determination of the metabolic capacity of cells. It measures oxidation-reduction reactions, which principally occur in the mitochondria of live cells. Cells were seeded in a black 96-well plate and incubated at 37°C for 4 hours after overlaying 100 μl of 2 times mitochondrial viability stain solution on each well, which contained 100 μl of media. Following a wash with PBS, optical density was measured by absorbance at 570 nm.

**Measurement of Mitochondrial Mass and Intra-cellular Reactive Oxygen Species.** MitoTracker green FM fluorescent probe (Invitrogen, Carlsbad, CA, USA) was used to measure mitochondrial mass and CM-H$_2$DCFDA fluorescent probe (Invitrogen) was used to measure intracellular reactive oxygen species (ROS) levels according to the manufacturer's protocol. Cells (1 × 10^5) in black 96-well plates were incubated with MitoTracker green FM fluorescent probe at a final concentration of 200 nM for 30 minutes or with CM-H$_2$DCFDA fluorescent probe at a final concentration of 20 μM and then washed with PBS. DCF and MitoTracker green FM fluorescence intensities were measured at an excitation wavelength of 480 nm and emission wavelength of 535 nm. The relative intensity of fluorescence was calculated and normalized by cell numbers.

**Measurement of Mitochondrial ROS Production.** MitoSOX Red (Invitrogen) was used to measure mitochondrial superoxide production according to the manufacturer's protocol. The cells were incubated with 5 μM MitoSOX reagent for 10 minutes at 37°C in the dark. Fluorescence intensity in each well was measured at an excitation wavelength of 510 nm and an emission wavelength of 590 nm. The relative intensity of MitoSOX Red fluorescence was calculated.

**Measurement of ATP Concentration.** ATP concentrations were measured using an ATP determination kit (Molecular Probes, Inc., Eugene, OR, USA) following the manufacturer's instructions. Cells (2.5 × 10^5) were lysed in a buffer (containing 20 mM Tris, pH 7.0; 0.5% NP-40; 25 mM NaCl; 2.5 mM EDTA; and 2.5 mM EGTA). Immediately, the samples were placed in a white 96-well plate and the reaction solution was added. After 5 minutes, luminescence was measured in an LMax microplate luminometer (Molecular Devices Corp., Sunnyvale, CA, USA). The relative ATP levels were normalized with the protein concentration measured with the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

**Measurement of Mitochondrial Membrane Potential.** Measurement of mitochondrial membrane potential (ΔΨm) was measured using the JC-1 dye (BioVision) and microplate assay. Cells seeded onto black 96-well plates were treated with siRNA at 37°C for 48 hours. JC-1 dye was used to determine the changes in the ΔΨm of hCECs. The cells were incubated with a final concentration of 1 μM JC-1 for 30 minutes at 37°C in the dark. Each well was analyzed by a spectrofluorometer (SFM 25, Kontron Instruments, Everett, MA, USA). Measurement of fluorescence intensity in each well was conducted at an excitation/emission wavelength of 495 nm/530 nm for the JC-1 monomer, and an excitation/emission wavelength of 525 nm/590 nm for JC-1 aggregates. Images were acquired using a live cell imaging system (Incucyte FLR 10x; Essen Bioscience, Ann Arbor, Michigan, USA). The ΔΨm was calculated by a red/green fluorescence ratio of JC-1. The relative intensity of fluorescence was calculated by setting the fluorescence intensity of the nontreated cultures to 100% after subtracting the corresponding blanks.

**Protein Expression Analysis by Western Blot.** Radioimmunoprecipitation assay buffer (Biosesang, Seoul, Korea) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (PhosSTOP, Roche, Basel, Switzerland) was used for isolation of total cellular proteins. Western blotting was conducted using standard protocols. The 5% skim milk or gelatin was used for blocking the nonspecific binding for 1 hour. Primary antibodies were rabbit anti-human TCF4 antibody (sc-13027, Santa Cruz Biotechnology, 1:200 dilution), mouse anti-human GFAP antibody (sc-58766, Santa Cruz Biotechnology, 1:1000 dilution), rabbit anti-human ERK1/2 antibody (ab17942, Abcam, Cambridge, MA, 1:1000 dilution), rabbit anti-human pERK1/2 antibody (ab4819, Abcam, 1:1000 dilution), rabbit anti-human GSK3β antibody (ab32391, Abcam, 1:1000 dilution), rabbit anti-human pGSK3β antibody (ab75745, Abcam, 1:1000 dilution), rabbit anti-human SIRT1 antibody (sc-15404, Santa Cruz, 1:1000 dilution), rabbit anti-human β-catenin antibody (ab325572, Abcam, 1:1000 dilution), mouse anti-human caspase-9 antibody (sc-56076, Santa Cruz, 1:1000 dilution), rabbit anti-human CDK1 antibody (ab131450, Abcam, 1:1000 dilution), rabbit anti-human cyclin D1 antibody (sc-718, Santa Cruz, 1:1000 dilution), anti-NADPH oxidase 4 antibody (NOX4; MABC616, Merck Millipore, Billerica, MA, USA, 1:500 dilution), anti-cyclin-dependent kinase inhibitor 2A antibody (CDKN2A; MABE1328, Merck Millipore, 1:500 dilution), rabbit anti-human AMP-activated protein kinase antibody (AMPK; sc-25792, Santa Cruz, 1:1000 dilution), rabbit anti-human-phospho-AMPK antibody (pAMPK; sc-101630, Santa Cruz, 1:1000 dilution), and rabbit anti-human LC3 antibody (PM036, MBL International, Woburn, MA, USA, 1:1000 dilution). The TCF4 antibody (sc-13027 [H-125]) used in this study targets amino acids 486-610 on the C-terminus of the protein. An alkaline phosphatase conjugated secondary antibody with a 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) color development substrate (Promega, Madison, WI) or a horseradish peroxidase (HRP) conjugated secondary antibody with a Miracle-StarWestern Blot Detection System (INiRON Biotechnology, Seoul, Korea) was used for detection of immunoreactive bands. Data were quantified by video image analysis. Protein bands were measured by densitometry.

**Role of TCF4 in Corneal Endothelial Cells In Vivo.** This study was approved by the Institutional Animal Care and Use Committee of Hallym University Medical Center. All procedures were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision

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**TCF 4 Regulates the Regeneration of CECs**

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Research. Female six-week-old Sprague-Dawley (SD) rats were used for this procedure. Seven SD rats were included in each group. Rats were maintained in a colony room with a 12/12-hour light/dark cycle at 25°C for 7 days before beginning the experiments.

In Vivo Transfection of TCF4 CRISPR/dCas9 Activation Plasmid Using Electroporation. A TCF4 CRISPR/dCas9 activation system (sc-400607-ACT) was used for TCF4 overexpression. TCF4 CRISPR activation plasmids (1 μg; α-TCF4 group) or control CRISPR activation plasmids (1 μg; control group) were injected into anterior chamber (intracameral injection) and transfected into corneal endothelium of rats using electroporation, as described previously.20 Electroporation was performed as follows: a 7-mm tweezer electrode (BTX, San Diego, CA, USA) was placed on each cornea, with the positive electrode on the plasmid-injected eye. The settings of electroporation were 140 V, 100 ms length, 950 ms interval, 5 pulses, and 100 V/cm². The siRNA for TCF4 was used for blocking TCF4 expression (si-TCF4 group). The siRNA for TCF4 (1 μg) was injected into anterior chamber and transfected into corneal endothelium of rats using electroporation as described. The next day, the corneal endothelium was cryoinjured. The metal rod (3-mm length, 950 ms interval, 5 pulses, and 100 V/cm²) was placed on the corneas for 10 seconds. Then, the corneas were washed using normal saline for 3 minutes. The corneas were photographed and obtained. The rats were euthanized on days 3, 7, and 14. The eyes were enucleated and fixed in 3.7% formaldehyde.

Clinical Evaluation and Histology. Corneal opacity was evaluated, and images were collected on days 0, 3, 7, and 14. Corneal opacity was graded as follows: grade 0 = clear cornea, grade 1 = mild corneal opacity and still allowing good visibility of details of the iris, grade 2 = moderate corneal opacity with partial masking of the iris, and grade 3 = severe corneal opacity without view of the iris.

Immunofluorescence Staining and Alizarin S Red Staining. The corneal endothelium was immunostained for TCF4. Briefly, the eyeballs were fixed with 4% paraformaldehyde in PBS and the corneas were excised from the eyeballs. The corneas were permeabilized in 0.3% Triton X-100 for 10 minutes, blocked with 5% skim milk at room temperature for 30 minutes, incubated with rabbit anti-TCF4 antibody (MA5-14975, Thermo Fisher Scientific) or rabbit anti-β-catenin antibody (ab325572, Abcam) for 3 hours, and then treated with FITC conjugated goat anti-rabbit antibody for 1 hour. After nuclear counterstaining with Hoechst 33342, the corneas were flat-mounted on the slide and covered with a coverslip. The 0.2% alizarin S red was injected into the anterior chamber and corneas were fixed using 4% paraformaldehyde in PBS. After washing, corneas were flat-mounted and observed using light microscopy (Leica DM2000, Leica Microsystems).

In Vivo Staining of MitoTracker Green FM Fluorescent Probe and MitoSOX Probe. The corneal endothelium was immunostained using MitoTracker green FM fluorescent probe and MitoSOX probe to evaluate mitochondrial mass and oxidative stress. Briefly, the eyeballs were obtained and the corneas were incubated with 200 nM MitoTracker green FM fluorescent reagent for 30 minutes and 5 μM MitoSOX™ reagent for 10 minutes at 37°C in the dark. After washing, corneas were flat-mounted and observed using light microscopy (Leica DM2000, Leica Microsystems).

Real-Time Reverse Transcription Polymerase Chain Reaction. RNA was extracted from the CECs of rats using the PureLink FFPE RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA concentrations were determined by ultraviolet spectrophotometry. The first strand complementary DNA was synthesized from 0.2 μg of total RNA with oligonucleotide primers using a commercially available kit (GoScript Reverse Transcription System; Promega Corporation, Madison, WI, USA). Complementary DNA samples were aliquoted and stored at −20°C until use. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) were performed in 20-μl volumes using the AccuPower 2X GreenStar qPCR Master Mix (Bioneer) with real-time qPCR Primer Assay for humans, and the following thermocycling parameters: 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, and finally 60°C for 60 seconds. SYBR green fluorescence was measured at the end of each cycle. The β-actin gene, a housekeeping gene, was used as a standard for normalization. Primer sequences for TCF4 were as follows: sense, 5′-ACATGCGATGGAAATCTGG-3′ and anti-sense, 5′-TGAATGTCTGTGCTTCGAAA-3′. Assays were performed in triplicate. Melting curve analysis was performed to ensure good-quality specific PCR products. RT-qPCR results were analyzed, using the relative standard curve method and compared and calibrated against the control group.

Statistical Analysis. All experiments were conducted in triplicate or quadruplicate and all values are represented as the mean ± standard deviation. Differences in results were determined by the Independent t-test or Mann-Whitney U test. Results were considered statistically significant at a P-value < 0.05.

RESULTS

Role of TCF4 in Cultured Human Corneal Endothelial Cells In Vitro

TCF4 in Cultured hCECs Does Not Affect the Expression of GSK3β and β-catenin. The hCECs were cultured and a mosaic morphological pattern was observed (Fig. 1A). siRNA for TCF4 (si-TCF4) decreased TCF4 expression (22.39 ± 9.57% compared to control) and the CRISPR/dCas9 TCF4 activation plasmid (pl-TCF4) resulted in TCF4 overexpression (174.89 ± 60.66%; Figs. 1B, 1C). β-catenin expression (Figs. 1D, 1E for si-TCF4 and Figs. 1F, 1G for pl-TCF4) and activation of glycogen synthase kinase 3 beta (GSK3β; Figs. 1H, 1I for si-TCF4 and Figs. 1J, 1K for pl-TCF4) was not different with TCF4 inhibition by si-TCF4 and with TCF4 overexpression (pl-TCF4). Gliarial fibrillary acidic protein (GFAP) expression was reduced by si-TCF4 and was elevated by pl-TCF4 (P = 0.011 in si-TCF4 and P = 0.020 in pl-TCF4, respectively, Independent t-test; Figs. 1E, 1M, 1N, 1O).

TCF4 Regulates Cell Proliferation and the Cell Cycle. Cell proliferation rate was measured by BrdU proliferation assay. The si-TCF4 reduced cell proliferation, whereas pl-TCF4 increased it (P = 0.026 and 0.038, respectively, Independent t-test; Fig. 2A). Cell cycle analysis using NucleoCounter showed that the percentage of cells in the S-phase was lower with si-TCF4 than in control cells (Figs. 2B, 2C). In contrast, an increase in S-phase cells was observed with pl-TCF4 (P = 0.022 and < 0.001, respectively; Figs. 2B, 2D). The expression of pERK1/2 was decreased by si-TCF4 (Figs. 2E, 2F) and increased
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**FIGURE 1.** Culture and identification of hCECs and Wnt signaling regulated by TCF4. (A) The hCECs were cultured in mosaic pattern. (B, C) TCF4 expression after treating siRNA for TCF4 (si-TCF4) and CRISPR/dCas9 activation system for TCF4 (pl-TCF4). (D–G) β-catenin expression was not different. (H–K) Activation of GSK3β was not different. (L–O) GFAP expression was regulated by TCF4. All the measurements were conducted in triplicate or quadruplicate. ***P < 0.001, **P < 0.01, and *P < 0.05 statistically significant by independent *t*-test.

with pl-TCF4 (P = 0.006 and 0.005, respectively; Figs. 2G, 2H). The si-TCF4 also reduced cyclin-dependent kinase 1 (CDK1) expression (Figs. 2I, 2J), whereas the opposite effect was observed with pl-TCF4 (P = 0.008 and 0.007, respectively; Figs. 2K, 2L). Similarly, cyclin D1 expression was decreased by si-TCF4 (Figs. 2M, 2N) and was increased upon pl-TCF4 (P = 0.018 and 0.048, respectively; Figs. 2O, 2P). Cyclin-dependent kinase inhibitor 2A (CDKN2A) expres-
TCF 4 Regulates the Regeneration of CECs

**FIGURE 2.** Cell proliferation and the cell cycle depending on TCF4 expression. (A) Cell proliferation rate by TCF4 inhibition (si-TCF4) or TCF4 overexpression (pl-TCF4). N = 7, P = 0.026, and 0.038. (B–D) Cell cycle analysis showed the percentage of cells in the S-phase. (E–H) The expression of phospho-extracellular signal–regulated kinase 1/2 (pERK1/2) was shown. (I–L) Cyclin-dependent kinase 1 (CDK1) expression is shown. N = 4, P = 0.008 and 0.007. (M–P) CDK2A expression. All the measurements were conducted in triplicate or quadruplicate. ***P < 0.001, **P < 0.01, and *P < 0.05 statistically significant by independent t-test.

expression was elevated by si-TCF4 (Figs. 2Q and 2R) and was reduced upon pl-TCF4 (P = 0.001 and 0.013, respectively; Figs. 2S, 2T).

**TCF4 Regulates Mitochondrial Functions.** A mitochondrial viability stain (ab129732, Abcam, Cambridge, England) was used for determination of the metabolic capacity of cells. Mitochondrial viability was reduced by si-TCF4 and elevated by pl-TCF4 (P = 0.008 and 0.037, respectively, independent t-test; Figs. 3A, 3B, 3C). Mitochondrial mass was decreased by si-TCF4 and increased by pl-TCF4 (P = 0.004 and < 0.001, respectively; Figs. 3D, 3E, 3F). Mitochondrial superoxide production was evaluated by MitoSOX Red fluorescence. Mitochondrial superoxide formation decreased with si-TCF4 and increased with pl-TCF4 (P = 0.033 and 0.027, respectively; Figs. 3G, 3H, 3I). MitoSOX red fluorescence co-localized with MitoTracker green fluorescence.
FIGURE 3. Role of TCF4 in mitochondrial functions. (A–C) Mitochondrial viability stain showed that mitochondrial viability was regulated by TCF4. N = 7, P = 0.008, and 0.037. (D–F) Mitochondrial mass was evaluated by MitoTracker Green fluorescence. Mitochondrial mass was regulated by TCF4. Bar scale = 50 μm. (G–I) Mitochondrial superoxide production was evaluated by MitoSOX Red fluorescence. Mitochondrial superoxide formation was regulated by TCF4. Bar scale = 50 μm. (J) Co-localization of MitoTracker green and MitoSOX. (K–M) Intracellular oxidative stress levels were evaluated by DCF fluorescence (green). Bar scale = 50 μm. N = 6, P = 0.026, and 0.001. (N, O) Relative adenosine triphosphate (ATP) production was evaluated. All the measurements were conducted in triplicate or quadruplicate. ***P < 0.001, **P < 0.01, and *P < 0.05 statistically significant by independent t-test. (Fig. 3J). Intracellular ROS levels were reduced by si-TCF4 and elevated by pl-TCF4 (P = 0.026 and 0.001, respectively; Figs. 3K, 3L, 3M). Relative adenosine triphosphate (ATP) production decreased upon si-TCF4 but was elevated by pl-TCF4 (P = 0.009 and 0.026, respectively, independent t-test; Figs. 3N, 3O). Activation of AMP-activated protein kinase (AMPK) decreased with si-TCF4 (Figs. 3P, 3Q), however, pl-TCF4 induced the opposite effect (P = 0.047 and 0.043, respectively; Figs. 3R, 3S).

TCF4 Controls Cell Viability. The ΔΨm was measured using JC-1 and was decreased by si-TCF4 and elevated by pl-TCF4 (P = 0.005 and 0.038, respectively, independent t-test, Figs. 4A, 4B). Cell viability was decreased by si-TCF4 and increased by pl-TCF4 (P = 0.001 and 0.005, respectively; Fig. 4C). Expression of caspase-9 was increased by si-TCF4 and decreased by pl-TCF4 (P = 0.015 and 0.003, respectively, independent t-test; Fig. 3D). Autophagy was measured by the ratio of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II to LC3-1 (Figs. 4E, 4F). si-TCF4 increased LC3-II expression (P = 0.003; Figs. 4E, 4F). SIRT1 expression was elevated by si-TCF4 and reduced by pl-TCF4 (P = 0.033 and 0.014, respectively; Figs. 4H, 4I, 4J, 4K). NADPH oxidase 4 (NOX4) expression was increased by si-TCF4 and decreased by pl-TCF4 (P = 0.001 and 0.002, respectively; Figs. 4L, 4M, 4N).

Role of TCF4 in Corneal Endothelial Cells In Vivo

In Vivo Transfection of TCF4 CRISPR/dCas9 Activation Plasmid and siRNA for TCF4 Using Electroporation. TCF4 CRISPR activation plasmid (sc-400607-AC1, 1 μg; a-TCF4 group), control CRISPR activation plasmid (1 μg; control group) or siRNA for TCF4 (1 μg) was injected into the anterior chamber. Then electroporation was performed for transfection into corneal endothelium of rats as follows. A 7-mm tweezertrode (BTX, San Diego, CA, USA) was placed on each cornea, with the positive electrode on the plasmid-injected eye. The settings of electroporation...
Figure 4. Regulation of TCF4 expression in mitochondria-induced cell death. (A, B) ΔΨm was measured using JC-1. Bar scale = 100 μm. (C) Cell viability was decreased by si-TCF4 and was increased by pl-TCF4. (D) Expression of caspase-9 was increased by si-TCF4 and decreased by pl-TCF4. (E–G) Autophagy was measured by the ratio of LC3-II/LC3-1 expression. The si-TCF4 increased LC3-II expression. (H–K) Sirtuin 1 (SIRT1) expression was elevated by si-TCF4 and reduced by pl-TCF4. (L–N) NOX4 expression increased with si-TCF4 and decreased with pl-TCF4. All the measurements were conducted in triplicate or quadruplicate. *** \( P < 0.001 \), ** \( P < 0.01 \), and * \( P < 0.05 \) statistically significant by independent t-test.
FIGURE 5. Effect of TCF4 CRISPR activation on corneal endothelial wound healing. (A, B) Corneal opacity in control, a-TCF4 group, and si-TCF4 group. (C) TCF4 expression (green) was increased in the cornea endothelium of the a-TCF4 group. Nuclear staining was performed with Hoechst 33342 (blue). (D) qRT-PCR showed the TCF4 expression levels. (E) β-catenin expression in the corneal endothelium. (F, G) Corneal endothelium stained with alizarin S red. Alizarin S red staining showed the increased wound healing in the corneal endothelium in the a-TCF4 group. The number of corneal endothelial cells was higher in the a-TCF4 group. (H) MitoTracker green fluorescence indicates mitochondrial mass and mitoSOX red fluorescence indicates oxidative stress levels. All the measurements were conducted in triplicate or quadruplicate. ***P < 0.001, **P < 0.01, and *P < 0.05 by independent t-test.

were as follows: 140 V, 100 ms length, 950 ms interval, 5 pulses, and 100 V/cm.20

Corneas were more transparent in the a-TCF4 group than in the control at 1 week and 2 weeks (P = 0.040 and 0.046, independent t-test; Figs. 5A, 5B). There was no difference in the si-TCF4 group compared to control. Immunofluorescence staining of TCF4 showed that TCF4 expression was observed in the corneal endothelium of the a-TCF4 group (Fig. 5C). However, it was not seen in the si-TCF4 group. TCF4 expression was not shown in stroma. RT-qPCR showed that TCF4 mRNA expression in CECs was lower in the si-TCF4 group than in the si-control (P = 0.010) and was higher in the a-TCF4 group than in its control group (P = 0.038; Fig. 5D). β-catenin is localized in cell membrane in control and si-TCF4 group and in the nucleus in a-TCF4 group at two weeks.

Alizarin S red staining showed that wound healing of corneal endothelium was promoted in the a-TCF4 group (Fig. 5E). There was no difference in the si-TCF4 group compared to control. The number of CECs was higher in the a-TCF4 group than in its control group at one week and two weeks (P = 0.020 and < 0.001, independent t-test; Fig. 5G). However, there was no difference in the si-TCF4 group compared to control. Mitochondrial mass and mitoSOX fluorescence intensity in rat corneal endothelium was similar to the results in in vitro experiments.
**DISCUSSION**

FECD is a corneal endothelial disease characterized by the progressive loss of hCECs that results in BK. TCF4 mutation is associated with the pathogenesis of FECD. TCF4 activates target genes associated with cell proliferation and play an essential role in the regeneration of the normal tissues. Activation of TCF4 may be a potential treatment to regenerate the corneal endothelium. Here, we used siRNA and CRISPR/dCas9 systems for TCF4 to investigate its role in CECs in vitro and in vivo. TCF4 was knocked down using siRNA for TCF4 instead of CRISPR/Cas9 to avoid off-target effects of the latter; the CRISPR/Cas9 system cuts and repairs DNA, resulting in occasional and unintended cell death.

In vivo, we found that the TCF4 CRISPR/dCas9 activation system (a-TCF4 group) effectively increased the TCF4 expression in the corneal endothelium of the rats. We performed a cryoinjury to destroy corneal endothelium. Corneal opacity occurred because CECs were destroyed by cryoinjury. Corneal opacity in the control group persisted until two weeks because the control group was not found to regenerate. In the a-TCF4 group, the corneal endothelium was regenerated and migrated to the center, making the cornea transparent. In the si-TCF4 group, the corneal opacity persisted because the corneal endothelium was not regenerated as in the control group. Our in vivo study showed that TCF4 activation in the corneal endothelium of rats promoted the migration of CECs and restored the transparency of the cornea, but si-TCF4 did not. TCF4 has been reported to induce cell migration and proliferation. After transfection of plasmid for a-TCF4, TCF4 expression was not shown in stroma. CECs and Descemet membrane may act as a barrier to penetrate the plasmids to stroma.

In vitro experiments were conducted to determine the mechanism of the in vivo results. Cultured hCECs show the mosaic pattern only when they are confluent. The transfections were performed at the 70% confluency, in that state the shape of hCECs did not show a mosaic. This study demonstrated that the expression of pGSK3β and β-catenin was not different by blocking TCF4 or by overexpressing TCF4. The pGSK3β and β-catenin are upstream of TCF4 signaling. If only downstream TCF4 is inhibited in normal cells, upstream β-catenin does not change. However, in vivo experiments showed that β-catenin was located at plasma membrane in control group and si-TCF4 group and at nucleus in a-TCF4 group. Without a Wnt signal, β-catenin is degraded via interactions with Axin, adenomatous polyposis coli, and the protein kinase GSK3β. Upon Wnt activation, β-catenin moves from the cytoplasm to the nucleus and forms the β-catenin/TCF4 complex that controls the transcription of crucial target genes. TCF4 is the major effector of the Wnt signaling pathway, and regulate the transcription of downstream targets when β-catenin accumulates in the nucleus. TCF4 is also involved in the enhanced reprogramming effect of Wnt signaling. In this study, TCF4 regulated the level of GFAF, which is associated with neural stem cells and glial cells. Inhibition of Wnt/β-catenin signal is associated with GFAF suppression. GFAF is also linked to cellular proliferation, and is regulated by the Wnt signaling pathway.

In this study, we found that TCF4 regulates cell proliferation and cell cycle progression in hCECs. The siRNA-mediated TCF4 inhibition led to decreased cell proliferation. TCF4 activates ERK1/2, and ERK1/2 has been reported to mediate cell proliferation and apoptosis. ERK1/2 activation and translocation to the nucleus is essential for G1 to S phase progression. Our results show that TCF4 overexpression progressed the cell cycle into S-phase, upregulated CDK1 and cyclin D expression, and downregulated CDKN2A expression. TCF4-inducible gene products include the cell cycle control proteins cyclin D1 and CDK1. The CDKs are a family of serine-threonine kinases controlling progression through the cell cycle. CDK1 is a major component of the cell cycle progression engine. Cyclin D1 contributes to the regulation of the progression of cells through the G1-phase of the cell cycle. CDKN2A inhibits progression from G1-phase to S phase and arrests cell proliferation. The hCECs in vivo are arrested at the G1-phase and have not stimulated the cell cycle, although hCECs do proliferate in vitro. Therefore, they may be able to proliferate in vivo by strong mitogenic stimulation or through changes in their microenvironment. TCF4 may be a molecular target of hCECs regeneration in vivo.

This study reveals that TCF4 regulates mitochondrial function and energy metabolism in hCECs. Wnt signaling pathway is a key regulator of mitochondrial function. Wnt signaling is important in hCEC. It has been reported that Wnt signaling is involved in the pathogenesis of FECD. TCF4 may be a potential target for the treatment of FECD.

In this study, we found that TCF4 regulates cell survival and oxidative stress sensing. SIRT1 is a NAD+-dependent deacetylase that controls many cellular processes, such as cellular proliferation, energy metabolism, and cell survival, through deacetylating a wide range of substrates that includes p53, FOXO, and NF-κB. SIRT1 positively regulates autophagy and mitochondrial function, which is essential for cell survival. NOX4 plays a role in cell survival and oxidative stress sensing. have an indirect, antioxidant function by regulating the NRF2 pathway, and also promotes the activation of autophagy.

We also found that TCF4 regulated cell death in hCECs. Apoptosis and autophagy are the major pathogenesis of corneal endothelial diseases. It is important to prevent hCEC death and to maintain the number of hCECs. Corneal endothelial diseases, including FECD and BK, are associated with altered autophagy. Thus, autophagy, which is related to mitochondrial functions, can protect cells against death in corneal endothelium and maintain the hCEC population. In this study, cell viability was reduced by blocking TCF4 and was increased by its overexpression. TCF4 is involved in cell survival and induction of anti-apoptosis mechanisms. Moreover, inhibition of TCF4 induces apoptosis of colon cancer cells. In this study, caspase-9 expression...
was elevated by blocking TCF4 and decreased by its over-expression, which is compatible with cell viability. Transfection of the gene lead to apoptosis due to an off-target toxic phenotype. Caspase-9 is activated during apoptosis, and is a key mediator of this cell death process. However, although caspase-9 is an initiator of apoptosis, it regulates crosstalk between apoptosis and autophagy. Caspase-9 facilitates autophagic flux by forming a complex with Atg7. Notably, we found that LC3-II expression, which is used to monitor autophagy, was increased by blocking TCF4. Autophagy is a self-degradative process involving the balance of energy sources, and protect cells against apoptotic cell death. The Wnt signaling pathway negatively regulates both basal and stress-induced autophagy.

In this study, the specific sequence of TCF4 was inhibited using siRNA and another specific sequence of TCF4 was activated using CRISPR/dCas9. The existence of more than 100 isoforms of TCF4 have been reported and its function may vary depending on the isoform. Future studies investigating the specific functions of each isoform of TCF4 are expected.

In conclusion, our study revealed that TCF4 regulates cell proliferation, mitochondrial functions, energy production, and cell death. Furthermore, in vivo activation of TCF4 using CRISPR/dCas9 system was effective to promote the proliferation of CECs and restore the transparency of cornea. As a result, TCF4 may be a potential target for the treatment of hCEC diseases. Gene therapy using TCF4 CRISPR/dCas9 may be an effective treatment for hCEC diseases.

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