SOX2 Activation Using CRISPR/dCas9 Promotes Wound Healing in Corneal Endothelial Cells

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Key Words. Sex-determining region Y-box 2 (Sox2) • Proliferation • Regeneration • Corneal endothelial cells

ABSTRACT

There are no effective treatments for corneal endothelial diseases, except for corneal transplantation, as human corneal endothelial cells (hCECs) do not regenerate. The regeneration of hCECs could be induced through regulation of the expression of specific genes. In this study, we investigated whether the overexpression of sex-determining region Y-box 2 (SOX2) could regenerate hCECs in vivo and in vitro. SOX2 was activated using the clustered regularly interspaced short palindromic repeats (CRISPR)/deactivated CRISPR-associated protein 9 (dCas9) activation system. Genes were transfected into the corneal endothelium of Sprague-Dawley rats. Central corneal thickness and opacity were measured, and alizarin red S staining was performed. Corneal opacity and central corneal thickness were reduced in the SOX2 group compared with the control group. Additionally, hCECs were cultured and analyzed after overexpressing SOX2. Cell viability, proliferation rate, and the number of cells in S-phase were increased after SOX2 overexpression (p < .05). Cyclin-dependent kinase 1 and cyclin D1 were found to be overexpressed (p < .05). WNT signaling was repressed, and the AKT pathway was activated by SOX2 overexpression. Mitochondrial oxidative stress and energy production were increased by SOX2 overexpression (p < .05). In conclusion, SOX2 activation promotes wound healing and regeneration in CECs. SOX2 activation using the CRISPR/dCas9 system may thus be useful for the treatment of hCEC diseases. STEM CELLS 2018; 36:1851–1862

SIGNIFICANCE STATEMENT

Cornea should be transparent for clear vision. Corneal endothelial injury, including surgery, trauma, and dystrophy, can lead to painful blindness. In these cases, the treatment option is only corneal transplantation. This study suggests that gene therapy using sex-determining region Y-box 2 activation clustered regularly interspaced short palindromic repeats/deactivated CRISPR-associated protein 9 system can regenerate the corneal endothelium and serve as an important treatment option.

INTRODUCTION

The wound healing process is important for the repair of damaged human corneal endothelium [1]. Human corneal endothelial cells (hCECs) play a critical role in regulating corneal transparency through pump functions [2]. CEC loss may occur from corneal trauma, intraocular surgery, or corneal endothelial dystrophy [1, 2]. CECs do not proliferate in vivo even if they are injured, and wound healing occurs by cell hypertrophy [3]. Severe damage of the corneal endothelium can lead to corneal endothelial dysfunction, which causes the cornea to swell and become painful and invisible [4]. There are currently no effective treatments for corneal endothelial diseases, except for corneal transplantation [5]. However, the regeneration of hCECs could be induced through the regulation of specific gene expression.

Sex-determining region Y-box 2 (SOX2) is a transcription factor essential for maintaining the self-renewal capacity and pluripotency of embryonic stem cells [6]. It plays a critical role in the maintenance of embryonic and neural stem cells [7], inhibits differentiation into the mesendoderm germ layer, and promotes differentiation into the neural ectoderm germ layer. SOX2 plays a pivotal role in the development of the anterior segment of the eye [8]. Mutations in this gene have been linked to bilateral anophthalmia, a severe structural eye deformity [9]. SOX2 is expressed in wounded CECs [10]. It is believed that the activation of SOX2 may be able to regenerate the corneal endothelium without interfering with the function...
of CECs. In this study, we investigated whether SOX2 promotes wound healing after corneal endothelial injuries in vivo, as well as its related mechanisms through in vitro experiments.

Clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9) system can be used to effectively modify genes through RNA-guided DNA targeting [11]. It has been developed to activate the expression of endogenous genes by targeting a fusion protein of deactivated Cas9 (dCas9) and a transactivation domain to synergistic activation mediators (SAM) via different combinations of guide RNAs (gRNAs) [12]. This new system can be used to activate transcription factors at the normal physiological site of the nucleus, rather than at ectopic sites [11]. In this study, we investigated the effect of SOX2 activation on wound healing in the corneal endothelium using the CRISPR/dCas9 activation system both in vivo and in vitro.

**MATERIALS AND METHODS**

**Activation of SOX2 Regenerates CECs In Vivo**
This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Hallym University Medical Center. All procedures were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Six-week-old Sprague-Dawley rats were used for this procedure. Four Sprague-Dawley rats were included in each group. Rats were maintained in a colony room with a 12-hour light/dark cycle at 25°C for 7 days before initiating experiments.

For electroporation, SOX2 CRISPR/dCas9 activation plasmids (0.1 nmol; SOX2 group) or control CRISPR/dCas9 activation plasmids (0.1 nmol; control group) were injected into the anterior chamber of Sprague-Dawley rats after paracentesis. Then, 7-mm Tweezertrodes (BTX, Holliston, MA) were placed on each cornea, with the positive electrode on the plasmid-injected eye. The parameters were set at 140 V, 100 milliseconds length, 950 milliseconds interval, five pulses, and 100 V/cm². Then, a corneal endothelial injury model was induced using cryoinjury 2 days after electroporation. Corneal endothelial injury model using cryoinjury has been established previously [13,14]. Briefly, the cornea was cryoinjured for 10 seconds in contact with a metal rod of 3-mm diameter that had been frozen in liquid nitrogen for 10 minutes and then irrigated with normal saline (day 0). Then, the corneas were evaluated.

**Clinical Evaluation and Histology.** Corneal opacity was graded with photographs at days 0, 3, 7, and 14. Corneal opacity was graded as follows: grade 0 = clear cornea; grade 1 = mild corneal opacity allowing good visibility of details of the iris; grade 2 = moderate corneal opacity with partial masking of iris; and grade 3 = severe corneal opacity without a view of iris. The cornea was evaluated using anterior segment optical coherence tomography (AS-OCT), Cirrus HD-OCT; Carl Zeiss Meditec, Jena, Germany). Central corneal thickness and corneal edema were measured using AS-OCT.

Rats were sacrificed at day 3, 7, or 14. Eyes were enucleated and fixed in 3.7% formaldehyde. H&E staining was performed. Sections on the slides were immunohistochemically stained with rabbit anti-α-smooth muscle actin antibody (Abcam, Cambridge, U.K.) and the ZytoChem Plus horseradish peroxidase Broad Spectrum Kit. Slides were mounted and observed using a light microscope (DM2000; Leica, Wetzelar, Germany).

Samples were flat-mounted to evaluate the patterns of the corneal endothelium. Vital staining was performed with 0.2% alizarin red S in 0.9% NaCl (pH 4.2) for 90 seconds. The corneas were then fixed in 2.5% glutaraldehyde. Then, the corneal buttons were removed, placed on cavity slides, and mounted under a coverslip (taped to the slide to flatten the cornea) with a drop of 0.9% NaCl. The endothelium was viewed under light microscopy (DM2000; Leica), and photographs of the central and peripheral zone of the endothelium were taken. Cells were counted at x400 magnification.

H&E staining was performed as the conventional methods. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using DeadEnd colorimetric TUNEL system (Promega, Madison, WI) as the manufacturer’s protocol.

Immunofluorescence staining was performed for SOX2, CAS9, and Ki-67. The eyeballs were fixed in 4% paraformaldehyde and the corneas were excised. The corneas were treated with 20 μg/ml proteinase K solution for 15 minutes and washed with phosphate buffered saline with tween-20 (PBST). The corneas were permeabilized with 0.5% Triton-X for 15 minutes, blocked with 5% skim milk, and incubated overnight with primary antibody at 4°C. The corneas were washed in PBS and incubated with fluorescein-labeled secondary antibodies for 2 hours at 25°C. The following primary antibodies were used: mouse anti-SOX2 antibody (sc-365823, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-CAS9 antibody (sc-517386, diluted 1:100; Santa Cruz Biotechnology), or rabbit anti-Ki67 antibody (ab15580, diluted 1:100; Abcam). The following secondary antibodies were used: Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen; diluted 1:200) or Alexa Fluor 488 chicken anti-mouse IgG (H + L) (Invitrogen; diluted 1:200). Counterstaining was performed with 5 μg/ml Hoechst 33342. The corneas were placed on cavity slides and mounted under a coverslip (taped to the slide to flatten the cornea) with a drop of 0.9% NaCl. The endothelium was viewed under light microscopy (DM2000; Leica), and photographs of the central and peripheral zone of the endothelium were taken.

**Activation of SOX2 Induces the Regeneration of Cultured hCECs**

**Transfection of SOX2 CRISPR/dCas9 Activation Plasmids into Cultured hCECs.** This study was reviewed and approved by the Institutional Review Board of Hallym University Kangnam Sacred Heart Hospital. This study was carried out in accordance with the Declaration of Helsinki for research involving human subjects. hCECs were cultured as previously described [3]. The cells were transfected with green fluorescent protein (GFP)-encoded plasmids (sc-108083; Santa Cruz Biotechnology, Dallas, TX) and UltraCruz transfection medium (sc-395739; Santa Cruz Biotechnology). Transfection efficiency was calculated as the percentage of GFP-expressing cells.

For overexpression of SOX2 in hCECs, the CRISPR/dCas9 activation system was used. SOX2 CRISPR/dCas9 activation plasmid (sc-423086-ACT; Santa Cruz Biotechnology) consisted of a pool of three plasmids designed to overexpress the SOX2 gene. The control CRISPR/dCas9 activation plasmid (sc-437275;
Santa Cruz Biotechnology) was used as a negative control. Plasmid transfection medium and UltraCruz transfection medium were used according to the manufacturer’s protocol. Briefly, cells (1 x 10^5 cells per well) were seeded on 6-well culture plates in 3 ml antibiotic-free Dulbecco’s modified Eagle’s medium 24 hours before transfection and grown to 70% confluence. Cells were transfected with 1 μg SOX2 CRISPR/dCas9 activation system (Santa Cruz Biotechnology) using UltraCruz Transfection Reagent (Santa Cruz Biotechnology) and incubated at 37°C with 5% CO2. Three days after transfection, cells were used for evaluation.

**T7 Endonuclease 1 (T7E1) Assay.** The T7E1 assay was performed using GenCrispr Mutation Detection Kit (Genscript, Piscataway, NJ) according to the manufacturer’s recommendation. Cells were harvested, and genomic DNA was isolated using QuickExtract DNA Solution (Epicenter Biotechnologies, Madison, WI). Then, PCR amplification was performed. PCR conditions were one step of 30 seconds at 98°C, followed by 40 cycles of 10 seconds at 98°C, 15 seconds at 55°C, and 30 seconds at 72°C, and a final elongation for 2 minutes at 72°C. PCR amplification and concentration were checked by spectrophotometry. Heteroduplex digestion was performed. Purified PCR products were heated at 95°C for 5 minutes, cooled down at a rate of 2°C/second from 95°C to 85°C and 0.1°C/second from 85°C to 25°C using a thermocycler. A mixture of annealed PCR product (19 μl) and T7E1 (1 μl) was incubated at 37°C for 15 minutes. Then, 1 μl protease K was added and samples were incubated for 5 minutes at 37°C to stop the reaction. Agarose gel (2%) electrophoresis was performed.

**Cell Viability and Shape.** Cell viability was measured using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) and calculated as the percentage relative to the control. Cell shape was observed using an inverted microscope, and representative photographs were taken.

**BrdU Cell Proliferation Assay.** Cell proliferation was evaluated using 5-bromo-2-deoxyuridine (BrdU) incorporation with the Cell Proliferation enzyme-linked immunosorbent assay (ELISA) colorimetric kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s protocol. The cell proliferation rate was measured using a colorimetric BrdU Cell Proliferation ELISA Kit (Roche Diagnostics) and calculated as a percentage relative to the control.

**Cell Cycle Analysis.** Cell cycle analysis was performed using the Muse Cell Analyzer (Millipore, Billerica, MA) and the Muse Cell Cycle Assay Kit according to the manufacturer’s protocol.

**Mitochondrial Oxidative Stress Measurement.** MitoSOX Red (final concentration, 2.5 μM; Invitrogen, Carlsbad, CA) was used to measure mitochondrial oxidative stress. The ADP/ATP Ratio Bioluminescence Assay Kit (Biovision, San Francisco, CA) was used to evaluate ATP production in mitochondria.

**Immunofluorescence.** Immunofluorescence was carried out as previously described [15]. Briefly, cultured hCECs on coverslips were transfected with a plasmid for 48 hours, fixed in 3.7% formaldehyde, and permeabilized with 0.5% Triton X-100. Cells were washed with phosphate-buffered saline (PBS) and incubated with blocking solution for 30 minutes. Cells were incubated with primary antibody at 4°C overnight. Cells were washed with PBS and incubated with fluorescein-labeled secondary antibodies for 1 hour at room temperature. The following primary antibodies were used: goat anti-aquaporin-1 (AQP-1) antibody (SC-9878, diluted 1:100) and rabbit anti-zonula occludens-1 (ZO-1) antibody (sc-10804, diluted 1:100). The following secondary antibodies were used: Alexa Fluor 568 goat anti-rabbit IgG (diluted 1:100; Invitrogen) or Alexa Fluor 488 chicken anti-mouse IgG (H + L) (Invitrogen, diluted 1:100). Samples were mounted on slides with antifade mounting medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Slides were viewed under a light microscope (DM2000; Leica).

**Western Blotting.** Western blotting was performed as previously described [16]. Samples were homogenized in RIPA buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor (Roche Diagnostics). The protein concentration was measured using the Pierce bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The primary antibodies used were rabbit anti-SOX2 antibody (ab5603, diluted 1:500; Millipore), rabbit anti-CDK1 antibody (ab131450, diluted 1:1,000; Abcam), rabbit anti-cyclin D1 antibody (sc-718, diluted 1:500; Santa Cruz Biotechnology), rabbit anti-GSK3B antibody (ab32391, diluted 1:500; Abcam), rabbit anti-AMPK antibody (sc-25792, diluted 1:500; Santa Cruz Biotechnology), mouse anti-pAMPK antibody (sc-101630, diluted 1:500; Santa Cruz Biotechnology), mouse anti-SIRT1 antibody (sc-15404, diluted 1:500; Santa Cruz Biotechnology), mouse anti-pAKT antibody (ab66138, diluted 1:500; Abcam), mouse anti-AKT antibody (diluted 1:1,000; Cell Signaling Technology, Danvers, MA), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody (LF-PA0212, diluted 1:5,000; Abfrontier, Seoul, Korea) as an internal control. Alkaline phosphatase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG (Bio-Rad Laboratories, Hercules, CA) was used as secondary antibody. Protein bands were detected using a 5-bromo-4-chloro-3-iodophosphate/nitro blue tetrazolium Color Development Substrate solution (Promega).

**RNA Analysis.** Total RNA was extracted using the ReliaPrep RNA Miniprep System (Promega). cDNA was generated using the Promega GoScript reverse transcription system according to the manufacturer’s protocol. Real-time quantitative PCR was performed on a LightCycler 480 (Roche Diagnostics) using MG 2x qPCR MasterMix (SYBR Green; MGmed, Seoul, Korea). Primer sequences are described below. All reactions were performed in triplicate, and data were analyzed according to the △△Ct method. The primer sequences used were as follows: β-actin (forward: 5’-AGAGCTACGCTGCGTACG-3’, reverse: 5’-AG CACTTGGCGGTACAG-3’), Wnt-3a (WNT3A; forward: 5’-AG TTTGCTGGATGTTCGTC-3’, reverse: 5’-CGTGGACTGTCGCTT GAG-3’), 5’-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1, p238050; Bioneer, Daejeon, Korea), blcin-1 (BECN1, p130865; Bioneer), β-catenin (p271730; Bioneer), and collagen VIII-α2 (COL8A2, p247970; Bioneer).

## RESULTS

**Activation of SOX2 Regenerates CECs In Vivo**

**Effect of SOX2 Activation Using SOX2 CRISPR/dCas9 Activation System.** Flat mounting showed that the transfection
Effect of SOX2 Activation on Corneal Opacity and Thickness. Corneal opacity was graded according to the photographs obtained at days 3, 7, and 14. Corneal opacity was lower in the SOX2 group than in the control group at days 2, 4, 7, 10, and 14 (p = .024 , .024 , .030 , .031 , and .017 , respectively; Fig. 2A). AS-OCT showed a thickened cornea and corneal edema (Fig. 2B). The central corneal thickness was thinner in the SOX2 group than in the control group at days 3, 7, and 14 (453.0 ± 25.79 μm vs. 385.25 ± 40.90 μm, 439.3 ± 48.34 μm vs. 268.0 ± 73.54 μm, and 453.0 ± 25.79 μm vs. 276.5 ± 76.53 μm; p = .003 , .001 , and .001 , respectively; Fig. 2C). Corneal edema showed a sharp decline in the SOX2 group (p < .001).

Effect of SOX2 Activation on Corneal Endothelial Wound Healing and Regeneration. Flat mounting also showed that CECs were damaged and that there were no cells in the center of the mass; large cells at a low cell density were found at the midperiphery, and small cells at a high cell density were found at the periphery in the control group at day 3 (Fig. 3A). At days 7 and 14, we found large cells at a low cell density in the center and small cells at a high cell density at the periphery in the control group. In the SOX2 group, the cell number was higher compared with the control group at days 3, 7, and 14 (2,917.33 ± 153.74 vs. 552.67 ± 38.28; 3,770.67 ± 618.09 vs. 966.67 ± 106.91; and 4,162.67 ± 560.93 vs. 1,726.67 ± 239.48; p < .001 , .002 , and .002 , respectively). High-magnification microscopy revealed that CECs in the control group showed a large and irregular morphology, whereas CECs in the SOX2 group showed a small and regular morphology (Fig. 3B). The cell number at the center was higher in the SOX2 group than in the control group (636.67 ± 85.33 vs. 89.33 ± 78.44 at day 3, 942.67 ± 154.52 vs. 203.67 ± 92.42 at day 7, and 957.33 ± 195.36 vs. 325.33 ± 85.44 at day 14; p = .001 , .002 , and .007 , respectively). Immunofluorescence staining showed that Ki67 was expressed in the nucleus in the cells of the SOX2 group but not in the cells of the control group (Fig. 3C). The number of Ki67-positive cells was higher in the SOX2 group compared with the control group (p = .002 ). H&E staining showed inflammatory cell infiltration and damaged corneal endothelial cells on the endothelium in the control group (Fig. 3D). However, a few damaged corneal endothelial cells were detected in the SOX2 group at day 3 and normal healthy endothelial cells were shown in the SOX2 group at days 7 and 14. TUNEL-positive apoptotic cells were detected in the control group at days 3 and 7, whereas in the SOX2 group, a few apoptotic cells were detected only at day 3 and no apoptotic cells were observed at days 7 and 14 (Fig. 3E).

Overexpression of SOX2 Induces Regeneration of Cultured hCECs

Transfection Efficiency and Effect of SOX2 Activation on Cell Viability and Proliferation. Cultured hCECs showed a mosaic pattern at 100% confluence and passage 0 (Fig. 4A). copGFP-encoding plasmids were transfected into the cells when they were at 70% confluence. Transfection efficiency was 59.12% ± 2.38% (Fig. 4B). T7E1 assay did not show the cleaved products in the control and SOX2 groups (Fig. 4C). SOX2 expression increased dramatically in the SOX2 cells (229.1% ± 11.3%; Fig. 4D). Cell viability was higher in the SOX2 cells than in the control cells (212.5% ± 34.5%, p < .001; Fig. 4E). Inverted microscopy showed that the confluence of the SOX2 cells was also higher than in the control cells (Fig. 4F). The BrdU cell proliferation rate was higher in the SOX2 cells compared with the control cells (2.34 ± 0.15-fold, p < .001; Fig. 4G). Cell cycle analysis showed that the number

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of cells in S-phase increased in the SOX2 cells compared with the control cells (12.06% \pm 1.07% vs. 10.03% \pm 0.25%, \(p = .020\); Fig. 3I). CDK1 and Cyclin D1 expression levels were elevated in the SOX2 cells compared with the control cells (\(p = .002\) and \(p < .001\), respectively; Fig. 4H). In contrast, CDKN2A expression decreased in the SOX2 cells compared with the control cells (\(p = .016\); Fig. 4J).

Effect of SOX2 Activation on Mitochondrial Oxidative Stress and Function. Mitochondrial vital staining fluorescence increased in the SOX2 cells compared with the control cells (\(p = .005\); Fig. 5A), and mitochondrial oxidative stress also increased in the SOX2 cells (\(p = .014\); Fig. 5B). However, the mitochondrial membrane potential remained unchanged (Fig. 5C). ATP production increased in the SOX2 cells compared with the control cells (1.21 \pm 0.34-fold, \(p = .019\); Fig. 5D). ATP5B expression increased in the SOX2 cells (\(p = .008\); Fig. 5E). PRKAA1 mRNA and pAMPK expression levels were reduced in the SOX2 cells compared with the control cells (\(p = .044\) and \(p = .048\), respectively; Fig. 5F and 5G). SIRT1 expression levels were also elevated in the SOX2 cells compared with the control cells (\(p = .002\); Fig. 5H). In contrast, BECN1 mRNA expression levels decreased in the SOX2 cells (\(p = .021\); Fig. 5I).

Effect of SOX2 Activation on Various Signaling Pathways. WNT3A mRNA expression levels decreased (\(p = .008\); Fig. 6A) and GSK3B mRNA increased (\(p = .029\); Fig. 5B) in the SOX2 cells, which was confirmed by Western blotting (\(p = .047\); Fig. 6C). \(\beta\)-catenin mRNA expression levels decreased in the SOX2 cells (\(p = .044\); Fig. 6D), which was confirmed by Western blotting (\(p = .004\); Fig. 6E). COL8A2 mRNA expression levels also decreased in the SOX2 cells (\(p = .001\); Fig. 6F). In contrast, pAKT and pFKHRL1 expression levels increased in the SOX2 cells (\(p = .034\) and .013, respectively; Fig. 6G, 6H).

Effect of SOX2 Activation on Differentiation Marker Expression. ZO-1 expression levels were similar in both groups (Fig. 7A), whereas AQP-1 expression levels increased in the SOX2 cells compared with the control cells (\(p = .049\); Fig. 7B).

**DISCUSSION**

There are no effective treatments for corneal endothelial diseases, except for corneal transplantation, because hCECs do not regenerate in vivo [3]. However, the regeneration of hCECs could be induced through regulated expression of specific genes. In this study, we used the CRISPR/dCas9 activation system [11], which has several advantages [17]. This transcriptional activation system functions across a wide range of cell types and species and provides many options for transcriptional and epigenetic manipulation [17]. The system induced the overexpression of our target transcription factor by increasing gene expression at its normal location, rather than at an ectopic site. SOX2 is the most likely candidate for corneal endothelial cell regeneration, contributing to proliferation, maintenance of stem cell function, and the development of cornea.

In this study, we used electroporation for in vivo transfection after injecting the plasmid vector into the anterior chamber of Sprague-Dawley rats and used cryoinjury to induce corneal endothelial damage. Electroporation was used for in vivo transfection because it is efficient and causes minimal tissue damage [18]. We obtained a transfection efficiency of 50.93% \pm 2.91%. Immunofluorescence staining for SOX2 and
CAS9 was performed to evaluate the efficiency of the SOX2 CRISPR/dCAS9 activation system. This study showed that SOX2 and CAS9 were expressed in the nuclei of the cells in the SOX2 group but not in the cells of the control group. In this study, we used the CRISPR/dCas9 activation system to overexpress SOX2 in cultured hCECs. SOX2 expression in cultured hCECs increased after the CRISPR/dCas9 activation system. Off-targeting of CRISPR/Cas9 system has been reported to cause nonspecific genetic modifications [19]. Several methods have been proposed to assess off-targeting [20]. T7E1 assay is generally used to validate gene cleavage using CRISPR/Cas9 system [21]. In this study, results of T7E1 assay showed no cleaved products, indicating that there is a little off-targeting effect. However, CRISPR/dCas9 activation system does not cleave the gene and activate the gene expression using SAM; therefore, the T7E1 assay has a limitation.

Figure 3. Effect of SOX2 activation on corneal endothelial wound healing and cell death. (A): Flat-mounted samples showed the density and shape of corneal endothelial cells (CECs) during the experimental period. The total cell density was higher in the SOX2 group compared with the control group during the experimental period. (B): High-magnification microscopy revealed the CECs at the corneal center. The cell number at the center was higher in the SOX2 group than in the control group during the experimental period. (C): Immunofluorescence staining revealed the presence of Ki67-stained cells in the SOX2 group but not in the control group. (D): In H&E staining, the inflammatory cells and damaged endothelial cell clusters are shown in the control group, which were not shown in the SOX2 group. (E): TUNEL staining showed the apoptotic cells on the corneal endothelium in the control group and a few apoptotic cells in the SOX2 group. * p values were calculated with Student’s t test; * p < .05.
In this study, cryoinjury was used for in vivo corneal endothelial decompensation model. Transcorneal freezing was used for making a corneal endothelial decompensation model. Cryoinjury effectively destroys the corneal endothelium and induces the bullous keratopathy [13, 14]. In this study, corneal opacity and edema decreased significantly in the SOX2 group, and flat-mounted slides of corneas stained with Alizarin red S showed that wound healing in the corneal endothelium was faster in the SOX2 group. CECs eliminated the corneal edema by expelling water that had entered the gaps between cells [1]. If the corneal endothelium is damaged and does not completely cover the posterior surface of the cornea, it will become

Figure 4. Transfection efficiency, cell viability and cell proliferation. (A): Human corneal endothelial cells were cultured and showed a mosaic pattern at 100% confluence and passage 0. (B): copGFP-encoding plasmids were transfected into the cells at 70% confluence. (C): SOX2 expression was sharply increased in the SOX2 cells (229.1% ± 11.3%). (D): T7 endonuclease 1 assay did not show the cleaved product. (E): Cell viability was higher in the SOX2 cells compared with the control cells ($p < .001$). (F): Inverted microscopy showed that the confluence of the SOX2 cells was higher compared with the control cells. (G): The 5-bromo-2-deoxyuridine (BrdU) cell proliferation rate results are shown for the SOX2 cells and the control cells. (H): CDK1 and Cyclin D1 expression using Western blotting in the SOX2 cells and the control cells ($p = .002$ and $p < .001$, respectively). (I): Cell cycle analysis showed the number of cells in the S-phase in the SOX2 cells and the control cells ($p = .016$). Bars show the mean, and error bars indicate the SD. $p$ values were calculated with Student’s t test; *$p < .05$. 

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edematous and opaque, as the barrier function of corneal endothelium fails; therefore, it cannot pump water out of the corneal stroma [22]. The small, regular shape of CECs covering the posterior surface of the cornea in the SOX2 group is essential to ensure that the cornea is transparent. In this study, immunofluorescence staining revealed the presence of Ki67-stained cells in the SOX2 group but not in the control group. Ki67 is a proliferation marker, levels of which increase in the S-phase of the cell cycle [23]. SOX2 promotes proliferation and migration via G1/S-phase transition and the AKT pathway.

Figure 5. Effect of SOX2 activation on mitochondrial oxidative stress and function. (A): Mitochondrial vital staining fluorescence in the SOX2 cells and the control cells is shown. (B): Mitochondrial oxidative stress increased in the SOX2 cells compared with the control cells. (C): Mitochondrial membrane potential in the SOX2 cells and the control cells. (D): ATP production in the SOX2 cells and the control cells is shown. (E): ATP5B expression was evaluated in the SOX2 cells and the control cells. (F, G): PRAKA1 mRNA and pAMPK expression in the SOX2 cells and the control cells are shown. (H): SIRT1 expression in the SOX2 cells and the control cells are shown. (I): BECN1 mRNA expression was evaluated in the SOX2 cells and the control cells. Bars show the mean, and error bars indicate the SD. p values were calculated with Student’s t test; *p < .05.
in vitro study showed that cell viability and proliferation rate both increased after SOX2 overexpression. Cell cycle analysis showed an increase in the number of cells in the S-phase and a decrease of cells in the G0/G1 phase. The G0/G1 phase is a resting phase wherein cells exit the cycle and do not proliferate [25]. During S-phase, DNA is replicated in proliferating cells [26]. Although the cell cycle of corneal endothelium in vivo stops at the G0/G1 phase [3], the overexpression of SOX2 may cause the cell cycle to progress. In this study, SOX2 activation increased the expression of CDK1 and Cyclin D1, which regulate cell cycle progression [27, 28]. SOX2 regulates the expression of CDK1 and cyclin D1. Cyclin D1 is a major mediator of SOX2 in cell proliferation [29]. The levels of CDKN2A, which inhibit the progression of the cell cycle and induce senescence [30, 31], decreased after SOX2 activation. SOX2 regulates proliferation and differentiation depending on the cell [7]; we found that it induced proliferation in hCECs. The expressions of CDK1 and cyclin D1 are affected by cell confluence [32, 33]. SOX2 induced cell proliferation enhanced cell confluence, which may have affected the expression of CDK1 and cyclin D1.

WNT signaling has been reported to regulate the fate of hCEC [34]. Dysregulation of WNT signaling has been suggested to be the main pathology of the hCEC disease [35]. The WNT signaling system was analyzed because WNT-induced epithelial-mesenchymal transition (EMT) can cause the morphological degeneration and loss of function of hCECs [36]. In this study, SOX2 repressed the WNT/β-catenin pathways and activated GSK3B. WNT3A and β-catenin are members of the WNT signaling pathway [37, 38]. Accumulation of cytosolic β-catenin is often a good indicator of WNT/β-catenin signaling activity [37]. GSK3B inactivates the WNT signaling pathway [39]. GSK3-mediated phosphorylation of β-catenin may not only induce degradation but also directly affect transcriptional activity [40]. SOX2 interacts with the WNT/β-catenin signaling network and can affect WNT signaling.

Figure 6. Effect of SOX2 activation on different signaling pathways. (A): WNT3A mRNA expression was evaluated in the SOX2 cells and the control cells. (B, C): GSK3B mRNA increased in the SOX2 cells, which was confirmed by Western blotting. (D, E): β-catenin mRNA expression decreased in SOX2 cells, which was confirmed using Western blotting. (F): COL8A2 mRNA expression in the SOX2 cells and the control cells is shown. (G, H): pAKT and pFKHR1 mRNA expression in the SOX2 cells and the control cells are shown. Bars show the mean, and error bars indicate the SD. p values were calculated with Student’s t test; *p < .05. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
SOX2 activation using the CRISPR/dCas9 activation system promotes wound healing and regeneration of hCECs and restores their function and shape. SOX2 activation may thus be useful for treating diseases in hCECs.
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AUTHOR CONTRIBUTIONS
Y.K.C., J.S.H., and T.Y.C. performed the experiments and analyzed the data. Y.J.S. designed and performed the experiments, analyzed and interpreted the data, assembled the input data, and wrote the manuscript. All authors discussed the results and implications and commented on the manuscript.

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