Generation of Human Corneal Endothelial Cells via In Vitro Ocular Lineage Restriction of Pluripotent Stem Cells

Jiagang J. Zhao and Natalie A. Afshari

Shiley Eye Institute, Department of Ophthalmology, University of California San Diego, La Jolla, California, United States

Correspondence: Jiagang J. Zhao, Shiley Eye Institute, Department of Ophthalmology, University of California San Diego, La Jolla, CA 92093-0946, USA;
j3zhao@ucsd.edu.
Natalie A. Afshari, Shiley Eye Institute, Department of Ophthalmology, University of California San Diego, La Jolla, CA 92093-0946, USA;
naafshari@ucsd.edu.

Submitted: May 28, 2016
Accepted: November 8, 2016

Citation: Zhao JJ, Afshari NA. Generation of human corneal endothelial cells via in vitro ocular lineage restriction of pluripotent stem cells. *Invest Ophthalmol Vis Sci*. 2016;57:6878–6884. DOI:10.1167/iovs.16-20024

Purpose. We generate a renewable supply of corneal endothelial cells (CEC) from human pluripotent stem cells (PSCs) under defined culture conditions.

Methods. Corneal endothelial cell induction was driven by small molecules in a stepwise fashion of lineage specification. During the initial phase, PSC fate was restricted to the eye field-like state and became eye field stem cells (EFSCs). In the second phase, PSC-derived EFSCs were further directed toward either neural crest lineage or retinal lineage. The CECs were directly induced from ocular neural crest stem cells (NCSCs) by suppressing TGF-β and ROCK signaling.

Results. Under chemically defined conditions, PSCs were massively converted into EFSCs and subsequently NCSCs. Eye field cell identity was characterized by the expression of key fate restriction factors for early eye field cells, such as *PAX6*, *LHX2*, and *VSX2*. The induction of ocular NCSCs was initiated by promoting WNT signaling in EFSCs. Within 2 weeks of induction, the majority of cells expressed the typical neural crest markers p75NTR and HNK-1. Eye field stem cell-derived NCSCs can be propagated and cryopreserved. Subsequently, a CEC monolayer was induced from adherent NCSCs in the presence of small molecular inhibitors to suppress TGF-β and ROCK signaling. The polygon-shaped CEC-like cells became visible after a week in culture. The NCSC-derived CECs expressed typical CEC markers, such as N-Cadherin and Na^+/K^+-ATPase.

Conclusions. A novel small molecule-based approach was developed to derive human CECs from PSCs via ocular lineage specification. Moreover, EFSC-derived NCSCs could serve as an immediate source cell for rapid CEC induction in vitro.

Keywords: pluripotent stem cell, corneal endothelial cell, neural crest, eye field, small molecule

Corneal endothelial dystrophy is a primary cause of loss of vision. Fuchs endothelial corneal dystrophy (FECD) is age-related and the most common corneal endothelial disorder leading to blindness. It affects 4% of the United States population over age 40 and likely millions of people worldwide.[1] Currently, restorative corneal transplantation is the only option to treat this disease, but the procedure faces many challenges, such as the shortage of healthy donor material for millions of patients around the world. To address this issue, it is imperative that an alternative source of transplantation material be developed.

Human corneal endothelium consists of a monolayer of orderly arranged polygonal cells that function as a barrier to separate the corneal stroma from the aqueous humor of the anterior chamber. It also serves as a nutritional gateway and regulates water content of the corneal stroma. Human corneal endothelial cells (CECs) are not proliferative and show no signs of functional regeneration in vivo.[2] Average cell density gradually decreases at a rate of 0.6% per year in a healthy cornea. Cell loss is more profound in aged, injured, or inflamed corneas. If cell density drops below a critical level, corneal physiologic function fails and corneal edema ensues, which leads to bullous keratopathy and loss of visual acuity. One of the major obstacles to conducting disease mechanistic studies in the human cornea and developing nonsurgical medical treatments, such as drug therapy, is the limited availability of CECs in culture due to poor mitotic activity and propagation of cells in vitro.[3] Therefore, to better study the disease mechanisms of corneal endothelial dystrophies and to alleviate the worldwide shortage of donor tissue, there is considerable interest in the development of in vitro expandable cell sources for engineering corneal endothelium.

Attempts to expand CECs from primary CECs isolated from human donor tissues in vitro have been made in the past.[4–6] However, massive production of CECs from a small number of healthy and qualified donor tissues remains a challenge due to the limited regenerative capacity of donor cells. In addition, cells from older donors grow slower, exhibit more heterogeneity, and are more prone to senescence compared to those from younger donors.[7,8] With recent advances in human pluripotent stem cell (PSC) technology, it now is plausible to derive an unlimited supply of CECs from PSCs in culture. A few studies have been reported recently.[9,10] Here, we report the development of a highly efficient, small molecule–based method for the induction of CECs from PSCs in vitro under a defined set of culture conditions. To emulate the natural developmental process of CEC cell fate specification, we first derived eye field stem cells (EFSCs) from PSCs, then directed EFSCs toward ocular neural crest stem cell (NCSC) fate, and subsequently differentiated ocular NCSCs to CECs. Using this three-step strategy, we were able to produce a highly homogenous and expandable monolayer of CECs in culture.

Methods

Cell Culture and Differentiation

Human PSCs H9 (WA9, WiCell) and iPSC, derived from BJ human fibroblasts (provided by the stem cell core of Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA), were cultured under feeder-free and serum-free conditions in StemPro hESC SFM medium (Thermo Fisher Scientific, Carlsbad, CA, USA) on plates coated with growth factor–reduced Matrigel (BD Biosciences, San Jose, CA, USA). After the undifferentiated PSCs reached approximately 80% confluence in culture, the medium was switched to serum-free N2B27 priming medium (Dulbecco's modified Eagle's medium [DMEM]/F12, N2, B27, 0.2% BSA, 2 mM L-GlutaMAX, 0.1 mM MEM nonessential amino acids, and 0.1 mM β-mercaptoethanol) supplemented with 20 ng/ml BFGF for 1 to 2 days. The nearly confluent monolayer culture of human (h) PSCs was further cultured in N2B27 priming medium supplemented with small molecule inhibitors (5 μM SB431542, 50 nM LDN193189, and 1 μM IWP2). The medium was changed daily for 6 days. The PSC-derived EFSCs can be maintained and expanded in this serum-free, inhibitor-supplemented priming medium. To induce retinal ganglion cell (RGC) differentiation from EFSC, the cells were cultured in the priming medium supplemented with a new combination of small molecule inhibitors, including 1 μM IWP2, 10 μM DAPT, and 200 nM PD173074 for over 2 weeks. For photoreceptor precursor differentiation, dissociated EFSCs were plated on a Matrigel-coated plate and cultured in neural induction medium as described previously[11] and supplemented with 1 μM IWP2, 10 μM DAPT, and 100 nM purmorphamine for 6 days. Subsequently, the culture was shifted to neural induction medium supplemented with 500 nM retinoic acid and 100 μM taurine for 7 days. To induce RPE differentiation, the induction medium (Glasgow minimal essential medium [GMEM], 10% knockout serum replacement, 0.1 mM MEM nonessential amino acids, 1 mM Na-Pyruvate, and 0.1 mM β-mercaptoethanol) supplemented with 10 mM nicotinamide and 100 ng/ml activin A was added to the monolayer culture of EFSCs for 1 week. Subsequently, the RPE precursors were matured in RPE medium, consisting of MEM-a modified medium, 5% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, and 1 mM Na-Pyruvate, supplemented with N1 and taurine, hydrocortisone, triiodo-thyronin (THT).[12] For ocular NCSC differentiation, EFSCs were plated at low density on a Matrigel-coated surface. The cells were cultured in neural crest induction medium (DMEM/F12:Neurobasal 50:50; N2, B27, 0.3 mM 2-phospho-L-ascorbic acid) and supplemented with 3 μM CHIR 99021. The cultures were passaged when they reached approximately 80% confluence. To direct CEC differentiation, ocular NCSCs were seeded at low cell density on a plate coated with FNC Coating Mix (US Biological, Marblehead, MA, USA). After 24 hours, the culture was switched to human CEC induction medium (human endothelial-SFM, 5% FBS, 0.3 mM 2-phosphate ascorbic acid, 1% penicillin/streptomycin) supplemented with small molecular inhibitors 1 μM SB431542 and 2.5 μM H-1125.[13]

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100-PBS for 5 minutes twice, and blocked in a solution of PBS containing 5% normal donkey serum and 0.2% TritonX-100%, followed by an overnight incubation in primary antibody solutions at 4°C. After three washes in PBS, cells were incubated with Alexa fluorescently conjugated secondary antibodies for another 90 minutes. After rinses and washes in PBS, cell nuclei were counterstained with 100 ng/ml Hoechst 33342 for 10 minutes. Primary antibodies and their working dilutions were as follows: rabbit anti-Pax6 (1:600, Covance, Princeton, NJ, USA), goat anti-Lhx2 (1:200, Santa Cruz Biotechnology, Dallas, TX, USA), sheep anti-Chx10 (1:300, Exalpha Biologicals, Inc., Shirley, MA, USA), goat anti-Nestin (1:350, Santa Cruz Biotechnology), mouse anti-Crx (1:200, Abnova, Taipei City, Taiwan), mouse anti-Ki67 (1:250, Santa Cruz Biotechnology), mouse anti-HNK-1 (1:200, Sigma-Aldrich Corp., St. Louis, MO, USA), rabbit anti-b-catenin (1:250, Santa Cruz Biotechnology), rabbit anti-p75NTR (1:1600, Cell Signaling Technology, Danvers, MA, USA), mouse anti-N-Cadherin (1:250, Santa Cruz Biotechnology), rabbit anti-ZO-1 (1:250, Life Technologies, Carlsbad, CA, USA), and mouse anti-Na^+/K^+ATPase a1 (1:200, Santa Cruz Biotechnology). The secondary antibodies used were the corresponding Alexa-488, -555, -633, or -647 fluorescent-labeled antibodies (1:1000; Life Technologies). The specific immunoreactivity of each antibody was confirmed by immunostaining using appropriate retinal tissues as positive controls under the same conditions. Labeled cells were imaged with either a laser-scanning confocal microscope (Olympus, Tokyo, Japan) or a fluorescence microscope (EVOS/Thermo Fisher Scientific).

Results

Induction of EFSCs From PSCs Under a Chemically Defined Condition

During the early stages of embryonic brain development, eye field formation is influenced by Wnt and BMP signaling gradients.[14,15] The gradient of Wnt signaling is important in establishing forebrain and midbrain identities. Downregulation of Wnt signaling leads to formation of the forebrain, where the eye field resides. Accordingly, treating human ESC-derived embryoid bodies with Dkk1, a potent WNT inhibitor, and noggin, a BMP antagonist, promotes the development of an eye field.[16,17] In addition, under dual SMAD inhibition by exposure to SB431542 (a selective and potent inhibitor of the TGF-β superfamily type I activin receptor-like kinases *ALK-4*, *-5*, and *-7*) and LDN193189 (a selective inhibitor of BMP type I receptors *ALK-2* and *ALK-3*, a noggin analog), ESCs are likely to become forebrain/eye field precursors that strongly express PAX6 and LHX2, two key early transcription factors responsible for eye field formation.[18,19] Therefore, we developed a chemically defined culture protocol to induce the differentiation of human PSCs toward eye field identity under synergistic inhibition of WNT and TGF-β/BMP signaling activities using the small molecule inhibitors IWP2 (inhibitor of Wnt Production-2, a Dkk1 analog), LDN193189, and SB431542 (referred to hereafter as IWP, LDN, and SB, respectively).[20] The human PSCs were seeded onto Matrigel-coated plates and cultured under feeder-free and serum-free conditions to near confluence. Subsequently, the culture was switched to eye field priming medium supplemented with the IWP-LDN-SB small molecule cocktail for 1 week. Under this treatment, the PSCs were converted into EFSCs en masse. The induction of EFSCs was confirmed by immunocytochemical labeling of key early eye field transcription factors.[16,21] The majority of cells were labelled positively for either PAX6, LHX2, or both, two key transcription factors expressed by early eye field progenitors (Figs. 1A, 1B). The PSC-derived EFSCs also expressed *CHX10* (also known as *VSX2*; Fig. 1D), a gene required for retinal progenitor proliferation.[22] The proliferative activity of EFSCs was evidenced by strong expression of Ki67 (Fig. 1E). Most PSC-derived EFSCs also expressed NESTIN, a typical marker of primitive neuroepithelial stem cells (Fig. 1F). The induction of

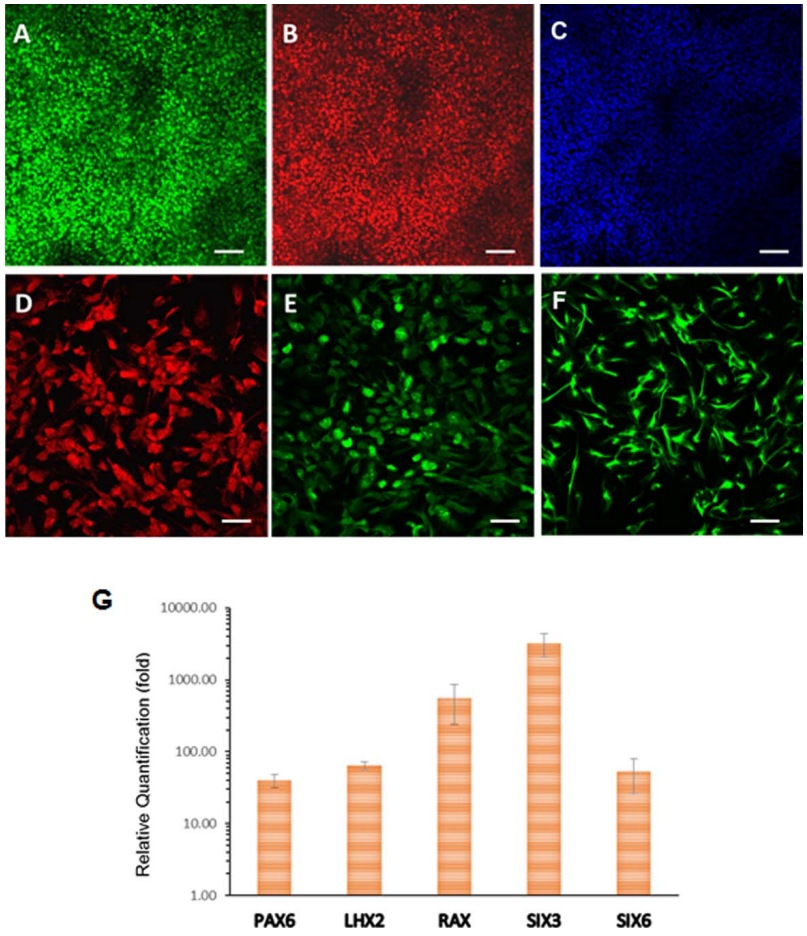

Figure 1. Induction of EFSC from human PSC. Confocal images of immunofluorescence staining of typical early eye field transcription factors PAX6 (**A**) and LHX2 (**B**) in EFSCs derived from PSCs after 1 week of induction. Cell nuclei were counterstained with Hoechst 33342 (**C**). Eye field stem cells were stained for a retinal progenitor early transcription factor CHX10 (**D**), a cell proliferation marker Ki67 (**E**), and a neuroectodermal cell marker NESTIN (**F**). *Scale bars*: (**A–C**) = 60 μm; and (**D–F**) = 30 μm. (**G**) Real-time quantitative PCR (qPCR) analysis of the induced gene expression of representative early eye field transcription factors.

EFSCs was further confirmed by real-time PCR to assess the expression of typical early eye field transcription factor genes, including *PAX6*, *RAX*, *LHX2*, *SIX3*, and *SIX6* (Fig. 1G). We found that the increased expression levels of these markers in EFSCs versus those in PSCs ranged from 40- to over 3000-fold. Moreover, the PSC-derived EFSCs were stable through at least five passages in culture and could be cryopreserved for long term use.

PSC-Derived EFSCs Can Be Directed Into the Specific Fates of Major Retinal Cell Types

To investigate whether EFSCs have the potential to give rise to different sublineages of eye cells in culture, we took a small molecule–based approach to mimic the inductive cues present in early eye development. We directed the differentiation of EFSCs toward specific retinal cell fates in vitro. Retinal ganglion cells are a major type of retinal neuron and have a critical role in transmitting visual signals from the retina to several regions of the brain. We first tested whether EFSCs could be instructed to differentiate into RGCs directly under chemically-defined conditions. Previous results demonstrate that inhibition of Notch and VEGFR signaling is important for RGC specification.[23,24] We formulated a cocktail of small molecule inhibitors, including IWP2, DAPT, and PD173074, that could inhibit the activities of WNT, NOTCH, and VEGFR signaling, respectively. The treatment rapidly converted EFSCs to a RGC fate. After 2 weeks of induction, the majority of cells were positive for TUJ1 and BRN3, markers of RGCs (Fig. 2A). Long axon-like processes were observed after extended differentiation in culture (~3 weeks; Zhao JJ, manuscript in preparation). Photoreceptors are the primary retinal neurons responsible for the initiation of visual signal transduction. We developed a modified in vitro photoreceptor differentiation method based on a previous report[25] and used small molecules to direct the differentiation process. The restriction of photoreceptor fate from EFSCs was achieved with a two-step process. During the initial phase, EFSCs were treated with the small-molecule inhibitors SB, CHIR99021, and DAPT to suppress ALK4/5/7, GSK-3, and Notch signaling activities, respectively. Furthermore, the Shh signaling pathway was stimulated by the addition of small molecule, purmorphamine.[26] Robust cell growth and proliferation, but not expression of photoreceptor-specific markers, were observed during this initial phase as previously described.[27] During the second phase, the culture was shifted to medium supplemented with retinoic acid and taurine as described previously,[25] which induced morphologic changes including extension of cellular processes in some cells after 1 week. To identify the fate of these differentiated cells, we examined the expression of photoreceptor-specific markers by immunocytochemistry. By day 14 after the initial induction, the pan-photoreceptor marker CRX was detected (Fig. 2B). The

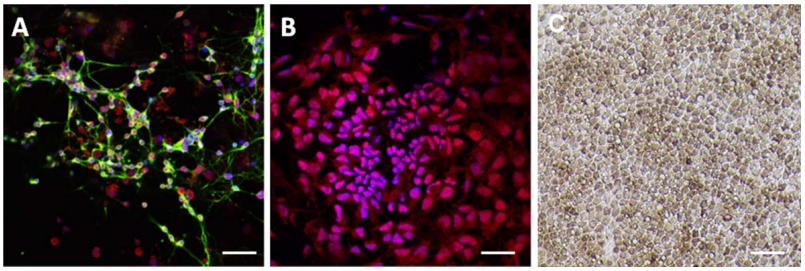

Figure 2. Characterization of EFSC differentiation to either neuronal or nonneuronal retinal cell fates under defined culture conditions. (**A**) Retinal ganglion cell differentiation from EFSCs. After 2 weeks of culture under conditions for RGC induction, differentiated cells displayed long neuronal processes and expressed typical RGC markers, such as BRN3 (*red*) and TUJ1 (*green*). Cell nuclei were counterstained with Hoechst 33342 (*blue*). (**B**) Photoreceptor induction was evidenced by immunocytochemistry on the expression of an early photoreceptor marker CRX (*red*), and cell nuclei were counterstained with Hoechst 33342 (*blue*). (**C**) Pigmented RPE appeared after prolonged RPE induction and maturation in culture. *Scale bars*: 30 μm.

cone cell–specific marker OPN1SW or blue Opsin, and rod cell–specific marker Rhodopsin as well as Recoverin also were detected (Zhao JJ, manuscript in preparation). The RPE, a monolayer of cells between the neural retina and choriocapillaris, is the first committed retinal cell type to appear in the outer layer of the early optic cup.[28] To test whether EFSCs also were capable of differentiating into nonneuronal RPE cells, we withdrew small molecule inhibitors from the adherent EFSC monolayer culture and shifted to RPE initiation medium as described previously.[29] The removal of SMAD signaling inhibition and addition of activin A were important for directing EFSC toward the RPE fate because activin A and BMP activities are required for RPE specification.[28–30] During the first 2 weeks of induction, low expression levels of an RPE-specific marker RPE65 were detected (Zhao JJ, manuscript in preparation). The formation of polygonally shaped cells and pigmentation of RPE were observed after further maturation in culture (Fig. 2C).

Directed Differentiation of EFSCs into Ocular NCSCs

Previous studies on fate maps in bird and mammalian eyes demonstrated that the corneal endothelium is derived solely from neural crest cells in the optic region.[10,31] The in vitro derivation of NCSCs from PSCs has been described previously.[32,33] We reasoned if EFSCs could be primed toward neural crest lineage, the resulting neural crest could retain certain ocular identity, which could be important for CEC induction. To direct EFSCs toward neural crest fate, we seeded EFSCs at low density and initiated neural crest lineage specification in NCSC induction medium supplemented with 3 μM CHIR 99021. After 1 week of induction, the majority of EFSCs started to express a typical NCSC surface marker HNK-1 (Fig. 3A). The subsequent analysis of EFSC-derived NCSCs showed the enrichment of NGF receptor (p75NTR)-expressing NCSCs in the culture (Fig. 3B). Immunostaining also detected the nuclear translocation of β-catenin (Fig. 3C), an indication of increased WNT canonical signaling during neural crest formation. We further asked if the ocular lineage specification of PSC resulted in the loss of its pluripotency by following the expression changes of stemness genes, such as *OCT4* and *SOX2*. While the expression of *OCT4* was turned down in EFSCs and NCSCs, the expression of *SOX2* showed some rebound in NCSCs after the initial loss in EFSCs (Fig. 4).

Differentiation of CECs from Ocular NCSCs

To test if CECs could be directly induced from the EFSC-derived NCSCs in vitro, we seeded NCSCs at low cell density. After 24 hours, the culture medium was changed to CEC induction medium in the presence of small molecular inhibitors 1 μM of SB431542 and 2.5 μM of H-1125, which suppress ALK4/5/7 and Rho-associated kinase (ROCK) signaling activities, respectively. We previously used ROCK inhibitor H-1125 instead of Y21632 to achieve a better wound healing effect in a rabbit corneal endothelium injury model.[34] We found that the addition of H-1125 could improve the in vitro differentiation of CECs from NCSCs. Within 1 week of induction, the cells displayed hexagonal/polygonal morphology (Fig. 5A). The NCSC-derived CECs remained proliferative and formed a relatively homogenous monolayer of cells after a few passages. To further characterize the NCSC-derived CEC sheets, we performed immunostaining that positively labeled the cells for several typical CEC markers, including tight junction protein 1 (TJP1 or ZO-1), Na^+/K^+ ATPase, and N-Cadherin (NCAD) (Figs. 5B, 5C, 5D). In addition, we also confirmed the increased mRNA expression of ATP1A1 and NCAD by real-time PCR in NCSC-derived CECs versus NCSCs (Fig. 5E).

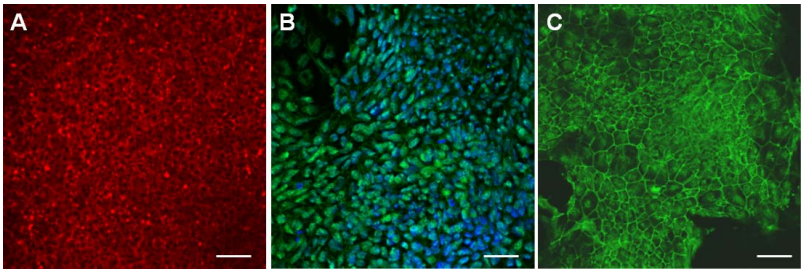

Figure 3. Induction of NCSCs from PSCs in vitro. The cells were stained positively for two typical NCSC markers, HNK-1 (*red*, [**A**]) and p75NTR (*green*, [**B**]). (**C**) Immunostaining for the nuclear translocation of β-catenin (*green*), an indication of increased WNT signaling. Cell nuclei were counterstained with Hoechst 33342. *Scale bars*: (**A**) 200 μm; (**B**, **C**), 50 μm.

Investigative Ophthalmology & Visual Science

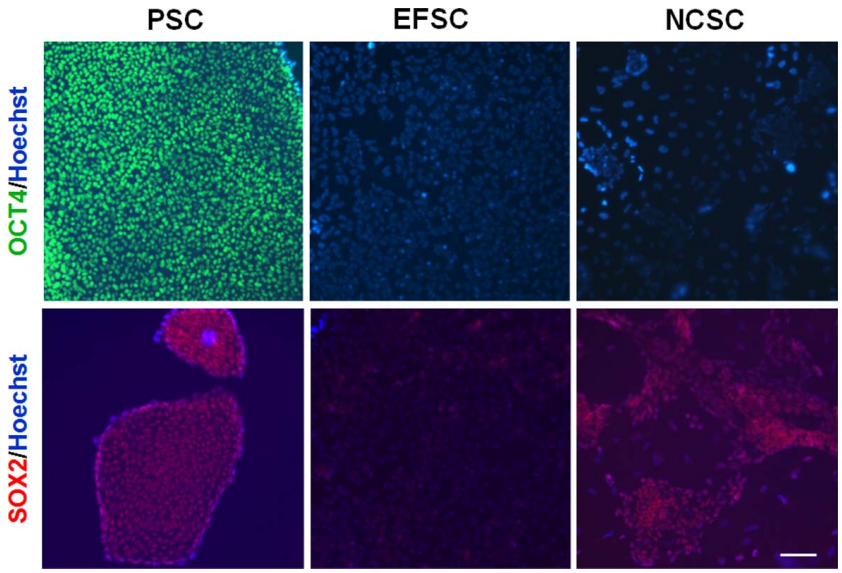

Figure 4. Expression-pattern shifts of pluripotency genes during the lineage specification.

Discussion

In this study, we developed a small molecule-based guidance protocol to generate eye field stem cells defined by the expression of multiple early eye field transcription factor genes (*PAX6*, *LHX2*, and *SIX6*, and so forth) and by their ability to further differentiate into retinal cells (RPE, photoreceptors, and RGCs) and ocular NCSCs. We further showed that ocular NCSCs can be converted into CECs, which express typical markers (Na/K ATPase and NCAD).

During the early stages of embryonic brain development, eye field formation is influenced by Wnt and BMP signaling gradients.[14,15] The gradient of Wnt signaling is important in establishing forebrain and midbrain identities. Downregulation of Wnt signaling leads to formation of the forebrain, where the eye field resides. Accordingly, treating human ESC-derived embryoid bodies with Dkk1, a potent Wnt inhibitor, and noggin, a BMP antagonist, promotes the development of an eye field.[16,17] In addition, under dual SMAD inhibition by exposure to SB431542 (a selective and potent inhibitor of the TGF-β superfamily type I activin receptor-like kinases *ALK-4*, *-5*, and *-7*) and LDN193189 (a selective inhibitor of BMP type I receptors *ALK-2* and *ALK-3*, a noggin analog), PSCs are likely to become forebrain/eye field precursors that strongly express PAX6 and LHX2, two key early transcription factors responsible for eye field formation.[18,19] Our approach used small molecules to target these pathways based on previously described principles and summarized in Figure 6. For example, simultaneous inhibition of TGF-β, BMP, and WNT signaling results in significantly elevated gene expression of early eye field transcription factors, while the increase of WNT signaling in the ocular niche environment is important for formation of the neural crest during eye development. Early work on fate maps in bird and mammalian eyes demonstrated that the

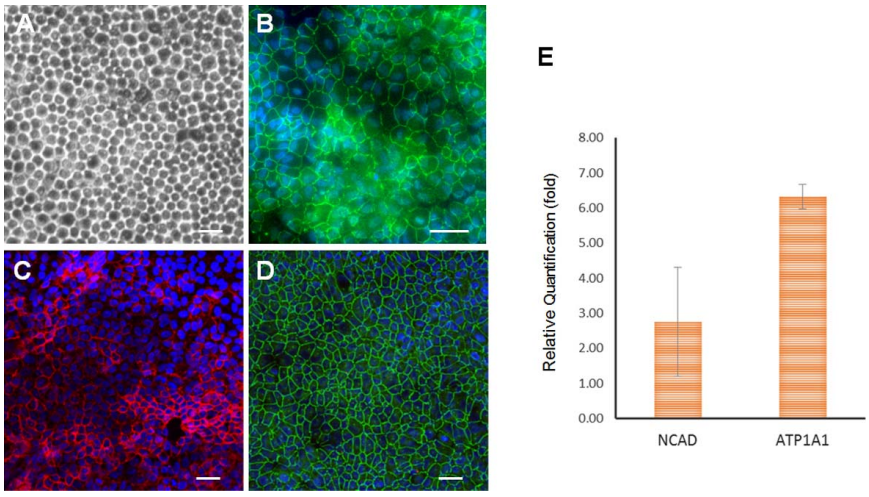

Figure 5. Morphology and characterization of human NCSC-derived CECs. (**A**) Representative phase-contrast micrograph of a confluent monolayer of human NCSC-derived CECs with light treatment with Accutase for 30 seconds. Immunofluorescent images of NCSC-derived CECs which express ZO-1 (*green*, [**B**]), Na^+/K^+ATPase (*red*, [**C**]), and N-cadherin (*green*, [**D**]), three indicative markers of the corneal endothelium. Cell nuclei were counterstained with Hoechst 33342. *Scale bars*: (**A**, **C**, **D**) 50 μm; (**B**) = 100 μm. (**E**) Real-time qPCR analysis of increased mRNA expression of representative CEC genes.

Investigative Ophthalmology & Visual Science

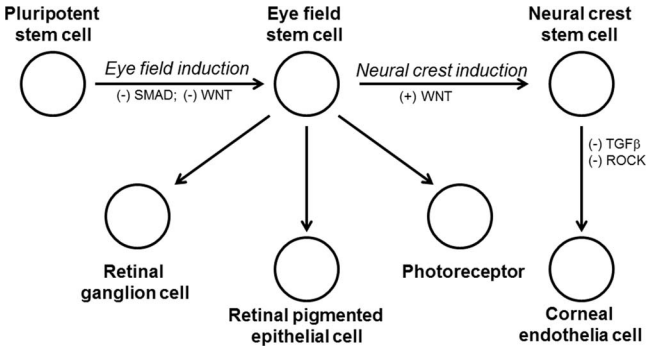

Figure 6. Schematic of fate restriction of major eye cell types under the small molecule-driven processes.

corneal endothelium is derived solely from neural crest cells.[31] The neural crest is a multifated and transient cell population in vertebrate embryos. Early studies on altered positions of neural crest subdomains along the anterior–posterior axis revealed the critical role of local cues for the fate of neural crest cells during their migration and at their destination sites.[35] It is plausible that NCSCs derived from eye field cells may carry a positional cue and are restricted toward the fate of ocular lineage. Thus, EFSC-derived NCSC is likely to be a robust cell source to give rise to CECs in vitro. During eye development, the separation of the lens from the surface ectoderm is an important step before cornea formation.[36] This separation may lead to reduced signaling activity of growth factors, such as FGF2 and TGF-β. Based on these observations, we targeted these signaling pathways by suppressing TGF-β pathway signaling with SB43542 and removing FGF2 from the differentiation culture medium during our in vitro CEC induction from NCSCs. In addition, Okumura et al.[36,37] have demonstrated that inhibition of the ROCK signaling pathway with small molecule Y-27632 resulted in inhibition of apoptosis, increased proliferation of CECs, and enhanced corneal endothelial wound healing in vitro and in vivo. In similar studies, we also observed that H-1152, a more potent ROCK inhibitor, exhibited more stimulatory effect on CEC migration, proliferation, and wound healing than Y-27632.[34] Therefore, we used H-1152 in our study to block ROCK activity during CEC induction and optimize expansion in vitro.

We showed here that by applying a small molecule–driven differentiation approach, the pluripotent state of human PSCs can be uniformly converted to a primitive eye field state. The human PSC-derived EFSCs have characteristics of tissue stem cells and can be directed to differentiate toward either ocular neural crest or retinal lineages in responding to the inductive cues provided in culture. Using this chemically defined 2D culture approach, we have been able to routinely generate highly enriched EFSCs and NCSCs of ocular lineage. We semi-quantitatively estimated the percentages of cell fate conversion from PSC to EFSC and subsequently to NCSC to be approximately 91 ± 3% and 88 ± 4%, respectively, based on cell counts of the staining images. A precise measurement of purity of population of a sublineage cell type must be developed by cell sorting that is based on the expression of a stable and cell type–specific marker. The present work represents an alternative approach to induce a specific type of eye cell directly in a robust and defined process. Particularly, we demonstrated that CECs can de directly induced from EFSC-derived NCSCs in vitro. Unlike previously reported attempts that mainly relied on spontaneous differentiation and conditioned medium to generate CECs,[9,10] our method is based on the delineated developmental process of CEC fate specification. We reasoned that restricting neural crest fate toward the ocular lineage may significantly facilitate CEC induction. Using this strategy, we were able to convert EFSC-derived NCSCs to a relatively homogenous and expandable monolayer of CECs in culture. The characteristic morphology of CEC sheets and the expression of CEC-specific proteins were observed. Our future studies will focus on evaluating the functional cell replacement of diseased CECs with induced CECs in animal models.

Acknowledgments

The authors thank Joanne Ho, MD, and Jeffrey Tsao for reviewing the manuscript.

Supported by Research to Prevent Blindness unrestricted grant and National Institutes of Health (NIH; Bethesda, MD, USA) Grant P30EY022589. UCSD confocal microscopy was supported by P30NS047101.

Disclosure: **J.J. Zhao**, None; **N.A. Afshari**, None

References

1. Musch DC, Niziol LM, Stein JD, Kamyar RM, Sugar A. Prevalence of corneal dystrophies in the United States: estimates from claims data. *Invest Ophthalmol Vis Sci*. 2011;52:6959–6963.
2. Bourne WM, Nelson LR, Hodge DO. Central corneal endothelial cell changes over a ten-year period. *Invest Ophthalmol Vis Sci*. 1997;38:779–782.
3. Joyce NC, Harris DL, Mello DM. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. *Invest Ophthalmol Vis Sci*. 2002;43:2152–2159.
4. Ishino Y, Sano Y, Nakamura T, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci*. 2004;45:800–806.
5. Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res*. 2003;22:359–389.
6. Peh GSL, Toh K-P, Wu F-Y, Tan DT, Mehta JS. Cultivation of human corneal endothelial cells isolated from paired donor corneas. *PLoS One*. 2011;6:e28310.
7. Miyata K, Drake J, Osakabe Y, et al. Effect of donor age on morphologic variation of cultured human corneal endothelial cells. *Cornea*. 2001;20:59–63.
8. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci*. 2000; 41:660–667.
9. McCabe KL, Kunzevitzky NJ, Chiswell BP, Xia X, Goldberg JL, Lanza R. Efficient generation of human embryonic stem cell-derived corneal endothelial cells by directed differentiation. *PLoS One*. 2015;10:e0145266.
10. Zhang K, Pang K, Wu X. Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. *Stem Cells Dev*. 2014;23:1340–1354.
11. Li W, Sun W, Zhang Y, et al. Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. *Proc Natl Acad Sci U S A*. 2011;108:8299–8304.
12. Maminishkis A, Chen S, Jalickee S, et al. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. *Invest Ophthalmol Vis Sci*. 2006;47:3612–3624.
13. Peh GSL, Chng Z, Ang H-P, et al. Propagation of human corneal endothelial cells: a novel dual media approach. *Cell Transplant*. 2015;24:287–304.
14. Del Barco Barrantes I, Davidson G, Gröne H-J, Westphal H, Niehrs C. Dkk1 and noggin cooperate in mammalian head induction. *Genes Dev*. 2003;17:2239–2244.

15. Cavodeassi F, Carreira-Barbosa F, Young RM, et al. Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. *Neuron*. 2005;47:43–56.
16. Lamba DA, Karl MO, Ware CB, Reh TA. Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc Natl Acad Sci U S A*. 2006;103:12769–12774.
17. Reh TA, Lamba D, Gust J. Directing human embryonic stem cells to a retinal fate. *Methods Mol Biol Clifton NJ*. 2010;636:139–153.
18. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol*. 2009;27:275–280.
19. Kriks S, Shim J-W, Piao J, et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature*. 2011;480:547–551.
20. Chen B, Dodge ME, Tang W, et al. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol*. 2009;5:100–107.
21. Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. Specification of the vertebrate eye by a network of eye field transcription factors. *Dev Camb Engl*. 2003;130:5155–5167.
22. Burmeister M, Novak J, Liang MY, et al. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat Genet*. 1996;12:376–384.
23. Austin CP, Feldman DE, Ida JA, Cepko CL. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Dev Camb Engl*. 1995;121:3637–3650.
24. Hashimoto T, Zhang X-M, Chen BY, Yang X-J. VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. *Development*. 2006;133:2201–2210.
25. Osakada F, Ikeda H, Mandai M, et al. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. *Nat Biotechnol*. 2008;26:215–224.
26. Sinha S, Chen JK. Purmorphamine activates the Hedgehog pathway by targeting Smoothened. *Nat Chem Biol*. 2006;2:29–30.
27. Czekaj M, Haas J, Gebhardt M, et al. In vitro expanded stem cells from the developing retina fail to generate photoreceptors but differentiate into myelinating oligodendrocytes. *PLoS One*. 2012;7:e41798.
28. Müller F, Rohrer H, Vogel-Höpker A. Bone morphogenetic proteins specify the retinal pigment epithelium in the chick embryo. *Development*. 2007;134:3483–3493.
29. Idelson M, Alper R, Obolensky A, et al. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. *Cell Stem Cell*. 2009;5:396–408.
30. Fuhrmann S, Levine EM, Reh TA. Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. *Dev Camb Engl*. 2000;127:4599–4609.
31. Gage PJ, Rhoades W, Prucka SK, Hjalt T. Fate maps of neural crest and mesoderm in the mammalian eye. *Invest Ophthalmol Vis Sci*. 2005;46:4200–4208.
32. Lee G, Kim H, Elkabetz Y, et al. Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells. *Nat Biotechnol*. 2007;25:1468–1475.
33. Dupin E, Sommer L. Neural crest progenitors and stem cells: From early development to adulthood. *Dev Biol*. 2012;366:83–95.
34. Meekins LC, Rosado-Adames N, Maddala R, Zhao JJ Rao PV, Afshari NA. Corneal endothelial cell migration and proliferation enhanced by rho kinase (ROCK) inhibitors in in vitro and in vivo models. *Invest Ophthalmol Vis Sci*. 2016;57:6731–6738.
35. Tuft SJ, Coster DJ. The corneal endothelium. *Eye*. 1990;4:389–424.
36. Okumura N, Ueno M, Koizumi N, et al. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. *Invest Ophthalmol Vis Sci*. 2009;50:3680–3687.
37. Okumura N, Koizumi N, Ueno M, et al. Enhancement of corneal endothelium wound healing by Rho-associated kinase (ROCK) inhibitor eye drops. *Br J Ophthalmol*. 2011;95:1006–1009.

Investigative Ophthalmology & Visual Science