Establishment of Functioning Human Corneal Endothelial Cell Line with High Growth Potential

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Abstract

Hexagonal-shaped human corneal endothelial cells (HCEC) form a monolayer by adhering tightly through their intercellular adhesion molecules. Located at the posterior corneal surface, they maintain corneal transulcency by dehydrating the corneal stroma, mainly through the Na\(^+\) and K\(^+\)-dependent ATPase (Na\(^+\)/K\(^+\)-ATPase). Because HCEC proliferative activity is low in vivo, once HCEC are damaged and their numbers decrease, the cornea begins to show opacity due to overhydration, resulting in loss of vision. HCEC cell cycle arrest occurs at the G1 phase and is partly regulated by cyclin-dependent kinase inhibitors (CKIs) in the Rb pathway (p16-CDK4/CyclinD1-pRb). In this study, we tried to activate proliferation of HCEC by inhibiting CKIs. Retroviral transduction was used to generate two new HCEC lines: transduced human corneal endothelial cell by human papillomavirus type E6/E7 (THCEC (E6/E7)) and transduced human corneal endothelial cell by Cdk4R24C/CyclinD1 (THCEH (Cyclin)). Reverse transcriptase polymerase chain reaction analysis of gene expression revealed little difference between THCEC (E6/E7), THCEH (Cyclin) and non-transduced HCEC, but cell cycle-related genes were up-regulated in THCEC (E6/E7) and THCEH (Cyclin). THCEH (Cyclin) expressed intercellular molecules including ZO-1 and N-cadherin and showed similar Na\(^+\)/K\(^+\)-ATPase pump function to HCEC, which was not demonstrated in THCEC (E6/E7). This study shows that HCEC cell cycle activation can be achieved by inhibiting CKIs even while maintaining critical pump function and morphology.

Introduction

Human corneal endothelial cells (HCEC) are hexagonal in shape and form a fragile monolayer lying posterior to the surface of the cornea. These cells maintain corneal transparency by their tight intercellular barrier and perform an ion transport pump function through Na\(^+\)/K\(^+\)-ATPase, which regulates the hydration of the corneal stroma [1,2]. If HCEC sustain damage, excessive hydration and opacity of the cornea occur, resulting in decreased vision.

Corneal endothelia are believed not to increase in adult humans, but have been successfully isolated and cultured by introducing stimulating agents such as epidermal growth factor, platelet-derived growth factor-BB, bovine pituitary extract and fetal bovine serum [17,18]. However, the number of cells with proliferative activity and the ability to respond to such agents is relatively low, and much variation in proliferative activity exists between donors of different ages [19,20]. Thus, there is a requirement to achieve a stable and effective culture of cells in terms of both cell proliferation and physiologic function.
The HCEC cell cycle is mainly regulated by the p53 and pRB pathways, both of which have been inactivated by human papilloma virus (HPV) type 16 E6/E7 to successfully immortalize cells. Kim et al. reported the establishment of an immortalized HCEC line using HPV type 16 E6/E7 on lyophilized human amniotic membrane [21]. However, several studies have reported carcinogenesis of the cell line established by viral oncogenes including HPV type 16 E6/E7 or SV40 large T antigen [22,23]. Therefore a corneal endothelial cell line developed in this way does not appear to be suitable for the treatment of human corneal diseases. To resolve this problem, we expressed mutant cyclin-dependent kinase (Cdk) 4 and CyclinD1 to inactivate the pRb pathway and generate corneal endothelial cell lines without transducing viral oncogenes.

**Results**

HCEC with Descemet’s membranes were proliferated slowly in a culture dish coated in type IV collagen. After two passages, the cells were transferred into 24-well dishes and transsected with a retroviral vector carrying E6/E7 or mutant Cdk4 and CyclinD1. Three cell lines were successfully generated, as shown in Fig. 1A, with obvious differences in growth (Fig. 1B). Protein expression from the transduced gene was confirmed by western blotting (Fig. 1C). As previously reported [21], THCEC (E6/E7) was immortalized, and THCEC (Cyclin) demonstrated the same proliferative capacity as THCEC (E6/E7), while primary cells grew more slowly even when cultured in 10% fetal bovine serum. These results indicate that induction of mutant Cdk4 and CyclinD1 is sufficient to generate a HCEC line that proliferates at a faster rate than the primary cell line.

Proliferation capacity was also confirmed by immunohistochemistry of Ki-67 (Fig. 2A). Expression of downstream genes of CyclinD1 which are associated with cell proliferation was analyzed by real-time polymerase chain reaction (PCR) (Fig. 2B). Positive staining of Ki-67, which is detected in the nucleus, was confirmed in both THCEC (Cyclin) and THCEC (E6/E7). Real-time PCR also revealed that CDC2 and PCNA, target genes of E2F (an upstream transcriptional factor), that are activated by CyclinD1, were up-regulated in THCEC (E6/E7) and especially in THCEC (Cyclin).

Expression of genes involved in active transmembrane transporter activity, including Na+/K+-ATPase, or cell adhesion, including ZO-1 and N-cadherin, were assessed by semi-quantitative reverse transcriptase (RT)-PCR (Fig. 3A). Expression of intercellular adhesion molecules was confirmed by immunohistochemistry (Fig. 3B-J). Semi-quantitative RT-PCR showed that there was no significant difference between the three cell lines regarding the expression of genes associated with several molecules of cell adhesion or of ion transporter channels, which are characteristically expressed by HCEC [21,24]. This was also confirmed by real-time PCR (data not shown).

ZO-1 and N-cadherin, key HCEC adhesion molecules [24], demonstrated positive staining at the intercellular junction in HCEH (Fig. 3F, I) and THCEC (Cyclin) (Fig. 3E, H), while neither ZO-1 nor N-cadherin was detected by semi-quantitative RT-PCR (Fig. 3A). Expression of intercellular adhesion molecules was confirmed by immunohistochemistry (Fig. 3B-J). Expression of genes specific to HCEC was not drastically different between the three immortalized cells. In the present study, expression of genes specific to HCEC was not drastically different between the three cell lines. However, key proteins including ZO-1 and N-cadherin that are important in forming intercellular contacts were detected, probably because of the unknown influence of viral oncogenes on post-translational modification, posttranslational import or protein stability/degradation.

We recently established genetically stable, non-transformed immortalized ovarian surface epithelium (OSE) cell lines without viral oncogenes by expressing mutant Cdk 4, CyclinD1 and hTERT, based on the hypothesis that inactivation of the pRb pathway and activation of telomerase are sufficient for OSE immortalization [27]. Meanwhile, Rane et al. demonstrated that mutant Cdk 4 (Cdk4R24C) is sufficient to induce carcinogenesis in several other tissues including those of the pancreas, pituitary and brain [28], and Joyce and colleagues showed that HCEG are arrested in the G1 phase and regulated by CKIs, p16INK4a and p21WAF1/Cip1 [29]. Considering the importance of maintaining...
morphology and physiologic function in HCEC, we only transduced mutant Cdk 4 and CyclinD1, not hTERT, in the present study. We believe that our careful method enabled THCEC (Cyclin) to form a fragile and regularly arranged monolayer complete with physiologic function.

Although THCEC (Cyclin) has similar characteristics to primary HCEC, immunohistochemistry and the Ussing chamber assay also highlighted the differences between the cells. ZO-1 protein was expressed around the nucleus of THCEC (Cyclin) but not in primary cells. Since semi-quantitative PCR detected almost the same level of mRNA expression between the cell lines, staining around the nucleus in THCEC (Cyclin) probably reflects an error in posttranslational import of ZO-1 protein. The Ussing chamber assay detected a similar pump function between THCEC (Cyclin) and primary cells, but the current in THCEC (Cyclin) was more variable than that of the primary cells, which might have been caused by reduced Na⁺/K⁺-ATPase activity, immature intercellular adhesion allowing irregular intercellular ion transport or differences in cellular density.

Cells established by a retrovirus carry a potential risk of promoting carcinogenesis [30], and direct transplantation to humans of cell sheets composed of such cells may lead to complex problems. Recently, to resolve this problem, several studies have reported the establishment of untransfected corneal endothelial cell lines [31,32,33], which are the most ideal cell lines for the treatment of human corneal disease. Meanwhile, alternative bioengineering approaches, including lipofection of p27kip1 siRNA [34], proteomics technology analyzing the difference between younger and older HCEC [35] and drug usage of promyelocytic leukemia zinc finger protein, a cell cycle transcriptional repressor and negative regulator [36], have also been introduced. The present findings support the idea that targeting the interaction between p16INK4a and Cdk4 using such methods is a promising strategy to generate HCEC with sufficient proliferative capacity and physiologic function.

Materials and Methods

Isolation and cell culture of human corneal cells

Ethics Statement. A cornea was excised from the surgically enucleated eye of a 2-year-old infant undergoing therapy for retinoblastoma, with the approval (approval number, #156) of the
Ethics Committee of the National Institute for Child and Health Development, Tokyo, Japan. Signed informed consent was obtained from the donor’s parents, and the surgical specimens were irreversibly de-identified. All experiments handling human cells and tissues were performed in line with the tenets of the Declaration of Helsinki.

The corneal piece, which was grossly normal with no pathological lesions, was cut 1.5 mm from the corneal limbus.

Figure 2. Evaluation of proliferative capacity. (A) Immunohistochemistry of Ki-67 in three cell lines. Positive staining of Ki-67, located in the nucleus, was obviously identified in THCEC (Cyclin) and THCEC (E6/E7), but rarely detected in HCEC. (B) Real-time PCR of downstream genes of cyclinD1 associated with proliferation. Gene expression levels of both CDC2 and PCAN were clearly higher than that of HCEC. The gene expression was much more activated in THCEC (Cyclin) in which the expression of E2F, an upstream transcriptional factor of two genes, was constitutively activated by transduced mutant Cdk4 and CyclinD1. doi:10.1371/journal.pone.0029677.g002

Figure 3. HCEC-associated genes and cytolocalization of junctional components expressed by cell lines. (A) Semi-quantitative reverse transcriptase polymerase chain reaction for HCEC-associated genes. Total RNA was prepared from cultured cells seven days after reaching confluency. No significant difference in mRNA expression was observed between the three cell lines. Compared with phase-contrast micrographs of (B) THCEC (Cyclin), (C) HCEC and (D) THCEC (E6/E7), cytolocalization was examined by immunofluorescence staining of ZO-1 (E, F, G) and N-cadherin (H, I, J). THCEC (E6/E7) did not stain positive for intercellular junctional molecules, while ZO-1 and N-cadherin stained positive at the junction in THCEC (Cyclin) and HCEC. doi:10.1371/journal.pone.0029677.g003
avoiding contamination of the trabecular meshwork tissue. HCEC with Descemet’s membrane were stripped from the posterior surface of the corneal tissue with sterile surgical forceps under a dissecting microscope. They were cut into two pieces and cultured in a cell culture dish covered with Type IV collagen in a growth medium (GM); Dulbecco’s modified Eagle’s medium (DMEM)/Nutrient mixture F12 (1:1) with high glucose supplemented with 10% fetal bovine serum, insulin-transferrin-selenium and MEM-NEAA (Gibco, Auckland, NZ). Cells were subcultured after reaching confluency by treating with trypsin/EDTA and seeded at a density of $5 \times 10^5$ cells/well in 6-well dishes.

Viral vector construction and viral transduction
Lentiviral vector plasmids, CSII-CMV-cyclin D1 and -CDK4R24C were constructed by recombination using the Gateway system (Invitrogen, Carlsbad, CA) as described previously [37]. Briefly, cDNAs of human cyclinD1 and a mutant form of Cdk4 (Cdk4R24C: an inhibitor resistant form of Cdk4, generously provided by Dr Hara) were recombined with a lentiviral vector, CSII-CMV-RfA (a gift from Dr Miyoshi), by LR reaction to create a Gateway expression plasmid (Invitrogen) according to the manufacturer’s instructions.

Previous work has described the production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein [37], the recombinant retrovirus vector plasmid, pCLXSN-16E6E7 encoding HPV16 E6/E7 [16E6E7] [38] and recombinant retroviruses [39]. Following the addition of recombinant viral fluid to cells seeded in 24-well dishes in the presence of 4 µg/ml polybrene, the cells were infected by the viruses. Stably transduced cells with an expanded life span were designated transduced.

Figure 4. Transmission electron microscopy of cell line intercellular junctions. The junctional complex was detected at the intercellular junction in THCEC (Cyclin) and HCEC. No component of the intercellular junction was found in THCEC (E6/E7), in which cells grew in multilayers without being inhibited by cellular contact (scale bar = 200 nm).
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Figure 5. The pump function of cell lines. Short-circuit currents representing Na$^+$/K$^+$-ATPase activity from corneal cell monolayers on the insert well area of 4.67 cm$^2$ were calculated before and after addition of the Na$^+$/K$^+$-ATPase inhibitor ouabain. (A) Representative tracings of short-circuit current ($\mu$A/well) obtained with cell monolayers of THCEC (Cyclin) (upper panel), HCEC (middle panel) and THCEC (E6/E7) (lower panel). THCEC (Cyclin) possessed equal transport activity to HCEC, whereas no pump function was detected in THCEC (E6/E7). (B) Time-course changes in the average short circuit current of cultured monolayers of cell lines at 1, 5, 10 and 20 min. Data shown are for (▲) THCEC (Cyclin) at PD8, (●) THCEC (Cyclin) at PD 21, (◆) HCEC and (■) THCEC (E6/E7); all data are expressed as mean±SD of four replicate experiments of each cell line.
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Table 1. Tumorigenesis assay of cell lines in BALB/C nude mice.

| Inoculated cells | Total dose (cell/mouse) | Number of mice (% mortality) | Number of mice with tumor |
|------------------|------------------------|------------------------------|--------------------------|
| THCEC (Cyclin)   | 1.2×10^6               | 3(0)                         | 0                        |
| THCEC (E6/E7)    | 1.2×10^6               | 3(0)                         | 0                        |
| HeLa cells       | 2.0×10^5               | 3(0)                         | 3                        |

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human corneal endothelial cell by E6/E7 (THCEC (E6/E7)) and transduced human corneal endothelial cell by Cdk4R24C/cyclinD1 (THCEC (Cyclin)).

Culture of transfected cell lines and growth curve

When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA, collected into tubes, and centrifuged. The cells were counted using a cell viability analyzer (Vi-CELL Cell Viability Analyzer, Beckman Coulter, Brea, CA), and population doubling (PD) was calculated. The pellets were suspended in growth medium, and the cells were passaged at a density of 5×10^2 cells/well in a 100-mm dish. The original cells were regarded as PD 2 (day 0).

Western blot analysis

Western blotting was conducted as described previously [40]. Antibodies against Cdk4 (ser473; Cell Signaling Technology, Danvers, MA), CyclinD1 (clone G124-326; BD Biosciences, Franklin Lakes, NJ), β-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA) were used as probes, and horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA) or anti-goat (sc-2033; Santa Cruz Biotechnology, Santa Cruz, CA) immunoglobulins were employed as secondary antibodies.

Immunocytochemistry

Cell lines were grown on Type IV collagen-coated glass dishes 14 days after reaching confluence and were fixed with 4% formaldehyde (pH 7.0) for 15 min at room temperature. Cell lines were then rehydrated in phosphate buffered saline (PBS), incubated with 0.2% Triton X-100 for 15 min and rinsed three times with PBS for 3 min each. After incubation with 2% BSA to block nonspecific staining for 30 min, cell lines were incubated with anti-ZO-1 (1:50; sc-8146; Santa Cruz Biotechnology, Santa Cruz, CA), anti-N-cadherin (1:50; sc-7939; Santa Cruz Biotechnology) and anti-Ki67 (1:100; ab15580; Abcam, Cambridge, UK) for 16 h at 4°C. After three washes with PBS, cell lines were incubated with the secondary antibody for 60 min, followed by counterstaining with 4',6-diamidino-2-phenylindole (1:200; sc-3398; Santa Cruz Biotechnology) for 10 min.

Semi-quantitative RT-PCR

Total RNA was extracted from 1×10^6 cultured HCEC using the RNaseasy Plus mini-kit (Qiagen, Germantown/Gaithersburg, MA) according to the manufacturer’s instructions and quantified by absorption at 260 nm. Total RNA was then reverse-transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo random hexamers. cDNAs of each component were amplified by PCR using specific primers and DNA polymerase. The reaction was first incubated at 95°C for 10 min, followed by 39 cycles at 98°C for 30 s, 58°C for 30 s and 74°C for 30 s. PCR primers are listed in Table 2.

Quantitative real-time RT-PCR

Total RNA extraction and reverse transcription into cDNA was carried out as above. Each quantitative real-time RT-PCR for target genes, including Cell Division Cycle 2 (CDC2) and proliferating cell nuclear antigen (PCNA), was performed using the Chromo4 real time detection system (Bio-Rad, Hercules, CA). For a 20 nl PCR, the cDNA template was mixed with the primers to final concentrations of 200 nM and 10 μl of SsoFast EvaGreen Supermix (BIO-RAD), respectively. The reaction was first incubated at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 57°C for 15 s, and 72°C for 20 s.

Transmission Electron Microscopy

Cell lines cultured on Type IV collagen-coated dishes were fixed in HEPES buffered 2% glutaraldehyde and subsequently post-fixed in 2% osmium tetroxide for 3 h on ice. Specimens were then dehydrated in graded ethanol and embedded in the epoxy resin. Ultrathin sections were obtained by ultramicrotomy and stained with uranyl acetate for 10 min and modified Sato’s lead solution for 5 min then submitted to TEM observation (JEOL).

Measurement of pump function

The pump function of confluent monolayers of HCEC was measured using an Ussing chamber as described previously [41]. Cells cultured on Snapwell inserts coated with Type IV collagen were placed in the Ussing chamber EM-CSYS-2 (Physiologic Instruments, San Diego, CA) with the endothelial cell surface side in contact with one chamber and the Snapwell membrane side in contact with another chamber. The chambers were carefully filled with Krebs-Ringer bicarbonate (120.7 mM NaCl, 24 mM NaHCO3, 4.6 mM KCl, 0.5 mM MgCl2, 0.7 mM Na2HPO4, 1.5 mM NaH2PO4 and 10 mM glucose bubbled with a mixture of 5% CO2, 7% O2 and 88% N2 to pH 7.4). The chambers were maintained at 37°C using an attached heater.

The short-circuit current was sensed by narrow polyethylene tubes positioned close to either side of the Snapwell, filled with 3 M KCl and 4% agar gel and connected to silver electrodes. These electrodes were connected to the computer through the Ussing system VCC-MC2 (Physiologic Instruments) and an iWorx 118 Research Grade Recorder (iWorx Systems, Dover, NH), and the short-circuit current was recorded by Labscribe2 Software for Research (iWorx). After the short-circuit current had reached a steady state, ouabain (final concentration, 1 mM) was added to the chamber, and the short-circuit current was re-measured. The pump function attributable to Na+/K+-ATPase activity was calculated as the difference in short-circuit current measured before and after the addition of ouabain.

Tumorigenesis assay

Cells were harvested by Trypsin/EDTA treatment, collected into tubes, and centrifuged, and the pellets were suspended in...
Table 2. Oligonucleotide sequences for RT-PCR.

| Name       | Sequence                                   | Size (bp) | Accession Number |
|------------|--------------------------------------------|-----------|-----------------|
| Collagen type IV | F: 5'-GGC ACC TGC CAC TAC TAC GC-3'  |          |                 |
|            | R: 5'-TCA CCA GGA GGT AGC CGA T-3'          | 472       | NM_001136200    |
| Keratin 12 | F: 5'-GAT GCT GAG CTC GA-3'                | 393       | NM_000223       |
|            | R: 5'-ACC TCG CCT ACA GCT TGA TA-3'        |           |                 |
| VDAC3      | F: 5'-TGA TCT TAT ATG CTA TGT TAC CG-3'    | 482       | NM_001135694    |
|            | R: 5'-TCA ATT TGA CTC CGT GTA GAA-3'       |           |                 |
| CLCN3      | F: 5'-AGG GGA TAG ACG GAT CAA-3'           | 204       | NM_001829       |
|            | R: 5'-GCT TCC ACC ACA ACG CAC TAA-3'       |           |                 |
| SLC4A4     | F: 5'-GTT CAG ATG ACT GGG GAT AGC          | 697       | NM_001136200    |
|            | R: 5'-CGA CCA TAA ACACAAAGC GTA A-3'       |           |                 |
| Na⁺/K⁺-ATPase| F: 5'-CCC AGG ACT CAT GGT TTTC-3'           | 482       | NM_000702       |
|            | R: 5'-GGA CCA AAG CTG ACC GTA AC-3'        |           |                 |
| N-cadherin | F: 5'-CAA CTT GCC AGA AAA CTC CAG G-3'     | 205       | NM_001792       |
|            | R: 5'-ATG AAA CCG GGC TAT CTC CTC-3'       |           |                 |
| β-catenin  | F: 5'-TAC CTC CCA AGT CCT GTA G-3'         | 180       | NM_001904       |
|            | R: 5'-TGA GCA GCA TCA AAT GTA GTA G-3'     |           |                 |
| P-120      | F: 5'-CCC CAG GAT CAC AGT CAC CT-3'        | 144       | NM_001085467    |
|            | R: 5'-CCG AGT GGT CCC ATC ATC TG-3'        |           |                 |
| ZO-1       | F: 5'-AGT CCC TTA CTC CCT GGC TGA-3'       | 180       | NM_003257       |
|            | R: 5'-TCT CTT AGC ATT ATG TGA GCT GC-3'    |           |                 |
| GAPDH      | F: 5'-GCT CAG ACA CCA TGG GGA TG-3'        | 474       | NM_002046       |
|            | R: 5'-GTG GTG CAG GCA TGG CTG A-3'         |           |                 |
| PCNA       | F: 5'-GGCAGAGCCATTCCAGCATGTG-3'            | 76        | NM_002592       |
|            | R: 5'-TCTCCGGCCCTGAGTTAGTGA-3'             |           |                 |
| CDC2       | F: 5'-GGATGCTTATGCAGGATGAGC-3'             | 100       | NM_001786       |
|            | R: 5'-CATGATGACCAGGGAGTAG-3'               |           |                 |

VDAC3: voltage-dependent anion channel 3, CLCN3: chloride channel protein 3, SLC4A4: sodium bicarbonate cotransporter membrane.
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Author Contributions

Conceived and designed the experiments: Tadashi Yokoi YS Tae Yokoi TK AU HN NA. Performed the experiments: Tadashi Yokoi YS Tae Yokoi HM SH MY TK HN NA. Analyzed the data: Tadashi Yokoi YS Tae Yokoi HM SH MY AU HN NA. Contributed reagents/materials/ analysis tools: Tadashi Yokoi YS SH MY TK HN NA. Wrote the paper: Tadashi Yokoi YS TK AU HN NA.

DMEM. The same volume of Basement Membrane Matrix (BD Biosciences) was added to the cell suspension. Cells (1.7 × 10⁶) of THCEC (Cyclin) and THCEC (E6/E7) were inoculated subcutaneously into dorsal flanks of each of three Balb/c nu/nu mice (CRIA, Japan) for 60 days. A total of 2.0 × 10⁶ DF1 cells per mouse were used as positive controls. The skin of dorsal flanks of inoculated mice was surgically opened and the tumorigenic status was examined.
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