Establishment of a Human Corneal Epithelial Cell Line Lacking the Functional TACSTD2 Gene as an In Vitro Model for Gelatinous Drop-Like Dystrophy

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Purpose. Gelatinous drop-like corneal dystrophy (GDLD) is characterized by subepithelial amyloid deposition that engenders severe vision loss. The exact mechanism of this disease has yet to be elucidated. No fundamental treatment exists. This study was conducted to establish an immortalized corneal epithelial cell line to be used as a GDLD disease model.

Methods. A corneal tissue specimen was obtained from a GDLD patient during surgery. Corneal epithelial cells were enzymatically separated from the cornea and were dissociated further into single cells. The epithelial cells were immortalized by the lentiviral transduction of the simian virus 40 (SV40) large T antigen and human telomerase reverse transcriptase (hTERT) genes. For the immortalized cells, proliferative kinetics, gene expressions, and functional analyses were performed.

Results. The immortalized corneal epithelial cells continued to proliferate despite cumulative population doubling that exceeded 100. The cells showed almost no sign of senescence and displayed strong colony-forming activity. The cells exhibited a low epithelial barrier function as well as decreased expression of tight-junction–related proteins claudin 1 and 7. Using the immortalized corneal epithelial cells derived from a GDLD patient, we tested the possibility of gene therapy.

Conclusions. We established an immortalized corneal epithelial cell line from a GDLD patient. The immortalized cells exhibited cellular phenotypes similar to those of in vivo GDLD. The immortalized cells are thought to be useful for the development of new therapies for treating GDLD corneas and for elucidation of the pathophysiology of GDLD.

Keywords: corneal dystrophy, gelatinous drop-like, immortalized cells

Gelatinous drop-like dystrophy (GDLD; OMIM #204870), which was first reported by Nakaizumi in 1914,[1] is characterized by amyloid deposition at the subepithelial region of the corneal stroma. The symptoms of GDLD patients, including severe photophobia, foreign-body sensation, and epiphora, usually appear in the first decade of life and engender blurred vision in late stages.[2–4] Reduction of the corneal epithelial barrier function and the resultant tear-fluid permeation into the corneal stroma are probably the primary pathologic events related to GDLD. However, the exact mechanism for the amyloid deposition remains unclear. GDLD is an inheritable disease with an autosomal recessive trait. It has been reported predominantly in Japan, with an estimated frequency of incidence of 1 in 30,000,[5,6] although it is extremely rare in the Western world, accounting for only a few reported cases to date.[2] The gene responsible for GDLD, discovered using linkage analysis and a candidate gene approach in 1999, was designated as tumor-associated calcium signal transducer 2 (TACSTD2) gene.[7,8]

GDLD appears to be an extremely refractory corneal disease. Several treatment remedies have been performed for GDLD-afflicted corneas such as penetrating or lamellar keratoplasty, laser photoablation, keratoprosthesis, and scraping of the abnormal subepithelial deposition.[9–11] However, in most GDLD patients, the disease symptoms generally recur within a few years after such interventions, thereby necessitating repeated keratoplasties.[12–14] No currently used treatment for GDLD is fundamental. All are merely supportive, aimed at providing temporary relief from disease symptoms. Disease models provide large amounts of invaluable information for the development of novel effective treatments. Wang et al. generated TACSTD2$^{-/-}$ mice as an animal model for GDLD. However, they described no apparent abnormality in the corneas of those mice.[15]

The TACSTD2 gene encodes a membrane glycoprotein[16] that transduces calcium signals as a cell surface receptor.[17] The TACSTD2 protein is expressed in normal epithelial cells of various types, such as those of the conjunctiva, skin, pharynx, esophagus, uterine cervix, and vagina.[18] In several epithelial tumor types, this often overexpressed protein has been regarded as playing a major role in tumorigenesis.[16,19] We have reported that the TACSTD2 protein is necessary for the

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epithelial barrier function of corneal epithelium through binding to CLDN1 and CLDN7 proteins.[18] Numerous mutations have been reported for this gene, two-thirds of which are nonsense or frameshift mutations.[20,21] Such mutations might cause the truncation of the TACSTD2 protein, which engenders the defect of a C-terminal transmembrane domain, eventually leading to the loss of function of the gene.

In this study, we established an immortalized human corneal epithelial cell line lacking the functional TACSTD2 gene for use as an in vitro model of the GDLD cornea. The cells were created by the lentiviral transduction of the simian virus 40 (SV40) large T antigen and human telomerase reverse transcriptase (hTERT) genes to corneal epithelial cells of a GDLD patient. The cells showed markedly lower epithelial barrier function as well as decreased expression of the tight-junction–related proteins claudin (CLDN) 1 and 7, which is consistent with our previous findings related to in vivo GDLD corneas. The cells are expected to be useful for developing novel effective treatments for GDLD corneas and for elucidating the pathophysiology of the disease.

Materials and Methods

Ethical Issues

Prior informed consent in accordance with the Declaration of Helsinki was obtained from the GDLD patient. All experimental procedures were approved by the Institutional Review Board for human studies of Kyoto Prefectural University of Medicine (C-1067).

Antibodies

All antibodies were raised against human antigens (Table 1).

Oligomers

All oligomers used for this study were synthesized by Life Technologies Corp. (Carlsbad, CA) (Table 2).

Abbreviations

The immortalized corneal epithelial cell line from the GDLD patient and the immortalized corneal epithelial cell line from a normal cornea are abbreviated, respectively, herein as imHCE_GDLD and imHCE_normal.

Culture of Human Corneal Epithelial Cells Derived From Normal and GDLD Corneal Tissue Specimen

A 59-year-old Japanese woman bearing a biallelic loss-of-function mutation of the TACSTD2 gene (p.Gln118X) underwent lamellar keratoplasty and keratoepithelioplasty. A specimen of her corneal tissue was obtained at the time of surgery. A normal corneal tissue specimen was taken from a cornea intended for research obtained from Northwest Lyons Eye Bank (Seattle, WA).

Corneal epithelial sheets were peeled from these two corneas via the application of 1000 protease units (PU)/mL of dispase (Dispase type II; Godo Shusei Co., Ltd., Tokyo, Japan) at 4°C overnight. The peeled epithelial sheets were then dissociated via the application of trypsin-like protease (TrypLE Express; Life Technologies Corp.) at 37°C for 5 minutes. The dissociated corneal epithelial cells were seeded on a collagen-coated six-well plate and were cultured in a supplemented corneal epithelial cell-oriented growth medium (CnT-20; Cellntec Advanced Cell System AG, Bern, Switzerland) under standard culture conditions.

Lentiviral Vector Construction and Transduction of SV40 Large T Antigen and hTERT Genes

The coding sequences of SV40 large T antigen and hTERT genes were amplified by PCR and were TA-cloned into a commercial lentiviral vector (pLenti6.3_V5-TOPO; Life Technologies Corp.) (Figs. 1A, 1B). The lentiviral vectors were transfected to 293T cells along with three packaging plasmids (pLP1, pLP2, and pLP/glycoprotein of the vesicular stomatitis virus; Life Technologies Corp.) using a commercial transfection reagent (Fugene HD; Promega Corp., Madison, WI). After 48 hours of transfection, the supernatant of the culture medium was harvested, centrifuged briefly, and stored in a freezer at –80°C. For lentiviral transduction, the virus-containing supernatant was added to the cultures of the dissociated corneal epithelial cells in the presence of 5 μg/mL polybrene.

Population-Doubling (PD) Analysis

Growth kinetics were measured using PD analysis, as described previously.[22] Briefly, cells (5×10^4 to 1×10^5) were seeded to a T-25 plastic flask and were then fed every other day. When the cells reached subconfluence (approximately 70% confluence), they were harvested using TrypLE Express, counted, and seeded again to a new T25 plastic flask. Increment PD per passage was calculated with a formula $\log_2$ (Ch/Cs), where Ch corresponds to the number of harvested cells, and Cs corresponds to the number of seeded cells.

Colony-Forming Assay

Colony-forming activity was investigated as described in a previous report.[23] Briefly, single-cell suspensions of the transduced or nontransduced cells were seeded at a density of 1×10^2, 1×10^3, or 1×10^4 cells per well on a six-well plate in the presence of Mitomycin C–treated feeder cells and allowed to grow for 7 to 10 days. The cells were then fixed with 10% buffered formalin for 10 minutes, stained with 1% rhodamine B solution for 10 minutes, washed, and photographed.

Senescence-Associated β-Galactosidase (SAβgal) Assay

SA-βgal activity was detected using a commercially available kit (Senescence Detection Kit; Bio Vision, Inc., San Francisco, CA). Briefly, the cells were fixed and then stained with a staining solution (containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside [X-gal]), included with the kit, at 37°C overnight. Then, they were photographed.

Telomere Repeat Amplification Protocol (TRAP) Assay

TRAP assay was performed according to a previous report, but with minor modifications.[24] Briefly, 2×10^5 cells were lysed. Telomerase substrate (TS) primer was elongated by the telomerase activity contained in the lysate. After purification, the reaction mixture was amplified by PCR using a 344 nM primer pair (TS primer and CX primer). The PCR products were then electrophoresed on a 10% nondenaturing acrylamide gel, stained (SYBR Green I; Takara Bio, Inc., Otsu, Japan), and photographed.

Immunostaining Analysis

Cells grown on a commercially available culture-glass slide (Nunc Lab-Tek Chamber Slide System; Thermo Fisher Scientif-

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Table 1. List of Antibodies Used for This Study

Antibody	Category	Company*	Clone/Cat No.	Dilution
CLDN1	MM	Abnova	1C5-D9	×200
CLDN4	MM	Life Technologies	3E2C1	×100
CLDN7	MM	Life Technologies	5D10F3	×100
TACSTD2	GP	R&D	AF650	×100
OCLN	GP	Santa Cruz	sc-8145	×50
ZO-1	MM	Life Technologies	ZO1-1A12	×200
SV40 large T	MM	Abcam	PAb416	×100
hTERT	MM	Novocastra	44F12	×50

Cat, catalog; MM, mouse monoclonal; GP, goat polyclonal.

* Abnova, Abnova Corp., Taipei, Taiwan; Life Technologies, Life Technologies Corp., Carlsbad, CA; R&D, R&D Systems, Minneapolis, MN; Santa Cruz, Santa Cruz Biotechnology, Inc., Dallas, TX; Abcam, Abcam plc., Cambridge, UK; Novocastra, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK.

ic, Inc., Rochester, NY) were fixed with Zamboni's fixative (phosphate-buffered combination of picric acid and paraformaldehyde) or 95% ice-cold ethanol, blocked with 1% skim milk, incubated overnight with a primary antibody at 4°C, washed with PBS, and incubated with a secondary antibody (Alexa Fluor 488-labeled anti-mouse or anti-goat IgG; Life Technologies Corp.) at room temperature for 1 hour. Subsequently, they were washed again with PBS, counterstained, mounted, covered with coverslips, and photographed using a fluorescence microscope (AX70 TRF; Olympus Corp., Tokyo, Japan) and a confocal laser scanning microscope (TCS-P2; Leica Microsystems, Wetzlar, Germany).

Western Blotting Analysis

Proteins were separated on a commercially available 4% to 20% gradient SDS-polyacrylamide gel (Mini-PROTEAN TGX; Bio-Rad Laboratories, Inc., Hercules, CA) and were transferred to a polyvinylidene difluoride membrane (Trans-Blot Turbo Transfer Pack; Bio-Rad Laboratories, Inc.). The blotted membrane was then blocked in TBS-T (Tris-buffered saline with 0.05% Tween 20) buffer containing 1% skim milk, incubated overnight with primary antibodies at 4°C, washed, incubated with a horseradish peroxidase–conjugated secondary antibody at room temperature for 1 hour, and washed again. A chemiluminescent reagent (ECL Advance Western Blotting Detection Kit; GE Healthcare, Little Chalfont, UK) was then applied onto the blotted membrane. The luminescent signal was detected using a chilled charge-coupled device (CCD) digital imaging camera (LAS-3000UVmini; Fujifilm Corp., Tokyo, Japan).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was reverse transcribed using a commercial reverse transcriptase (Superscript III; Life Technologies Corp.). The cDNA was amplified by PCR and then electrophoresed on a 2% agarose gel.

Measurement of Trans-Epithelial Resistance (TER)

Epithelial cells were cultured on 12-well porous membrane filters (Transwell, 0.4 μm pore; Corning, Inc., Corning, NY). Two days after the cells had reached 100% confluence, the culture medium was switched to a serum-containing, high-calcium medium (1 mM) to promote epithelial barrier formation. Resistance between the upper and lower chambers of the porous filter was measured using a volt–ohm meter (EVOM; World Precision Instruments, Sarasota, FL). The TER was then calculated by multiplying the measured resistance (ohms) by the culturing area of the filter (1.12 cm^2).

Results

Characteristics of the Established Corneal Epithelial Cell Line From the GDLD Patient

The immortalized corneal epithelial cell line from the GDLD patient (imHCE_GDLD) exhibited a small, square, cell shape (Fig. 2A). When the cells reached confluence, they demonstrated an organized cobblestone-like appearance that is typical of epithelial-type cells. That cell shape resembled that of the immortalized corneal epithelial cell line from the normal cornea (imHCE_normal). The imHCE_GDLD cells were found to be completely devoid of the TACSTD2 protein, as judged from immunostaining analysis (Fig. 2B), although the cells did express the TACSTD2 gene at the RNA level (Fig. 2C). The expressed TACSTD2 mRNA was found to harbor the p.Gln118X mutation (Fig. 2D). We initially hypothesized that this discrepancy was attributable to the inability of the antibody to react to the truncated TACSTD2 protein produced by the nonsense mutation of the gene at the upstream region of its transmembrane domain. However, results showed that

Table 2. List of Oligomers Used for This Study

Primer	Sequence
SV40 large T_forward	5′-GGCGCCATGGATAAAGTTTTAAACAGAGAGGA-3′
SV40 large T_reverse	5′-TTATGTTTCAGGTTCAGGGGGAG-3′
hTERT_forward	5′-AGCAGGCACCATGCCGCGCGCTCC-3′
hTERT_reverse	5′-GCTGGGTTCTAGTCCAGGATGGTC-3′
TACSTD2_forward	5′-CTGACCTCCAAGTGTCTGCTG-3′
TACSTD2_reverse	5′-GTCCAGGTCTGAGTGGTTGAAG-3′
TS	5′-AATCCGTCGAGCAGAGTT-3′
CX	5′-CCCTTACCCTTACCC TTACCCTAA-3′

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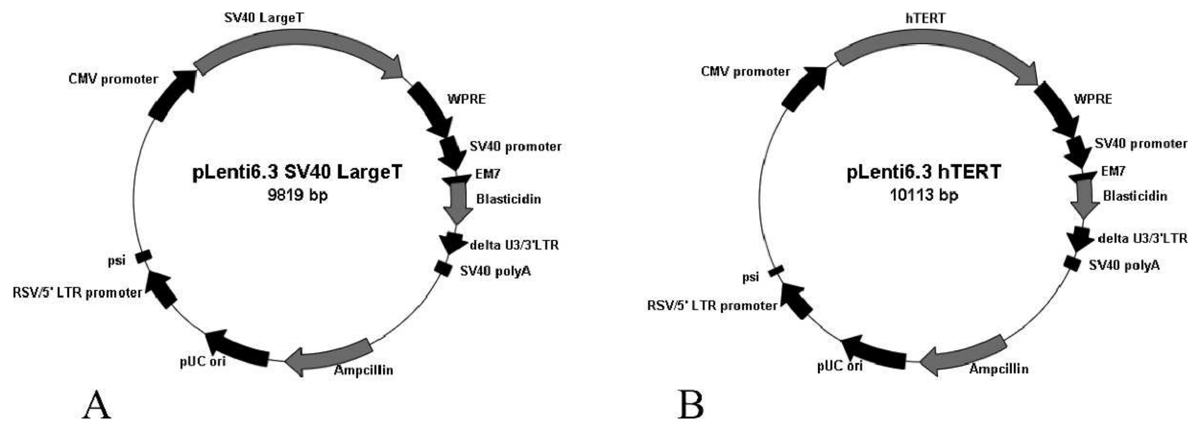

FIGURE 1. Schematic representation of the structure of lentivirus vectors expressing the SV40 large T antigen (**A**) and the hTERT genes (**B**).

the goat polyclonal antibody can recognize the TACSTD2 protein with the p.Gln118X nonsense mutation (Supplementary Fig. S1). Therefore, our current hypothesis is that the TACSTD2 protein with the p.Gln118X mutation is secreted to the extracellular space or is degraded within the cells. The imHCE_GDLD cells were found to express the SV40 large T antigen and hTERT genes (Figs. 2E, 2F), indicating that our lentiviral transduction process is efficient.

Cell Proliferative Kinetics, Senescent Status, Telomerase Activity, and Colony-Forming Activity in the Immortalized Corneal Epithelial Cell Line From the GDLD Patient

The cell proliferative kinetics was investigated using PD analysis (Fig. 3A). The imHCE_GDLD cells continued to proliferate after the cumulative PDs exceeded 104.7, although the nontransduced corneal epithelial cells from the GDLD patient stopped proliferating when the cumulative PDs reached 19.7.

Although their cumulative PDs exceeded 100, the imHCE_GDLD cells were rectangular and small (Fig. 3B). The nontransduced corneal epithelial cells from the GDLD patient exhibited almost identical cell shape and cell size to those of the imHCE_GDLD cells in their early stage of culture, but they gradually became more flattened and larger as their cumulative PDs increased. Their flattened cell shape appeared to be typical of senesced cells. Therefore, we tested the cellular senescent status by evaluating the SA-βgal activity. As depicted in Figure 3B, most nontransduced corneal epithelial cells from the GDLD patient exhibited blue staining in their cytoplasmic area when their cumulative PDs were 19.4. However, the imHCE_GDLD cells exhibited nearly unstained clear cell bodies, although some cells were stained faintly in blue when their cumulative PD was 54.1. Those results indicate clearly that the imHCE_GDLD cells were almost completely out of the senescent stage, irrespective of their PDs, although the nontransduced corneal epithelial cells from the GDLD patient entered the senescent stage as their PDs increased.

We regarded it as important to confirm whether the imHCE_GDLD cells acquired telomerase activity because the telomerase is a holoenzyme comprising proteins (dyskerin; DKC1 and telomerase protein component 1; TEP1)[25,26] and RNA (telomerase RNA component; TERC).[27] Therefore, the forced expression of the hTERT gene does not necessarily guarantee the acquisition of telomerase activity. The imHCE_GDLD cells showed clear laddering of the multiple-sized TRAP products (Fig. 3C). The intensity and the degree of extension to long fragments in the TRAP ladder of the imHCE_GDLD cells were at almost the same level as those of HeLa cells (well-known cancer cells that have been maintained continuously for more than 60 years)[28] and HCE-T cells (commonly used immortalized corneal epithelial cells established more than 20 years ago that have passed for many years).[29] Those results indicate that the forcedly expressed hTERT protein, which was expressed also under the regulation of inauthentic virus-origin promoter (cytomegalovirus promoter), integrated into a functionally competent telomerase complex and elongated the telomeric sequence of the chromosomal ends.

We also performed a colony-forming assay to examine the cell-proliferation competence of the imHCE_GDLD cells expanded from single cells. The imHCE_GDLD cells produced multiple-cell expansion foci that were larger and more numerous than the nontransduced corneal epithelial cells from the GDLD patient (Fig. 3D). The results strongly suggest that the imHCE_GDLD cells can be maintained for a much longer period.

Epithelial Barrier Function and Expression of Tight-Junction–Related Proteins in the imHCE_GDLD Cells

We next investigated whether the imHCE_GDLD cells had appropriate cellular features to be used as an in vitro model of a GDLD cornea. We initially investigated the epithelial barrier function of the imHCE_GDLD cells by measuring the TER. As portrayed in Figure 4A, the epithelial barrier function of the imHCE_GDLD cells was significantly lower than that of the imHCE_normal cells ($P < 0.05$, Student's *t*-test). We also investigated the expression of the tight-junction–related proteins in the imHCE_GDLD cells. The CLDN1 and CLDN7 protein expression levels were found to be reduced significantly more in the imHCE_GDLD cells than in the imHCE_normal cells (Fig. 4B), which is consistent with our earlier observation that the expression level of these proteins was decreased significantly in an in vivo GDLD cornea.[18] In addition, the immunostaining pattern of the CLDN1 and CLDN7 proteins was dramatically different from that in the imHCE_normal cells. The other three tight-junction–related proteins including CLDN4, occludin (OCLN), and zonula occludens-1 (ZO-1) were expressed both in the imHCE_GDLD and the imHCE_normal cells at an

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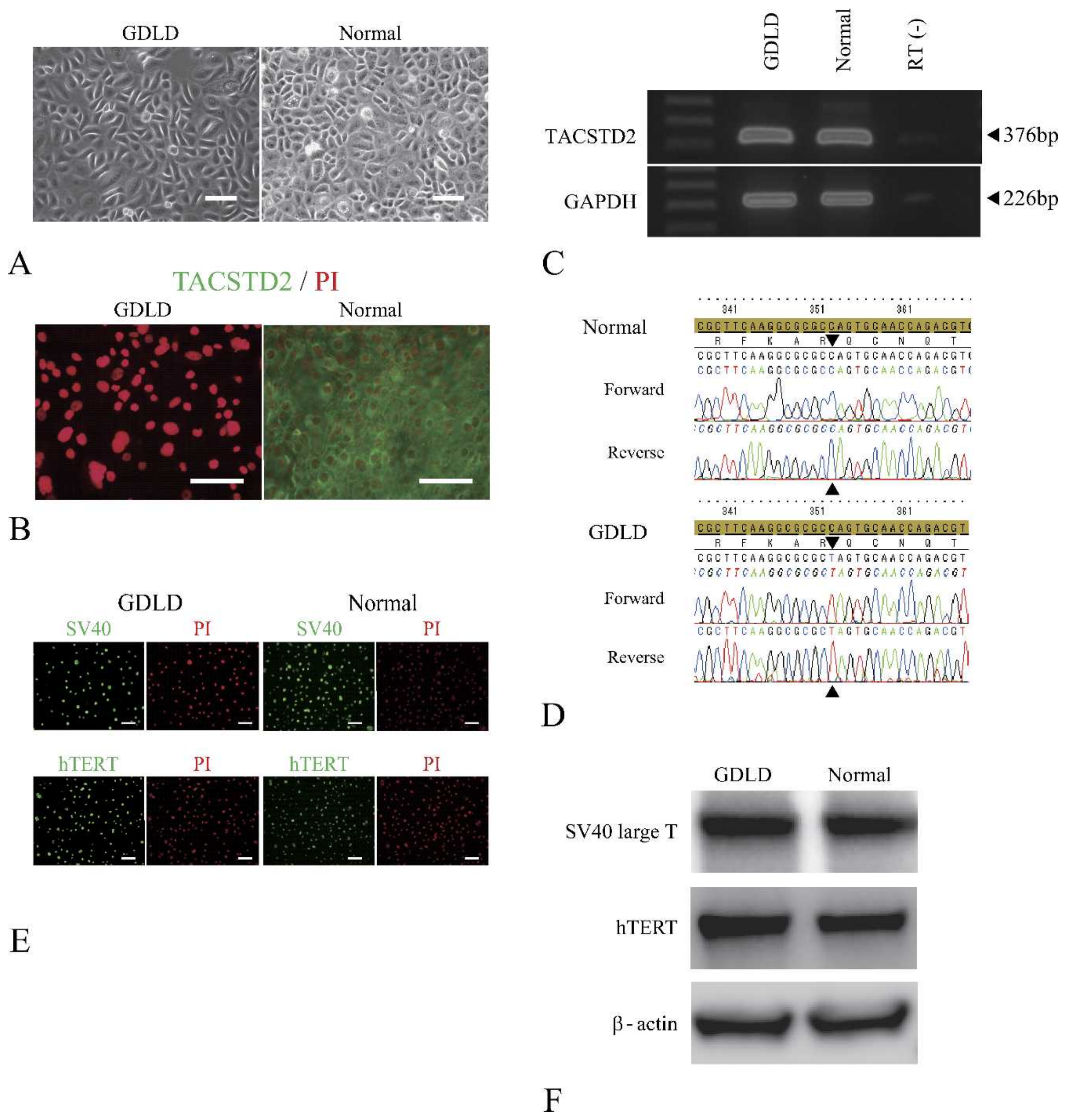

FIGURE 2. Characteristics of the imHCE_GDLD cells and of the imHCE_normal cells. (A) Both cell lines exhibited similar cell size and cell shape, demonstrating an organized cobblestone appearance by phase contrast microscopy. (B) The imHCE_GDLD cells were devoid of the TACSTD2 protein, but the imHCE_normal cells expressed the protein. (C) At the RNA level, both cell lines expressed the TACSTD2 gene almost at the same level. GAPDH was investigated as an internal control. (D) Sequencing analysis detected the p.Gln118X mutation in the RT-PCR product of the imHCE_GDLD cells. Both cell lines expressed the SV40 large T antigen and hTERT proteins, as judged by immunostaining (E) as well as Western blot (F) analyses. *Scale bars* in the immunostaining analysis correspond to 100 μm. The immortalized corneal epithelial cell line from the GDLD patient and the immortalized corneal epithelial cell line forming the normal cornea are abbreviated respectively as imHCE_GDLD and imHCE_normal.

almost identical level and with an almost identical immunolocalization pattern (Fig. 4C). Confocal microscopy analysis revealed that the immunolocalization of the CLDN1 and CLDN7 proteins exhibited a pattern with dots, many of which seemed to exist on the plasma membrane in the imHCE_GDLD cells, although it exhibited the membrane-bound pattern in the imHCE_normal cells (Fig. 4D).

Preliminary Study of Gene Therapy for the Treatment of GDLD Corneas

Finally, we conducted a preliminary study to investigate whether gene therapy is beneficial for the treatment of GDLD corneas. We expected that exogenous introduction of the wild type TACSTD2 gene might normalize the disease situation

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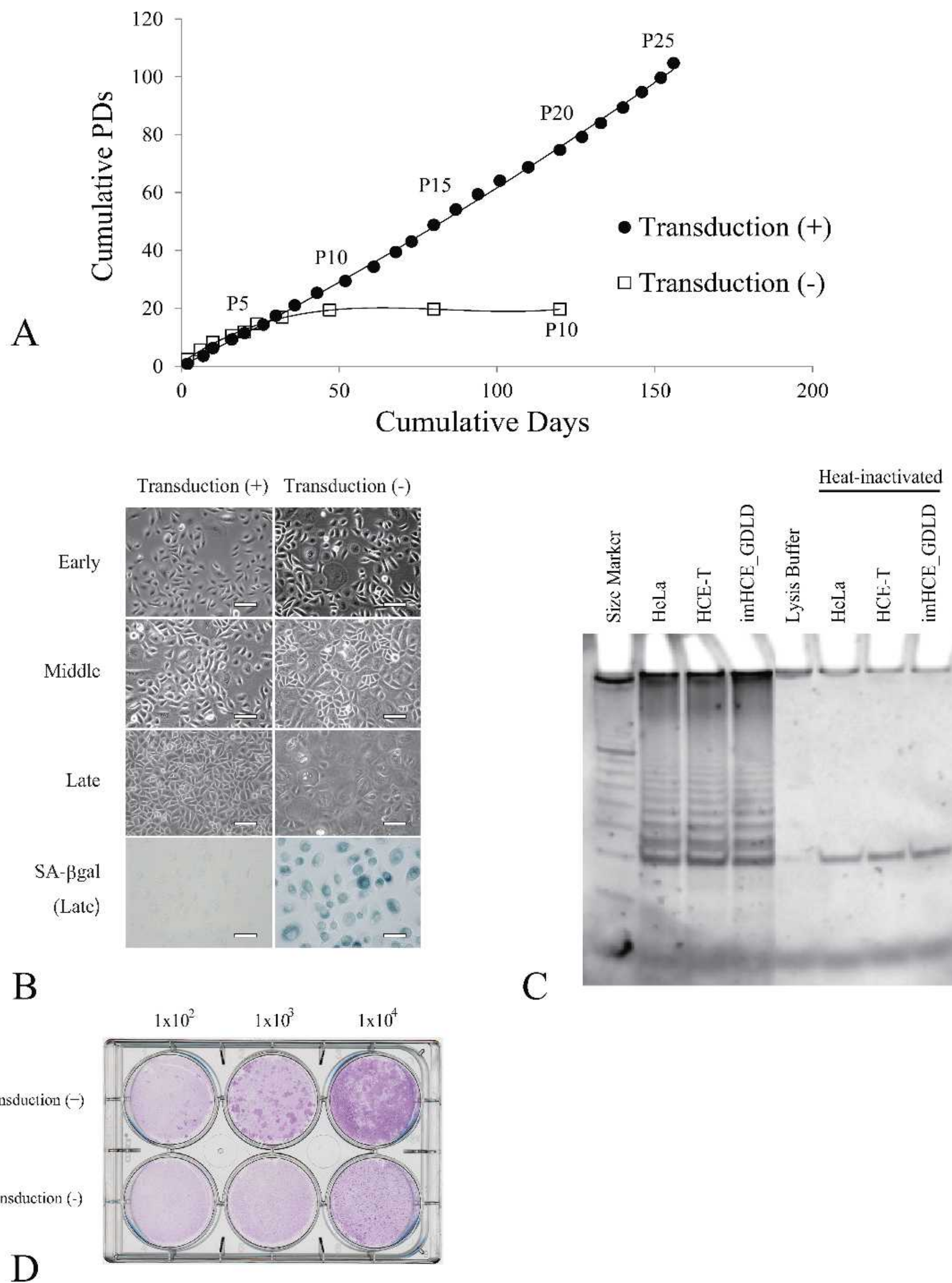

FIGURE 3. Cell growth kinetics, senescence status, telomerase activity, and colony-forming activity of the immortalized corneal epithelial cells of the GDLD patient. (**A**) PD analysis revealed that the GDLD corneal epithelial cells continued to proliferate with the transduction of the two genes (SV40 large T antigen and hTERT genes), whereas the nontransduced cells stopped proliferating at their early stage of culture. (*P numbers* represent the number of passages, not the PD number.) (**B**) The morphology and the senescence status were assessed in the transduced cells or in nontransduced cells at the early (3.5 PDs and 3.2 PDs, respectively), middle (11.5 PDs and 11.8 PDs, respectively), and late (54.1 PDs and 19.4 PDs, respectively) stages of culture. The transduced cells maintained their small and square cell morphology, although the nontransduced cells gradually exhibited large and flattened cell morphology. The senescence status was assessed using SA-βgal assay in both transduction (+) and (–) cells at their late (54.1 PDs and 19.4 PDs, respectively) stages of culture. Most of the nontransduced cells exhibited apparent *blue staining* in their cytoplasmic area, whereas the transduced cells did not. *Scale bars*: 100 μm. (**C**) Telomerase activity was assessed using TRAP assay for HeLa cells (*lanes 2* and *6*), immortalized human corneal epithelial cells (HCE-T, *lanes 3* and *7*), and the immortalized GDLD corneal epithelial cells (imHCE_GDLD, *lanes 4* and *8*). For the negative controls, lysis buffer (*lane 5*) and heat-inactivated lysates of the above cell types (*lanes 6–8*) were investigated. *Lane 1* shows the size marker. The HeLa cells, the HCE-T cells, and the immortalized GDLD corneal epithelial cells exhibited clear laddering of TRAP products, indicating the telomerase activity in these cells. (**D**) Colony-forming assay was performed for the transduced or nontransduced corneal epithelial cells from the GDLD patient. The transduced cells produced multiple-cell expansion foci that were significantly larger and more numerous than the nontransduced cells.

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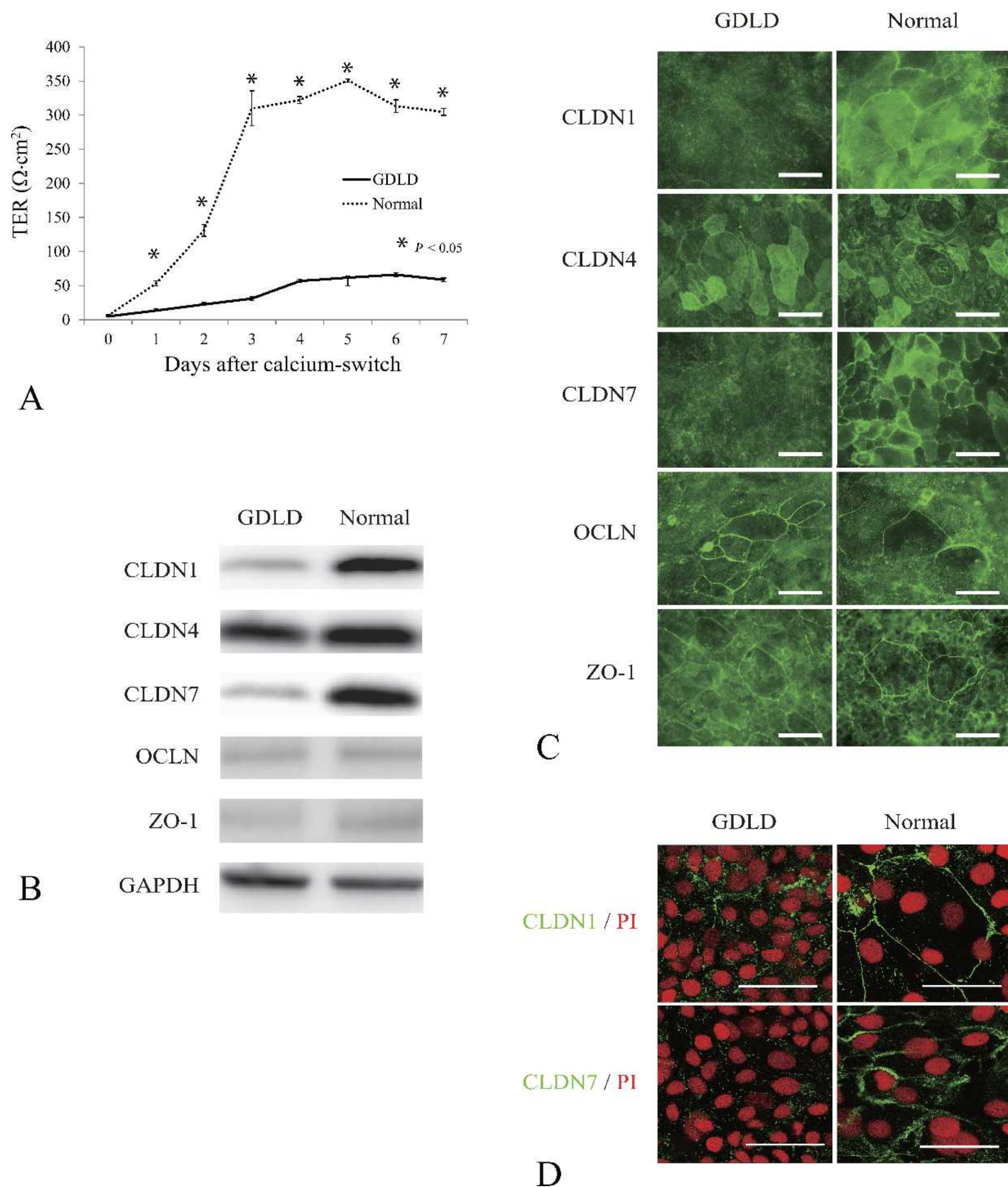

Figure 4. Epithelial barrier function and the expression of tight-junction–related proteins were investigated in imHCE_GDLD. (**A**) The epithelial barrier function was investigated by measuring the trans-epithelial resistance (TER). The TER of the imHCE_GDLD cells was significantly lower than that of the imHCE_normal. The experiments were done in triplicate, with data presented as mean ± SD. $^*P < 0.05$ (Student's *t*-test). Expression of the tight-junction–related proteins including CLDN 1, 4, and 7; OCLN; and ZO-1 were analyzed using Western blot (**B**) and immunostaining (**C**) analyses in the imHCE_GDLD and imHCE_normal cells. (**B**) The expression levels of the CLDN1 and CLDN7 proteins were significantly lower in the imHCE_GDLD cells than in the imHCE_normal cells. (**C**) The immunostaining patterns of the CLDN1 and CLDN7 proteins in the imHCE_GDLD cells differed dramatically from those in the imHCE_normal cells. The other three tight-junction–related proteins exhibited an almost identical immunostaining pattern and an almost identical expression level in both the imHCE_GDLD and imHCE_normal cells. GAPDH was investigated as a loading control in Western blotting. *Scale bars*: 100 µm. (**D**) Subcellular localization of the CLDN1 and CLDN7 proteins was investigated further using confocal microscopy. The immunolocalization patterns of the CLDN1 and CLDN7 proteins were dot-like with many dots presumably lining the plasma membrane in the imHCE_GDLD cells, but with a membrane-bound pattern in the imHCE_normal cells. *Scale bars*: 50 µm. The immortalized corneal epithelial cell line from the GDLD patient and the immortalized corneal epithelial cell line from the normal cornea are abbreviated as imHCE_GDLD and imHCE_normal, respectively.

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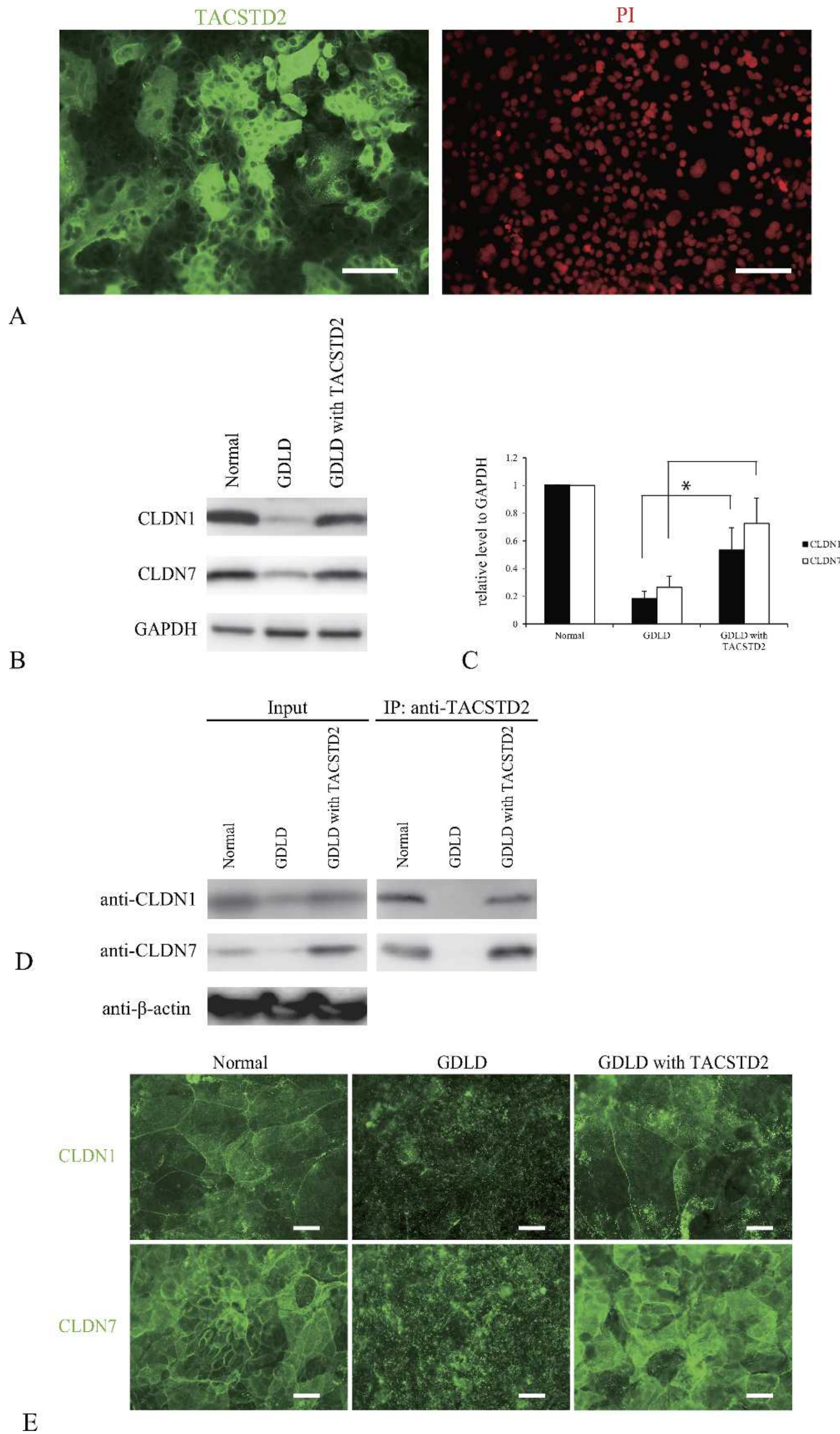

FIGURE 5. Preliminary study of gene therapy for GDLD cornea. The imHCE_GDLD was transduced with a lentiviral vector expressing the wild-type TACSTD2 protein. (**A**) Approximately 70% of the imHCE_GDLD cells were positive for the TACSTD2 protein. Transduction efficiency was estimated by counting the TACSTD2 positive cells from randomly selected immunostaining images. *Scale bars*: 100 μm. (**B**) Western blot analysis demonstrates the remarkable upregulation of the CLDN1 and CLDN7 proteins after the transduction of the wild-type TACSTD2 gene in the imHCE_GDLD cells. (**C**) Results of Western blot analyses were quantitated using densitometry. The bar graph shows the relative level of the CLDN1 and CLDN7 proteins to the GAPDH protein. $^{*}P < 0.05$ (Student's *t*-test). (**D**) Immunoprecipitation assay revealed that the TACSTD2 protein binds to the CLDN1 and CLDN7 proteins. (**E**) Results of the immunostaining analysis against the imHCE_normal, the imHCE_GDLD, and

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the TACSTD2 gene transduced imHCE_GDLD cells. The transduction of the wild-type TACSTD2 gene almost completely normalized the subcellular localization of the CLDN1 and CLDN7 proteins in the imHCE_GDLD. *Scale bars*: 50 μm. The immortalized corneal epithelial cell line from the GDLD patient and the immortalized corneal epithelial cell line from the normal cornea are abbreviated as imHCE_GDLD and imHCE_normal, respectively.

occurring in GDLD cornea because GDLD is a monogenic disorder caused by the biallelic loss of function mutation of the TACSTD2 gene. The imHCE_GDLD cells were transduced with the wild-type TACSTD2 gene by the lentivirus, with transduction efficiency as high as approximately 70% (Fig. 5A). The exogenous transduction of the wild-type TACSTD2 gene significantly increased the expression levels of the CLDN1 and CLDN7 proteins (Figs. 5B, 5C). The exogenously transduced wild-type TACSTD2 protein was found to be bound to the CLDN1 and CLDN7 proteins, as judged by immunoprecipitation analysis (Fig. 5D). In addition, the exogenous transduction of the wild-type TACSTD2 gene almost completely normalized the subcellular localization of the CLDN1 and CLDN7 proteins (Fig. 5E).

Discussion

In this study, we established the immortalized corneal epithelial cells from a GDLD patient by the transfection of SV40 large T antigen and hTERT genes. The cell line has high proliferation activity after their cumulative PDs exceed 100 and exhibits significant reduction of the barrier function, which is the major characteristic of corneal epithelial cells in a GDLD patient. Furthermore, the cell line exhibited decreased expression of the CLDN1 and CLDN7 proteins as well as the altered subcellular localization of these proteins, which shows good agreement with the in vivo GDLD cornea. Therefore, the established cell line, which well reflects the disease situation of the GDLD cornea, might be a good in vitro model for a GDLD cornea.

Normal human cells usually stop dividing at approximately 40 to 60 PDs, depending on several factors such as the age of their origin, the cell type, and the culture condition.[30] In general, the expansion of the life-span of a cell encounters a two-step barrier: senescence (M1) and crisis (M2).[31] The two replicative barriers have been shown to play a crucial role mainly in limiting the progress of tumorigenesis,[32] which is a life-threatening situation in most multicellular organisms. The M1 replicative barrier is known to be operated by two crucial tumor-suppressor genes: the retinoblastoma (RB) gene and the p53 gene. The nuclear Rb protein binding prevents accessibility of the transcription factor E2F to nuclear cyclins (E and A). Upon stimulation by growth factor receptor signals, the Rb protein is phosphorylated. It releases E2F, which can enable the activation of these S phase–related cyclins.[33] The M2 replicative barrier is known to be achieved by the shortening of telomeres, which might be attributable to the silencing of the hTERT gene expression in stem cells and others in the human body, with the notable exception being expression in germline cells.[24] Some virus-derived proteins such as the SV40 large T antigen and human papilloma virus E6/E7 proteins are known to bind to the RB and p53 proteins to abrogate their tumor-suppressive activity, resulting in the bypass of cells from the M1 stage.[34,35] The hTERT gene is known to be the rate-limiting factor in the telomerase activity, whereas other components of the telomerase-holoenzyme complex are known to be expressed constitutively in cells of many types.[36] Reportedly, the forced expression of only the hTERT gene was sufficient for the acquisition of telomerase activity.[37]

Several reports have described the immortalization of cells by single-gene transduction with SV40 large T antigen[29,35] or hTERT gene.[37,38] However, because each of the two replicative barriers M1 and M2 has strong inhibitive power to suppress cell expansion, it might be generally accepted that single-gene transduction with either of the two genes has markedly lower potential to achieve immortalization than that with both genes. It can be speculated that the reported immortalization by the single-gene transduction with either of the two genes might be at least partially attributable to the spontaneous repression of either or both RB and p53 gene(s), or the spontaneous activation of the endogenous hTERT gene, possibly because of gene mutation[39] or epigenetic alteration.[40] Since the starting cell number of our GDLD corneal epithelial cells was limited, we theorized that the immortalization process might fail if its efficiency was not high. Therefore, we chose to use both genes to easily overcome the M1 and M2 replicative barriers.

As for the development of a new therapy, the transduction of the wild-type TACSTD2 gene to the patient's corneal epithelial cells can be a promising therapy because GDLD is a monogenic disorder caused by the biallelic loss of function mutation of the TACSTD2 gene. Therefore, the transduction of the wild-type TACSTD2 gene might normalize the disease situation in the GDLD corneal epithelial cells. However, before the clinical application of the gene therapy, several issues must be resolved. Physicians must ascertain the optimal dosage of the transduced gene because, in general, faint expression would have little therapeutic effect, although overexpression might engender unanticipated side effects. For the TACSTD2 gene, overexpression of the gene might present a risk for tumorigenesis because the gene is presumably oncogenic.[41] We performed a preliminary experiment as for the gene therapy of GDLD. Approximately 70% imHCE_GDLD cells were transduced with the wild-type TACSTD2 gene. The gene transduction normalized the disease situation of corneal epithelial cells of GDLD, with increased expression of the CLDN1 and 7 proteins and altered subcellular localization of the two proteins from cytoplasm to plasma membrane. Unfortunately, after multiple repetitions of experiments, which yielded all of these promising data, we were unable to obtain the normalization of TER, perhaps because of the insufficiency of the transduction efficiency. Direct gene correction by artificial nucleases such as zinc finger nuclease and TAL effector nuclease presents another avenue for gene therapy.

In summary, the results of this study demonstrate the establishment of an immortalized corneal epithelial cell line from this GDLD patient. Currently, we are only halfway along in our understanding of GDLD pathophysiology. Moreover, no single prominent advance has occurred during the last decade in the development of novel effective treatments for GDLD. We hope that this newly established cell line will help foster breakthroughs in the examination of these important issues.

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