

RESEARCH ARTICLE

Generation of *TGFBI* knockout ABCG2+/ABCB5+ double-positive limbal epithelial stem cells by CRISPR/Cas9-mediated genome editing

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Abstract

Corneal dystrophy is an autosomal dominant disorder caused by mutations of the transforming growth factor β -induced (*TGFBI*) gene on chromosome 5q31.8. This disease is therefore ideally suited for gene therapy using genome-editing technology. Here, we isolated human limbal epithelial stem cells (ABCG2+/ABCB5+ double-positive LESC) and established a *TGFBI* knockout using RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing. An LESC clone generated with a single-guide RNA (sgRNA) targeting exon 4 of the *TGFBI* gene was sequenced in order to identify potential genomic insertions and deletions near the Cas9/sgRNA-target sites. A detailed analysis of the differences between wild type LESC and the single LESC clone modified by the *TGFBI*-targeting sgRNA revealed two distinct mutations, an 8 bp deletion and a 14 bp deletion flanked by a single point mutation. These mutations each lead to a frameshift missense mutation and generate premature stop codons downstream in exon 4. To validate the *TGFBI* knockout LESC clone, we used single cell culture to isolate four individual sub-clones, each of which was found to possess both mutations present in the parent clone, indicating that the population is homogenous. Furthermore, we confirmed that *TGFBI* protein expression is abolished in the *TGFBI* knockout LESC clone using western blot analysis. Collectively, our results suggest that genome editing of *TGFBI* in LESC by CRISPR/Cas9 may be useful strategy to treat corneal dystrophy.

Introduction

Corneal dystrophies are slowly progressive, symmetric, and unrelated to systemic or environmental factors [1]. Bilateral corneal deposits resulting from these conditions can cause photophobia, tearing, pain, and eventually reduce visual acuity [2]. The advent of genetic analysis has allowed the identification of transforming growth factor β -induced gene (*TGFBI*)

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mutations that are associated with specific corneal dystrophies. For example, p.Arg124Leu is found in Reis-Bücklers corneal dystrophy (RBCD), p.Arg555Gln leads to Thiel-Behnke corneal dystrophy (TBCD), p.Arg124Cys causes lattice corneal dystrophy type 1 (LCD1), p.Arg555Trp results in granular corneal dystrophy type 1 (GCD1), and p.Arg124His occurs in granular corneal dystrophy type 2 (GCD2) [1]. Overall, 57 mutations in the *TGFBI* gene have been associated with corneal dystrophies.

Based on published studies, p.Arg124His (GCD2) is the most frequently observed mutation in the Asian population, with the second most common mutation likely to be either p.Arg124Cys (LCD1) [3] or p.Arg555Trp (GCD1) [4]. A Japanese report, for example, found that the GCD2 mutation accounted for up to 72% of patients in their study population [3, 4]. Our group has identified 21 individuals that are homozygous for this mutation in Korea and calculated that heterozygotes are likely to account for 1 out of every 870 Korean people [5, 6]. China was distinct from other Asian countries in that the GCD1 mutation was most frequently identified in *TGFBI* corneal dystrophy patients, followed by the LCD1 and GCD2 mutations [7]. Further, in Western countries, LCD1 was most common genetic variant in this disease.

The corneal epithelium arises from, and is maintained by, limbal epithelial stem cells (LESCs) in the basal layer of the corneal limbus. These multiply slowly giving rise to transient amplifying cells (TACs), which migrate superficially while becoming more and more differentiated [8–10]. Limbal stem cell deficiency (LSCD) can arise for a number of reasons, including burn, injury, and infection. Due to a lack of corneal donor tissue and the decreased of graft survival after penetrating keratoplasty, stem cell therapies based on the autologous or homologous expansion of LESCs has been proposed in severe cases of LSCD [11].

LESCs are identified by expression of $\Delta Np63\alpha$ along with a high nuclear to cytoplasmic ratio [12, 13]. ABCG2 (ATP binding cassette sub family G member 2) positivity detected in LESCs as well as several other cells exist in the suprabasal limbus and these markers used to identify the LESC population based on their staining ability in clusters of stem-like cells in the limbus [14, 15].

ABCB5 (ATP-binding cassette subfamily B member 5) is a regulator of limbal stem cell behavior and is required for corneal development [16]. ABCB5 was mainly expressed in basal layer cells of the mouse limbus. In human eyes, ABCB5⁺ cells were located in the basal layer of the limbus and co-expressed $\Delta Np63\alpha$ —a known expressed in epithelial stem cells [16, 17], including human limbal stem cells [18, 19].

Recently, we isolated ABCG2⁺/ABCB5⁺ LESCs and confirmed differentiation of LESC into corneal epithelial cell [17]. The ABCG2⁺/ABCB5⁺ LESCs that we established displayed powerful stem cell activity, continuous growth, and high telomerase activity. Moreover, ABCG2⁺/ABCB5⁺ LESCs expressed the core transcription factors Oct4, Sox2, c-Myc, and Klf4, which are also expressed in multipotent stem cells [17]. These data indicate that the ABCG2⁺/ABCB5⁺ LESCs that we established have powerful stem cell activity and may be used to regenerate corneal epithelia. Based on these data, knock out of mutant *TGFBI* in ABCG2⁺/ABCB5⁺ LESC from corneal dystrophy patients may be treatment strategy for corneal dystrophy patients.

Recently, an RNA-mediated adaptive immune system found in bacteria and archaea, known as clustered regularly interspaced short palindromic repeats (CRISPR) has been used to develop a revolutionary technology for gene editing in cells and organisms [20–25]. This CRISPR/Cas9 system uses the bacterial Cas9 protein, combined with a short single-guide RNA (sgRNA), which together can be used to generate targeted double-stranded breaks in the genomic DNA [26]. Additionally, cytoplasmic microinjections of *in vitro* transcribed mRNA combined with the CRISPR/Cas9 technology have been successfully used for genome

modifications (correction of genetic disorders or disruption of the mutated gene) in cells, as well as in several types of mammalian embryos [27–30].

Because corneal dystrophy is commonly caused by dominant mutations in the *TGFBI* gene, we hypothesize that this disease is suited for gene therapy with genome-editing technology. Here, we present the use of CRISPR/Cas9 gene editing to knock out endogenous human *TGFBI* expression at the genome level in ABCG2+/ABCB5+ double-positive LSCs, resulting in the establishment of a *TGFBI* gene knockout clone. Our results suggest that genome editing of *TGFBI* in human LSCs by CRISPR/Cas9 may be useful strategy to treat corneal dystrophy.

Materials and methods

ABCG2+/ABCB5+ double-positive LSCs culture

Human corneal tissue was harvested from healthy corneas from the eye bank after penetrating or lamellar keratoplasty. The age, gender and health of donors are listed in Table 1. Donor confidentiality was maintained in accordance with the Declaration of Helsinki and was approved by the Severance Hospital IRB Committee (CR04124), Yonsei University. ABCG2+/ABCB5+ double-positive LSCs were isolated as previously described.[17] In brief, Fresh corneoscleral tissue was cut into four similar segments in a 60-mm culture dish containing HBSS (HBSS: Hank’s balanced salt solution), and each segment was digested with 15 mg/mL Dispase II (Roche, Rotkreuz, Switzerland) in SHEM (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) with 100 mM sorbitol (Sigma-Aldrich, St Louis, MO) at 4°C for 18 hours. Under a dissecting microscope, an already loose limbal epithelial sheet was separated by inserting and sliding a noncutting flat stainless-steel spatula into a plane between the limbal epithelium and the stroma. Isolated limbal epithelial cells were seeded on specific matrix [5% matrigel (BD Biosciences, Bedford, MA) and 0.05 mg/ml human fibronectin (Sigma-Aldrich, St Louis, MO)] coating plate and cultured with CnT-20 medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland). After 3 days, limbal epithelial cells were cultured with 10% Serum DMEM (Invitrogen, Carlsbad, CA) medium and changed at every 2 days. After 8–10 days, highly proliferative cell colonies were washed with PBS two times and treated with 1 mL Accutase (Sigma-Aldrich, St Louis, MO). Single cells were seeded onto plates coated with a matrix of Matrigel and fibronectin and cultured in DMEM containing 10% FBS. The highly proliferative cells that attached to the new plate were designated limbal epithelial stem cells (LSCs). After 48 hours, The LSCs were treated with Accutase and sorted by FACS analysis using ABCG2+ antibodies (Abcam, Cambridge, MA) and ABCB5+ antibodies (Thermo fisher

Table 1. Human donor information.

	Male (N = 3)	Female (N = 3)
Mean age	37.6±11.02	31±7.8
Systolic blood pressure (mmHg)	120±9.8	115±10.5
Diastolic blood pressure(mmHg)	75±12.5	73±11.3
BMI (kg/m ²)	19.9±3.4	20.1±5.9
Hemoglobin (g/dl)	14.5±0.7	14.0±0.4
Glucose level(mg/dl)	81±5.9	80±4.7
Creatinine (mg/dl)	0.9±0.11	0.8±0.12
AST(IU/L)	21±2.2	20±2.3
ALT(IU/L)	20±2.4	19±2.1
γGTP(IU/L)	45±3.5	23±2.7
Proteinuria	Normal	Normal

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scientific, Rockford, IL). The ABCG2+/ABCB5+ double-positive cells were cultured using mass culture methods and were named ABCG2+/ABCB5+ double-positive LESC.

LESC transfection

Transfections were performed in 24-well cell culture plates with confluencies of approximately 60–70% using 1.5 µg of the *TGFBI*-targeting sgRNA (TCAGCTGTACACGGACCGCACGG) plasmid, 1 µg of the Cas9 plasmid, and 10 µl of Lipofectamine Reagent (Invitrogen, Carlsbad, CA), or negative control using only 1 µg of the Cas9 plasmid, and 10 µl of Lipofectamine Reagent, according to the manufacturer's instructions. Transfected cells were cultured for 24 h; these were then harvested, diluted in cell culture medium to approximately 1 cell/100 µl, and re-plated in 96-well cell culture plates. Once individual colonies were apparent in 66 of two 96-well plate, these were cultured in separate wells of 24-well plates and, subsequently, further expanded in 6-well and 60 mm plates until cell numbers were sufficient for genomic DNA extraction and western blot analyses.

Design of sgRNA/Cas9 vectors

Basically, we focused on granular corneal dystrophy type 2 (GCD2). Substitution of arginine for histidine at codon 124 (p.Arg124His) is associated with this disease. Therefore, we chose exon 4 as the target of sgRNA in both NHEJ to knockout and HDR to correct the mutation. Custom-designed CRISPR/Cas9 vectors, targeting one specific region of exon 4 of the *TGFBI* gene, were obtained from Toolgen (South Korea). First, four sgRNA-target sites were selected by an algorithm, and one of the sgRNA-target sites was selected by an algorithm that suggests sites with minimal risk to generate off-target effects and by mismatched sensitive nuclease assay (T7E1 assay). Selected sgRNA-target sites have the best cutting efficiency and fewer predicted off-target sites. Single-guide (sg) RNAs targeting the *TGFBI* gene were under the regulation of a U6 promoter, whereas expression of the Cas9 enzyme was driven by a cytomegalovirus promoter.

DNA mismatch-specific (T7E1) endonuclease assay

LESCs transfected with the *TGFBI*-targeting sgRNA plasmid and the Cas9 plasmid were harvested after 3 days of growth, and genomic DNA was extracted using the QIAamp DNA Blood Kit (QIAGEN, Valencia, CA). A region of the *TGFBI* gene exon 4 was amplified with genomic DNA-specific primers (forward primer, 5' -GTTTCACGTAGACAGGCATTTGA-3'; reverse primer, 5' -GCCTTTTCTAAGGGGTTAAGGA-3'). Homoduplex PCR products were then denatured and rehybridized using step-down annealing conditions to generate homo- and heteroduplexes, and the duplex mixture was treated with T7E1 nuclease for 1 h at 37°C (New England Biolabs, Ipswich, MA). The reaction was stopped using 1.5 µl of 0.25 M EDTA, and the products were analyzed on a 2% agarose gel.

Western blot

Cells were grown to near confluency in 60 mm plates. Growth medium was then removed, and the cells were rinsed twice with phosphate buffered saline (PBS) prior to lysis with a radio-immunoprecipitation assay (RIPA) buffer, supplemented with phosphatase and protease inhibitors. Insoluble cell debris was removed by centrifugation for 15 min at 13,000 rpm and 4°C, and the protein levels were adjusted based on a bicinchoninic acid colorimetric (BCA) assay. Equal amounts of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF)

membranes. These membranes were blocked in tris-buffered saline with Tween 20 (TBST) containing 5% skim milk and incubated with the primary anti-hTGFBI antibody (1:3,000, Abfrontier, Seoul, South Korea) overnight at 4°C. Blot membranes were then incubated with anti-rabbit secondary antibody (1:5,000, Thermo scientific, Rockford, IL, USA) in 5% skim milk in TBST, and the immunoreactive bands were visualized with a chemiluminescent reagent as recommended by Amersham Biosciences, Inc.

Statistical analysis

Each experiment was repeated at least three times in triplicate. Data were performed using GraphPad Prism 5 software. Measurements are presented as means \pm SE. Comparisons between two groups were analyzed by Student's *t*-test. Multiple comparisons were performed by one-way ANOVA followed by either Dunnett's or Tukey's post hoc test.

Results

CRISPR/Cas9-mediated modification of *TGFBI* in LESC

We first identified an appropriate target site in *TGFBI* for gene editing by screening 20 bp target sequences and 3 bp protospacer associated motif (PAM) sequences in exon 4 of the human *TGFBI* gene that was filtered by the Toolgen company to minimize off-target cross-reactivity. From a list of several candidates, we chose a target sequence spanning bases 354–373 of the human *TGFBI* cDNA. On the minus strand, these nucleotides are positioned immediately 5' to the trinucleotide PAM sequence "CGG" (Fig 1a). Once identified, an oligo pair containing the guide sequence was cloned into the pRGEN-U6 vector to produce the pRGEN-U6-TGFBI plasmid; we also obtained the pRGEN-Cas9-CMV expression vector, which expresses the Cas9 enzyme, from the Toolgen company.

We chose to use primary human ABCG2+/ABCB5+ double-positive LESC as the model system for generating *TGFBI* knockout cells using the CRISPR/Cas9 system. Novel limbus-derived, highly proliferative ABCG2+/ABCB5+ double-positive LESC were established in our previous research.[17] To test the effectiveness of the *TGFBI*-targeting sgRNA at triggering Cas9-mediated gene editing at the target site, LESC were transiently co-transfected with the pRGEN-U6-TGFBI sgRNA plasmid and the pRGEN-Cas9-CMV vector. This resulted in the generation of a heterogeneous total population of edited and non-edited cells. At 72 h post-transfection, we extracted total genomic DNA from this population and PCR-amplified a genomic region containing the target site in exon 4 of *TGFBI*. We then denatured and re-annealed the PCR amplicons in a thermal cycler to generate heteroduplex pairs and subjected these rehybridized products to digestion with T7E1 nuclease. The T7E1 enzyme selectively recognizes and cleaves mismatched bracket sites and heteroduplexes harboring indels [31]. Since CRISPR/Cas9 complexes trigger double-stranded breaks and imperfect non-homologous end joining (NHEJ) near the PAM [21], we predicted that T7E1 digestion of mismatches in the target site should generate DNA fragments of ~227 bp and ~284 bp in size. As shown in Fig 1b, the results of this T7E1 efficiency assay suggested that the *TGFBI*-targeting sgRNA was functional.

We therefore attempted to isolate single LESC clones targeted by the *TGFBI* sgRNA by co-transfecting LESC with the sgRNA and Cas9 protein expression plasmids and culturing individual clones derived from a single cell each in 96-well plates. These clones were allowed to grow until colonies formed, with fresh media provided every 2 days. Single clones were then dissociated and moved to 24-well plates and, subsequently, to 6-well plates. Genomic DNA was isolated from each clone, and PCR reactions were performed as described above. The

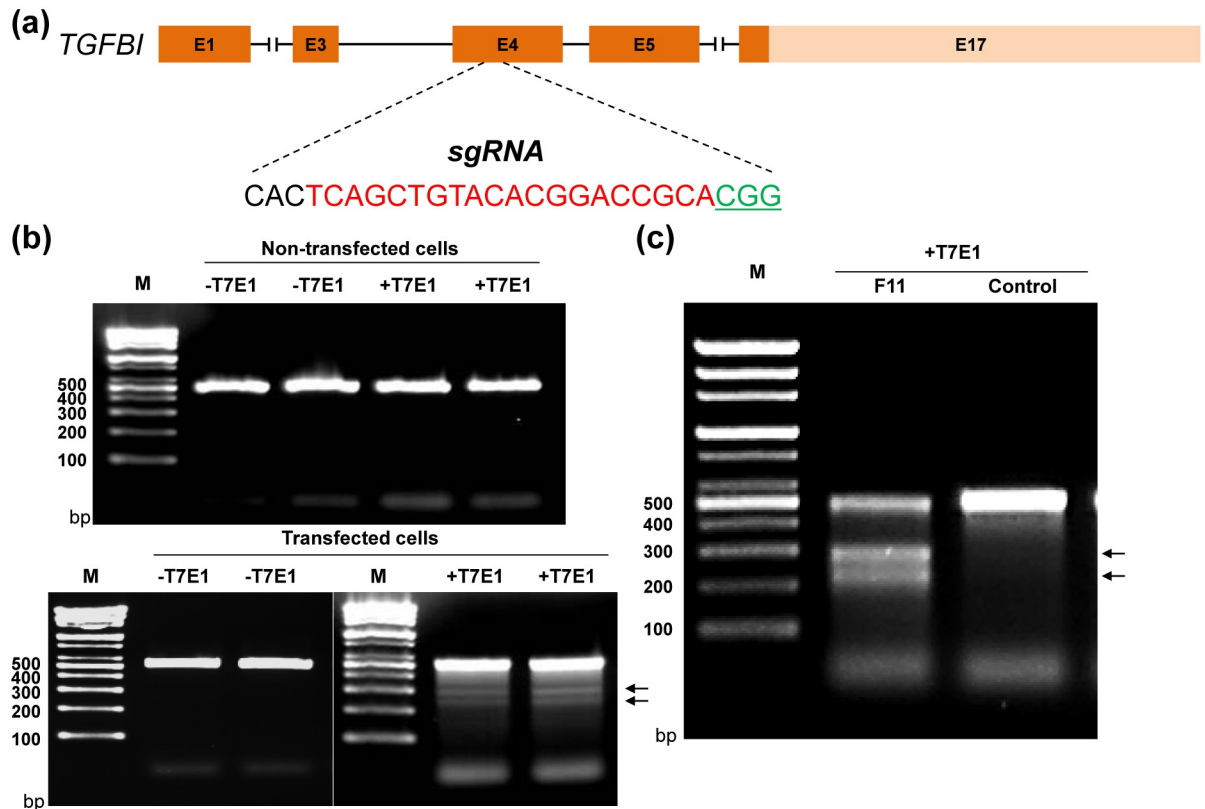


Fig 1. Evaluation of the sgRNA/Cas9-mediated *TGFBI* modifications in LESC. (a) Schematic diagram of the *TGFBI* partial protein coding region and the locus targeted by the sgRNA/Cas9 complex. The sgRNA-targeting site is presented in red, and the PAM sequence is shown in green and underlined. Amino acid sequence was presented under nucleotide sequence. (b) Targeting efficiency test for the genome editing constructs. Genomic PCR (gPCR) products spanning *TGFBI* exon 4 were amplified from a heterogeneous population of LESC that were transfected with or without the *TGFBI*-targeting sgRNA and Cas9 expression plasmids. Denaturation of these products, followed by rehybridization and treatment with T7E1 nuclease for 1 h at 37°C results in bands of ~280 bp and ~220 bp, indicated with arrows. This is consistent with the predicted cleavage sizes of 284 bp and 227 bp. M, marker. (c) Screening of single clones from LESC transfected with *TGFBI*-targeting sgRNA and Cas9 expression plasmids. Single cells were cultured in 96-well plates until colonies were visible, with media changes every 2 days. These were dissociated and moved first to 24-well plates and then to 6-well plates, and gPCR products spanning exon 4 of *TGFBI* were amplified from individual single cell clones. The gPCR products were denatured, rehybridized, and treated with T7E1 nuclease for 1 h at 37°C, and the products were analyzed on a 2% agarose gel. LESC clone F11 was found to be T7E1 nuclease positive, producing cleavage products of ~280 bp and ~220 bp. Arrows indicate bands resulting from T7E1 nuclease cleavage.

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products were rehybridized, treated with T7E1 nuclease for 1 h at 37°C, and analyzed on a 2% agarose gel. We found that the F11 single LESC clone was T7E1 nuclease positive, and produced a PCR product that was cleaved to yield products of ~280 and ~220 bp (Fig 1c).

We next compared the indel sequences found near the *TGFBI* sgRNA target site in the F11 single LESC clone to the corresponding region in wild-type LESC using Sanger sequencing, and observed overlapping peaks in the sequencing chromatographs (Fig 2). From these data, we determined that two different mutations were present, an 8 bp deletion and a 14 bp deletion flanked by one point mutation, and these were present in both the forward (Fig 2a) and reverse (Fig 2b) sequencing reactions. We did not observe a single wild type *TGFBI* sequence in either the forward or reverse sequencing results from the F11 single LESC clone. Intriguingly, these mutations both lead to a frameshift missense mutation and the formation of a premature stop codon downstream in exon 4.

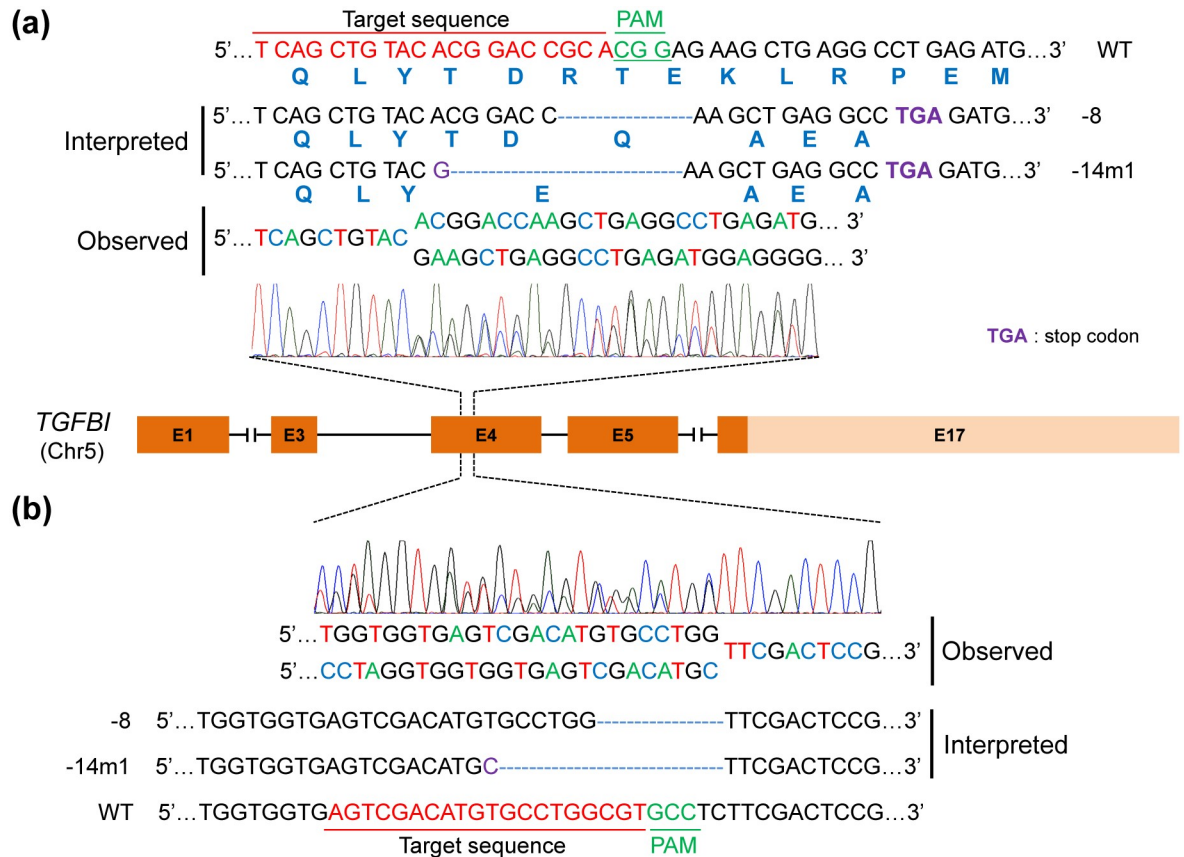


Fig 2. Insertion/Deletion (indel) analysis for the F11 single LESC clone. Sanger sequencing of gPCR products spanning exon 4 of *TGFBI* and containing the *TGFBI* sgRNA target site to identify indels in the F11 single LESC clone. (a) Results of the forward sequencing reaction. The wild-type (WT) sequence was identified in control LESC, and two distinct mutant sequences, an 8 bp deletion and a 14 bp deletion flanked by a single point mutation, were identified in the F11 clone. These mutations each lead to a frameshift missense mutation and the generation of a premature stop codon downstream of the target site in exon 4. The 20-nt sgRNA target site, 3-nt PAM sequence, and stop codon site are highlighted in red, green, and purple, respectively. Deletions (-) or nucleotide mutation (m) are shown to the right of each allele. (b) Results of the reverse sequencing reaction. The WT sequence was identified in control LESC, and the two mutant sequences described in (a, b) were identified in the F11 single LESC clone.

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Validation of *TGFBI* knockout LESC

Because our F11 single LESC clone generated by the *TGFBI*-targeting sgRNA contained two distinct types of mutation (i.e., an 8 bp deletion and a 14 bp deletion with an adjacent point mutation, Fig 2a and 2b), we wondered whether this clone has mono-allelic CRISPR-mediated 8bp deletion or 14bp deletion with single point mutation or rather, if some cells has bi-allelic 8bp deletion and others has bi-allelic 14bp deletion with single point mutation. To answer this question, we subcloned single cells from the F11 single LESC clone in 96-well plates and obtained four single cell subclones, which we termed F11-1, -2, -3, and -4. We then extracted genomic DNA from these subclones and amplified PCR products spanning exon 4 of *TGFBI*. PCR products from each single cell clone, as well as a mixture of the PCR product obtained from wild type LESC and a single cell clone, were rehybridized, treated with T7E1 nuclease, and analyzed on a 2% agarose gel. We predicted that if the F11 single LESC clone has a mono-allelic CRISPR-mediated 8bp deletion or 14bp deletion with single point mutation, the T7E1 assay would be positive for both the single cell clones and the mixed reaction, containing PCR products from wild type LESC and the single cell clones. Conversely, if some cells has bi-

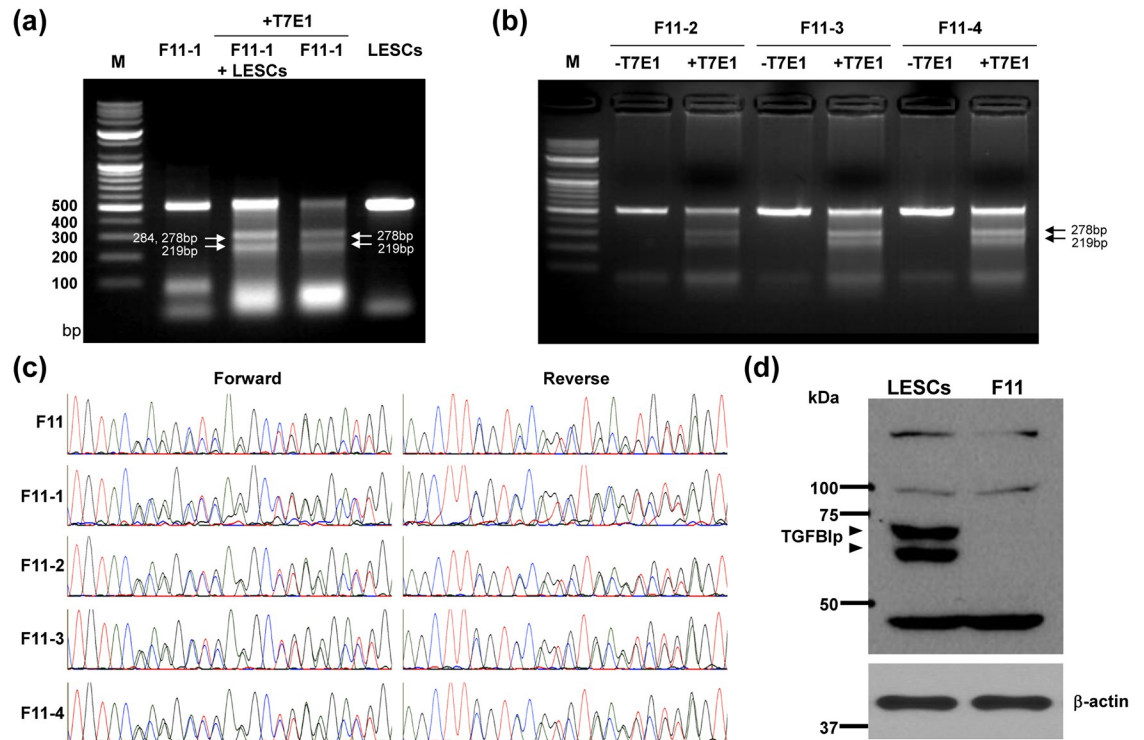


Fig 3. Validation of *TGFBI* knockout LESC clones. (a, b) The F11 LESC clone was single-cell cultured in 96-well plates, and individual subclones were allowed to reach 80% confluency, with media changes every 2 days. Genomic DNA was extracted from four F11 subclones, F11-1, -2, -3, and -4, and gPCR products spanning exon 4 of *TGFBI* were amplified. These were denatured, rehybridized, and treated with T7E1 nuclease for 1 h at 37°C, both individually and when mixed with the gPCR products from WT LESC, and the resulting fragments were analyzed on a 2% agarose gel. Cleavage products of 278 bp and 219 bp were present after T7E1 nuclease treatment of products from individual F11 subclones, and fragments of 284 bp, 278 bp, and 219 bp were generated by T7E1 nuclease treatment of mixed reactions with gPCR products from WT LESC. M, marker. (c) Results of forward and reverse sequencing reactions of gPCR products spanning *TGFBI* exon 4 from single subclones of the F11 LESC clone. Both mutant sequences found in the F11 parent were identified in all single cell subclones. (d) *TGFBI* protein levels in cell lysates (35 µg) from WT LESC and the F11 single LESC clone were determined by Western blot analysis using an anti-*TGFBI* primary antibody and an anti-β-actin antibody, as a loading control. Arrowheads indicate the position of the band corresponding to the *TGFBI* protein. Intact *TGFBI* protein is absent in the F11 clone.

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allelic 8bp deletion and others has bi-allelic 14bp deletion with single point mutation, some of which contain the 8 bp deletion and some of which contain the 14 bp deletion/single point mutation, the T7E1 assay would be positive only for the mixed reactions containing PCR products from wild type LESC and the single cell clones. We found that both the PCR products from the single cell clones and the mixed PCR products from wild type LESC and the single cell clones were cleaved by T7E1 nuclease (Fig 3a and 3b), suggesting that the F11 clone has mono-allelic CRISPR-mediated 8bp deletion or 14bp deletion with single point mutation.

To further validate our hypothesis, we analyzed sequences near the *TGFBI* sgRNA target site in the F11 single LESC clone and in the four single cell subclones, F11-1, -2, -3, and -4, using Sanger sequencing (Fig 3c). We found that all cells possessed the same sequences and mutations, that is, the 8 bp deletion and the 14 bp deletion flanked by a single point mutation, near the *TGFBI* sgRNA target site. Therefore, we conclude that the F11 single LESC clone is composed of homogeneous population of cells that contain two distinct *TGFBI* loci.

The predicted consequence of both the 8 bp deletion and the 14 bp deletion flanked by a single point mutation in the F11 clone is a frameshift, which generates four new amino acids and a premature stop codon downstream of alanine 127 or alanine 125, respectively (Fig 2).

Thus, this LESC clone lacks the intact wild-type allele, and all of the genomic DNA sequences that were identified contained deletions that create nonfunctional gene products. To confirm the loss of TGFBI protein (TGFBIp) expression in the knockout cells, we performed a western blot analysis on total protein lysates from normal LESC clones and the F11 single LESC clone using the human TGFBI polyclonal antibody and the β -actin antibody, as a loading control (Fig 3d). Our data show the complete lack of detectable TGFBIp expression in the F11 single LESC clone, thus confirming the generation of a *TGFBI* knockout cell line using the CRISPR/Cas9 system.

In Fig 3D, TGFBIp showed two bands. Based on our previous studies, TGFBIp showed one or two bands (68kDa and additional smaller size bands) by western blotting, dependent on the cell conditions or cell type [32–35]. However, we are unsure why TGFBIp showed one or two bands. There are two possibilities. One is that the cells express two isoforms of TGFBIp depending on cell conditions or cell type. Another is that the cells express an enzyme that modifies TGFBIp depending on the cell condition or cell type. Our current results suggest that the cells express an enzyme that modifies TGFBIp under different cell conditions. (unpublished data). Therefore, TGFBIp showed one or two bands on the western blot. We are currently verifying this hypothesis.

Discussion

Transforming growth factor beta-induced gene (*TGFBI*; *BIGH3*; *bigh3*) encodes transforming growth factor beta-induced protein (TGFBIp), composed of 683 amino acid residues. TGFBIp is known to be expressed ubiquitously in various organs including pancreas, heart, skin, liver [36], bone [37], endometrium [38], kidney [39], tendon [40], and blood plasma [41].

TGFBIp is thought to play important roles in physio-pathologic responses by mediating cell adhesion [36, 42–44] migration [42, 43], proliferation and differentiation [44]. TGFBIp mediates cell adhesion and/or spreading through integrins $\alpha1\beta1$, $\alpha3\beta1$, $\alpha\nu\beta3$, $\alpha\nu\beta5$, $\alpha6\beta4$, and $\alpha\text{m}\beta2$ [43, 45–49] and also associated with metastasis and suppression of malignant tumors [42, 50, 51]. Recently, our report suggested that TGFBIp increases migration, adhesion and differentiation of lymphatic endothelial cells so that inhibition of TGFBIp expression resulted in reduction of tumor lymphangiogenesis. These effects finally inhibited the metastasis of TGFBIp-producing tumors [34, 35]. We also reported that TGFBIp increases the migration and adhesion of endothelial progenitor cells through integrins $\alpha4$ and $\alpha5$ [52]. In the cornea, TGFBIp is expressed mainly in the epithelium [53], and up-regulated significantly during wound healing of the cornea, and increased the mucins expression [54, 55].

In cornea, mutation of *TGFBI* gene induces 5q31-linked autosomal dominant corneal dystrophies [56]. These diseases are characterized by accumulation of deposits in the cornea, often culminating in blindness due to the accumulation of protein deposits in the cornea. Munier et al. recognized the relationships between *TGFBI* mutations and specific corneal dystrophies [1]: p.Arg124Leu is found in Reis-Bücklers corneal dystrophy (RBCD), p.Arg555Gln leads to Thiel-Behnke corneal dystrophy (TBCD), p.Arg124Cys causes lattice corneal dystrophy type 1 (LCD1), p.Arg555Trp results in granular corneal dystrophy type 1 (GCD1), and p.Arg124His occurs in granular corneal dystrophy type 2 (GCD2) [1]. Overall, 57 mutations in the *TGFBI* gene have been associated with corneal dystrophies.

Immunohistological studies showed that wild-type TGFBIp exists mainly in the extracellular space of corneal epithelial cells [53], while mutant TGFBIp is abundant in the pathologic deposits in TGFBIp-related corneal dystrophies [53]. TGFBIp presents in both a free soluble form and a covalently bound state [57]. The soluble TGFBIp may serve a regulatory function, while the bound state TGFBIp may exhibit as anchors for cells in the ECM. Therefore,

interaction between TGFBIp and collagen is important for understanding the pathobiology of *TGFBI*-linked corneal dystrophies. However, the role of wild- and mutant-type TGFBIp in corneal epithelial cells is largely unknown, despite its clear expression in the cornea.

Over the years, our understanding of the pathogenesis of *TGFBI*-related corneal dystrophies has advanced significantly, but much remains to be learned. Currently, several surgical techniques have tried to treating visually significant deposits in corneal dystrophy patients. However, recent efforts have focused on the development of topical medications that might prevent the deposition of mutant TGFBIp and/or dissolve existing deposits. Gene therapy using RNA interference (RNAi), which can silence a disease-associated mutant allele, has been investigated to treat diseases such as corneal dystrophy. In using of RNAi for gene suppression, there are two commonly used methods: small interfering RNAs (siRNAs), and short hairpin siRNAs (shRNAs). Yuan *et al.* generated a shRNA, which was able to reduce the levels of TGFBIp in a transformed HEK 293 cell line transfected with a *TGFBI* expression plasmid [58]. Courtney *et al.* further developed an allele-specific siRNA targeting the TGFBI-Arg124Cys LCD1 variant, and this was able to reduce both the mutant *TGFBI* expression and amyloid aggregate formation *in vitro* [59]. Because TGFBIp plays multiple physiological roles, however, the non-specific nature of this siRNAs raises concerns regarding the safety of their clinical application.

The CRISPR/Cas9 system has, in recent years, been extensively applied for gene editing in various organisms [22, 28, 60, 61]. This system uses the Cas9 protein combined with a sgRNA, which together, promote targeted double-stranded breaks in the genomic DNA [26]. Genome modification by CRISPR/Cas9 has dramatically accelerated in the genomic editing field and has successfully been employed to correct the Duchenne muscular dystrophy of mouse [29]. Ultimately, gene therapy with tools such as CRISPR/Cas9 system may provide an effective treatment strategy to repair the gene sequences mutated in *TGFBI*-related corneal dystrophies.

LESCs are located in the basal layer of the corneal limbus [62, 63] and are responsible for the repair [64, 65] and maintenance of the corneal surface [66, 67]. Disease and injury can lead to a deficiency of LESCs, resulted the corneas becoming opaque, vascularized, and inflamed. Cultured LESC therapy was first described in 1997 [68], and LESCs cultured from either patients or donors have been used to successfully treat LSCD.

Corneal dystrophy is commonly caused by dominant mutations in the *TGFBI* gene, and thus, we hypothesize that this is a disease ideally suited for gene therapy with genome editing technology. Here, we co-transfected human ABCG2+/ABCB5+ double-positive LESCs with plasmids expressing the *TGFBI*-targeting sgRNA (pRGEN-U6-TGFBI) and the Cas9 protein (pRGEN-Cas9-CMV) and isolated a single *TGFBI* gene knockout LESC clone. This clone (F11) was shown to comprise a homogeneous population of cells, each of which contains two distinct *TGFBI* loci, one with an 8 bp deletion and another that has a 14 bp deletion flanked by a single point mutation. Intriguingly, both these mutations lead to a frame-shift missense mutation and generate premature stop codons downstream in exon 4. In addition, we performed whole-genome sequencing to analyze the CRISPR/Cas9-system based off-target effects. However, F11 single clone cells showed no off-target effects on the genome. Therefore, we suggest that the selected sgRNA is safe for use in the treatment of stem cells from patient's. The successful knockout of *TGFBI* was confirmed by western blot, which showed the complete lack of detectable TGFBIp expression in the F11 single LESC clone. These findings confirm the generation of a *TGFBI* knockout LESC cell line using the CRISPR/Cas9 system, and to our knowledge, our study is the first report describing the successful targeting of *TGFBI* using this technology.

Collectively, our results suggest that CRISPR/Cas9 genome editing targeting the *TGFBI* gene in human ABCG2+/ABCB5+ double-positive LESCs may be applied therapeutically in

corneal dystrophy patients. Specifically, we predict that autologous transplantation of LESC clones containing either a *TGFBI* gene knockout or a corrected *TGFBI* allele represents a feasible treatment strategy for corneal dystrophy patients. We also anticipate that the cell clones described in this report will be useful to the research community studying the pathogenesis of corneal dystrophy diseases. Importantly, the CRISPR/Cas9-mediated genome editing described here can now easily be adapted for the generation of additional *TGFBI* knockout clones in other cell lines, and future studies will be aimed at correcting the *TGFBI* gene mutations present in corneal dystrophy using advanced CRISPR/Cas9 systems. When combined with more conventional *in vitro* cell manipulation approaches, these new tools may not only facilitate the identification of both the cellular function of *TGFBI* and signaling pathways critical for corneal dystrophy diseases but also provide new treatment options for patients suffering from this disease.

Conclusions

Genome editing of *TGFBI* in human ABCG2+/ABCB5+ double-positive LESC clones by CRISPR/Cas9 may be a useful strategy to treat corneal dystrophy, and these new tools may not only facilitate the identification of both the cellular function of *TGFBI* and signaling pathways critical for corneal dystrophy diseases but also provide new treatment options for patients suffering from this disease.

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