

genes were cloned into pENTR1A vector (Invitrogen), resulting in pENTR-TERT-IRES-EGFP. The HSV-TK gene was cloned into pENTR1A vector, resulting in pENTR-TK. LR recombination reactions were performed with the entry vectors (pENTR-TERT-IRES-EGFP and pENTR-TK) and the destination vectors (pLentiNeo and pLentiPuro), respectively, and the expression vectors (pLenti-TERT-IRES-EGFP-Neo and pLenti-TK-Puro) were created. The lentivirus was produced by co-transfecting 293T cells with the lentiviral expression vector and pLP/VSVG (encoding the VSV-G envelope protein), along with the packaging constructs pLP1 and pLP2 (Invitrogen), and the concentrated lentivirus was prepared by ultracentrifugation for 2 h at 20,000 rpm.

### 2.3. Infection of lentiviral vector

TERT + EGFP + TK-transduced human corneal stromal cells were produced in two steps. First, human corneal stromal cells were infected with lentivirus (pLenti-TERT-IRES-EGFP-Neo). After infection, selection by 800 µg/mL G418 (Invitrogen) was performed and TERT + EGFP-transduced human corneal stromal cells were produced. Second, TERT + EGFP-transduced human corneal stromal cells were infected with lentivirus (pLenti-TK-Puro). After infection, selection by 1 µg/mL puromycin (Sigma) was performed and TERT + EGFP + TK-transduced human corneal stromal cells were produced. The cells were maintained in DMEM/10% FBS.

### 2.4. Ganciclovir treatment

TERT + EGFP- or TERT + EGFP + TK-transduced human corneal stromal cells ( $2 \times 10^6$  cells/cm<sup>2</sup>) were cultured in 24-well culture dishes. After 1 day culture, ganciclovir ( $0, 10^{-7}, 10^{-6}, 10^{-5},$  or  $10^{-4}$  M) was added to each cell. Viable cells were counted following trypan blue staining at 0, 2, 4, 6, 8, 10, and 12 days after the start of ganciclovir treatment. The medium containing ganciclovir was changed every 2 days. The cell numbers are shown as the percentage of each value at time 0. Each value is expressed as the mean ± standard error (SE.) of three wells.

### 2.5. Preparation of human corneal epithelial cell sheet

Corneas were obtained from an eye bank in the USA. The remaining corneal scleral rims after keratoplasty were incubated with 2.4 U/mL Dispase solution (BD Biosciences) for 1 h at 37 °C and treated with 0.02% EDTA solution (Nacalai Tesque) for 2 min at room temperature. The epithelial cells including limbal zones were scraped with sterile surgical forceps. The collected cells were incubated with 0.25% trypsin-EDTA (Invitrogen) for 15 min at 37 °C; culture medium (DMEM/F-12 [Invitrogen] with 5% FBS, 1 nM cholera toxin [Calbiochem], 10 ng/mL human recombinant epidermal growth factor [Sigma], insulin-transferrin-selenium-G supplement [Invitrogen], 100 U/mL penicillin, and 100 µg/mL streptomycin [Invitrogen]) then was added to stop the enzyme activity. The epithelial cell suspensions ( $1-7 \times 10^5$  cells/mL) were co-cultured with MMC-treated TERT + EGFP + TK-transduced human corneal stromal cells ( $2 \times 10^4$  cells/cm<sup>2</sup>) on type I collagen gel (Collagen Gel Culturing Kit; Nitta Gelatin). MMC-treated TERT + EGFP + TK transduced human corneal stromal cells were prepared by treatment with MMC (8 µg/mL) for 2 h at 37 °C. After 2 to 3 weeks in culture, the epithelial cells were treated with  $10^{-4}$  M ganciclovir for 6 or 7 days. The medium containing  $10^{-4}$  M ganciclovir was changed every 2 to 3 days.

### 2.6. PCR analysis of feeder cell contamination

After treatment with  $10^{-4}$  M ganciclovir, part of the human corneal epithelial cell sheet was harvested. TERT + EGFP + TK-transduct-

ed human corneal stromal feeder cells were collected as a control. Genome DNAs were extracted with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The PCR reaction was performed as follows. The thermal cycling conditions were 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and 10 min at 72 °C. The long terminal repeat primers were specific for lentivirus vector integrated into human corneal stromal feeder cells. The forward primer was 5'-AAGGCTAATCTACTCCAA-3', and the reverse primer was 5'-TGCCTCAGAGAGCTCTGTTT-3'. The GAPDH control primers were as follows. The forward and reverse primers were 5'-TCCAGAATCATCCTCGCTCTA-3', and 5'-TGTTGAAGTCAGAGGACACCTG-3', respectively. PCR products were electrophoresed on a 2% agarose gel.

### 2.7. Transplantation of human corneal epithelial cell sheet in mice

Animal experiments were performed according to the Guidelines for Animal Experiments of Osaka University. Eight-week-old male ICR mice were obtained from SLC. Corneas were wounded using standard protocols [18,19]. Briefly, the entire corneal surface including the limbal region was exposed for 30 s to 50% ethanol, and a superficial keratectomy was performed. A human corneal epithelial cell sheet 5 mm in diameter then was trephined and transplanted onto the cornea; the lids were closed using 8.0 nylon sutures (Alcon). Antibiotics and steroids were applied postoperatively. The mice were sacrificed 7 days after transplantation.

### 2.8. Immunohistochemistry

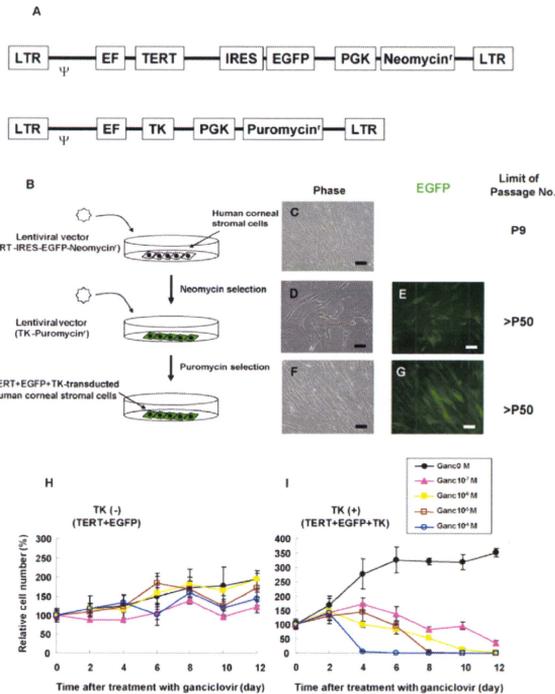
Cultivated human corneal epithelial cell sheets and mouse eyes transplanted with human corneal epithelial cell sheets were fixed with 4% paraformaldehyde in PBS for 30 min and for 3 to 4 h, respectively. After fixation, the specimens were embedded in Technovit 8100 (Heraeus Kluser) according to the manufacturer's instructions. The sections (5 µm) were processed for hematoxylin and eosin (HE) staining and indirect immunohistochemistry. The sections were incubated in 0.05% trypsin-EDTA (Invitrogen) for 10 min at 37 °C and washed in PBS. The sections then were incubated in blocking buffer (Brockcade [DS Pharma Biomedical] and 0.3% TritonX-100 in PBS) for 1 h at room temperature. The sections then were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal anti-cytokeratin 3 (1:50, Progen), and murine monoclonal anti-human mitochondria (1:10, Millipore). The secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (1:200, Invitrogen). Cell nuclei were counterstained with 0.1 µg/mL 4',6'-diamidino-2-phenylindole (DAPI, Sigma).

## 3. Results

### 3.1. Development of genetically modified human corneal stromal cells

To eliminate unknown infections and contamination from xenogenic feeder cells, three genes (the human-derived telomerase reverse transcriptase (TERT) gene, the enhanced green fluorescent protein (EGFP) gene and the herpes simplex virus thymidine kinase (HSV-TK) gene) using two lentiviral vectors (Fig. 1A) were transduced into human corneal stromal cells anatomically present in contact with a target cultured epithelial cell layer. Fig. 1B shows the developmental procedures of TERT + EGFP + TK-transduced human feeder cells. Feeder cells infected with two lentiviral vectors exhibited the same fibroblast-like morphology and contact inhibition as primary human corneal stromal cells (Fig. 1C, D and F).

We then studied the proliferation and visualization of human feeder cells transduced with the TERT and EGFP genes. The primary human corneal stromal cells had a limited lifespan and were



**Fig. 1.** Development of human genetically modified feeder cells. (A) Lentiviral vectors used to express *TERT*, *EGFP*, and *TK*. LTR, long terminal repeat; Ψ, packaging signal; EF, human elongation factor 1α subunit) gene promoter; neomycin', neomycin resistance gene; TK, herpes simplex virus thymidine kinase; puromycin', puromycin resistance gene. (B) Methods of development of genetically modified feeder cells. (C) The primary human corneal stromal cells. (D, E) *TERT* + *EGFP*-transduced human corneal stromal cells. (F, G) *TERT* + *EGFP* + *TK*-transduced human corneal stromal cells. Scale bar, 100 μm. (H, I) The time course of effects of ganciclovir (ganc) on *TERT* + *EGFP*- (H) and *TERT* + *EGFP* + *TK*- (I) transduced human corneal stromal cells.

not maintained over nine passages; however, *TERT* + *EGFP*- and *TERT* + *EGFP* + *TK*-transduced human feeder cells were maintained over 50 passages by the *TERT* gene and were visualized by the *EGFP* gene (Fig. 1E and G).

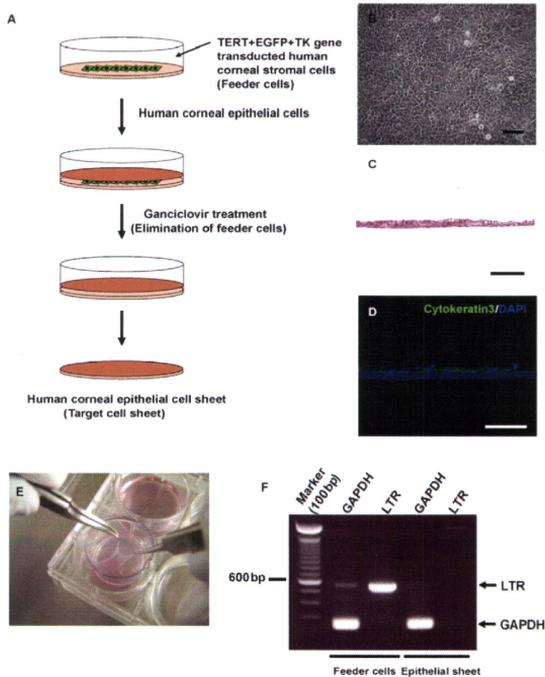
We also investigated the susceptibility of human feeder cells transduced with the *TK* gene to ganciclovir, which has specific cytotoxicity to cells expressing the *TK* gene. *TERT* + *EGFP*-transduced human corneal stromal cells (cells without the *TK* gene) were grown in five ganciclovir concentrations ( $0$ ,  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ , or  $10^{-4}$  M; there was no effect of ganciclovir on growth (Fig. 1H)). However, *TERT* + *EGFP* + *TK*-transduced human feeder cells were killed by ganciclovir in a dose-dependent manner (Fig. 1I). In the  $10^{-4}$  M ganciclovir group, all cells died 6 days after treatment with or without mitomycin C treatment (Supplementary Fig. S1A–D). We also investigated the characteristics of *TERT* + *EGFP* + *TK*-transduced human feeder cells after long-term passage.

*TERT* + *EGFP* + *TK*-transduced cells over 50 passages (52nd cell passage) have fibroblastic morphology (Supplementary Fig. S2A), are visualized by *EGFP* expression (Supplementary Fig. S2B), and are sensitive to treatment with ganciclovir for 6 days (Supplemen-

tary Fig. S2C). These results indicated that *TERT* + *EGFP* + *TK*-transduced human corneal stromal cells are immortalized by the *TERT* gene, visualization by the *EGFP* gene, and killed by the *TK* gene.

### 3.2. Human corneal epithelial cell sheet cultured on genetically modified human corneal stromal feeder cells

Fig. 2A shows the harvesting procedures for target cell sheet cultured on genetically modified feeder cells. Human corneal epithelial cells were co-cultured in direct contact with MMC-treated genetically modified (*TERT* + *EGFP* + *TK*-transduced) human feeder cells for 2 to 3 weeks. A target epithelial cell sheet was harvested after killing and eliminating the genetically modified feeder cells using ganciclovir for 6 or 7 days. Human corneal epithelial cells with a squamous shape could be cultured on genetically modified feeder cells (Fig. 2B). Cultivated corneal epithelial cell sheets formed five to seven layers in a hematoxylin and eosin (HE) staining study (Fig. 2C) and expressed cytokeratin 3, a specific marker of corneal epithelial cells, in an immunohistochemical study (Fig. 2D). These results suggested that human corneal epithelial cells



**Fig. 2.** Target cell sheet production on genetically modified feeder cells and transplantation *in vivo*. (A) Methods of harvest of human corneal epithelial cell sheet. (B) Microphotographs of human corneal epithelial cells prepared on genetically modified human corneal stromal feeder cells. Scale bar, 100  $\mu$ m. (C, D) Histology of a human corneal epithelial cell sheet. A human corneal epithelial cell sheet is stained with HE (C) and cytokeratin 3 antibody (D). The nuclei are counterstained with DAPI (D). Scale bar, 100  $\mu$ m. (E) A human corneal epithelial cell sheet is harvested easily with surgical forceps. (F) PCR analysis of a corneal epithelial cell sheet for contamination of genetically modified feeder cells. PCR is performed against a long terminal repeat (LTR) sequence (554 bp) of lentiviral vector integrated into genetically modified feeder cells and a GAPDH sequence (256 bp) as a positive control.

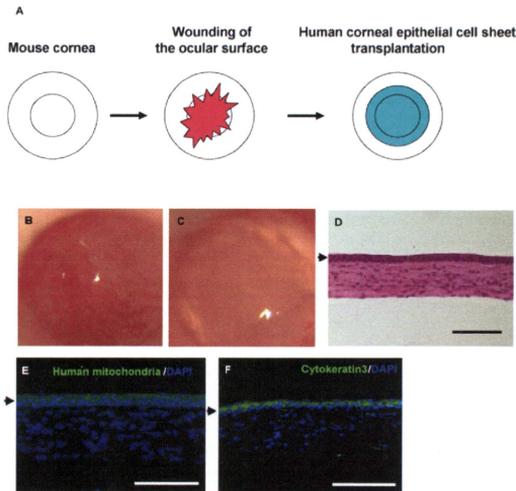
produced by direct contact culture with genetically modified feeder cells are similar to those observed *in vivo*. Moreover, the cultivated human corneal epithelial cell sheets, produced by direct contact culture with genetically modified feeder cells, were harvested easily with surgical forceps (Fig. 2E).

We then investigated contamination of genetically modified feeder cells in harvested target cell sheet. Visually confirmation of *EGFP*, or PCR analysis against the long terminal repeat (LTR) sequence of lentiviral vector integrated into the genetically modified feeder cells showed that the harvested corneal epithelial cell sheets had no contaminated genetically modified feeder cells (Fig. 2F). These results suggested that human corneal epithelial cell sheets can be prepared on genetically modified feeder cells and harvested without contaminating the feeder cells.

### 3.3. Cultivated human corneal epithelial cell sheet transplantation *in mice*

We investigated whether human corneal epithelial cell sheets produced by direct contact culture with genetically modified feeder cells can be transplanted onto the ocular surface *in vivo*.

Fig. 3A shows the transplantation procedures for cultivated human corneal epithelial cell sheets. The murine ocular surface including the limbal region, which includes corneal stem cells, was wounded by 50% ethanol. The wounded corneal surfaces were covered by conjunctival cells with vascularization and severe opacity (Fig. 3B). The cultivated human corneal epithelial cell sheet was transplanted just after removing the corneal epithelial layer. The transplanted corneal surfaces were transparent and there was no migration of conjunctival cells with vascularization 7 days after transplantation (Fig. 3C). HE staining showed three to five layers of cells in the corneal epithelial layer (Fig. 3D) and confirmed that those cells were transplanted human corneal epithelial cells, and immunohistochemical examinations were performed against human mitochondria and cytokeratin 3. In the murine eyes transplanted with human corneal epithelial cell sheets, both human mitochondria (Fig. 3E) and cytokeratin 3 (Fig. 3F) were positive, and the epithelial cells on the murine cornea were derived from human and mature. These results suggested that human corneal epithelial cell sheets produced by direct contact culture with genetically modified feeder cells could be applied *in vivo*.



**Fig. 3.** Human corneal epithelial cell sheet transplantation in mice. (A) A schematic of human corneal epithelial cell sheet transplantation in mice. (B) A photograph of a murine cornea after wounding of the ocular surface. (C) A photograph of a murine cornea 7 days after transplantation. (D–F) Histology of a murine cornea 7 days after transplantation of a human corneal epithelial cell sheet on a denuded ocular surface. The transplanted mouse cornea is stained with HE (D), and human mitochondria (E) and cytokeratin 3 antibodies (F). The nuclei are counterstained with DAPI (E, F). The arrowheads indicate a transplanted human corneal epithelial cell sheet. Scale bar, 100  $\mu$ m.

#### 4. Discussion

A new culture technique reported here produced human eliminable feeder-assisted target cell sheets that use human eliminable feeder cells with gene modification-transduced *TERT*, *EGFP*, and *TK* genes for immortalization, labeling, and suicide as needed. Using this technique, target cell sheets could be produced as one sheet and successfully transplanted *in vivo* in a corneal model.

To use human corneal stromal cells as feeder cells, obtaining a large number of corneal stromal cells is more desirable for preparing the feeder cells. We transduced the *TERT* gene as an immortalization gene into the corneal stromal cells and expected unlimited propagation of *TERT*-transduced cells. Our data showed the *TERT*-transduced corneal stromal cells were maintained for more than 50 passages; stromal cells without *TERT* gene transduction were maintained for fewer than nine passages.

Previous reports have shown that telomerase expression in human somatic cells does not induce changes associated with malignant transformation [20,21]. The current data showed that genetically modified human corneal stromal cells, including cells after long-term passages, possessed the same characteristics of fibroblastic morphology and contact inhibition as primary human corneal stromal cells and that the *EGFP* and *TK* genes also were expressed in feeder cells that underwent long-term passage. Based on these results, genetically modified feeder cells did not show risks of becoming cancerous. Therefore, the genetically modified feeder cells that we produced are a safe and stable immortalized cell line. Moreover, these genetically modified feeder cells can be preserved frozen and are accessible whenever needed.

When target cell sheets produced by direct contact culture with genetically modified feeder cells are transplanted *in vivo*, genetically modified feeder cells should not be contaminated in target cell

sheets. Therefore, we focused on the *EGFP* gene, a marker gene to visually confirm feeder cells based on the *EGFP* color, and the *TK* gene, a suicide gene, to eliminate feeder cells using ganciclovir. We transduced the *EGFP* and *TK* genes into the human corneal stromal cells and visually confirmed contamination of *EGFP*-transduced feeder cells and complete elimination of *TK*-transduced feeder cells by ganciclovir when they were unneeded. Namely, we produced corneal epithelial cell sheets by direct contact culture with genetically modified human corneal stromal feeder cells, because genetically modified feeder cells can be eliminated by ganciclovir. Moreover, PCR analysis showed that the harvested corneal epithelial cell sheets had no contaminated genetically modified feeder cells.

We have successfully performed the sheet culture of not only corneal epithelial cells, but also human corneal endothelial cells, conjunctival epithelial cells, fibroblast cells, or something in direct contact with genetically modified feeder cells (data not shown), indicating that they have the possibility of producing various cell sheets that may be applied to various disease models.

To use the cell source with lentiviral gene transduction for practical transplantation remains to be controversial because of its biological risks and ethical issues, however, no lentiviral components such as long terminal repeats (LTR) were detected in the harvested cell sheets for practical transplantation (Fig. 2F). In addition, auto-transplantation without gene transduction is desirable to avoid immunogenic rejection. Although these eliminable feeder cells are derived from allogenic immortalized cells and lentivirally modified with gene transduction, only autogenic target cell sheet for practical transplantation can be harvested after complete removal of these feeder cells without lentiviral components. Therefore, this culture method, using these genetically modified feeder cells through lentiviral gene transfer, does not have ethical and immunogenic problems, validating that our technique is clinically safe.

This culture method may be applicable for producing any cultivated cell production that needs feeder cells, such as embryonic stem cells [22–24] or induced pluripotent stem cells [25,26]. Furthermore, by introducing stimulating genes, such as *growth factor* or *differentiation factor gene*, into these feeder cells, we can proliferate target cells that do not proliferate and induce stem cells that cannot be differentiated into target cells. Genetically modified human eliminable feeder cells could be new promising tools for cultivated cell sheet transplantation.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.079.

#### References

- G.G. Gallico 3rd, N.E. O'Connor, C.C. Compton, et al., Permanent coverage of large burn wounds with autologous cultured human epithelium, *N. Engl. J. Med.* 311 (1984) 448–451.
- M. Yamato, M. Utsumi, A. Kushida, et al., Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without disperse by reducing temperature, *Tissue Eng.* 7 (2001) 473–480.
- I.R. Schwab, Cultured corneal epithelia for ocular surface disease, *Trans. Am. Ophthalmol. Soc.* 97 (1999) 891–896.
- N. Koizumi, T. Inatomi, A.J. Quantock, et al., Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits, *Cornea* 19 (2000) 65–71.
- R.J. Tsai, L.M. Li, J.K. Chen, Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells, *N. Engl. J. Med.* 343 (2000) 86–93.
- N. Koizumi, T. Inatomi, T. Suzuki, et al., Cultivated corneal epithelial stem cell transplantation in ocular surface disorders, *Ophthalmology* 108 (2001) 1569–1574.
- P. Rama, S. Bonini, A. Lambiase, et al., Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency, *Transplantation* 72 (2001) 1478–1485.
- J. Shimazaki, M. Aiba, E. Goto, et al., Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders, *Ophthalmology* 109 (2002) 1285–1290.
- K. Nishida, M. Yamato, Y. Hayashida, et al., Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface, *Transplantation* 77 (2004) 379–385.
- K. Nishida, M. Yamato, Y. Hayashida, et al., Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium, *N. Engl. J. Med.* 351 (2004) 1187–1196.
- T. Sumide, K. Nishida, M. Yamato, et al., Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces, *FASEB J.* 20 (2006) 392–394.
- Y. Shiroyanagi, M. Yamato, Y. Yamazaki, et al., Urothelial regeneration using viable cultured urothelial cell sheets grafted on demucosalized gastric flaps, *BJU Int.* 93 (2004) 1069–1075.
- S. Miyagawa, Y. Sawa, S. Sakakida, et al., Tissue cardiomyoplasty using bioengineered contractile cardiomyocyte sheets to repair damaged myocardium: their integration with recipient myocardium, *Transplantation* 80 (2005) 1586–1595.
- M.J. Martin, A. Muotri, F. Gage, et al., Human embryonic stem cells express an immunogenic nonhuman sialic acid, *Nat. Med.* 11 (2005) 228–232.
- H. Miyashita, S. Shimamura, K. Higa, et al., A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations, *Tissue Eng. Part A* 14 (2008) 1275–1282.
- H. Kawasaki, K. Mizuseki, S. Nishikawa, et al., Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity, *Neuron* 28 (2000) 31–40.
- H. Miyoshi, U. Blomer, M. Takahashi, et al., Development of a self-inactivating lentivirus vector, *J. Virol.* 72 (1998) 8150–8157.
- G. Cotsarelis, S.Z. Cheng, G. Dong, et al., Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells, *Cell* 57 (1989) 201–209.
- F. Majo, A. Rochat, M. Nicolas, et al., Oligopotent stem cells are distributed throughout the mammalian ocular surface, *Nature* 456 (2008) 250–254.
- X.R. Jiang, G. Jimenez, E. Chang, et al., Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype, *Nat. Genet.* 21 (1999) 111–114.
- C.P. Morales, S.E. Holt, M. Ouellette, et al., Absence of cancer-associated changes in human fibroblasts immortalized with telomerase, *Nat. Genet.* 21 (1999) 115–118.
- J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, et al., Embryonic stem cell lines derived from human blastocysts, *Science* 282 (1998) 1145–1147.
- B.E. Reubinoff, M.F. Pera, C.Y. Fong, et al., Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro, *Nat. Biotechnol.* 18 (2000) 399–404.
- S.E. Lanzendorf, C.A. Boyd, D.L. Wright, et al., Use of human gametes obtained from anonymous donors for the production of human embryonic stem cell lines, *Fertil. Steril.* 76 (2001) 132–137.
- K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* 126 (2006) 663–676.
- K. Takahashi, K. Tanabe, M. Ohnuki, et al., Induction of pluripotent stem cells from adult human fibroblasts by defined factors, *Cell* 131 (2007) 861–872.

# Fluoroquinolone antibacterial eye drops: effects on normal human corneal epithelium, stroma, and endothelium

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**Background:** In vitro studies have suggested the corneal cytotoxicity of third-generation fluoroquinolone levofloxacin (LVFX) and fourth-generation fluoroquinolone moxifloxacin hydrochloride (MFLX) among fluoroquinolone antibacterial eye drops. This study investigated the effects of these two eye drops on the human cornea in vivo.

**Methods:** We evaluated 30 healthy adults (19 men and 11 women,  $38.3 \pm 6.3$  years old). Each subject received an LVFX ophthalmic solution 0.5% in one eye and an MFLX ophthalmic solution 0.5% in the other eye three times daily for 7 days. Functional and morphological corneal changes before and after instillation were evaluated through ophthalmic examinations including breakup time of tear film (BUT) as measured by fluorescein staining and DR-1, Schirmer I test, Heidelberg Retina Tomograph II Rostock Cornea Module (HRTII-RCM), specular microscope, and Pentacam examination.

**Results:** Both the LVFX and MFLX groups had no significant change in each examination before and after instillation. There was also no statistically significant difference in measurements after the 7-day instillation between the groups.

**Conclusion:** Our study results suggest that as with LVFX, MFLX used in a normal clinical setting is unlikely to cause any obvious adverse effects on human normal cornea.

**Keywords:** cornea, fluoroquinolones, ocular surface, toxicity

## Introduction

Fluoroquinolone antibacterial eye drops are a broad spectrum antibacterial that effectively inhibit two enzymes, bacterial topoisomerase IV and topoisomerase II (DNA gyrase). They are widely used for ocular infections and perioperative prophylaxis in ophthalmic surgery because of these advantages.

Fluoroquinolones are relatively safe, although they have shown inhibitory activity against mammalian topoisomerase II, which is related to bacterial DNA gyrase.<sup>1</sup> It is therefore also possible that fluoroquinolone antibacterial eye drops have some influence on corneal cells. In vitro studies of fluoroquinolone using human corneal keratocyte cultures suggest that levofloxacin (LVFX) and moxifloxacin (MFLX) may be cytotoxic.<sup>2</sup> However, the effects of these drugs in vivo remain to be determined.

In the present study, using a moxifloxacin ophthalmic solution 0.5% (MFLX) (Vegamox; Alcon Japan, Tokyo, Japan), a fourth-generation fluoroquinolone with excellent aqueous penetration<sup>3,4</sup> and a levofloxacin ophthalmic solution 0.5% (LVFX) (Cravit, Santen Pharmaceutical, Osaka, Japan), a third-generation fluoroquinolone used widely in Japan, we investigated the effects of these fluoroquinolone antibacterial eye drops on normal human cornea in terms of the break-up time of tear film (BUT) and

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the morphological appearance of the corneal epithelium, stroma, and endothelium.

## Methods

The protocol for this research project has been approved by the Ethics Committee of Tohoku University Graduate School of Medicine and it conforms to the provisions of the Declaration of Helsinki in 1995. This study gave informed consent and patient anonymity was preserved. We recruited 40 healthy adult volunteers from the public at the Department of Ophthalmology, Tohoku University Graduate School of Medicine. Among them, 30 volunteers were judged eligible for participation through the screening test and, after providing informed consent, they were randomly assigned to two groups. Subjects in one group received MFLX in their left eye or LVFX in their right eye and those in the other group received MFLX in their right eye or LVFX in their left eye. The study was conducted in a single blind fashion where only the investigator was masked to the allocated study eye drops.

Subjects received one drop of MFLX or LVFX in either eye three times a day for 7 days. Before and after instillation of the study eye drops, the effects of each study drug on the corneal epithelium, stroma, and endothelium were evaluated through the method described later in this paper.

The exclusion criteria for this study included a history of hypersensitivity to fluoroquinolones, corneal epithelial defects (including superficial punctate keratopathy (SPK)), conjunctivitis, a history of herpetic keratitis, abnormal lacrimation (a Schirmer I test measurement of 5 mm or less or a BUT measurement of 5 seconds or less), use of any antibacterial eye drops within 1 week of study entry, use of eye drops other than the study eye drops, use of contact lenses within 2 weeks of study entry, intraocular pressure of 21 mmHg or more, inability of the subject to provide written informed consent, and any other reason judged ineligible for participation in the study by the investigator.

## Study eye drops

The eye drops used in the present study were MFLX and LVFX, both of which did not contain benzalkonium chloride and are commercially available in Japan.

## Evaluations

Two weeks before study entry and on the last day of study (after 7 days of instillation), subjects underwent a general ocular examination including slit-lamp microscopy, evaluation of the influence on the corneal

epithelium by fluorescein staining,<sup>5</sup> BUT measurement with fluorescein staining and with a DR-1 (Tear film lipid layer interferometry system, Kowa, Nagoya, Japan), and a Schirmer I test. In addition to these tests, corneal function and morphological changes were observed using a specular microscope (Topcon SP-3000F, Topcon Ltd, Tokyo, Japan), Pentacam (Oculus, Wetzlar, Germany), and Heidelberg Retina Tomograph II Rostock Cornea Module (HRTII-RCM) (Heidelberg Engineering, Dossenheim, Germany). On the last day of the study, treatment compliance and the development of any adverse events were confirmed. In addition, subjects were asked to complete a questionnaire to assess their satisfaction with the study eye drops (irritating sensation and drop comfort upon instillation) using a visual analog scale (VAS) (0 to 100 mm). Table 1 summarizes the observation items and details of each item.

The DR-1 is a system designed to observe interference colors generated by a specular light from the tear film lipid layer and classify the status of dry eye into five grades for evaluation. Normal eyes exhibit a gray interference color that is distributed uniformly, whereas dry eyes frequently show two or more interference colors (Grade 3, 4, and 5). In severe dry eyes, tear production with blinking is inadequate and the corneal surface is partially exposed (Grade 5).<sup>6</sup> HRTII-RCM allows noninvasive in vivo observation of the corneal microstructure including epithelium, stroma, and endothelium.<sup>7</sup> The range of the captured HRTII-RCM picture was 400 × 400 μm, and the resolution power of the image was 384 × 384 pixels. The Pentacam is a rotating single Scheimpflug camera system. It computes anterior and posterior curvatures and provides complete corneal pachymetry of the entire cornea.

## Statistical analysis

Subject data were entered and analyzed using the following software: Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA) and JMP Version 6 (SAS Institute Japan Ltd, Tokyo, Japan).

Comparison of the two study eye drops was performed by the paired *t*-test. Measurements are represented in mean ± standard deviation. The grading of the tear film lipid layer was made with the Mann–Whitney *U*-test. Comparison of the patients with epithelial defects before and after treatment was performed using Fisher's exact test.

## Results

The 30 subjects consisted of 19 men and 11 women (mean age ± standard deviation: 38.3 ± 6.3 years). Throughout the

**Table 1** The observation items and details of each item

Observation item	Timing of observation		Observation of cornea			Endothelium
	2 weeks before study entry	After 7-days of instillation	Epithelium	Stroma	Evaluation	
Informed consent	○					
Subject background	○					
Concomitant drugs	○	○				
Visual acuity test	○	○				
Treatment compliance	○	○				
Slit-lamp microscopy	○	○	✓			Observation of the corneal epithelium
Anterior-segment pictures (fluorescein staining) (BUT)	○	○	✓			Evaluation of the influence on the corneal epithelium
Specular microscope	○	○			✓	Observation of the corneal endothelium. Measurement of endothelial cell density, and coefficient of variation (CV)
DR-1 (BUT)	○	○	✓			Evaluation of influence on the corneal epithelium based on tear film lipid layer grading
Schirmer I test (BUT)	○	○	✓			Lacrimation function based on tear volume
Pentacorn (Corneal thickness)	○	○		✓		Measurement of corneal thickness (influence on the corneal endothelium)
HRT II-RCM	○	○		✓		Morphological observation of the corneal stroma
Adverse events	○	○				Evaluation of cell density
Questionnaire to assess satisfaction with use		○				Assessment of irritating sensation and drop comfort upon instillation using a VAS

study, they had favorable treatment compliance and each subject instilled the assigned study eye drops reliably.

## Influence on the corneal epithelium BUT

BUT scores measured by fluorescein staining before and after the 7 days of instillation were  $13.8 \pm 5.8$  seconds and  $11.9 \pm 6.5$  seconds, respectively, for eyes instilled with MFLX (MFLX group) and  $13.6 \pm 5.4$  seconds and  $10.4 \pm 6.0$  seconds, respectively, for eyes instilled with LVFX (LVFX group). No significant change was observed in either group before or after the 7 days of instillation, and no statistically significant difference between the groups was observed in measurements after the 7 days of instillation ( $P = 0.4376$ , *t*-test).

BUT scores measured by DR-1 before and after the 7-day instillation were  $29.0 \pm 12.4$  seconds and  $22.8 \pm 13.6$  seconds, respectively, for the MFLX group and  $29.4 \pm 14.2$  seconds and  $22.3 \pm 14.3$  seconds, respectively, for the LVFX group. No significant change was observed in either group before or after the 7-days of instillation, and no statistically significant difference between groups was observed in measurements after the 7-days of instillation ( $P = 0.8795$ , *t*-test).

Grades of the tear film lipid layer using the DR-1 before and after the 7 days of instillation were  $2.2 \pm 0.5$  and  $2.8 \pm 0.6$ , respectively, for the MFLX group and  $2.1 \pm 0.6$  and  $2.8 \pm 0.4$ , respectively, for the LVFX group. No statistically significant difference between the groups was observed in grades after the 7-days of instillation ( $P = 0.9709$ , *U*-test). Changes in the grade were seen in 56.7% of subjects (17/30) in the MFLX group and in 66.7% of subjects (20/30) in the LVFX group, all changes were slight.

Schirmer I test scores before and after the 7 days of instillation were  $20.9 \pm 9.3$  mm and  $18.4 \pm 11.2$  mm, respectively, for the MFLX group and  $19.3 \pm 9.9$  mm and  $19.2 \pm 11.3$  mm, respectively, for the LVFX group. No significant change was observed in either group before or after the 7 days of instillation, and no statistically significant difference between the groups was observed in scores after the 7 days of instillation ( $P = 0.3325$ , *t*-test).

## Corneal epithelial defects according to area and density grading

The severity of superficial punctate keratitis (SPK) can be classified into nine grades according to the area and density (AD) grading which is the combination of the area (A) and density (D)

Group	Before instillation	After 7 days	<i>P</i> -value
<b>MFLX</b>	0/30	8/30	0.675
<b>LVFX</b>	0/30	7/30	

AD grading of each eye

LVFX		MFLX	
Before instillation	After 7 days	Before instillation	After 7 days
A0D0	A1D1	A0D0	A1D1
A0D0	A1D1	A0D0	A1D1
A0D0	A1D1	A0D0	A1D1
A0D0	A1D1	A0D0	A1D1
A0D0	A1D1	A0D0	A1D1
A0D0	A1D1	A0D0	A1D1
A0D0	A1D1	A0D0	A1D1
		A0D0	A1D1

**Figure 1** Corneal epithelial defects according to AD grading. After 7 days of instillation, corneal epithelial defects were observed in eight eyes of the subjects receiving MFLX and seven eyes of the subjects receiving LVFX. AD grading of the 15 eyes was A1D1 for 14 eyes and A2D1 for the remaining eye. No statistically significant difference between the groups was observed in the incidence of epithelial defects ( $P = 0.675$ , *t*-test).

of the lesion stained as dots by fluorescein staining (Figure 1).<sup>7</sup> In this study, the severity of corneal epithelial defects was evaluated using AD grading. Before instillation, corneal epithelial defects were not observed in any subject of either group, however, after the 7 days of instillation, corneal epithelial defects were observed in eight eyes of the subjects receiving MFLX and seven eyes of the subjects receiving LVFX. Among these subjects, four had mild epithelial defects in both eyes. AD grading of the 15 eyes was A1D1 for 14 eyes and A2D1 for the remaining eye. There was a statistically significant difference between groups before and after the instillation of MFLX ( $P = 0.0046$ , Fisher's exact test). In addition, there was a statistically significant difference between groups before and after the instillation of LVFX ( $P = 0.0105$ , Fisher's exact test). No statistically significant difference between the groups was observed in the incidence of epithelial defects ( $P = 0.675$ , *t*-test).

### Influence on the corneal stroma

#### Cell density of the corneal stroma

Changes in cell density of the superficial, middle, and deep layers of the corneal stroma were observed.

Cell density of the superficial layer of the corneal stroma before and after the 7 days of instillation was  $643 \pm 53$  cell/mm<sup>2</sup> and  $633 \pm 42$  cell/mm<sup>2</sup>, respectively, for the MFLX group and  $625 \pm 42$  cell/mm<sup>2</sup> and  $630 \pm 36$  cell/mm<sup>2</sup>, respectively, for the LVFX group. No statistically significant difference between the groups was observed in measurements after the 7 days of instillation.

Cell density of the middle layer of the corneal stroma before and after the 7 days of instillation was  $488 \pm 20$  cell/mm<sup>2</sup> and  $487 \pm 22$  cell/mm<sup>2</sup>, respectively, for the MFLX group and  $481 \pm 26$  cell/mm<sup>2</sup> and  $485 \pm 25$  cell/mm<sup>2</sup>, respectively, for the LVFX group. No statistically significant difference

between the groups was observed in measurements after the 7 days of instillation.

Cell density of the deep layer of the corneal stroma before and after the 7 days of instillation was  $428 \pm 22$  cell/mm<sup>2</sup> and  $421 \pm 21$  cell/mm<sup>2</sup>, respectively, for the MFLX group and  $423 \pm 27$  cell/mm<sup>2</sup> and  $424 \pm 25$  cell/mm<sup>2</sup>, respectively, for the LVFX group. No statistically significant difference between the groups was observed in measurements after the 7 days of instillation.

No significant change was observed in any layer before or after the 7 days of instillation, and no statistically significant difference between the groups was observed in measurements after the 7 days of instillation.

No Langerhans cells or inflammatory cells appeared in any of the layers.

## Influence on the endothelium

### Endothelial cell density

Endothelial cell density before and after the 7 days of instillation was  $2817.9 \pm 250.2$  cell/mm<sup>2</sup> and  $2778.0 \pm 221.5$  cell/mm<sup>2</sup>, respectively, for the MFLX group and  $2816.1 \pm 255.7$  cell/mm<sup>2</sup> and  $2808.9 \pm 261.9$  cell/mm<sup>2</sup>, respectively, for the LVFX group. No significant change was observed in either group before or after the 7 days of instillation, and no statistically significant difference between the groups was observed in measurements after the 7 days of instillation ( $P = 0.3195$ , *t*-test).

In addition, the coefficient of variation (CV) before and after the 7 days of instillation was  $29.9\% \pm 5.3\%$  and  $30.3\% \pm 5.1\%$ , respectively, for the MFLX group and  $30.9\% \pm 6.7\%$  and  $29.7\% \pm 5.2\%$ , respectively, for the LVFX group. No significant change was observed in either group before or after the 7 days of instillation, and no statistically significant difference between the groups was observed in measurements after the 7 days of instillation ( $P = 0.2295$ , *t*-test).

### Measurement of corneal thickness

Corneal thickness measured with Pentacam before and after the 7 days of instillation were  $561.9 \pm 38.6$   $\mu$ m and  $561.8 \pm 39.2$   $\mu$ m, respectively, for the MFLX group and  $558.4 \pm 37.6$   $\mu$ m and  $559.5 \pm 39.7$   $\mu$ m, respectively, for the LVFX group. No major change was observed in either group before or after the 7 days of instillation, and no statistically significant difference between the groups was observed in measurements after the 7 days of instillation ( $P = 0.6305$ , *t*-test).

## Safety

No serious adverse events were reported in either group. No clinically important changes in general ocular findings were also noted.

## Satisfaction with the study eye drops

All 30 subjects were asked to complete a questionnaire to assess their satisfaction with the study eye drops (irritating sensation and drop comfort upon instillation) using a VAS (0 to 100 mm). The mean value (95% confidence interval) of "irritating sensation" was 23.0 mm (14.1–31.8 mm) for MFLX and 31.0 mm (22.4–39.6 mm) for LVFX, showing a trend slightly favoring MFLX, however, no statistically significant difference was noted ( $P = 0.1658$ , Wilcoxon rank-sum test). The mean value (95% confidence interval) of "drop comfort upon instillation" was 20.5 mm (12.9–28.1 mm) for MFLX and 25.7 mm (17.1–34.4 mm) for LVFX, also showing a trend slightly favoring MFLX, however, no statistically significant difference was noted ( $P = 0.4539$ , Wilcoxon rank-sum test).

## Discussion

Although LVFX is a third-generation fluoroquinolone with a relatively broad spectrum of antibacterial activity, it has the disadvantage of being less active against gram-positive cocci. The minimum inhibitory concentrations of fourth-generation fluoroquinolones, MFLX and gatifloxacin (GFLX), against staphylococci and streptococci are higher than those of LVFX, and these fluoroquinolones are characterized by their broader antibacterial spectrum. In particular, since MFLX is known to have an approximately two- to three-fold lower ocular penetration than other antibacterial eye drops,<sup>3,4</sup> this drug is expected to provide excellent clinical benefits. The superior penetration of MFLX is considered to be due to its unique physicochemical structure that combines high lipophilicity for enhanced corneal penetration with high aqueous solubility at physiological pH.<sup>3,4</sup> However, it has not yet been clearly defined whether its excellent penetration could have some influence on the cornea.

The effect of fourth-generation fluoroquinolones, Zymar<sup>®</sup> (gatifloxacin ophthalmic solution, Allergan, Inc, Irvine, CA) and VIGAMOX<sup>®</sup> (moxifloxacin ophthalmic solution; Alcon Laboratories, Inc, Fort Worth, TX), on the corneal epithelial cells and intercellular space was investigated in an in vivo study using rabbits, evaluating the effect on tight junctions using ZO-1 immune staining. It was reported that in rabbits treated with VIGAMOX<sup>®</sup>,

corneal epithelial cell integrity was maintained with no effect on tight junctions, whereas in rabbits treated with Zymar<sup>®</sup>, decreased corneal epithelial cells and breakdown of tight junctions were observed, suggesting that benzalkonium chloride, an additive in Zymar<sup>®</sup>, was responsible for this effect.<sup>8</sup> In the present study to objectively evaluate the in vivo effect of the fluoroquinolone eye drops in normal human eyes, although some subjects experienced mild corneal epithelial defects according to the AD grading, no statistically significant difference was observed between the two eye drops, LVFX and MFLX. Moreover, no obvious adverse effects of either drug on the corneal epithelium, stroma, and endothelium were noted in other evaluations. Furthermore, there were no issues concerning safety or subjective satisfaction with use of the study eye drops.

In vitro studies using cell cultures of human corneal keratocyte reported based on the proportion of concentration-time exposures in which fluoroquinolones were cytotoxic, ciprofloxacin was the most cytotoxic to human corneal keratocyte (96%), followed by MFLX (67%), ofloxacin (62.5%), GFLX (58%), and LVFX (42%) concluding that fluoroquinolones may differ in their cytotoxic potential to human corneal keratocytes.<sup>9</sup> However, this study revealed that there was no statistically significant difference between the groups before and after the 7 days of instillation. We also find that no Langerhans cells or inflammatory cells displayed in any of the stromal layers. These results suggest that both LVFX and MFLX affect no toxicity to the cornea. In addition, it is difficult to consider that MFLX has some influence on the corneal stroma under normal conditions. In the present study, we instilled the fluoroquinolone antibacterial eye drops into normal human eyes, and have concluded that these fluoroquinolone, used in a normal clinical setting, are less likely to cause such histological changes due to cytotoxicity. Therefore, the use of fluoroquinolone eye drops on the normal cornea for sterilization before intraocular surgery or prophylaxis against postoperative infection should not raise concerns about cytotoxicity in corneal cells.

Only healthy volunteers were included in this study. Thus, there are limitations in applying our findings to patients with dry eyes or abnormal epithelia. Furthermore, our results may have been different if the frequency of instillation had been increased (eg, instillations every hour for corneal ulcers). In severe cases with corneal abnormalities, the effects of antibiotics should be checked carefully. There may be limitations in comparing two antibiotics using some examinations with a high degree of variability in this

study, such as BUT. However, there is no clear difference between two antibiotics in the series of our routine eye examinations here.

A randomized comparative study of MFLX 1% ophthalmic solution, in which the concentration is twice that of the product marketed in Japan, in patients with bacterial keratitis, the safety of MFLX was comparable to the comparators, OFLX 0.3% ophthalmic solution and tobramycin (1.33%)/cephazolin (5%) ophthalmic solution, and the time to cure in non-exiting patients tended to be shorter for MFLX than for OFLX.<sup>9</sup> Moreover, a study of MFLX 0.5% ophthalmic solution instilled into the anterior chamber at the time of cataract surgery reported no statistically significant differences in corneal endothelial cell density after surgery or in corneal thickness after 1 month.<sup>10</sup> It is inferred, based on the results of these studies, that high concentrations of MFLX in the corneal tissue would not cause any clinical adverse effects.

## Conclusion

Although minor corneal SPK was observed in approximately 25% of cases, no adverse corneal effects appeared between the 2 antibiotics upon examination with instillation of LVFX and MFLX three times daily over 7 days.

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## Disclosure

The authors report no conflicts of interest in this work.

## References

1. Hussy P, Maass G, Tummeler B, Grosse F, Schomburg U. Effect of 4-quinolones and novobiocin on calf thymus DNA polymerase alpha primase complex, topoisomerases I and II, and growth of mammalian lymphoblasts. *Antimicrob Agents Chemother*. 1986;29:1073-1078.
2. Bezwada P, Clark LA, Schneider S. Intrinsic cytotoxic effects of fluoroquinolones on human corneal keratocytes and endothelial cells. *Curr Med Res Opin*. 2008;24:419-424.
3. O'Brien TP, Arshinoff SA, Mah FS. Perspectives on antibiotics for postoperative endophthalmitis prophylaxis: potential role of moxifloxacin. *J Cataract Refract Surg*. 2007;33:1790-1800.
4. Robertson SM, Curtis MA, Schleich BA, et al. Ocular pharmacokinetics of moxifloxacin after topical treatment of animals and humans. *Surv Ophthalmol*. 2005;50 Suppl 1:S32-S45.
5. Miyata K, Amano S, Sawa M, Nishida T. A novel grading method for superficial punctate keratopathy magnitude and its correlation with corneal epithelial permeability. *Arch Ophthalmol*. 2003;121:1537-1539.

6. Yokoi N, Takehisa Y, Kinoshita S. Correlation of tear lipid layer interference patterns with the diagnosis and severity of dry eye. *Am J Ophthalmol.* 1996;122:818-824.
7. Jalbert I, Stapleton F, Papas E, Sweeney DF, Coroneo M. In vivo confocal microscopy of the human cornea. *Br J Ophthalmol.* 2003; 87:225-236.
8. Ly LT, Cavanagh HD, Petroll WM. Confocal assessment of the effects of fourth-generation fluoroquinolones on the cornea. *Eye Contact Lens.* 2006;32:161-165.
9. Constantinou M, Daniell M, Snibson GR, Vu HT, Taylor HR. Clinical efficacy of moxifloxacin in the treatment of bacterial keratitis: a randomized clinical trial. *Ophthalmology.* 2007;114:1622-1629.
10. Espiritu CR, Caparas VL, Bolinao JG. Safety of prophylactic intracameral moxifloxacin 0.5% ophthalmic solution in cataract surgery patients. *J Cataract Refract Surg.* 2007;33:63-68.

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## Irreversible optical clearing of sclera by dehydration and cross-linking

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## ABSTRACT

This study manipulates both clear cornea and opaque sclera by two dehydration processes for revealing the relationship between altered tissue structures and change in optical functions. In contrast to the high levels of light scattering in dehydrated tissues by critical point dry, a simple dehydration at 4–8 °C effectively and significantly improved their visible-light transmission, even in the sclera, with accompanying dense fiber packing. Further improvement in visible-light transmission, from 40–50% to 80–90%, has been achieved by flattening tissue surface with cover glasses during dehydration at low temperature. Such optical clearing of sclera by dehydration is reversible. However, chemical cross-linking effectively stabilizes their densely packed microscopic structures and visible-light transmission at over 50% irreversibly, even at wet conditions. Interestingly, the repetition of both low temperature dehydration/cross-linking treatments effectively reduced the required amounts of cross-linking reagents to keep a high transparency. Wet transparent cross-linked sclera can also show a characteristic strong tensile strength. Furthermore, rabbit corneal epithelium has regenerated on the transparent sclera with cross-linking *in vitro*.

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## 1. Introduction

Structural characters in tissues are strongly linked with their naturally intrinsic functions. Recently, several studies have reported that tissue intrinsic light scattering properties can be altered artificially. Reversible optical clearing of non-light transmissive fibrous tissues such as skin have been investigated with chemical reagents (i.e. dimethyl sulfoxide and glycerol) [1–4], and mechanical compression [5,6] *in vitro* and *in vivo* as an attempt for future optical therapies and diagnosis (i.e. optical coherence tomography). Several researchers suggest that the potential mechanisms of optical clearing are thought to be dehydration and refractive index matching in matrix. These methods also cause structural changes in tissues, especially the significant reduction of thickness by compression, indicating the potential importance of structure for optical clearing.

On the other hand, one of the highly illustrative *in vivo* examples of structural difference affecting optical character is the relationship between transparent cornea and cloudy sclera, the same collagen fibrous membranes with approx. 500 μm thick, adjoined on the surface of the eye. In the cornea, collagen molecules self-assemble into fibrils with a remarkably uniform diameter (31 nm,

in humans), organizing the tissue-specific lateral arrangement of collagen fibrils with some degree of short-range spatial order [7–10]. In contrast, scleral coat is composed of disturbed fiber structures with inhomogeneous microfibril diameter ranging from 30 nm to 300 nm [8]. Such the difference in spontaneous structures has a huge bearing on their completely opposite characters against visible-light transmission *in vivo*.

The principal purpose of this study is to clarify specific structures that permit the visible-light transmission in the tissues. This study applied two types of dehydration processes, critical point drying and low temperature drying at 4–8 °C, to give a change in structures in both cornea and sclera *ex vivo*. The former method is widely applied to dehydrate biological tissues with maintaining their original structures before their scanning electron microscope observations [11–13]. In contrast, the latter method was reported as a simple method to vitrify boiled white egg [14] and purified atelocollagen solution [15,16]. Interestingly vitrified white egg and atelocollagen are known to permit visible-light transmission, but these methods have never been applied for the optical clearing of tissues. In the field of ophthalmology, tissues visible-light transmission is important, but there was assumption that the cornea was clear and sclera was clouded *in vivo*. Even clear cornea *in vivo* is gradually swelled and clouded in aqueous solution after its isolation. This study verified the cornea and sclera treated by the two processes described above from molecular level to macroscopic level by UV/Vis-spectroscopy and electronic microscopy.

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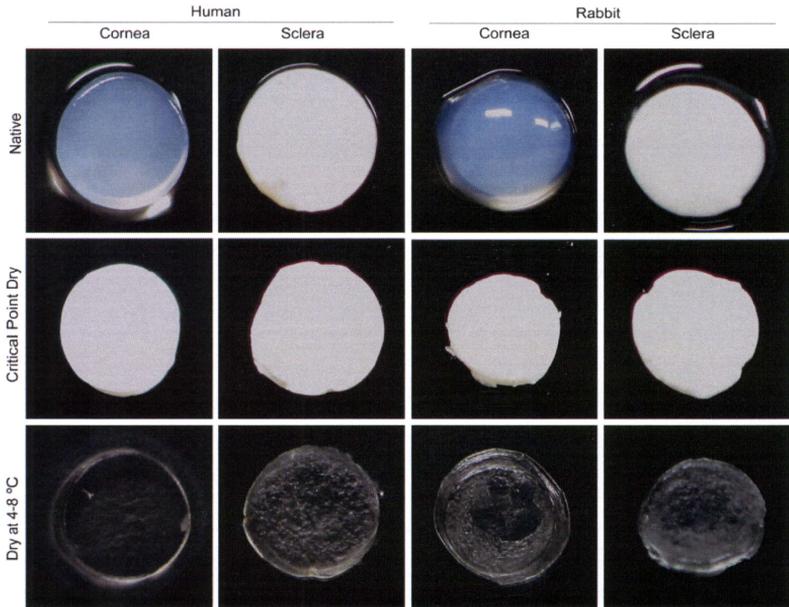


Fig. 1. Photography of cornea and sclera treated by drying processes. Human cornea, human sclera, rabbit cornea, and rabbit sclera are shown on the first, second, third, and fourth column, respectively. Native wet tissues, critical point dried tissues, and low temperature dried tissues are shown on the first, second, and third row, respectively.

Furthermore, this study also examined the following experiments to maintain the high visible-light transmission of dehydrated tissues, because the dried tissues swelled and clouded by rehydration in aqueous solution. As an additional investigation for structural contribution to optical clearing and further developments for the expansion of its possible applications, chemical cross-linking was performed on transparent dried sclera for fixing its specific structural character. It is extremely important to maintain the transparency irreversibly in aqueous solution, because the cornea of the living body always contact with aqueous humor in the anterior chamber. Moreover, transparent sclerotic coat could be used as a substitution for the cornea, and the tissue will be an ultimate solution for the donor shortage of corneal stroma. This study investigated corneal epithelial regeneration on irreversibly optically cleared sclera for examining their cell compatibility for further ocular application.

## 2. Materials and methods

### 2.1. Dry treatments of cornea and sclera

Human cornea and sclera were prepared from donated corneoscleral buttons (Eye Bank, SightLife, Seattle, USA). Rabbit cornea and sclera were obtained from Japanese white rabbit eyeball (Kitayama Labes, Ina, Japan). Tissues were trepanned (3 mm in diameter) and cut into adequate peaces for following analysis. All tissues were soaked in normal saline and followed by immersion in distilled water. Critical point dry or natural dry was applied for these rinsed tissues. Critical point dry was performed with a critical point dryer (HCP-2) (Hitachi, Hitachi, Japan) with carbon dioxide. In this case, the tissue was treated with elevated ethanol and isoamyl

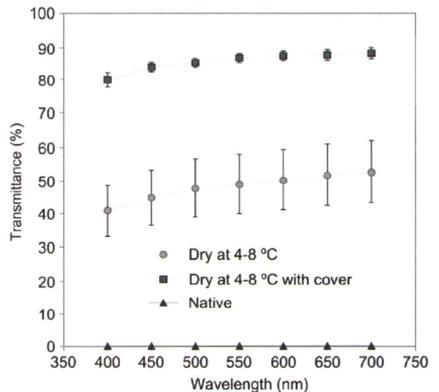


Fig. 2. Transmittance of rabbit sclera prepared by drying process. The closed triangles, squares, and circles represent wet sclera, sclera dried at 4–8 °C, and a sclera dried at 4–8 °C with cover, respectively.

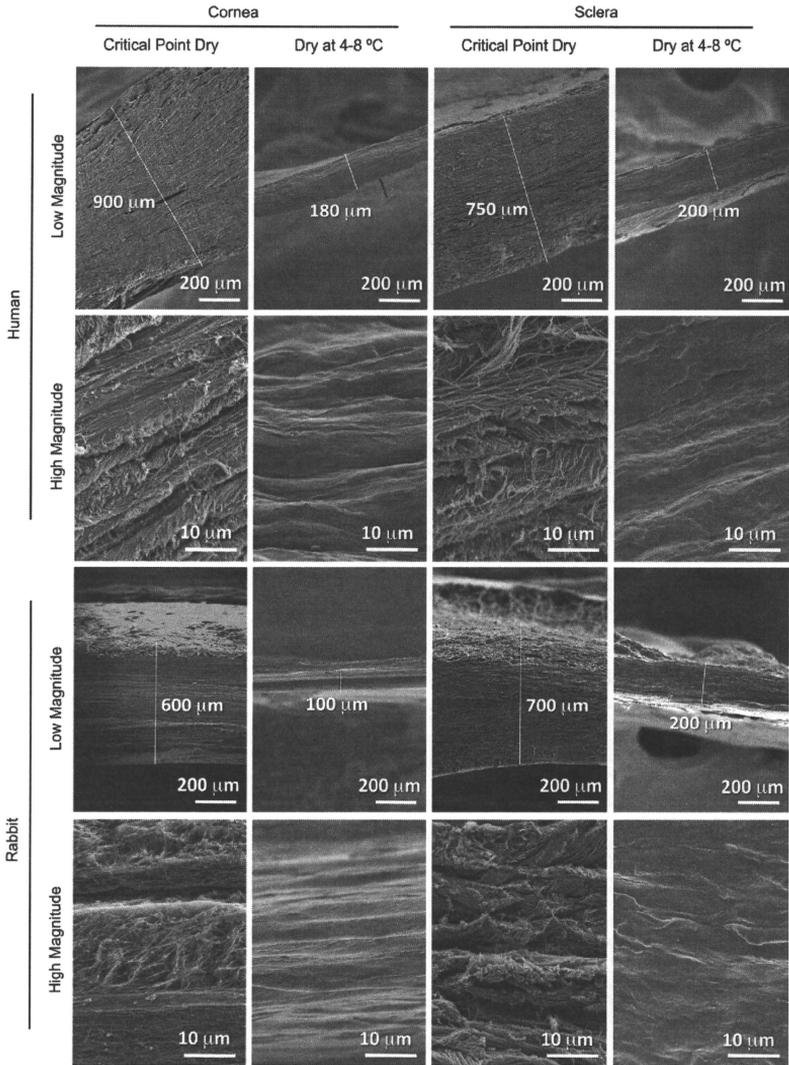
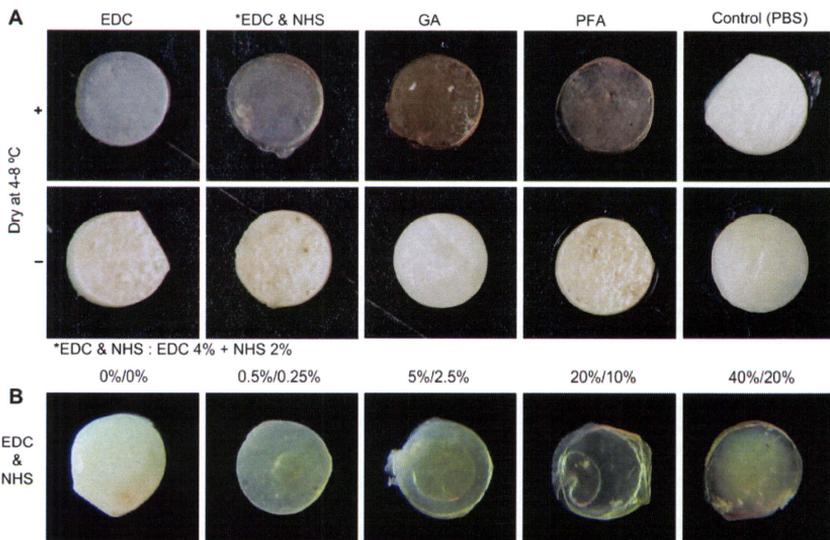


Fig. 3. SEM imaging of fiber structure on the cross-section of corneal stroma and sclera which were dried by low temperature dry treatment or critical point dry treatment.



**Fig. 4.** Stabilization of optically cleared human sclera at wet condition by chemical cross-linking. Effect of various types of cross-linking reagents, which are 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), Glutaraldehyde (GA), and paraformaldehyde (PFA) (A). Concentration effect of EDC and NHS (B).

acetate before the dry process. Low-temperature dry was conducted by putting the rinsed tissues on slide glass in a cold room at 4–8 °C until the color changes were observed (typically for 24 h).

## 2.2. Chemical cross-linking for maintaining the transparency of tissue

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Wako pure chemicals, Osaka, Japan), N-hydroxy succinimide (NHS) (Thermo science, Rockford, IL, USA), glutaraldehyde (GA) (Wako pure chemicals), paraformaldehyde (PFA) (Wako pure chemicals) was used as cross-linking reagents. Native and low-temperature dried human sclera buttons (3 mm in diameter) were treated with 0–200 mg/mL of the cross-linking reagents (EDC, EDC with NHS, GA, and PFA) for 2 h. For quantitative analysis, the larger pieces of low-temperature dried rabbit sclera (approx.  $5 \times 15$  mm) were treated with a mixture of EDC and NHS (the molecular ratio of EDC/NHS: 2/1) at room temperature for 2 h on slide glasses. The cross-linking reagents were washed out with normal saline and distilled water. Repetition of the low-temperature drying and the cross-linking/washing processes were quantitatively examined using rabbit sclera.

## 2.3. Evaluation of light transmission

Macroscopic images of tissues (mainly, 3-mm buttons) were obtained with a digital camera (PowerShot) (Canon, Tokyo, Japan). Transmission of samples was taken at room temperature with a UV/Vis-spectroscope (UV-2550/2450) (Shimadzu, Kyoto, Japan). Only rabbit sclera was evaluated, because the examined area of human samples was too small to perform UV/Vis-spectroscopy. The prepared sample was sandwiched between two slide glasses with a 500- $\mu$ m thick-silicon rubber spacer. For wet samples, normal saline was filled in narrow spaces between slide glasses, which were sealed with elastic films. For dried samples, no liquid was filled in the space.

## 2.4. Scanning electronic microscope (SEM) imaging of tissues

Tissues were cut to expose the cross-sections and treated by conventional specimen preparation procedure. Wet tissues were dried with a critical point dryer as described above. The prepared dried tissues were coated with osmium with an osmium coater (HPC-30) (Vacuum Device, Ibaragi, Japan) and observed with SEM (S-3200H) (Hitachi) at 5 kV.

## 2.5. Mechanical testing of tissues

The tensile strength of tissue containing Dulbecco's phosphate buffered saline (PBS) was measured with a Table-Top Universal Testing Instruments (EZ Test) (Shimadzu) at a rate of 10 mm/min. Edges were molded into a dumb-bell shape to ensure grip. The center part of sample under measurement was rectangular (6–10-mm length, 3-mm width, and 400- $\mu$ m thickness). Stress was monitored as a function of strain, and average elastic modulus was obtained by calculating the slope of the stress-strain curves before breaking point.

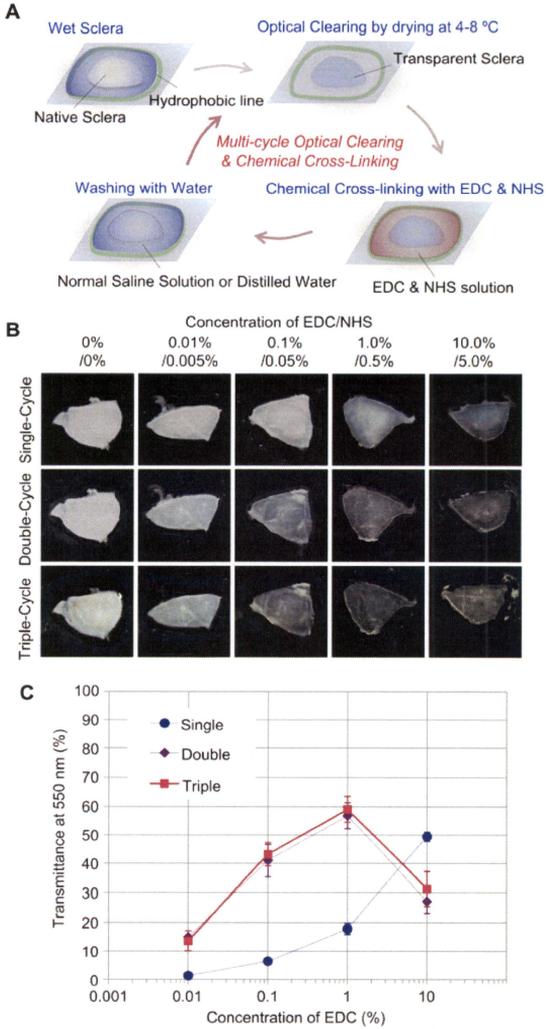
## 2.6. Corneal epithelial cell culture on optical transmission sclera

For examining whether doubly cross-linked rabbit sclera could support the proliferation of corneal epithelia, epithelial cells were cultured on the tissue with NIH/3T3 as feeder cells. Before the cell culture, doubly cross-linked rabbit sclera with 1% EDC/0.5% NHS was treated with 100 mmol/L arginine for 60 min at 37 °C. For preparing NIH/3T3 feeder layers, subconfluent NIH/3T3 fibroblasts were incubated with 16 mg/mL mitomycin C (MMC) for 2 h at 37 °C, then trypsinized, and seeded onto 6-wells plates at a density of  $2 \times 10^4$  cells/cm<sup>2</sup>. Corneoscleral buttons were obtained from Japanese white rabbit eyeball by following previous method [17] with partial revision. From the isolated cornea by cutting along the limbus between cornea and sclera, epithelial cells were collected by peeling off all epithelial layers after treatment with 3 mg/mL protease (Dispase II) (Roche Diagnostics, Mannheim, Germany) at 4–8 °C for overnight. Collected materials were put in a dish containing trypsin/EDTA for 20 min for preparing single cell suspensions. Cells ( $2 \times 10^4$ ) were seeded on the prepared scleral tissues with a 10-mm cloning ring on culture insert. The cells were cultured with MMC-treated NIH/3T3 feeder layers for 12 days.

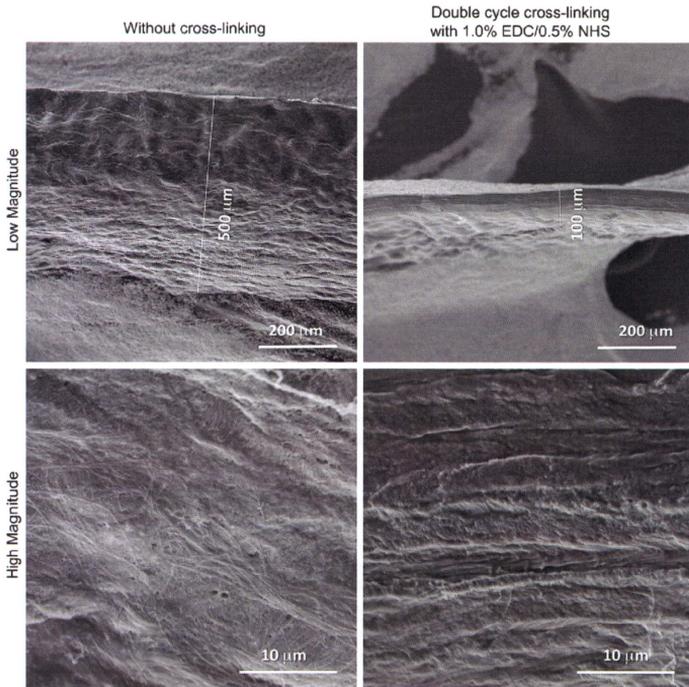
The obtained samples were washed with PBS and fixed with 4% PFA. For the SEM analysis, the samples were additionally fixed with 4% GA and dried with a critical point dryer. Paraffin blocks of the specimens were prepared for HE staining.

## 3. Results

Drastic changes in macroscopical visible inspection were observed at small rabbit and human cornea/sclera bottoms (3 mm in diameter) that obtained by two different types of drying



**Fig. 5.** Stabilization of optically cleared sclera at wet condition by multi-cycle low temperature dry and chemical cross-linking. Scheme of multi-cycle low temperature dry and chemical cross-linking (A). Photograph (B) and light transparency (C) of rabbit sclera treated by low temperature dry and chemical cross-linking with variously concentrated cross-linking reagents. Dehydrated sclera was treated with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) (the molecular ratio of EDC/NHS: 2/1) for 2 h at room temperature.



**Fig. 6.** SEM imaging of fiber structure on the cross-section of human and rabbit sclera which treated with double-cycle optical clearing (low temperature dry and cross-linking) with or without cross-linking.

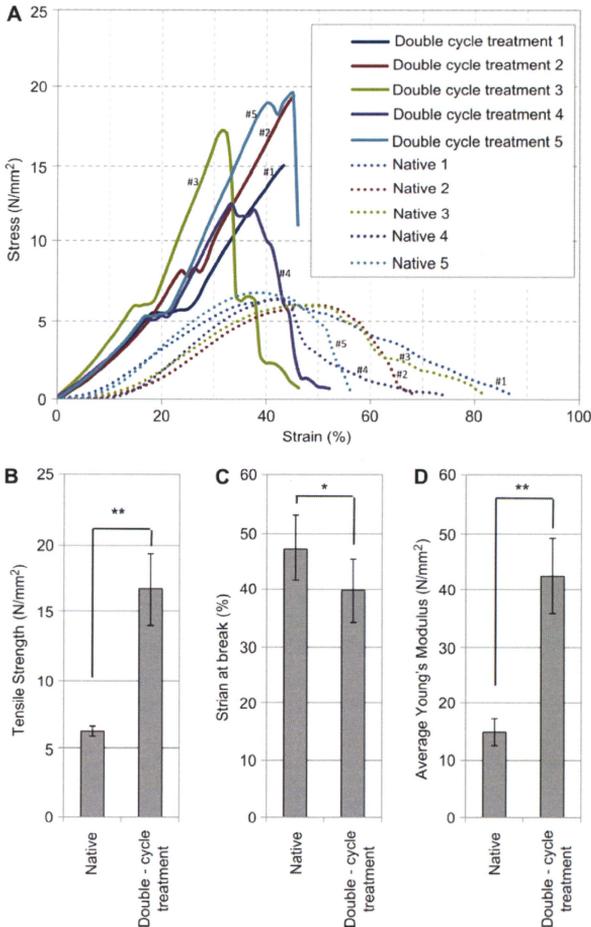
processes (Fig. 1). Trepanned cornea swelled and lost its transparency in PBS within several minutes, but still clearer than that of wet sclera (the first row, Fig. 1). Visible-light transmission of the swelled cornea was decreased by critical point dry treatment to the level of sclera (the second row, Fig. 1). In contrast, the transparency of all tissues including sclera turned to be transparent by low temperature drying treatment at cold room (4–8 °C) for one day (the third row, Fig. 1).

The quantitative analysis of visible-light transmission of rabbit sclera shows a significantly increase in visible-light transmission to 40–50% from 0 to 1% by low temperature drying treatment in cold room (4–8 °C) (Fig. 2). For the further improvement of visible-light transmission, applied glass covers were used during low temperature drying treatments for flattening the irregular surface. The rabbit sclera gradually turned to be transparent from the peripheral part to central part for 1–2 weeks. Interestingly, the visible-light transmission of the resultants with cover glass was significantly improved up to 80–90%.

SEM analysis of the cross-section of dried tissue clearly visualized differences in the tissue structures of both thickness and microscopic fiber morphology in both critical point dried tissues and tissues dried at low temperature without glass cover (Fig. 3). All

critical point dried tissues exhibited more than 600 μm thickness with lacunal structure. In contrast, all optical transmission tissues dried at low temperature exhibited more than 200 μm thickness with dense fiber packing.

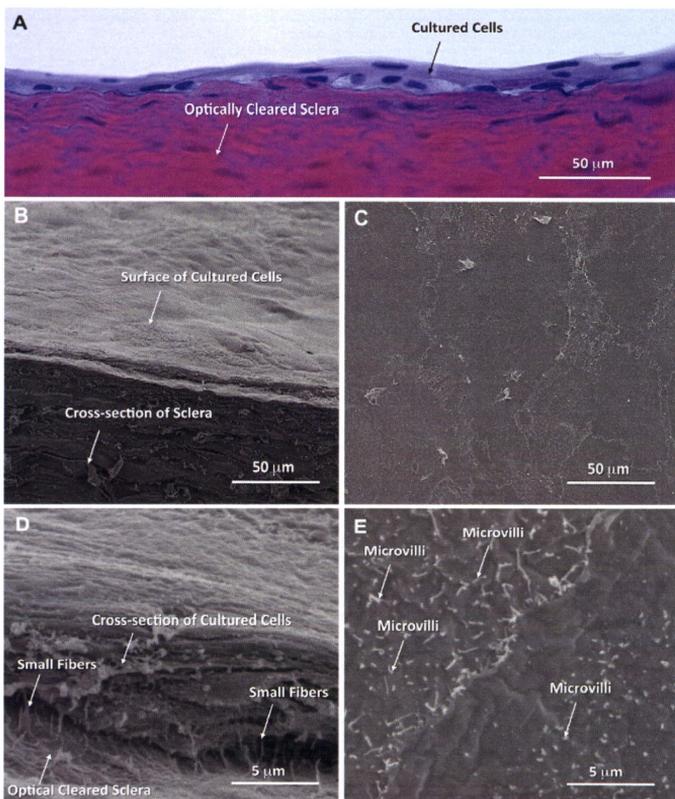
Both hydration processes with low temperature and chemical cross-linking treatments affected the transparency of human sclera as shown in Fig. 4A. The samples without dry treatment were clouded regardless of cross-linking agents (the second row, Fig. 4A). However, all samples hydrated with chemical cross-linking after low-temperature drying exhibited a color change and an improvement in light transmission (the first row, Fig. 4A), although the light transmission decreased to the level of native sclera without any cross-linking reagents (the control, the first row on the 5th column, Fig. 4A). Color of GA treated sclera was yellow, but no color change was found in the sample without dry process. Additionally, the effect of concentration of EDC/NHS on dehydrated sclera at low temperature was examined (Fig. 4B). The concentration of cross-linking reagents lower than 20%/10% (EDC/NHS) allowed the transparency of wet cross-linked sclera to increase with increasing cross-linking reagents, but the concentration over 40%/20% (EDC/NHS) allowed the sclera to be significantly clouded.



**Fig. 7.** Effect of double-cycle optical clearing (low temperature dry and cross-linking) on the mechanical properties of sclera. Stress-strain curves (A), tensile strength (B), strain at break (C), and average Young's modulus before break (D).  $n = 5$ , one-tail unpaired  $t$ -test (\* = significant difference  $p < 0.05$ , \*\* = significant difference  $p < 0.01$ ).

As a further quantitative study on these optical clearing treatments (low-temperature dry, chemical cross-linking, and washing) for improving transparency at wet conditions, these optical clearing processes were repeated on rabbit sclera (Fig. 5A). The control sclera swelled and clouded in PBS (the first column, Fig. 5B). The transparency of the sclera in PBS increased with swelling with increasing the concentration of cross-linking reagent (Fig. 5B and C). By the single-cycle treatment of low temperature drying/chemical cross-linking, the transparency of the rabbit sclera increased up to 50%

with increasing the concentration of cross-linking reagent. Repetition of the cycle treatment effectively increased the transparency when the concentration of EDC/NHS was 0.01%/0.005%, 0.1%/0.05%, and 1.0%/0.5%. In contrast, the transparency decreased when the concentration of EDC/NHS was 10.0%/5.0%. No significant differences in transparency were found between the double and the triple-cycle treated scleras. The maximum transparency of sclera was higher than 55%, when sclera was treated with the double or triple-cycle process with 1% EDC/0.5% NHS. The double-cycle



**Fig. 8.** Cultured rabbit corneal epithelial cells on irreversibly optically cleared sclera. HE staining of the cross-section (A), SEM images of the cross-section (B) and the flat mounted cell surface (C) at low-magnification, and images of the cross-section (D) and the flat mounted cell surface (E) at high-magnification.

process with 1% EDC/0.5% NHS was the best condition for providing the highest transmission to sclera and eliminating amounts of cross-linking reagent. When the concentration of EDC/NHS is high (10%/5%), the transparency significantly decreased with the possible structural distortion of sample.

The microscopical structure of the cross-section of sclera treated by the double-cycle treatment with 1% EDC/0.5% NHS was investigated by SEM after the critical point drying of the wet resultants (Fig. 6). This double-cycle treatment allowed the sclera thickness to be approx. 100 μm, which was less than 20% of the control sclera thickness without EDC and NHS (approx. 500 μm). In addition, the fiber structure in cross-linked rabbit sclera was observed to be much denser than that of non-cross-linked rabbit sclera.

On mechanical functional analysis, the double-cycle treated sclera with 1% EDC/0.5% NHS showed a sharper stress–strain curve in tensile test than that of native wet tissue (Fig. 7A). The applied

stress at break point significantly increased by approx. 100% by this double-cycle treatment (Fig. 7B). In contrast, the strain at break point was significantly decreased by approx. 20% (Fig. 7C). In addition, the most illustrative change was found on the averaged Young's modulus, which was significant increase by approx. 180% (Fig. 7D).

For investigating epithelial cell compatibility on the double-cycle treated rabbit sclera, seeded cells were rounded and floating, and the culture medium showed no color change for 3 days. In contrast, on arginine treated sclera, seeded epithelial cells proliferated and reached confluence with the color change of medium. After being cultured for 12 days, the regenerated epithelia on the sclera was fixed and analyzed. The light transmission of sclera was found to be stable during this cell culture experiments by visible inspection. Optical microscope and SEM showed that the surface of sclera was covered with 2–3 layered cells (Fig. 8A, B, and C). Morphology of the cultivated cell layer was flat and smooth at low magnification (Fig. 8B