Human Conjunctival Epithelial Sheets Grown on Poly(lactic-co-glycolic) Acid Membranes and Cocultured With Human Tenon’s Fibroblasts for Corneal Repair

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Submitted: July 29, 2017
Accepted: February 11, 2018

PURPOSE. We determine the feasibility of human conjunctival epithelial cells (hCjECs) on poly(lactic-co-glycolic) acid (PLGA) membranes for corneal epithelium regeneration. hCjECs on PLGA or polyester (PET) membranes with or without coculture of human Tenon’s fibroblasts (hTFs) were compared in vitro, and to determine whether epithelial sheets grown on PLGA membranes can repair injured rabbit corneal epithelium by transplantation for 2 weeks in vivo.

METHODS. Primary hCjECs were cultured on PLGA or the original PET membrane-based transwell inserts with or without coculture of hTFs on the floor of the culture plate. Cell behaviors, such as proliferation and differentiation, were compared. For in vivo assessment, the corneas of rabbits were burned, and PLGA-based epithelial sheets then were transplanted for 2 weeks before histologic staining was conducted and analyzed to determine the effectiveness of the repair.

RESULTS. Primary human epithelial cells on the PLGA membrane showed an increased proliferation when cocultured with fibroblasts, which was confirmed by CCK-8 analysis, and upregulation of Ki67, with the expression of the epithelial marker CK19. The stratified squamous cell marker MUC1 and conjunctival cell marker MUC5AC also were expressed in the epithelial sheet. The epithelial defect in the burned corneas was decreased in the PLGA-based epithelial sheet treatment group (6.1% ± 1.6% of the area) compared to that in the no-treatment group (30.5% ± 6.3%) 2 weeks postoperatively.

CONCLUSIONS. We developed a coculture system using a human feeder cell layer and PLGA membrane-based transwell inserts to create human conjunctival epithelial sheets. This system represents a promising strategy to regenerate corneal epithelium by transplantation.

Keywords: corneal epithelium, tissue engineering, human conjunctival epithelial cell
transwell coculture system induced paracrine factors from fibroblasts that affected epithelial cell proliferation and differentiation.

The original transwell permeable membrane was made of polyester (PET), which was used as a scaffold for epithelial sheets and retinal epithelium in rabbits, and as a tool for a coculture system. However, PET is not biodegradable in vivo and, therefore, requires an additional process to remove it from the transplantation site. Among the various biomaterials, the application of the Food and Drug Administration (FDA)-approved biodegradable polymer poly(lactic-co-glycolic) acid (PLGA) has shown potential as a scaffold for tissue engineering, including corneal epithelium regeneration. However, in vitro and in vivo evaluation of hCjECs on PLGA membranes for corneal epithelium regeneration has never been reported to our knowledge.

In this study, primary hCjECs were cultured on a biodegradable PLGA membrane and evaluated in vivo to determine their feasibility for corneal epithelium regeneration. For epithelial proliferation and stratification, the PET membrane of the transwell inserts was removed and replaced with the PLGA membrane as a scaffold material. Then, we cultured primary hCjECs on the PLGA membrane-based transwell inserts, with or without coculture of hTFs on the floor of the culture plate, and transplanted the resulting epithelial sheets onto the damaged cornea of the rabbit.

**MATERIALS AND METHODS**

**PLGA Membrane-Based Transwell Inserts**

Electrospun membranes were prepared as described previously (Fig. 1, Supplementary Fig. S1A). In brief, a solution of 50/50 \(\text{dl-lactide/glycolide copolymer (PLGA, 80,000 molecular weight (MW))}\) (Corbion, Amsterdam, The Netherlands) at 15% (wt/vol) in 20% dimethylformamide/80% tetrahydrofuran (both from Daejung, Gyeonggi-do, South Korea) was prepared by stirring at 80°C for at least 6 hours. For electrospinning, the solution feed had a flow rate of 0.5 mL/h, a DC voltage of 18 kV, and a working distance of 15 cm using an electrospinning machine (NanoNC Co., Ltd., Seoul, South Korea). The electrospun polymer membranes were collected as a random mesh on the surface of the collector. The membranes were dried overnight in a fume hood and then dried further in a vacuum chamber for the removal of the remaining solvent. The dried electrospun membranes were compressed with a vacuum at 55°C for 1 hour to form a flat surface. The electrospun, vacuum-compressed membranes were assessed using scanning electron microscopy (Seron, Gyeonggi-do, South Korea).

To prepare PLGA membrane-based transwell inserts, PET membranes of 6-, 12-, or 24-well Transwell inserts (Corning,
Table 2. Experimental Groups In Vivo

<table>
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<th>Group Name</th>
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<td>Control</td>
<td>Chemical burn, no implant</td>
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<tr>
<td>PLGA</td>
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<tr>
<td>Tissue sheet</td>
<td>PLGA membrane + hCjECs (PEF group)</td>
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In brief, conjunctival tissues were incubated for 1 hour at 37°C with 0.1% Protease (Sigma-Aldridk Korea Ltd., Yongin, South Korea) in a 1:1 of Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies Korea, Seoul, Korea). The transwell inserts then were coated with human fibronectin (Corning) in PBS, at 1 μg/cm² at room temperature (RT) for 1 hour, and then stored in a sterile container at 4°C until use.

Coculture of Primary hCjECs and hTFs

Figures 1B and 1C, and Tables 1 and 2 show the schematic diagram of experimental groups. The study protocol was approved by the institutional review board of Asan Medical Center (2014-0665). Primary hCjECs and hTFs were isolated from donated eyes and cultured as described previously.

For sterilization, the PLGA membrane-based transwell inserts were incubated with 70% ethanol under UV-C light for 30 minutes and washed three times with sterile PBS (pH 7.4; Gibco, Life Technologies Korea, Seoul, Korea). The transwell inserts were then coated with human fibronectin (Corning) in PBS, at 1 μg/cm² at room temperature (RT) for 1 hour, and then stored in a sterile container at 4°C until use.

Table 3. Gene Sequence

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td></td>
<td>Antisense: 5'-GACGTCCTCACAAATGTG-3'</td>
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<tr>
<td>Ki67</td>
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</tr>
<tr>
<td></td>
<td>Antisense: 5'-GTCGACCCCCCTCTTCTT-3'</td>
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In brief, conjunctival tissues were incubated for 1 hour at 37°C with 0.1% Protease (Sigma-Aldridk Korea Ltd., Yongin, South Korea) in a 1:1 of Dulbecco’s modified Eagle’s medium (DMEM; Gibco, New York, NY, USA) and Ham’s nutrient mixture F12 (F12; Gibco) with 1% penicillin-streptomycin (Gibco). The cells were scraped using a scraper, washed three times, and suspended. The cells were preplated on 60-mm culture dishes. For cultivation of nonattached hCjECs, the culture medium used was bronchial epithelial growth medium (Clonetics Corp, Walkerville, MD, USA) supplemented with insulin (5 μg/mL; Clonetics Corp.), hydrocortisone (0.5 μg/mL; Clonetics Corp.), epinephrine (0.5 μg/mL; Clonetics Corp.), triiodothyronine (6.5 ng/mL; Clonetics Corp.), transferrin (10 ng/mL; Clonetics Corp.), retinoic acid (10 ng/mL; Clonetics Corp.), bovine pituitary extract (0.13 mg/mL; Clonetics Corp.), gentamicin:amphotericin (50 μg/mL:50 μg/mL; Clonetics Corp.), human epidermal growth factor (10 ng/mL; Sigma-Aldrich Korea Ltd.), and bovine serum albumin (0.15 mg/mL; Sigma-Aldrich Korea Ltd.). The culture medium was changed 1 day after seeding and every other day thereafter until the cultures reached 60% to 70% confluence for 5 to 6 days, at which time they were dissociated with 0.25% trypsin-EDTA (Clonetics Corp.).

For making the hCjECs sheet, hCjECs were seeded at 5 × 10⁴ cells/cm² onto PET (T) or PLGA (P) membrane-based transwell inserts and cultured alone (TE and PE groups, respectively) or cocultured with hTFs (TET and PEF groups, respectively, Fig. 1C). To prepare a lethally-treated feeder cell layer, hTFs were cultured on the bottom of 6-, 12-, or 24-well cell culture plates with a seeding density of 2 × 10⁴ cells/cm². One day after seeding, hTFs were treated with 2.5 μg/mL mitomycin-C (Kyorai Hakko Kirin Korea, Seoul, South Korea) for 2 hours at 37°C. For coculture, the transwell inserts were placed into the hTF culture plates. Table 1 provides a description of the in vitro experiments.

Cell Viability

The viability of the cells on the membrane substrates was evaluated using a live/dead assay (Life Technologies Korea) at 7 and 14 days. In brief, all samples were incubated at 37°C and 5% CO₂ for 30 minutes in the live/dead solution and then observed using a fluorescence microscope (Carl Zeiss Meditec, Jena, Germany). Digital images were captured and processed using ImageJ (National Institutes of Health [NIH], Bethesda, MD, USA) image processing software (see Supplementary data). Finally, an index of live cells was constructed from the ratio of the live cell number to the total cell number.

Cell Proliferation

The proliferation of the cells on the sheets was determined in 24-well plates using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) 1, 3, 7, 9, and 14 days after cell seeding. The 24-well transwell plates containing the hCjECs and hTFs were incubated at 37°C in a humidified cell incubator containing 5% CO₂ for 2 hours, and the absorbance of the supernatant was measured using a plate reader (Sunrise; Tecan, Männedorf, Switzerland), which correlated with the amount of cell proliferation.

Relative Gene Expression in the In Vitro Experiments

Epithelial membranes were lysed on day 10 for total RNA extraction using TRIzol Reagent (Life Technologies Korea), according to the manufacturer’s instructions. cDNA was used as a template for the different groups, and quantitative real-time PCR was performed using the 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). All experiments were conducted in triplicate, and Ki67 (proliferation) and MUC1 (function) expression levels were analyzed. Table 3 shows the primer sequences. The 2⁻ΔΔCT method was used to calculate relative changes in gene expression.

In Vivo Assessment

The animal experiment protocol was reviewed and approved by the institutional animal care and use committee of Asan Medical Center (IACUC No., 2016-02-161). All animals were treated in accordance with the principles of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult New Zealand white rabbits (n = 12) were obtained from Orient Bio (Seongnam-si, Korea). The nictitating membrane was excised, a paper disk with 0.5 N sodium hydroxide was applied to the cornea and limbal zones of the left eye for 30 seconds, and the eyes were then washed with normal saline. hCjECs on the PLGA membranes (PEF group) were cut with a 10-mm diameter punch and apposed onto the
denuded corneal surfaces of four rabbits using forceps (PLGA-tissue sheet group). Eight 10–0 nylon sutures were used to attach each PLGA-based epithelial tissue sheet to the de-epithelialized rabbit cornea. This then was covered with a therapeutic soft contact lens and secured with four peripheral anchoring sutures. Finally, total tarsorrhaphy was performed with 6–0 nylon sutures. Four rabbits were transplanted similarly with PLGA membranes (PLGA-alone group) without epithelial tissue, and four rabbits received no surgery but underwent all other procedures (control group). After the

![Figure 2](http://arvojournals.org/)

**Figure 2.** (A–H) Live/dead fluorescence images of hCjECs on the PLGA and PET membranes on days 7 (A–D) and 14 (E–H). Green fluorescent cells are alive, red fluorescent nuclei indicate dead cells. Scale bars: 200 µm. (I) Quantification from the live/dead assay using ImageJ software (NIH). An index of live cells (% cell viability) was constructed from the ratio of live to total cell numbers. (Data represent mean ± SD, **P < 0.01; *P < 0.05). (J–M) Cell proliferation results from CCK-8 analysis of hCjECs on the PLGA and PET membranes on days 1, 3, 7, 9, and 14. *P < 0.05, **P < 0.01.

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</tr>
<tr>
<td>CK19</td>
</tr>
<tr>
<td>MUC5AC</td>
</tr>
<tr>
<td>Human mitochondria</td>
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<tr>
<td>Ki67</td>
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surgery, the rabbits were treated with topical antibiotics (0.5% levofloxacin; Samil Co., Ltd., Seoul, Korea) and triamcinolone acetonide (0.2 mL, injected subconjunctivally; Bristol-Myers Squibb Co., Tokyo, Japan). The corneas of the rabbits were analyzed with fluorescein staining under blue light and harvested on postoperative day (POD) 14.

Hematoxylin and Eosin Staining

The hCjEC cultured membranes from the transwell inserts or harvested corneas were fixed in 4% paraformaldehyde at 4°C for 1 hour or overnight and then transferred to 30% (wt/vol) sucrose (Duchefa Biochemie, Amsterdam, The Netherlands) and stored at 4°C until the samples submerged. Then, the samples were embedded in an optimal cutting temperature compound (Tissue-Tek; Sakura Fine Technical Co., Ltd., Tokyo, Japan), frozen, and stored at −80°C. Subsequently, 10-μm thick sections were obtained using a cryostat, followed by standard hematoxylin and eosin (H&E) staining. The H&E-stained sections were viewed using bright-field light microscopy (EVOS; Life Technology).

Immunofluorescence Staining

The sections were blocked and incubated for 1 hour at 37°C with the following primary antibodies: stratified squamous conjunctival marker, MUC1 (1:100); conjunctival epithelial marker, CK19 (1:100); goblet cell and mucin marker, MUC5AC (1:100); human mitochondria (1:100) for distinguish human cells; and proliferation marker, Ki67 (1:100; Table 4). The sections then were incubated further for 1 hour at room temperature with the respective secondary antibodies (1:100). F-actin in the tissue sheets was visualized with a 1:40 dilution of Alexa Fluor 594-phalloidin, and cell nuclei were counter-stained using 4',6-diamidino-2-phenylindole (DAPI). Table 4 lists all antibodies used. The sections were viewed using an LSM780 confocal microscope (Carl Zeiss Meditec).

Image Analysis

Fluorescent images were captured and analyzed using a digital software (Zen2011; Carl Zeiss Meditec), and fluorescence quantification was performed using a FIJI/ImageJ2 software (Supplementary Data). We defined regions of interest (ROIs) in three immunofluorescence figures for every group and obtained the integrated density for each ROI and each molecule (CK19, MUC5AC, human nuclei, and Ki67) to indicate their expression levels per frame analyzed. We also defined ROIs for each fluorescent signal and divided them by the DAPI ROI area to obtain percentage area data and the integrated intensity (Supplementary Data).

Statistical Analysis

All data are reported as means ± SD. Differences among the experimental groups were analyzed using ANOVA and a paired t-test using Excel and Origin, and a value of P < 0.05 was considered statistically significant.
RESULTS

PLGA Membrane-Based Transwell Inserts for Culture of Primary hCjECs and Coculture With hTFs

The electrospun membranes had a microscale thickness (25–40 μm, data not shown) and was composed of PLGA nanofilaments in a random direction (Fig. 1A, Supplementary Fig. S1A). After vacuum-assisted compression, the thickness of the PLGA membrane was reduced to approximately half of the original thickness (12–20 μm, Supplementary Fig. S1A) with flattened nanofibers (Supplementary Fig. S1B). The PLGA membrane-based transwell inserts were easy to handle with forceps and consisted of a semitransparent membrane 24 hours after seeding (Supplementary Figs. S1C, S1D).

**Figure 4.** Immunofluorescent analysis of primary human conjunctival epithelial sheets on the PLGA and original PET membranes (A). Green: CK19, Red: MUC5AC, Blue: DAPI. Scale bars: 200 μm. The graph represents the mean ± SD of the integrated fluorescence density of CK19 (B) and MUC5AC (C) expressed in epithelial sheets on days 7 and 14 (n = 3-4/group; data represent mean ± SD, *P < 0.05, **P < 0.01).
Cell Proliferation and Viability on PLGA and PET Membranes

Figures 2A through 2H show representative microscopic fields with the visualization of live and dead cells. The quantified data of Figures 2A through 2H summarizing the cell viability from all experimental groups in vitro are summarized in Figure 2I. Moreover, cell viability was consistently >80% on days 7 and 14 under all conditions.

The CCK-8 cell proliferation assay showed that hCjEC proliferation, when grown on PET and PLGA membranes, was significantly higher in the fibroblast coculture (TEF and PEF) groups than those without hTFs (TE and PE) on Days 9 and 14 (Figs. 2J, 2K). Comparing membrane-type effects, hCjEC cell proliferation without fibroblast coculture in the TE group was higher compared to that observed in the PE group (Fig. 2L). However, hCjEC cell proliferation with cocultured fibroblasts did not significantly differ between the TEF and PEF groups after 7 days of in vitro culture (Fig. 2M).

Morphology of Primary hCjECs on PET and PLGA Membranes

Figure 3 shows the histologic appearance of the hCjEC sheets. In the TEF and PEF groups, the cell sheets had two to three cell layers (Figs. 3A, 3B). Primary hCjECs form clusters and small colonies when seeded on both PLGAs, with differences in hCjEC morphology (Figs. 3C, 3D, 4A) on day 7. Furthermore, MUC1 was expressed on the surface of epithelial sheets on day 7 (Figs. 3E, 3F). These results showed that epithelial sheets in the PEF group could form a stratified epithelium and synthesize MUC1 mucin in vitro.

Epithelial Differentiation of Conjunctival Cells

Immunofluorescence revealed that hCjECs grown on the PLGA and PET membranes contained CK19- and MUC5AC-positive cells (Fig. 4A), indicating that there was a mixed cell population undergoing a variable degree of differentiation and proliferation. CK19 is a stratified conjunctival and limbus cell marker, and MUC5AC has been identified in human conjunctival goblet tissue. There were changes in CK19 and MUC5AC expression between hCjECs cultured on the PLGA and PET membranes. CK19 and MUC5AC were coexpressed with conjunctival epithelial markers, even in morphologically distinguishable goblet cells. Between days 7 and 14, the integrated density of CK19 in hCjECs significantly increased on the PLGA membrane, whereas it remained unchanged on the PET membrane (Fig. 4B). The integrated density of MUC5AC in hCjECs decreased on the PLGA membranes on days 7 and 14 (Fig. 4C).

Quantitative (q) RT-PCR of Ki67 and MUC1 revealed the effects of the substrate materials and the hTF feeder cells on the proliferation and differentiation of hCjECs, respectively (Fig. 5). hCjECs cultured on the PLGA membrane showed a much higher Ki67 expression compared to those on the PET membrane, with and without hTF feeder coculture (TE, 1; TEF, 1.4 ± 0.7; PE, 14.4 ± 5.6; and PEF, 13.4 ± 0.9). In contrast, Muc1 expression in cocultured hCjECs was increased on the PET membrane but decreased on the PLGA membrane (TE, 1; TEF, 3.5 ± 0.4; PE, 6.0 ± 1.4; and PEF, 1.0 ± 0.2).

In Vivo Experiments to Repair Injured Corneas

All experimental groups (control, PLGA, and tissue sheet groups) were confirmed at the surgical site on postoperative day 7 (physical examination, data not shown). On postoperative day 14, all experimental groups were analyzed with fluorescein staining on the burned corneas (Fig. 6). Representative photographs indicated that the transplanted PLGA membranes were partially degraded within 2 weeks (Figs. 6E, 6F). The percentage area of the epithelial damage was expressed as the percentage of the fluorescein-stained area to the total area on day 14 (n = 3–4 per group; control, 30.5 ± 6.3; PLGA-alone, 23.3 ± 20.4; PLGA-tissue sheet, 6.1 ± 1.6; Fig. 6).

Representative histological images showed that normal epithelium covered normal stroma (Fig. 7A, Supplementary Fig. S2A). The tissue sheet group showed re-epithelialization, represented by multilayered squamous-like corneal epithelium from the limbus of the cornea (Fig. 7C, Supplementary Fig. S2C), compared to the control group (Fig. 7B). Immunofluorescence images showed negativity for MUC5AC on the surface of the harvested rabbit cornea (Figs. 7D, 7E). This result revealed that human-derived goblet cells expressing MUC5AC were not detected on the harvested rabbit corneas. The transplanted epithelial sheets uniformly stained positively for...
the human mitochondrial antibody (Fig. 7G), showing that the epithelial cells in the rabbit cornea originated from the transplanted hCjECs. Ki67, which is a proliferation marker, was located in the only basal cell layer of the tissue sheet-treated rabbit cornea (Fig. 7I).

**DISCUSSION**

Epithelial cells were cultured in the transwell inserts to satisfy the requirements for submerged cultivation and multilayered epithelium differentiation by the air-liquid interface. However, the transwell system could be applied only for drug and cell studies in vitro, with no in vivo equivalents. PLGA membrane-based transwell inserts were designed with a bifunctional purpose, not only as a substrate membrane for the air-liquid interface of the cultures in vitro but also as a biodegradable scaffold for corneal tissue in vivo. Our study design that used a transwell insert-based coculture system reduced the chance of mixing between hCjECs on the insert membrane and hTF cells on the bottom surface of the culture dish. Consequently, the transwell insert system can reduce cellular and xeno interference. Similar transwell insert-based coculture systems have been reported with murine fibroblast (3T3) cell lines as the feeder cell layer to assist epithelial cell proliferation, while fibroblasts on collagen gels were stimulated by epithelial cells and showed myodifferentiation.

In this study, primary hCjECs were obtained from cadavers and required confirmation of the properties of cell proliferation and maturation and generation of the stratified structure. We analyzed the gene expression related to the proliferation and maturation using Ki67 and MUC1. MUC1 is expressed on the stratified and mature epithelial cells and expressed throughout the entire ocular surface in healthy eyes. Moreover, murine fibroblasts have been used as a feeder layer to cultivate human epithelial cells to mimic the in vivo environment. However, the harvest of epithelial cells requires splitting of the feeder layer, with the possibility of residual fibroblasts in the collected epithelial cells. To avoid the risk of residual interspecies cells transmitting pathogens, hTFs are safer than mouse fibroblasts as feeder cells when considering the clinical translation of membranes with cultured primary hCjECs, and our results showed that they effectively support hCjEC growth and viability.

From our in vitro results, hCjEC proliferation was promoted by coculture with hTFs. Cocultures with fibroblast feeders enhance epithelial cell proliferation. hTFs release interferon-beta and keratinocyte growth factor (KGF). KGF sequentially inhibited maturation and enhanced proliferation of dermal epithelial cells. Similarly, reduced maturation and enhanced proliferation of hCjECs may be due to the hTF feeders. The effects of hTF were associated with the enhancement of CK19 and F-actin and upregulation of Ki67 activity in the in vitro cultures (Fig. 5). MUC1, a mature epithelial cell marker, was expressed at the highest level in the PE group and was less expressed in the PEF group compared to that in the TEF group. On the PLGA membrane, the differentiation of hCjECs was dominant in conditions with no feeder cultures, whereas Ki67 expression did not show any significant differences. Because the PLGA was coated with fibronectin, which is a major component of cell adhesion and basement membranes, this may improve proliferation on the PLGA membranes. However, MUC1 expression was not significantly different in the conjunctival epithelial cells on the surface of rabbit eyes on postoperative days 0 (A–C) and 14 (D–I). The dashed lines showed the transplanted PLGA membrane and on the burned rabbit eyes. The percentage of epithelial damage was expressed as the percentage of fluorescein stained areas on day 14 related to the total area (n = 3–4/group; data represent mean ± SD, *P < 0.05).
PLGA membranes at day 10. These results are similar to the results using the MUC5AC staining (Figs. 4A, 4C). Figure 4 illustrates how the MUC5AC intensity of the PEF group was decreased from days 7 to 10. These figures showed that conjunctival epithelial cells cocultured with fibroblasts on the PLGA membranes showed proliferative rather than maturational properties.

Two weeks postoperatively in vivo, PLGA membranes were not fully degraded as described earlier. PLGA has properties of biodegradation by hydrolysis for 1 to 24 months.23 One limitation of this study was that the evaluation period of the xeno-transplantation was short for confirming transparency of the burned rabbit eyes. Further long-term evaluations of allografts with primary rabbit epithelial cells are needed to check functional vision. Future studies should determine whether the PLGA membrane has biodegraded.

Tseng et al.48 reported that conjunctival goblet cell possibly is correlated with neovascularization. In results of immunofluorescence staining, human-derived MUC5AC was not expressed on the transplanted sheets in vivo, which corresponded with a previous report.49 Also, during our experiment, neovascularization signs were not observed. Also, transplantation of conjunctival epithelial cells showed that the observed clinical signs are an improvement of corneal epithelial transparency, superficial corneal neovascularization, and epithelial irregularity in a clinical study.50 Ki67 expression (Figs. 7H, 7I) of hCJECs was observed to be high in a cultivated sheet and a transplanted sheet, suggesting that the proliferation was well maintained after transplantation, as described in previous reports.51,52 Our results confirmed that Ki67 is located in stroma adjacent to the epithelium, whereas Ki67 was localized in the basal layer of the epithelium.53,54 This similar localization can be checked in the reports of

Figure 7. H&E images of normal (A), nontreated (control) (B), and PLGA-tissue sheet-treated (C) rabbit corneas on postoperative day 14, showing regeneration of epithelium treated with PLGA-tissue sheets. Scale bars: 100 μm. Immunofluorescence images showing differences in MUC5AC expression (D, E), human mitochondria (F, G), and Ki67 (H, I) in cross-sections of the control and tissue sheet groups. EP and ST indicate each epithelium and stroma of the harvested rabbit cornea. Green: Human mitochondria, Red: MUC5AC and Ki67, Blue: DAPI. Scale bars: 100 μm. (J) Quantification of the mean percent area of MUC5AC, human mitochondria fluorescence, and Ki67 within cell sheet ROIs in the burn and tissue sheet groups. (n = 4; data represent average ± SD, *P < 0.05).
Ortillés et al., Wilson et al., and Nagata et al. reported that Ki67 was also expressed in stroma and epithelium by transplantation. A system could enable a promising strategy to regenerate corneal tissue, especially in burns. Ki67 expression was decreased (Supplementary Fig. S3, Figs. 7H, 7I).

In summary, PLGA membrane-based transwell inserts were used to generate epithelial sheet in vitro and in vivo. This system could enable a promising strategy to regenerate corneal epithelium by transplantation.

Acknowledgments

The authors thank the Confocal Laser Scanning Microscopy (CLSM) core facility at the Convergence Medicine Research Center (CREDIT), Asan Medical Center for support and instrumentation. Supported by the ASAN Institute for Life Sciences, ASAN Medical Center, Seoul, Korea (Grant Numbers, 2016-7206, 2017-7206) and by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (HI17C0193).

Disclosure: S. Hong, None; J.H. Yun, None; E.-S. Kim, None; J.S. Kim, None; H. Tchah, None; C. Hwang, None.

References


