Research Article

Establishment of Novel Limbus-Derived, Highly Proliferative ABCG2⁺/ABCB5⁺ Limbal Epithelial Stem Cell Cultures

Eung Kweon Kim,^{1,2} Ga-Hyun Lee,¹ Boram Lee,¹ and Yong-Sun Maeng^{1,3}

¹Department of Ophthalmology, Corneal Dystrophy Research Institute, Yonsei University College of Medicine, Seoul, Republic of Korea

²Institute of Vision Research, Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul, Republic of Korea

³Department of Obstetrics and Gynecology, Institute of Women's Life Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea

Correspondence should be addressed to Yong-Sun Maeng; maengmatthew@gmail.com

Received 26 June 2017; Revised 27 September 2017; Accepted 12 October 2017; Published 5 November 2017

Academic Editor: Ming Li

Copyright © 2017 Eung Kweon Kim et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Homeostasis and regeneration of corneal epithelia are sustained by limbal epithelial stem cells (LESCs); thus, an LESC deficiency is a major cause of blindness worldwide. Despite the generally promising results of cultivated LESC transplantation, it has been limited by variations in long-term success rates, the use of xenogeneic and undefined culture components, and a scarcity of donor tissues. In this study, we identified the culture conditions required to expand LESC *in vitro* and established human limbus-derived highly proliferative ABCG2⁺/ABCB5⁺ double-positive LESC. These LESC exhibited the LESC marker profile and differentiated into corneal epithelial cells. In addition, cultured LESC expressed high levels of the stem cell markers Sox2, Oct4, c-Myc, and Klf4, had high telomerase activity, and had stable, normal genomes. These results suggest that our novel cultivation protocol affects the phenotype and differentiation capacity of LESC. From the limbus, which contains a heterogeneous cell population, we have derived highly proliferative ABCG2⁺/ABCB5⁺ double-positive cells with the ability to differentiate into corneal epithelial cells. This study opens a new avenue for investigation of the molecular mechanism of LESC maintenance and expansion *in vitro* and may impact the treatment of corneal disease, particularly corneal blindness due to an LESC deficiency.

1. Introduction

A surgical strategy for restoring the corneal epithelial surface in patients that lack sufficient limbal epithelial stem cells (LESCs) is the transplantation of ex vivo expanded LESC, which is one of the few adult human stem cell therapies currently being used [1–4]. This therapeutic approach typically involves harvesting a small limbal sample from the patient or a donor followed by cell expansion to generate an epithelial sheet on a transplantable carrier, such as an amniotic membrane [5–10], fibrin gel, or temperature-responsive polymer [11]. Although successful repopulation of the ocular surface has been described for up to 1 year after transplantation, studies have indicated that epithelial viability is not sustained for very long [12] and that donor cells do not survive more than 9 months after transplantation [13, 14]. These

failures may have resulted from depletion of LESC in culture due to improper culture conditions. Most culture methods, including explant and airlift cultures, promote the proliferation and terminal differentiation of transient amplifying cells (TACs) rather than retaining LESC [15]. However, long-term restoration of the damaged ocular surface requires the preservation of LESC during culture and after grafting [4, 16]. Since the pioneering work in 1975 by Rheinwald and Green [17], studies have shown that long-term survival and serial expansion of LESC are possible if they are cocultured with fibroblast feeder cells [18]. Three types of clonogenic cells, which give rise to holoclones, meroclones, and paroclones, were identified by clonal analysis of human keratinocytes cultured on feeder layers [19]. Holoclone-forming cells have all of the hallmarks of LESC, including the capacity to self-renew and a high potential to proliferate, whereas

meroclonal and paraclonal are generated by different stages of TACs and have limited capacities for proliferation. This discovery was followed by the identification of holoclone-forming cells in the limbal epithelium and the development of a culture system that enriches for LESC by growing them clonally on feeder layers before seeding them onto fibrin gels to produce epithelial sheets [20, 21]. Consistently, keratinocytes cultured by this method have been used to restore massive epidermal defects permanently and to restore the corneal surface of patients with complete LESC deficiencies [1, 22–24]. Nevertheless, the question of whether the transplanted cell sheets actually contain LESC has not been addressed and the widespread use of this promising cultivation technique has been hampered by the lack of a standardized cultivation protocol.

In this study, we evaluated the effects of several culture variables on the growth and retention of LESC in culture to develop an optimal cultivation protocol that promotes the expansion and maintenance of LESC for therapeutic applications. We developed a culture method to establish human limbus-derived, highly proliferative ABCG2⁺/ABCB5⁺ double-positive LESC cultures. The LESC that we cultured by this method were confirmed to have the LESC marker profile and exhibited the potential to differentiate into corneal epithelial cells. Moreover, these LESC expressed high levels of stem cell markers, including Sox2, Oct4, c-Myc, and Klf4 [25, 26], displayed high telomerase activity, and had stable, normal genomes. Using the limbus, which contains a heterogeneous cell population, as a cell source and our specific culture conditions, we were able to establish a novel and highly proliferative ABCG2⁺/ABCB5⁺ double-positive stem cell population with the capacity for corneal epithelial differentiation. Thus, our proposed culture system may be essential for the long-term clinical success and stable regeneration of corneal epithelia to treat corneal blindness due to an LESC deficiency.

2. Materials and Methods

2.1. Cell Culture and Establishment of ABCG2⁺/ABCB5⁺ Double-Positive LESC. Human corneal tissues were harvested from healthy corneas that were deposited in an eye bank after penetrating or lamellar keratoplasty. Donor confidentiality was maintained in accordance with the Declaration of Helsinki, and the research protocol below was approved by the Severance Hospital IRB Committee (CR04124) of Yonsei University.

- (1) Within 4 hours after penetrating keratoplasty, fresh corneoscleral rim tissue was placed in a 60 mm culture dish containing Hank's Balanced Salt Solution (HBSS) and was cut into four equal segments of the limbus (limbus portion including the small cornea region). Any remaining iris and endothelial cells were rubbed off with a cotton tip.
- (2) Each segment was digested with 15 mg/mL of dispase II (Roche, Rotkreuz, Switzerland) in supplemented hormonal epithelial media (SHEM; CELLnTEC

Advanced Cell Systems AG, Bern, Switzerland) with 100 mM sorbitol (Sigma-Aldrich, St. Louis, MO) at 4°C for 18 h to separate the stroma from the rest of the tissue.

- (3) Under a dissecting microscope, an already loose limbal epithelial sheet was separated from the tissue by inserting and sliding a noncutting flat stainless steel spatula into the plane between the limbal epithelium and the stroma.
- (4) The isolated limbal epithelial cell (LEC) clusters were seeded onto a 60 mm plate coated with 5% Matrigel (BD Biosciences, Bedford, MA) and 0.05 mg/mL human fibronectin (Sigma-Aldrich, St. Louis, MO) and cultured in CnT20 medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland).
- (5) After 3 days, the LECs were cultured in 10% fetal bovine serum (FBS) Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA), and the medium was changed every 2 days.
- (6) After 8–10 days, highly proliferative cell colonies appeared in the culture plates.
- (7) Highly proliferative cell colonies were washed with PBS two times and treated with 1 mL Accutase (Sigma-Aldrich, St. Louis, MO), prewarmed at 37°C, and shaken evenly 5–6 times. The Accutase was then discarded, and cells were treated again with 1 mL Accutase and shaken evenly 4–5 times. Accutase was then discarded again, and cells were incubated for 3–5 min at 37°C in an incubator. Colonies were digested separately. Single cells were seeded onto plates coated with a matrix of Matrigel and fibronectin and cultured in DMEM containing 10% FBS. The highly proliferative cells that attached to the new plate were designated limbal epithelial stem cells (LESC).
- (8) After 48 hours, the LESC were treated with Accutase and sorted by FACS analysis using ABCG2⁺ antibodies (Abcam, Cambridge, MA) and ABCB5⁺ antibodies (Thermo Fisher Scientific, Rockford, IL).
- (9) The ABCG2⁺/ABCB5⁺ double-positive cells were seeded onto a plate coated with a matrix of Matrigel and fibronectin and cultured in 10% FBS DMEM. When the plate was full of cells, the cells were treated with Accutase and seeded onto a plate coated with a matrix of Matrigel and fibronectin and cultured in 10% FBS DMEM. Then, the ABCG2⁺/ABCB5⁺ double-positive cells were cultured using mass culture methods and were named ABCG2⁺/ABCB5⁺ double-positive LESC.

2.2. Real-Time Quantitative Reverse Transcriptase PCR (Real-Time qRT-PCR). Total RNA was isolated from corneal epithelial cells differentiated from ABCG2⁺/ABCB5⁺ LESC with TRIzol reagent (Invitrogen, Carlsbad, CA). mRNA expression of human GAPDH, $\Delta np63\alpha$, ABCG2, CK3,

CK19, *Integrin* $\alpha 9$, CK12, OCT4, SOX2, NANOG, *c-MYC*, and *KLF4* was measured using the Power SYBR Green RNA-to-CT™ 1-Step kit (Applied Biosystems, Foster City, CA, USA) and StepOnePlus™ (Applied Biosystems) according to the manufacturer's instructions. The PCR protocol was 48°C for 30 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Expression results were based on cycle threshold (Ct) values. The differences between the Ct values for the experimental genes and the reference gene *GAPDH* were calculated and graphed as ratios of experimental RNAs to the calibrated sample. The primers used for gene amplification are listed in Supporting Information Table S1 available online at <https://doi.org/10.1155/2017/7678637>. Three independent experiments were performed, and statistical analysis was carried out using the Newman-Keuls multiple comparison test.

2.3. Immunofluorescence Staining. Cells were fixed in 3.7% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS), and preincubated in a blocking solution of PBS containing 5% normal donkey serum and 0.05% Tween-20. Then, cells were incubated with primary antibody for 2 h at room temperature. The primary antibodies used included anti-human p63 α antibody (Cell Signaling, Beverly, MA), anti-human ABCG2 antibody (Abcam, Cambridge, England), anti-human ABCB5 antibody (Invitrogen, Carlsbad, CA), anti-human CK19 antibody (Abcam, Cambridge, England), anti-human CK3 antibody (Abcam, Cambridge, England), and anti-human desmoglein 3 antibody (Novus Biologicals, Littleton, Co.). Then, cells were labeled with a fluorescein-conjugated secondary antibody (Molecular Probes, Eugene, OR) and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Samples were observed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Differentiation of LSCs into Corneal Epithelial Cells. ABCG2⁺/ABCB5⁺ LSCs were seeded in a 12-well plate at a density of 3×10^4 cells per well and cultured in 10% FBS DMEM. At 90% cell confluence, DMEM was replaced with CnT30 medium (CELLnTEC Advanced Cell Systems AG). Negative control cultures were maintained in 10% FBS DMEM. Culture media was changed every 2 days for 5 days, and then the cells were fixed in 3.7% formaldehyde and immunostained with the appropriate antibodies.

2.5. Differentiation of LSCs into Corneal Epithelial Cells on Transwell Filters. ABCG2⁺/ABCB5⁺ LSCs (1×10^5) were seeded onto 0.4 μ m 12-well transwell filters in 10% FBS DMEM. At 90% cell confluence, DMEM was replaced with CnT30 medium. Negative control cultures were maintained in 10% FBS DMEM. Cell differentiation was performed under immersed conditions. Culture media was changed every 2 days for 5 days, and then the cells were fixed in 3.7% formaldehyde and immunostained with the appropriate antibodies.

After 6 days, the transwell filters were fixed in 3.7% formaldehyde, embedded into paraffin, and sliced into 8 μ m-thick sections. The sections were washed in PBS, blocked in 5%

normal donkey serum and 0.1% Triton X-100, and incubated overnight in primary antibody 2 h at room temperature. The primary antibodies included anti-human p63 α (Cell Signaling), anti-human ABCG2 (Abcam), anti-human ABCB5 (Invitrogen), anti-human CK3 (Abcam), and anti-human desmoglein 3 (Novus Biologicals). Then, the sections were labeled with a fluorescein-conjugated secondary antibody (Molecular Probes), and nuclei were counterstained with DAPI. Samples were observed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Differentiation of LSCs into Corneal Epithelial Cells on Amniotic Membranes. A total of 1×10^5 LSCs were seeded on a 60 mm plate and infected with GFP⁺ lentivirus (MOI = 10) for 16 h. After 24 h, the transduction efficiency was evaluated based on the number of GFP-positive LSCs scored under a fluorescence microscope. Under these conditions, the transduction efficiency was $35\% \pm 1.2\%$. However, to acquire pure GFP⁺ LSCs, LSCs were selected with puromycin (lentiviral vector containing the puromycin selection marker). Thus, we used pure GFP⁺ LSCs in this experiment. We also compared the proliferation and differentiation efficiency of LSCs infected with no lentivirus, GFP-negative lentivirus, or GFP-positive lentivirus. However, we did not find any significant differences in proliferation or differentiation of LSCs under each condition.

GFP lentivirus-infected ABCG2⁺/ABCB5⁺ LSCs (1×10^5) were seeded onto 1 cm² human amniotic membranes in 10% FBS DMEM. After 3 days, DMEM was replaced with CnT30 medium (CELLnTEC Advanced Cell Systems AG). Culture media was changed every 2 days for 10 days. Amniotic membranes were fixed in 3.7% formaldehyde and immunostained with the appropriate antibodies.

2.7. Growth Assay. For serial propagations, ABCG2⁺/ABCB5⁺ LSCs and primary LECs were seeded at densities of 4×10^4 cells per well on 12-well plates coated with a matrix of Matrigel and fibronectin. After 2 days, cells were confluent and passaged to new plates at a 1:3 ratio, which allowed cells to achieve confluence within 2 more days. Cells were passaged every 2 days for 92 days.

2.8. Telomerase Activity Assay. Telomerase activity in LSCs and LECs was analyzed with the TRAPEZE® Telomerase Detection Kit (S7700-KIT; Millipore Company, Purchase, NY) according to the manufacturer's instructions. In brief, a cell pellet containing 500–1000 cells was resuspended in 50 μ L of 1X CHAPS lysis buffer, incubated on ice for 30 min, and centrifuged. Then, 5 μ L of the supernatant was transferred into a fresh tube and incubated with 5 μ L of master mix A, which consisted of TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.05% (v/v) Tween 20, 1 mM EDTA, and 0.01% BSA; TRAPEZE Telomerase Detection Kit), dNTPs, TS primer, and dH₂O. The reaction was incubated at 30°C for 30 min, at 94°C for 1 min, and then kept on ice. Then, 10 μ L of master mix C (TRAP buffer, 0.01% BSA, ACX primer, Taq polymerase, 15% glycerol, SYBR green, and dH₂O) was added, and PCR was performed in a thermocycler (Applied Biosystems Veriti Thermal Cycler;

ABI) as follows: 94°C for 2 min and 40 cycles of 94°C for 10 s, 50°C for 5 s, and 72°C for 10 s. The differences between the Ct values for the LSCs and LECs were calculated and graphed.

2.9. Metaphase Chromosome Spread Assay. Cells were arrested in metaphase by incubation in culture media with 0.05 µg/ml colcemid (final concentration) for 1 h. The cell suspensions were then treated with 75 mM KCl for 30 min at room temperature and fixed with Carnoy's solution (3:1 methanol:acetic acid, v/v). To form chromosome spreads, cell suspensions were dropped onto glass slides, air-dried, and stained with DAPI. Images were obtained by fluorescence microscopy. Twenty cells were analyzed in each group.

2.10. Flow Cytometric Analysis of the Cell Cycle. For analysis of DNA content, 1×10^5 cells were harvested, washed with PBS, resuspended in 2 ml of an ice-cold 70% ethanol and 30% PBS solution, and incubated on ice for 30 min. Cells were then harvested by centrifugation and stained with 15 µg/mL propidium iodide in PBS with 0.1 mg/mL RNase A for 30 min at 37°C. At least 10,000 cells were acquired per sample. Data were collected with CellQuest™ software and analyzed with ModFitLT.

2.11. Clonal Analysis. Clonal expansion of ABCG2⁺/ABCB5⁺ LSCs was performed by seeding a single-cell suspension at 1×10^3 cells/cm² on a plate coated with a matrix of Matrigel and fibronectin and culturing cells in 10% FBS DMEM. Culture media was changed every 2 days, and colony formation was monitored daily by phase contrast microscopy. Cells were fixed in 3.7% formaldehyde and immunostained with the appropriate antibodies 8 days after seeding.

2.12. Statistical Analysis. All of the experiments were repeated at least three times. Data were expressed as mean ± standard error, and statistical comparisons between groups were performed by one-way ANOVA followed by the Tukey's test.

3. Results

3.1. Establishment of Highly Proliferative LESC Cultures. To obtain highly proliferative and marker-specific pure LSCs, we cultured human-derived LECs in various conditions. Matrigel has been used as an extracellular matrix for culturing LSCs [27], but Matrigel alone is not sufficient to culture LSCs. Fibronectin has also been used to culture various stem cells [28–31]. In our study, we used Matrigel, fibronectin, and a mixture of Matrigel and fibronectin as extracellular matrices for culturing LSCs. Furthermore, LSCs have been shown to maintain their stemness in cultures without serum, but in a medium with serum, LSCs differentiated into corneal epithelial cells [32]. We cultured LECs in CnT20 medium without serum and in 10% serum DMEM (Supplementary Figure S1). However, highly proliferative colonies formed when a mixture of Matrigel and fibronectin was used as the matrix and when cultured for over 15 days in 10% serum DMEM (Supplementary Figure S1). To reduce the

culture time and to increase the numbers of highly proliferative colonies, isolated LECs were seeded onto a plate coated with a mixture of Matrigel and fibronectin and cultured in CnT20 for 3 days. Then, the medium was replaced with 10% serum DMEM and changed every 2 days and cells were cultured for 9 days. For 9 days, medium was changed every 2 days, but cells were not passaged. During the 9 days, small colonies appeared on the plates and grew large. Among the various colony morphologies, we observed fast-growing, multilayered LESC-like colonies (Figure 1(a)).

ABCG2 and ABCB5 are known LESC markers [33, 34]. To increase the purity of the LSCs, we labeled them with antibodies to ABCG2 and ABCB5 and performed FACS analysis (Figure 1(b)). The cells isolated by FACS had the high proliferation phenotype (Figure 1(b)), and we named these cells ABCG2⁺/ABCB5⁺ double-positive LSCs. Our results demonstrate that we identified the specific culture conditions required to isolate and expand marker-specific LSCs *in vitro*.

3.2. Marker Analysis of LSCs and Differentiation of ABCG2⁺/ABCB5⁺ LSCs into Corneal Epithelial Cells. To characterize the established ABCG2⁺/ABCB5⁺ LSCs, we analyzed their marker expression profiles by RT-qPCR and immunostaining. ABCG2⁺/ABCB5⁺ LSCs expressed LESC marker CK19, p63α, ABCG2, and integrin α9 [35–37] mRNAs when cultured in 10% serum DMEM (Figure 2(a)). Immunostaining also showed expression of p63α, ABCG2, and CK19 in ABCG2⁺/ABCB5⁺ LSCs (Figure 2(b)). When ABCG2⁺/ABCB5⁺ LSCs were cultured in differentiation media CnT20 and CnT30, expression of the stem cell markers CK19, p63α, ABCG2, and integrin α9 decreased (Figure 2(a)). In contrast, expression of the corneal epithelial cell markers CK3 and CK12 increased when ABCG2⁺/ABCB5⁺ LSCs were cultured in CnT20 and CnT30 (Figure 2(c)). These results demonstrate that ABCG2⁺/ABCB5⁺ LSCs express LESC-specific markers and differentiate into corneal epithelial cells.

3.3. Differentiation of LSCs into Corneal Epithelial Cells on Transwell Filters and Amniotic Membranes. LSCs have the potential to differentiate into corneal epithelial cells *in vitro* and *in vivo* [37–39]. To confirm the potential of ABCG2⁺/ABCB5⁺ LSCs to differentiate into corneal epithelial cells, we seeded ABCG2⁺/ABCB5⁺ LSCs in 12-well plates and changed the DMEM medium to CnT30 medium. When cultured in 10% serum DMEM, ABCG2⁺/ABCB5⁺ LSCs expressed the stem cell-specific markers ABCG2 and P63α, but when cultured in CnT30, P63α expression decreased and expression of the corneal epithelial cell marker CK3 increased (Figure 3(a)). Furthermore, we developed a new differentiation system that mimics *in vivo* differentiation conditions. The cornea consists of five layered cell in our body, and limbal LSCs move to the cornea and differentiate into corneal epithelial cells [39, 40]. To mimic *in vivo* conditions, we seeded ABCG2⁺/ABCB5⁺ LSCs onto 12-well transwell filters and cultured them in 10% serum DMEM or CnT30 for 5 days. For 5 days, medium was changed every 2 days, but cells were not passaged. Under these conditions,

FIGURE 1: Establishment of limbus-derived highly proliferative LECs. (a) Cultivation of highly proliferative LECs from limbal epithelial cells. (b) Isolation of ABCG2⁺/ABCB5⁺ double-positive LECs. Scale bars = 500 μm, 200 μm, and 100 μm.

cells displayed multilayer growth without cell death. ABCG2⁺/ABCB5⁺ LECs cultured on transwell filters in 10% serum DMEM displayed multilayer cell growth and expressed the stem cell markers ABCG2 and P63α. However, expression of stem cell markers decreased and expression of the corneal epithelial cell marker CK3 increased ABCG2⁺/ABCB5⁺ LECs cultured on transwell filters in CnT30 (Figure 3(b)). To analyze the transwell-cultured ABCG2⁺/ABCB5⁺ LECs in detail, transwell-cultured ABCG2⁺/ABCB5⁺ LECs were embedded in paraffin and were stained immunohistochemically. Immunohistochemistry showed that the multilayered cell growth of ABCG2⁺/ABCB5⁺ LECs on transwell filters resembles the cornea *in vivo* (Figure 4(a)). In addition, immunostaining showed that ABCG2⁺/ABCB5⁺ LECs on transwell filters expressed the stem cell markers ABCG2, ABCB5, and P63α when cultured in 10% serum DMEM and expressed the corneal epithelial cell markers CK3 and desmoglein 3 and when cultured in CnT30 (Figures 4(b) and 5). Finally, we evaluated the differentiation potential of ABCG2⁺/ABCB5⁺ LECs on amniotic membranes. GFP lentivirus-infected LECs were seeded onto

amniotic membranes and cultured for 10 days in CnT30, and they differentiated into corneal epithelial cells and expressed the corneal epithelial cell marker CK3 (Figure 6). Collectively, our results suggest that ABCG2⁺/ABCB5⁺ LECs can differentiate into corneal epithelial cells and that our newly developed differentiation system can mimic *in vivo* differentiation and can be used to analyze the differentiation potential of LECs.

3.4. Stem Cell Potential of LECs. To evaluate the stem cell potential of the ABCG2⁺/ABCB5⁺ LECs, we analyzed the growth of LECs and ABCG2⁺/ABCB5⁺ LECs. LECs were cultured for approximately 15 days and passaged 3 times. After this time, LEC proliferation decreased and growth stopped after 30 days and 5 passages (Figure 7(a)). In contrast, ABCG2⁺/ABCB5⁺ LECs showed continuous growth over 90 days and 50 passages (Figure 7(a)). Phase contrast image shows the maintenance of LECs (Figure 7(a)). Furthermore, because telomerase activity is associated with cell proliferation in cultured cells [41] and in some stem cells [42], we examined telomerase activity in ABCG2⁺/ABCB5⁺

FIGURE 2: Marker analysis of ABCG2⁺/ABCB5⁺ LSCs. (a, c) Total mRNA was isolated from ABCG2⁺/ABCB5⁺ LSCs cultured in 10% DMEM, CnT20, or CnT30, and gene expression was assessed by RT-PCR. ** $p < 0.01$ versus DMEM (b) ABCG2⁺/ABCB5⁺ LSCs were immunofluorescently stained with antibodies to p63 α , ABCG2, and CK19. Scale bar = 50 μ m. w/o Ab: without primary antibody + secondary antibody.

LESCs and found high telomerase activity (Figure 7(b)). However, telomerase activity was not detected in primary LECs (Figure 7(b)). After culturing ABCG2⁺/ABCB5⁺ LSCs for 90 days, metaphase chromosome spread analysis was performed to detect chromosome instabilities. The chromosome spread assay has the powerful ability to analyze individual cells for genome aberrations, including insertions, deletions, and rearrangements involving one or more chromosomes [43]. No genomic insertions, deletion, or rearrangements were detected in ABCG2⁺/ABCB5⁺ LSCs; however, genetic abnormalities were detected in SW620 colon cancer cells (Figure 7(c)). In addition, cell cycle analysis by flow

cytometry showed an increase in the S-phase of ABCG2⁺/ABCB5⁺ LSCs, which has also been seen in some stem cells, but the cell cycle of LECs was found to be normal (Figure 7(d)).

Some stem cells express core transcription factors, such as Oct4, Sox2, Nanog, c-Myc, and Klf4 [44–46]. To assess expression of core transcription factors in ABCG2⁺/ABCB5⁺ LSCs, we isolated mRNA from ABCG2⁺/ABCB5⁺ LSCs cultured in different conditions and performed RT-qPCR. ABCG2⁺/ABCB5⁺ LSCs expressed Oct4, Sox2, c-Myc, and Klf4 mRNAs when cultured in 10% serum DMEM, but expression of these markers decreased when the cells

FIGURE 3: Differentiation of $ABCG2^+/ABCB5^+$ LSCs into corneal epithelial cells. (a) $ABCG2^+/ABCB5^+$ LSCs were cultured in 10% DMEM or CnT30 and stained with cell-specific markers. (b) $ABCG2^+/ABCB5^+$ LSCs were cultured on transwell filters in 10% DMEM or CnT30 and stained with cell-specific markers. Scale bars = 100 μm and 200 μm .

were cultured in CnT20 or CnT30 differentiation media (Supplementary Figure S2). To further elucidate the stem cell character of $ABCG2^+/ABCB5^+$ LSCs, we examined the colony-forming activity of $ABCG2^+/ABCB5^+$ LSCs. Immunostaining showed strong colony formation by $ABCG2^+/ABCB5^+$ LSCs (Supplementary Figure S3) indicating that $ABCG2^+/ABCB5^+$ LSCs have significant stem cell activity and may be used to regenerate corneal epithelia. Moreover, $ABCG2^+/ABCB5^+$ LSCs may be multipotent and may be able to differentiate into other cell lineages in addition to corneal epithelial cells.

4. Discussion

Many researchers have attempted to retain LSCs in culture, but have been unsuccessful. Since the pioneering work in 1975 by Rheinwald and Green [17], studies have shown that long-term survival and serial expansion of LSCs are possible if they are cocultured with fibroblast feeder cells. Nevertheless, the question of whether the transplanted cell sheets actually contain LSCs has not been addressed and the widespread use of this promising cultivation technique has been hampered by the lack of a standardized cultivation protocol. To expand cells and generate epithelial sheets, fibrin gels, temperature-responsive polymers, and amniotic membranes

have been used [11], but epithelial cell viability was not sustained for very long [12], and no donor cells survived 9 months after transplantation [13, 14]. These failures may have resulted from depletion of LSCs in culture due to improper culture conditions. Rather than favoring retention of LSCs, most culture methods promote the proliferation and terminal differentiation of transient amplifying cells (TACs) [15]. Long-term restoration of damaged ocular surfaces requires retention of a sufficient amount of LSCs during culturing and after grafting [4, 16] to ensuring successful regeneration of the ocular surface [47, 48].

In this study, we developed an optimal method to expand and increase the survival and proliferation of LSCs derived from a small limbal biopsy. Matrigel and fibronectin were used as matrices for culturing LSCs and other stem cells [27–31], but Matrigel or fibronectin alone is not sufficient to culture LSCs. In this study, highly proliferative LESC colonies formed when we used a mixture of Matrigel and fibronectin as the extracellular matrix.

LSCs are located in the basal region of the limbus and require a specific environment for survival and growth. To mimic the in vivo environment, we used various extracellular matrix components (Matrigel, collagen, gelatin, fibronectin, fibrin, etc.) for the in vitro culture of LSCs. However, we did not identify the optimal conditions for LESC culture.

FIGURE 4: Differentiation of $\text{ABCG2}^+/\text{ABCB5}^+$ LESC into corneal epithelial cells with a transwell system. (a) Sections of $\text{ABCG2}^+/\text{ABCB5}^+$ LESC cultured on transwell filters were stained with hematoxylin and eosin. (b) $\text{ABCG2}^+/\text{ABCB5}^+$ LESC were cultured onto transwell filters in 10% DMEM, and transwell sections were stained with cell-specific markers. Scale bar = 100 μm . w/o Ab: without primary antibody + secondary antibody.

FIGURE 5: Differentiation of $\text{ABCG2}^+/\text{ABCB5}^+$ LESC into corneal epithelial cells with a transwell system. $\text{ABCG2}^+/\text{ABCB5}^+$ LESC were cultured onto transwell filters in CnT30, and transwell sections were stained with cell-specific markers. Scale bar = 100 μm . w/o Ab: without primary antibody + secondary antibody.

FIGURE 6: Differentiation of $ABCG2^+/ABCB5^+$ LESC into corneal epithelial cells on an amniotic membrane. GFP-positive $ABCG2^+/ABCB5^+$ LESC were cultured on an amniotic membrane and were stained with antibodies to CK3. Scale bar = 200 μ m.

Matrigel has been used as an extracellular matrix for culturing LESC [27]. However, Matrigel alone is not sufficient for the culture of LESC under our conditions. We have had much experience in the culture of many types of stem cells (mesenchymal stem cells, endothelial stem cells, neuronal stem cells, embryonic stem cells, etc.) and have found that fibronectin has good effects on stem cell proliferation. Therefore, we mixed Matrigel and fibronectin, and highly proliferative LESC colonies formed when we used this mixture as the extracellular matrix. Other combinations of extracellular matrix components did not yield sufficient LESC culture. These results suggested that stimulation by fibronectin may recover the signal required for LESC growth, which could not be obtained by Matrigel alone.

In addition, LESC are known to maintain their stemness in a medium without serum, but a medium with serum induces differentiation of LESC into corneal epithelial cells [32]. We cultured LECs in CnT20 medium without serum and in 10% serum DMEM without extracellular matrices and did not observe highly proliferative cell phenotypes. However, highly proliferative cell colonies formed when LECs were cultured with a matrix of Matrigel and fibronectin in 10% serum DMEM indicating that specific culture conditions, including the compositions of the extracellular matrix and the cell culture medium, are required for efficient growth of undifferentiated LESC. In this study, we demonstrated

that corneal LESC can be consistently expanded *in vitro* using a mixed extracellular matrix and a medium containing serum. Meyer-Blazejewska et al. proposed an improved culture protocol in 2010 [37]. There are three major differences between our method and the method of Meyer-Blazejewska et al. First, we cultured the cells on a mixture of Matrigel and fibronectin, whereas they cultured the cells on a 3T3 feeder cell layer. Second, we used CnT20 and DMEM containing 10% serum, whereas they used MCDM151, Epilife, DMEM/F12, PCT, or D-KSFM with several growth factors. Third, we used transwell filters and amniotic membranes for *in vitro* differentiation, whereas they used fibrin gel. Most importantly, the clonal growth phenotype was very different. Under our conditions, LESC showed rapid growth and multilayered colonies on the plates. However, they showed only monolayer colonies. Collectively, these results suggested that our method for cultivation of $ABCG2^+/ABCB5^+$ LESC was different from that of Meyer-Blazejewska et al. and that our established LESC exhibited different characteristics, although some markers were similar.

The isolated and cultured $ABCG2^+/ABCB5^+$ LESC retained their viability and stemness as confirmed by the presence of the stem cell markers CK19, p63 α , ABCG2, and integrin α 9. We confirmed *in vitro* differentiation of LESC into corneal epithelial cells by the presence of the markers CK12, CK3, and desmoglein3. These results suggest that

FIGURE 7: ABCG2⁺/ABCB5⁺ LSCs are potent stem cells. (a) Growth analysis of LSCs and LECs. (b) Telomerase activity in ABCG2⁺/ABCB5⁺ LSCs and in LECs (500, 1000 cells) was measured by TRAP assay. ** $p < 0.01$ versus LEC (c) metaphase chromosome spreads of SW620 colon cancer cells and ABCG2⁺/ABCB5⁺ LSCs. (d) Cell cycle analysis of ABCG2⁺/ABCB5⁺ LSCs and LECs by propidium iodide staining and flow cytometry.

ABCG2⁺/ABCB5⁺ LSCs express LESC-specific markers and can differentiate into corneal epithelial cells. Moreover, our newly developed transwell filter differentiation system mimics *in vivo* differentiation and may be used to analyze the differentiation potential of LSCs *in vivo*.

The ABCG2⁺/ABCB5⁺ LSCs that we established displayed powerful stem cell activity, continuous growth, and high telomerase activity without chromosome instability. In addition, cell cycle analysis by flow cytometry showed that the S-phase of ABCG2⁺/ABCB5⁺ LSCs increased, which has been observed in other stem cells, but the cell cycle of LECs remained normal. In general, fate-determined normal cells (limbal epithelial cells, fibroblasts, endothelial cells, skin epithelial cells, etc.) exhibited a slow cell cycle, and about 20–30% of cells were in the S-phase, similar to LECs. However, stem cells (mesenchymal stem cells, embryonic stem cells, etc.) showed a rapid cell cycle and increased percentage of cells in the S-phase (over 50% of cells were in the S-phase).

ABCG2⁺/ABCB5⁺ LSCs grew very rapidly and showed an increase in the proportion of cells in the S-phase (over 50% of cells were in the S-phase), similar to other stem cells. Therefore, we suggested that ABCG2⁺/ABCB5⁺ LSCs had stem cell characteristics.

Moreover, ABCG2⁺/ABCB5⁺ LSCs expressed the core transcription factors Oct4, Sox2, c-Myc, and Klf4, which are also expressed in multipotent stem cells. These data indicate that the ABCG2⁺/ABCB5⁺ LSCs that we established have powerful stem cell activity and may be used to regenerate corneal epithelia. Moreover, ABCG2⁺/ABCB5⁺ LSCs may be multipotent and may be able to differentiate into additional cell lineages.

In conclusion, our results show that with the appropriate methods, including the appropriate matrix and medium, human limbus-derived, highly proliferative ABCG2⁺/ABCB5⁺ double-positive LSCs can be cultured. The cultured LSCs exhibited the LESC marker profile and the

ability to differentiate into corneal epithelial cells. Moreover, the LSCs expressed high levels of the multipotent stem cell markers Sox2, Oct4, c-Myc, and Klf4, displayed high telomerase activity, and were found to have a stable, normal genome. These results suggest that our novel culture system may be essential for long-term clinical success and stable regeneration of corneal epithelia to treat corneal blindness due to an LESC deficiency.

5. Conclusions

In summary, we described an improved cultivation protocol using biopsies from the limbus, appropriate extracellular matrices, and appropriate culture media to clonally expand marker-specific-isolated stem cells and to subsequently subcultivate highly proliferative cell colonies on a mixed Matrigel and fibronectin extracellular matrix in a defined environment to support the expansion and retention of stem cells. Whether this culture technique enhances the therapeutic potential of LESC transplantation remains to be evaluated. Nevertheless, this culture system may represent a new starting point for establishing a true stem cell-based therapy for long-term ocular surface reconstruction. Moreover, for extended survival of stem cells in a cultured graft, factors that reproduce the niche environment must be integrated into the culture system in the future.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by a Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A1B03933337).

References

- [1] M. De Luca, G. Pellegrini, and H. Green, "Regeneration of squamous epithelia from stem cells of cultured grafts," *Regenerative Medicine*, vol. 1, no. 1, pp. 45–57, 2006.
- [2] A. J. Shortt, G. A. Secker, M. D. Notara et al., "Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results," *Survey of Ophthalmology*, vol. 52, no. 5, pp. 483–502, 2007.
- [3] J. T. Daniels, M. Notara, A. J. Shortt, G. Secker, A. Harris, and S. J. Tuft, "Limbal epithelial stem cell therapy," *Expert Opinion on Biological Therapy*, vol. 7, no. 1, pp. 1–3, 2007.
- [4] G. Pellegrini, M. De Luca, and Y. Arsenijevic, "Towards therapeutic application of ocular stem cells," *Seminars in Cell & Developmental Biology*, vol. 18, no. 6, pp. 805–818, 2007.
- [5] R. J. Tsai, L. M. Li, and J. K. Chen, "Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells," *The New England Journal of Medicine*, vol. 343, no. 2, pp. 86–93, 2000.
- [6] N. Koizumi, T. Inatomi, T. Suzuki, C. Sotozono, and S. Kinoshita, "Cultivated corneal epithelial stem cell transplantation in ocular surface disorders," *Ophthalmology*, vol. 108, no. 9, pp. 1569–1574, 2001.
- [7] J. Shimazaki, M. Aiba, E. Goto, N. Kato, S. Shimmura, and K. Tsubota, "Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders," *Ophthalmology*, vol. 109, no. 7, pp. 1285–1290, 2002.
- [8] M. Grueterich, E. M. Espana, A. Touhami, S. E. Ti, and S. C. Tseng, "Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency," *Ophthalmology*, vol. 109, no. 8, pp. 1547–1552, 2002.
- [9] V. S. Sangwan, G. K. Vemuganti, S. Singh, and D. Balasubramanian, "Successful reconstruction of damaged ocular outer surface in humans using limbal and conjunctival stem cell culture methods," *Bioscience Reports*, vol. 23, no. 4, pp. 169–174, 2003.
- [10] T. Nakamura, T. Inatomi, C. Sotozono, N. Koizumi, and S. Kinoshita, "Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease," *Acta Ophthalmologica Scandinavica*, vol. 82, no. 4, pp. 468–471, 2004.
- [11] 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*, vol. 77, no. 3, pp. 379–385, 2004.
- [12] J. Shimazaki, F. Maruyama, S. Shimmura, H. Fujishima, and K. Tsubota, "Immunologic rejection of the central graft after limbal allograft transplantation combined with penetrating keratoplasty," *Cornea*, vol. 20, no. 2, pp. 149–152, 2001.
- [13] S. M. Daya, A. Watson, J. R. Sharpe et al., "Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction," *Ophthalmology*, vol. 112, no. 3, pp. 470–477, 2005.
- [14] J. R. Sharpe, S. M. Daya, M. Dimitriadi, R. Martin, and S. E. James, "Survival of cultured allogeneic limbal epithelial cells following corneal repair," *Tissue Engineering*, vol. 13, no. 1, pp. 123–132, 2007.
- [15] W. Li, Y. Hayashida, H. He, C. L. Kuo, and S. C. Tseng, "The fate of limbal epithelial progenitor cells during explant culture on intact amniotic membrane," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 2, pp. 605–613, 2007.
- [16] G. Pellegrini, P. Rama, F. Mavilio, and M. De Luca, "Epithelial stem cells in corneal regeneration and epidermal gene therapy," *The Journal of Pathology*, vol. 217, no. 2, pp. 217–228, 2009.
- [17] J. G. Rheinwald and H. Green, "Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells," *Cell*, vol. 6, no. 3, pp. 331–343, 1975.
- [18] S. C. Tseng, F. E. Kruse, J. Merritt, and D. Q. Li, "Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media," *Current Eye Research*, vol. 15, no. 9, pp. 973–984, 1996.
- [19] Y. Barrandon and H. Green, "Three clonal types of keratinocyte with different capacities for multiplication," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 8, pp. 2302–2306, 1987.

- [20] G. Pellegrini, C. E. Traverso, A. T. Franzi, M. Zingirian, R. Cancedda, and M. De Luca, "Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium," *The Lancet*, vol. 349, no. 9057, pp. 990–993, 1997.
- [21] G. Pellegrini, R. Ranno, G. Stracuzzi et al., "The control of epidermal stem cells (holoclones) in the treatment of massive full-thickness burns with autologous keratinocytes cultured on fibrin," *Transplantation*, vol. 68, no. 6, pp. 868–879, 1999.
- [22] 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*, vol. 72, no. 9, pp. 1478–1485, 2001.
- [23] V. Ronfard, J. M. Rives, Y. Neveux, H. Carsin, and Y. Barrandon, "Long-term regeneration of human epidermis on third degree burns transplanted with autologous cultured epithelium grown on a fibrin matrix," *Transplantation*, vol. 70, no. 11, pp. 1588–1598, 2000.
- [24] F. Mavilio, G. Pellegrini, S. Ferrari et al., "Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells," *Nature Medicine*, vol. 12, no. 12, pp. 1397–1402, 2006.
- [25] M. Wernig, A. Meissner, R. Foreman et al., "In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state," *Nature*, vol. 448, no. 7151, pp. 318–324, 2007.
- [26] J. B. Kim, H. Zaehres, G. Wu et al., "Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors," *Nature*, vol. 454, no. 7204, pp. 646–650, 2008.
- [27] N. Ahmadiankia, M. Ebrahimi, A. Hosseini, and H. Baharvand, "Effects of different extracellular matrices and co-cultures on human limbal stem cell expansion in vitro," *Cell Biology International*, vol. 33, no. 9, pp. 978–987, 2009.
- [28] Y. S. Maeng, J. Y. Kwon, E. K. Kim, and Y. G. Kwon, "Heterochromatin protein 1 alpha (HP1 α : CBX5) is a key regulator in differentiation of endothelial progenitor cells to endothelial cells," *Stem Cells*, vol. 33, no. 5, pp. 1512–1522, 2015.
- [29] L. Zhou, X. Chen, T. Liu et al., "SIRT1-dependent anti-senescence effects of cell-deposited matrix on human umbilical cord mesenchymal stem cells," *Journal of Tissue Engineering and Regenerative Medicine*, 2017.
- [30] K. M. French, J. T. Maxwell, S. Bhutani et al., "Fibronectin and cyclic strain improve cardiac progenitor cell regenerative potential in vitro," *Stem Cells International*, vol. 2016, Article ID 8364382, 11 pages, 2016.
- [31] K. Xue, X. Zhang, L. Qi, J. Zhou, and K. Liu, "Isolation, identification, and comparison of cartilage stem progenitor/cells from auricular cartilage and perichondrium," *American Journal of Translational Research*, vol. 8, no. 2, pp. 732–741, 2016.
- [32] A. Mikhailova, T. Ilmarinen, A. Ratnayake et al., "Human pluripotent stem cell-derived limbal epithelial stem cells on bioengineered matrices for corneal reconstruction," *Experimental Eye Research*, vol. 146, pp. 26–34, 2016.
- [33] B. R. Ksander, P. E. Kolovou, B. J. Wilson et al., "ABCB5 is a limbal stem cell gene required for corneal development and repair," *Nature*, vol. 511, no. 7509, pp. 353–357, 2014.
- [34] M. Morita, N. Fujita, A. Takahashi et al., "Evaluation of ABCG2 and p63 expression in canine cornea and cultivated corneal epithelial cells," *Veterinary Ophthalmology*, vol. 18, no. 1, pp. 59–68, 2015.
- [35] R. Albert, Z. Vereb, K. Csomos et al., "Cultivation and characterization of cornea limbal epithelial stem cells on lens capsule in animal material-free medium," *PLoS One*, vol. 7, no. 10, p. e47187, 2012.
- [36] H. Ouyang, Y. Xue, Y. Lin et al., "WNT7A and PAX6 define corneal epithelium homeostasis and pathogenesis," *Nature*, vol. 511, no. 7509, pp. 358–361, 2014.
- [37] E. A. Meyer-Blazejewska, F. E. Kruse, K. Bitterer et al., "Preservation of the limbal stem cell phenotype by appropriate culture techniques," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 2, pp. 765–774, 2010.
- [38] V. Holan, P. Trosan, C. Cejka et al., "A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction," *Stem Cells Translational Medicine*, vol. 4, no. 9, pp. 1052–1063, 2015.
- [39] N. Di Girolamo, "Moving epithelia: tracking the fate of mammalian limbal epithelial stem cells," *Progress in Retinal and Eye Research*, vol. 48, pp. 203–225, 2015.
- [40] F. Castro-Munozledo, "Review: corneal epithelial stem cells, their niche and wound healing," *Molecular Vision*, vol. 19, pp. 1600–1613, 2013.
- [41] L. L. Smith, H. A. Collier, and J. M. Roberts, "Telomerase modulates expression of growth-controlling genes and enhances cell proliferation," *Nature Cell Biology*, vol. 5, no. 5, pp. 474–479, 2003.
- [42] E. Hiyama and K. Hiyama, "Telomere and telomerase in stem cells," *British Journal of Cancer*, vol. 96, no. 7, pp. 1020–1024, 2007.
- [43] B. Howe, A. Umrigar, and F. Tsien, "Chromosome preparation from cultured cells," *Journal of Visualized Experiments*, vol. 83, article e50203, 2014.
- [44] L. A. Boyer, T. I. Lee, M. F. Cole et al., "Core transcriptional regulatory circuitry in human embryonic stem cells," *Cell*, vol. 122, no. 6, pp. 947–956, 2005.
- [45] S. Corbinea, B. Lassalle, M. Givélet et al., "Spermatogonial stem cells and progenitors are refractory to reprogramming to pluripotency by the transcription factors *Oct3/4*, *c-Myc*, *Sox2* and *Klf4*," *Oncotarget*, vol. 8, no. 6, pp. 10050–10063, 2017.
- [46] L. Liu, X. Wei, J. Ling, L. Wu, and Y. Xiao, "Expression pattern of Oct-4, Sox2, and c-Myc in the primary culture of human dental pulp derived cells," *Journal of Endodontics*, vol. 37, no. 4, pp. 466–472, 2011.
- [47] Y. Barrandon, "Crossing boundaries: stem cells, holoclones, and the fundamentals of squamous epithelial renewal," *Cornea*, vol. 26, no. 9, Supplement 1, pp. S10–S12, 2007.
- [48] S. Shimmura and K. Tsubota, "Surgical treatment of limbal stem cell deficiency: are we really transplanting stem cells?," *American Journal of Ophthalmology*, vol. 146, no. 2, pp. 154–155, 2008.