

Healing Ability of Central Corneal Epithelium in Rabbit Ocular Surface Injury Models

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Received: December 7, 2021

Accepted: June 2, 2022

Published: June 30, 2022

Keywords: limbal stem/progenitor cells; central corneal epithelial cells; corneal wound healing

Citation: Zhang W, Lan X, Zhu J, Zhang C, Huang Y, Mo K, Tan J, Guo H, Huang H, Li M, Ouyang H, Wang L. Healing ability of central corneal epithelium in rabbit ocular surface injury models. *Transl Vis Sci Technol.* 2022;11(6):28. <https://doi.org/10.1167/tvst.11.6.28>

Purpose: Wound healing of the corneal epithelium mainly involves two types of cells: limbal stem/progenitor cells (LSCs) and differentiated central corneal epithelial cells (CECs). The healing ability of CECs is still debatable, and its correlated transcriptomic alterations during wound healing are yet to be elucidated. This study aimed to determine the healing ability and mechanisms underlying the actions of CECs using rabbit ocular surface injury models.

Methods: A central corneal ring-like residual epithelium model was used to investigate the healing ability of CECs. Uninjured and injury-stimulated LSCs and CECs were collected for transcriptomic analysis. The analysis results were verified by quantitative reverse transcriptase polymerase chain reaction, immunofluorescence staining, and two types of rabbit corneal injury models.

Results: During wound healing, the upregulated genes in LSCs were mostly enriched in the mitotic cell cycle–related processes, but those in CECs were mostly enriched in cell adhesion and migration. CECs could repair the epithelial defects successfully at one-time injuries. However, after repetitive injuries, the CECs repaired notably slower and failed to completely heal the defect, but the LSCs repaired even faster than the one-time injury.

Conclusions: Our results indicated rabbit CECs repair the epithelial defect mainly depending on migration and its proliferative ability is limited, and LSCs are the main source of regenerative epithelial cells.

Translational Relevance: This study provides information on gene expression in the corneal epithelium during wound healing, indicating that regulation of the cell cycle, cell adhesion, and migration may be the basis for future treatment strategies for corneal wound healing.

Introduction

The cornea is the outermost tissue layer of the eyeball. In contrast to other organs, it has unique transparent and avascular properties, which are essential for clear vision. Corneal epithelium plays an essential role in protecting the eyes against UV rays, chemical injury, and pathogens from the external environment. The integrity of the corneal epithelium is important for the (lymph)angiogenic privilege of the cornea and further for the clear vision.^{1,2} The corneal epithelium has to

continuously self-renew to maintain a smooth optical surface.³ There are 12.7 million people globally with moderate to severe vision loss (vision less than 20/60) resulting from corneal opacity, which can be surgically corrected, and are actively waiting for a corneal transplant.⁴ However, due to the shortage of corneal graft tissue, approximately 53% people in the world have no access to corneal transplantation.⁵ Deeper understanding of the corneal wound-healing process can help avoid corneal opacity and can be used to develop new treatment strategies to ease the shortage of corneal graft tissue.

Corneal epithelial stem/progenitor cells are mainly derived from the corneoscleral limbus, so they are also known as limbal stem/progenitor cells (LSCs).^{6,7} Daughter cells of LSCs could migrate into the central cornea and differentiate into cytokeratin 12(KRT12)-positive committed epithelial cells.^{8,9} Previous studies proposed the X, Y, Z hypothesis for corneal epithelial maintenance: the transient amplifying (TA) cells, which are the daughter cells of LSCs, migrate centripetally (Y component) into the basal layer of the corneal epithelium and are then differentiated into the upper layers of the corneal epithelium (X component) to become postmitotic cells and are finally lost from the ocular surface (Z component).¹⁰

Corneal epithelial stem cells are believed to reside exclusively in the limbus, and the central corneal epithelium is thought to have no stem/progenitor cells that have little multiplication capacity compared to peripheral epithelial cells and LSCs.^{11,12} In 1998, Lehrer et al.¹³ unveiled that all slow-cycling cells are preferentially located in the limbus under resting conditions, while most TA cells are located in the peripheral corneal epithelium. Furthermore, Bojic et al.¹⁴ found that CD200, which enables enrichment of quiescent corneal stem cells with holoclone-forming potential, was exclusively located at the limbus. However, accumulating evidence indicates that central corneal epithelial cells (CECs) may have multiplication capacity, contributing to closure of small corneal wounds.^{15,16} Majo et al.¹⁷ suggested that the central corneal epithelium contains stem cells because CECs can give rise to large colonies, and serially transplanted mouse CECs can be regenerated. Chang et al.¹⁸ collected cells from the human central cornea and limbus for sphere-forming assays and found that both limbal and central epithelial cells were capable of forming spheres. The clonogenic sphere-forming ability of the central epithelium indicated that it contained cells with stem/progenitor properties. Nasser et al.¹⁹ found that following surgical deletion of the LSC pool, corneal-committed cells could dedifferentiate into bona fide LSCs that retained normal tissue dynamics and marker expression. However, whether stem cells exist within the central cornea remains debatable.^{20,21}

Wound healing is a convoluted and well-orchestrated process initiated in response to tissue injury, which involves diverse cellular and molecular interactions. Investigating the mechanisms of wound healing is helpful for understanding many biological processes such as regeneration, senescence, and cancer.^{22–24} The different outcomes of wound healing could lead to perfect regeneration or scar tissues.²⁵ Current studies mostly focused on comparing the differences between LSCs and CECs under resting

conditions^{26,27} without defining the different characteristics of gene expression between LSCs and CECs during wound healing. In view of this, this study aimed to determine the healing ability of CECs and the dynamic changes in genes during corneal wound healing.

Methods

Animal and Ethics Statement

This study was approved and supervised by the Animal Care and Use Committee of Zhongshan Ophthalmic Center. All the animal experiments were conducted according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Healthy adult male New Zealand white rabbits (weight 2.1–2.5 kg) were obtained from the Xinhua Laboratory Animal Base (Guangzhou, China).

Ocular Surface Injury Models

Two types of rabbit ocular surface injury models were used in this study. These rabbits were generally anesthetized with 3 mg/kg xylazine hydrochloride and 20 mg/kg ketamine hydrochloride and topically anesthetized by 0.5% proparacaine hydrochloride (Alcon, Fort Worth, TX, USA). To avoid the influence of circadian rhythm, all injuries were performed at the same time of day. In the corneal central ring-like residual epithelium model (model 1), the margins of the central epithelial injury were demarcated by the trephines 3 mm and 7.5 mm in diameter. In the whole central corneal epithelium injury model (model 2), the margins of the epithelial injury were demarcated by the trephines 12 mm in diameter. Corneal epithelium was scraped off by a dulled blade.

Culture of Human LSCs and Three-Dimensional Differentiation In Vitro

Human eyeballs were obtained from the eye bank (Guangdong, China). Limbus regions were taken and cut into small pieces for further procedures. The cell culture procedure was the same as previously reported.⁷ Briefly, after the limbus digestion by 0.2% collagenase IV at 37°C for 2 hours, the cluster obtained was further digested with 0.25% trypsin-EDTA to obtain singlet. Primary cells were then seeded on cell culture vessels coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and cultured in the human LSC medium.⁷

Three-dimensional differentiation was performed on an eight-well chamber. Dissociated single LSC cells were embedded in Matrigel at 2×10^4 cells per 50 mL gel. After 14 to 18 days' culture in differentiation medium CnT-30 (Cellntec, Bern, Switzerland), three-dimensional structures were formed.

RNA Extraction and Messenger RNA Sequencing

The samples of uninjured corneal limbal epithelium (360-degree peritomy, lamellar dissection of the limbal regions, and width of 2 mm) and the corneal central epithelium (7.5 mm in diameter) were collected in healthy rabbits. Correspondingly, the recovered tissue from the previous injured area, including limbal and central corneal epithelium, was collected as mentioned above after a 16-hour injury. Total RNA was extracted from more than three biological repetitions by RNeasy kits (Qiagen, Hilden, Germany), respectively. The preparation of the complementary DNA (cDNA) library and RNA sequencing (RNA-seq) were performed by the Annoroad Gene Technology Co., Ltd. (Beijing, China) on the HiSeqX-ten platform using the PE150 strategy (Illumina, San Diego, CA, USA), and 6 G reads were obtained of each sample. Original sequence was filtered to obtain high-quality clean data as in a previous report.²⁸ The subsequent analysis was performed with the clean data.

Gene Expression Analysis

RNA-seq data were analyzed following RSEM (RNA-Seq by Expectation-Maximization) quantification.²⁹ The sequence was mapped according to the reference genome OryCun2.0 (GCA_000003625.1) that was downloaded from the Ensembl website (<http://www.ensembl.org/>). DESeq2 R package was used to analyze the different expression of genes. The `prcomp` function was used to do principal component analysis (PCA). The volcanic maps were drawn by imageGP (<http://www.ehbio.com/ImageGP/index.php/Home/Index/Volcanoplot.html>).

Enrichment Analysis

The criterion for selecting the significantly differential expressed genes was set at $|\log_2 \text{fold change}| > 1$ and $q < 0.05$. Gene ontology enrichment analysis was performed at <https://biit.cs.ut.ee/gprofiler/gost> and illustrated by the `ggplot2` package in R.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

RNA was reverse transcribed into cDNA referring to the instruction of the superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). OligoArchitect online (<http://www.oligoarchitect.com/LoginServlet>) was used to design the primers of target genes (Supplementary Table S1). Following the SYBR real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster, CA, USA), the expression level of genes in each transcript was normalized by housekeeping gene GAPDH.

Immunohistochemistry

Uninjured and injured (16 hour) rabbit corneas were excised and fixed with 10% formaldehyde in $1 \times$ phosphate-buffered saline, then embedded in paraffin and sectioned at $5 \mu\text{m}$ on a microtome. After sections were deparaffinized and rehydrated, antigen retrieval was performed. The sections next were permeabilized with 0.3% Triton X-100 for 15 minutes and blocked by 3% bovine serum albumin for 1 hour at room temperature (RT). Subsequently, these sections were incubated with primary antibody (Supplementary Table S2) overnight at 4°C , with fluorescently labeled secondary antibody for 1 hour at room temperature and with nuclear dye Hoechst 33,258 (Thermo Fisher, Waltham, MA, USA) for 20 minutes at RT.

Data Analysis

For bar graphs, mean \pm SEM value was used to present the data of each group. Statistical analyses were performed using SPSS 26.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The data were assessed by the Shapiro–Wilk normality test. The data not following a Gaussian distribution were analyzed using the Kruskal–Wallis test. Otherwise, data were analyzed by Student's *t*-tests. $P < 0.05$ was considered statistically significant (asterisks indicate significant difference).

Results

Healing Ability of Rabbit CECs

To validate the healing ability of CECs without the influence of the LSCs, we established a ring-like central corneal epithelium residual model (model 1) of rabbits, which consisted of a circular central injury

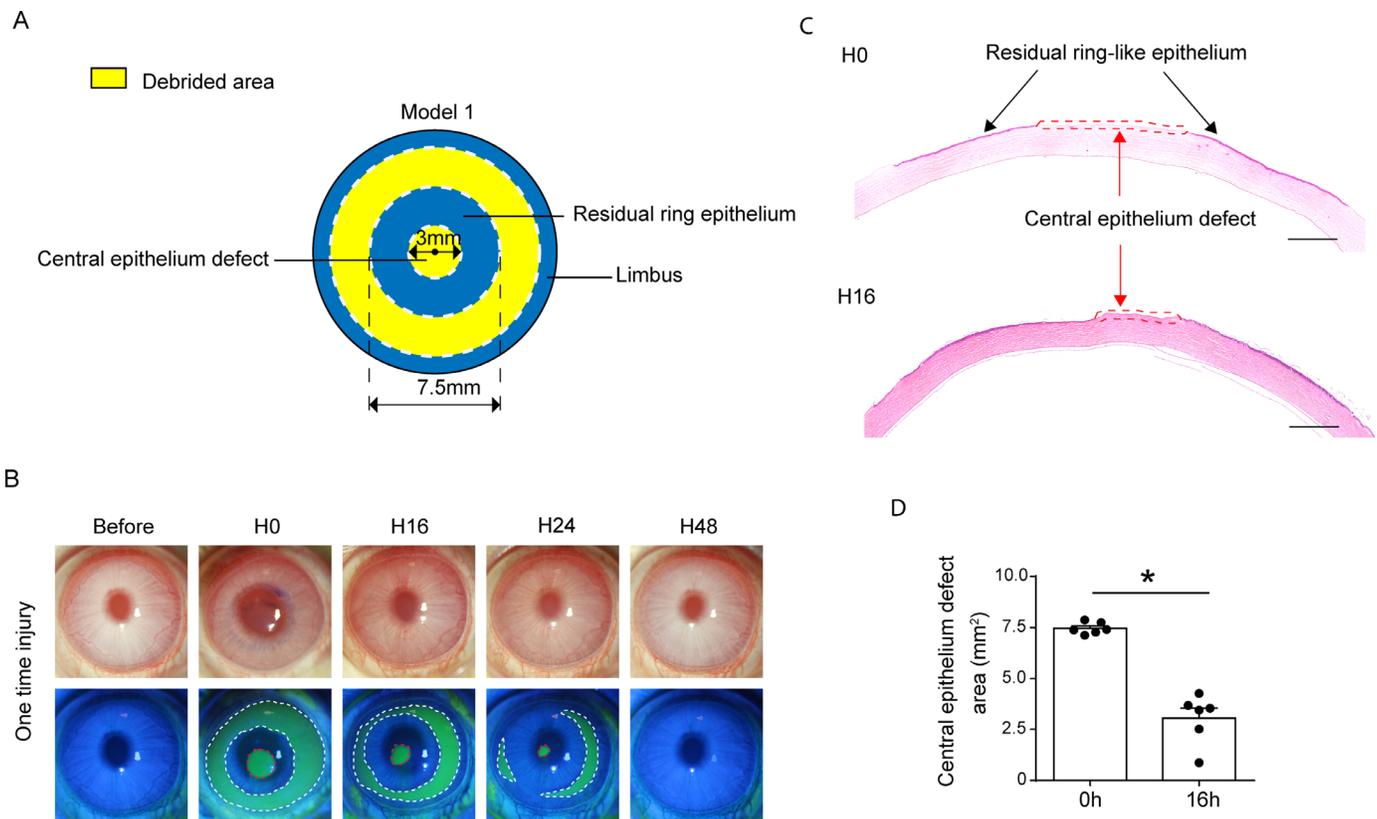


Figure 1. Corneal central ring-like epithelium residual model (model 1) and evaluation of the healing ability of CECs. (A) A diagram of model 1 (yellow area indicates the debridement area, and the blue area is the uninjured area). (B) Observation by slit-lamp microscopy under bright light (upper panel) and cobalt blue light with fluorescein sodium stain (lower panel). Central (red dotted line) and peripheral (white dotted line) corneal epithelial defect area decreased by the time. (C) Representative hematoxylin and eosin images of injured rabbit corneas at 0 and 16 hours. At 16 hours, the central epithelial defect area (red arrows) decreased and the central residual ring-like epithelium (black arrows) had not merged with the cells derived from the limbus. Scale bars: 500 μm . (D) Quantification of the central epithelial defect area. The defect area was significantly decreased at 16 hours postinjury ($*P < 0.05$, $n = 6$).

(3 mm diameter) surrounded by a ring-like residual central epithelium (3–7.5 mm diameter), and a ring-like peripheral epithelium injury (yellow concentric area) without hurting the limbus, as demonstrated in Figure 1A.

After 16 hours of recovery, 2% fluorescein staining imaging and measurement showed that central defect areas were significantly decreased, but there still existed an integrated ring-like defect in the peripheral epithelium, which indicated that LSCs had not moved in or contributed to the repair of the central epithelial defect. Therefore, the recovered central corneal epithelium was only healed by CECs. After 24 hours of recovery, the peripheral epithelial defect ring was no longer intact, indicating that epithelial cells derived from LSCs may have been mixed with CECs (Fig. 1B), and the origin of the healing epithelium became indistinguishable. Hence, we chose 16 hours as the time point for collecting recovery tissue for messenger RNA (mRNA)-seq analysis. Furthermore, para-

fin sections of the cornea with hematoxylin and eosin staining confirmed the corneal epithelial defect and central residual ring-like epithelium after wound formation, and the peripheral and central epithelium were still separated from each other until 16 hours after injury (Fig. 1C). Analysis of the central corneal area revealed that the central defect areas were significantly decreased at 16 hours (Fig. 1D). These results indicate that rabbit CECs have a healing ability and contribute to central corneal healing independent of LSCs.

Transcriptomic Difference Between LSCs and CECs During Corneal Epithelium Healing

Based on the above animal experimental results, total RNA from two independent samples of injured central (Injured-C) and limbal corneal epithelium (Injured-L) were extracted at 16 hours postinjury.

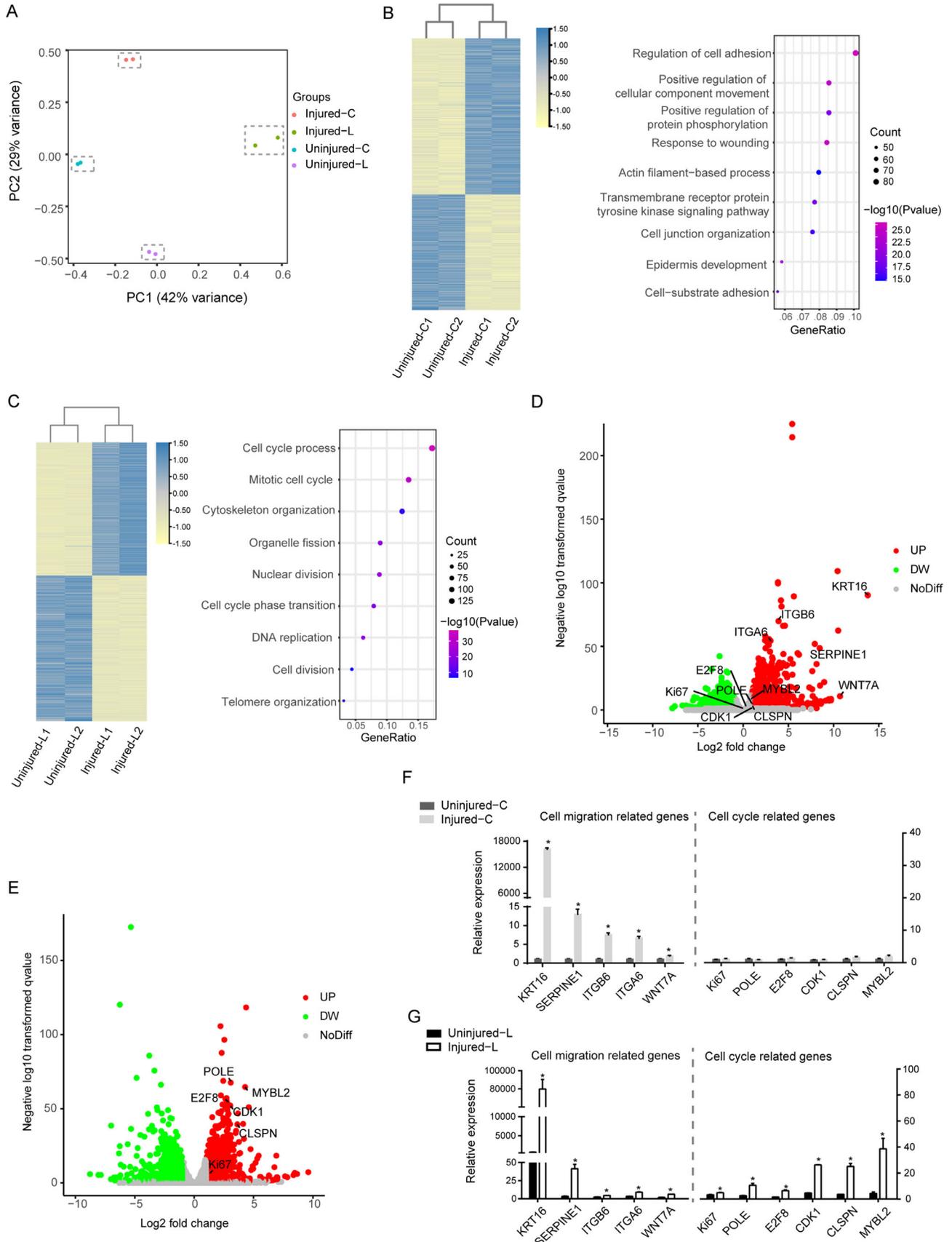


Figure 2. RNA-seq comparative analysis between the limbal and central corneal epithelium at 16 hours postinjury in model 1. (A) Principal component analysis of the sequencing samples. (B) A heatmap of all the DEGs (*left panel*) and a bubble chart of the upregulated DEG enriched



← pathways (right panel) of the central corneal epithelium after injury (\log_2 fold change cutoff = 1 and q value cutoff = 0.05). (C) A heatmap of all the DEGs (left panel) and a bubble chart of the upregulated DEG enriched pathways (right panel) of the limbal corneal epithelium after injury (\log_2 fold change cutoff = 1 and q value cutoff = 0.05). (D) Volcano plot of the significant DEGs between the injured and uninjured central corneal epithelium. (E) Volcano plot of the significant DEGs between the injured and uninjured limbal corneal epithelium. (F) qRT-PCR relative expression analysis of selected genes in the injured central corneal epithelium compared to the uninjured ones ($n = 3$; P value was calculated between Injured-C and Uninjured-C groups; $*P < 0.05$). (G) qRT-PCR relative expression analysis of selected genes in the injured/uninjured limbal corneal epithelium compared to the Uninjured-C ($n = 3$; P value was calculated between Injured-L and Uninjured-L groups; $*P < 0.05$).

Correspondingly, total RNA from two independent samples of native central (Uninjured-C) and limbal corneal epithelium (Uninjured-L) were extracted as controls. These four groups of samples were used for mRNA-seq analysis. The results of PCA showed clear heterogeneity among the four groups of sequencing samples (Fig. 2A). There were 1460 differentially expressed genes (DEGs) between Injured-C and Uninjured-C groups. Using the 844 upregulated genes in Injured-C to perform the gene ontology biological process (GO-BP) enrichment analysis, the results revealed that these upregulated genes were highly enriched in terms of cell adhesion (count = 85, $P < 0.05$) and positive regulation of cellular component movement (count = 72, $P < 0.05$), which are

presented in Figure 2B. Among the 1930 significant DEGs between the Injured-L and Uninjured-L, the 928 upregulated genes in Injured-L were highly enriched in the terms of cell cycle process (count = 133, $P < 0.05$) and mitotic cell cycle (count = 104, $P < 0.05$) by the GO-BP analysis (Fig. 2C).

We then selected several genes for experimental verification, including genes from the upregulated DEGs in Injured-C, which belong to the terms of cell adhesion and immigration, such as *KRT16*, *ITGB6*, *ITGA6*, *SERPINE1*, and *WNT7A* (Fig. 2D), and genes from the upregulated DEGs in Injured-L, which belong to the terms of cycle process and mitotic cell cycle, such as *Ki67*, *POLE1*, *E2F8*, *CDK1*, *CLSPN*, and *MYBL2* (Fig. 2E). Subsequently, the

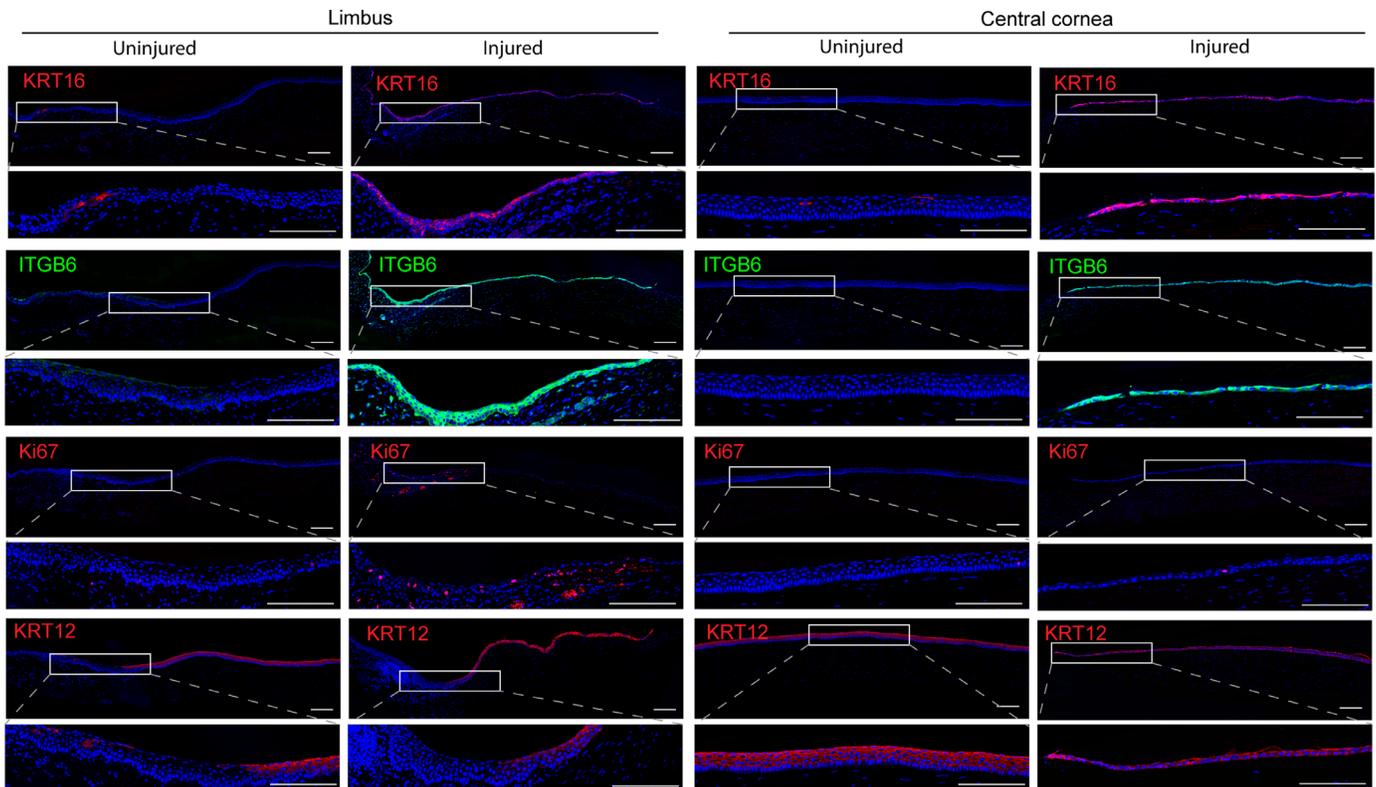


Figure 3. Representative immunofluorescence images of different gene expression (KRT16, ITGB6, Ki67, and KRT12) in uninjured and injured (16 hours) rabbit corneas. Scale bars: 200 μ m.

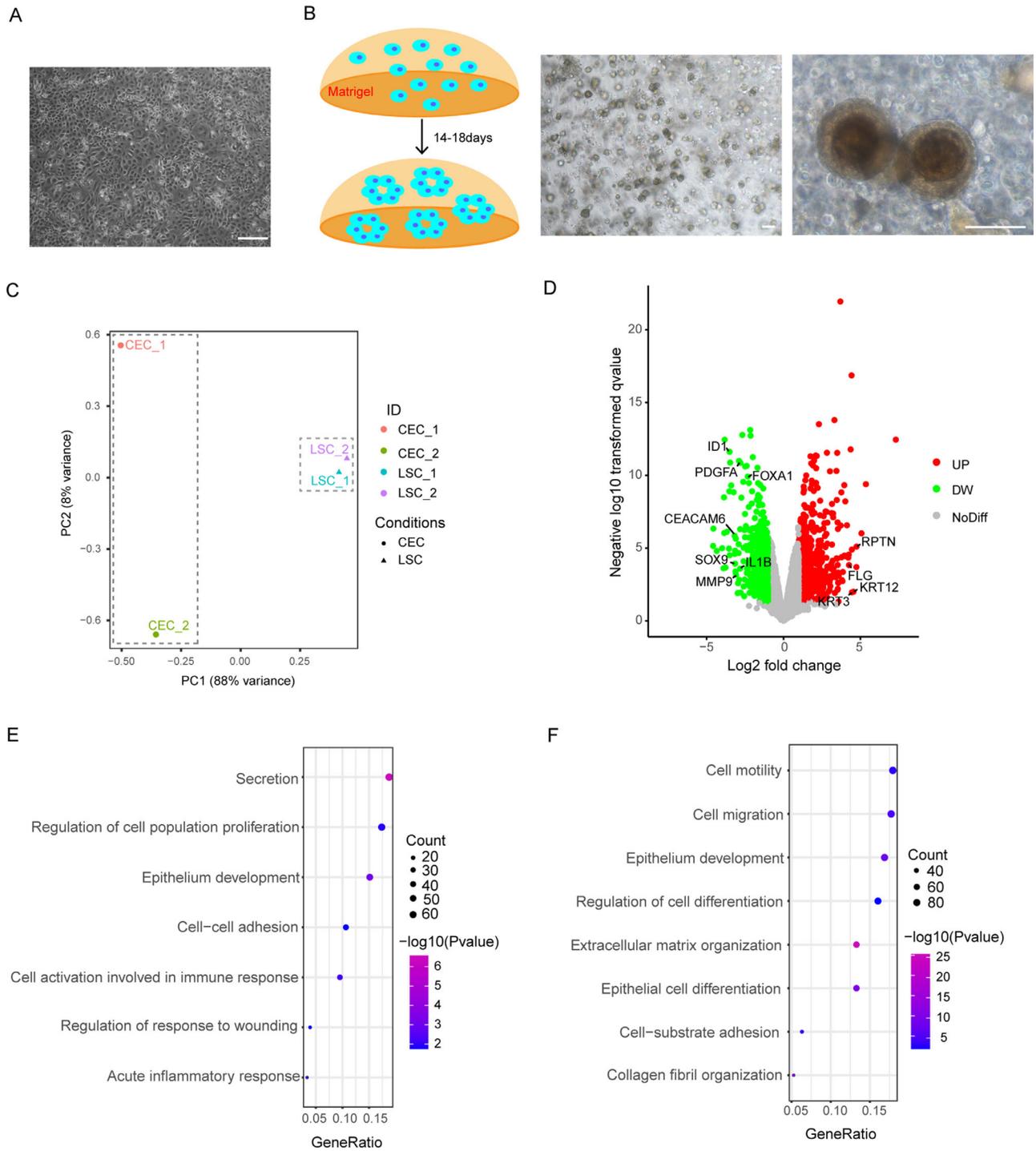


Figure 4. Microarray comparative analysis between the cultured human LSCs and in vitro differentiated CECs. (A) Primary human LSCs cultured in LSC media. Scale bar: 100 μ m. (B) Three-dimensional (3D) differentiation model of human LSCs into CECs. Schematic drawing of a CEC 3D differentiation culture system (left panel). Small clusters of CECs could be observed from day 5 (middle panel) and mature CEC spheres could be observed from day 14 (right panel, enlarged image). Scale bars: 100 μ m. (C) Principal component analysis of the sequencing samples. (D) Volcano plot of the significant DEGs between the cultured CECs and LSCs (\log_2 fold change cutoff = 2 and q value cutoff = 0.05). (E) A bubble chart of the downregulated DEG enriched pathways of the CECs (\log_2 fold change cutoff = 2 and q value cutoff = 0.05). (F) A bubble chart of the upregulated DEG enriched pathways of the CECs (\log_2 fold change cutoff = 2 and q value cutoff = 0.05).

expression of these selected genes was quantified in the injured and uninjured samples using quantitative reverse transcriptase PCR (qRT-PCR) analysis. qRT-PCR results showed that the expression of selected genes presented the same differential trend as the mRNA-seq results. The genes related to cell adhesion and migration were significantly upregulated in the injured central corneal epithelium, but the cycle process and mitotic cell cycle-related genes were not changed (Fig. 2F). Intriguingly, all selected genes were significantly upregulated in the injured limbal corneal epithelium (Fig. 2G).

Validation of the Selected Genes Expression In Vivo

In the uninjured and injured (16 hour) corneal tissues, we verified the expression of selected upregulated genes, such as *KRT16*, *ITGB6*, and *Ki67* (a marker of proliferative cells), by immunofluorescent staining, using KRT12 as a marker of corneal-committed cells to distinguish the limbal and central epithelium. KRT16 and ITGB6 were positively expressed in the injured limbal and central corneal epithelium, and only a few cells positively expressed KRT16 and ITGB6 in the uninjured limbal and central corneal epithelium (Fig. 3). The Ki67 positively expressed cells were significantly increased in the

injured limbal epithelium but not in the central epithelium (Fig. 3). Of note, regardless of the status of injury, almost all central corneal epithelium was KRT12 positive (Fig. 3). All these gene expression results were in accordance with the results of RNA-seq and qRT-PCR.

Gene Expression Analysis of Cultured Human LSCs and the In Vitro Three-Dimensional Differentiated CECs

To verify whether in vitro LSC differentiation presented similar transcriptional alteration as in this in vivo cornea wound-healing model, human LSCs were cultured and successfully differentiated to form colony spheres as reported (Figs. 4A, 4B),⁷ and our previously published microarray data⁷ were reanalyzed. First, comparative analyses using PCA revealed distinct mRNA expression patterns of the differentiation groups to LSC groups (Fig. 4C). Genes that were significantly upregulated (fold change ≥ 2 and $q < 0.05$) included genes associated with epithelial cell differentiation and cell migration, such as *KRT3*, *KRT12*, *RPTN*, and *FLG* (Fig. 4D). Significantly differentially downregulated genes (fold change ≤ -2 and $q < 0.05$) included genes associated with cell proliferation and adhesion, such as *ID1*, *PDGFA*, *FOXA1*, *CEACAM6*, *SOX9*, *IL1B*, and *MMP9* (Fig. 4D). GO-BP analysis

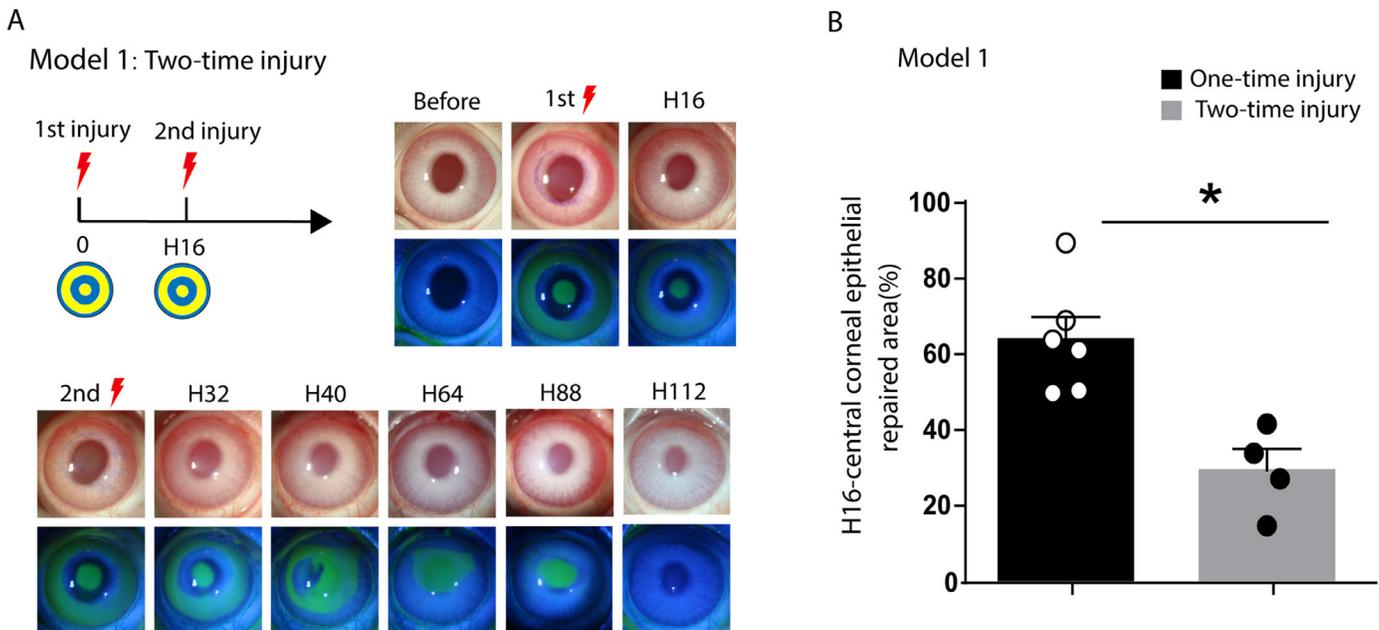


Figure 5. Evaluation of the healing ability of CECs in two-time injury of model 1. (A) Repetitive injury in model 1. The second injury was performed at 16 hours after the first injury (schematic diagram, upper-left panel). Representative slit-lamp images of the eyes in model 1 (upper-right and lower panels). (B) Quantification of the percentage of central epithelial repaired area 16 hours after the first and second injuries (* $P < 0.05$; one-time injured group, $n = 6$; two-time injured group, $n = 4$).

of DEGs between LSCs and differentiation groups of the microarray data also showed that several biological processes involving epithelial cell differentiation were significantly overrepresented in differentiation-upregulated genes, including extracellular matrix organization, collagen fibril organization, epithelium development, cell migration, and cell-substrate adhesion, which is similar to the central corneal wound-healing scenario. Differentially downregulated genes (fold change ≤ -2 and $q < 0.05$) were enriched in biological processes of secretion, epithelium development, acute inflammatory response, and regulation of cell population proliferation (Figs. 4E, 4F), which is similar to the limbal corneal wound-healing scenario.

Different Healing Abilities of Central and Limbal Corneal Epithelium in Repetitive Injury Models

The cell cycle-related genes were not upregulated in the CECs during wound healing, indicating that the replication capacity of CECs may be limited. To verify this assumption, we established two types of repetitive ocular injuries in rabbit models. First, based on previously established model 1, we performed a second injury at 16 hours postinjury when the central residual ring-like epithelium had not mixed with the limbus-derived cells (Fig. 5A). With the slit-lamp observation, the central corneal epithelium failed to resurface the central defect and completely mixed with

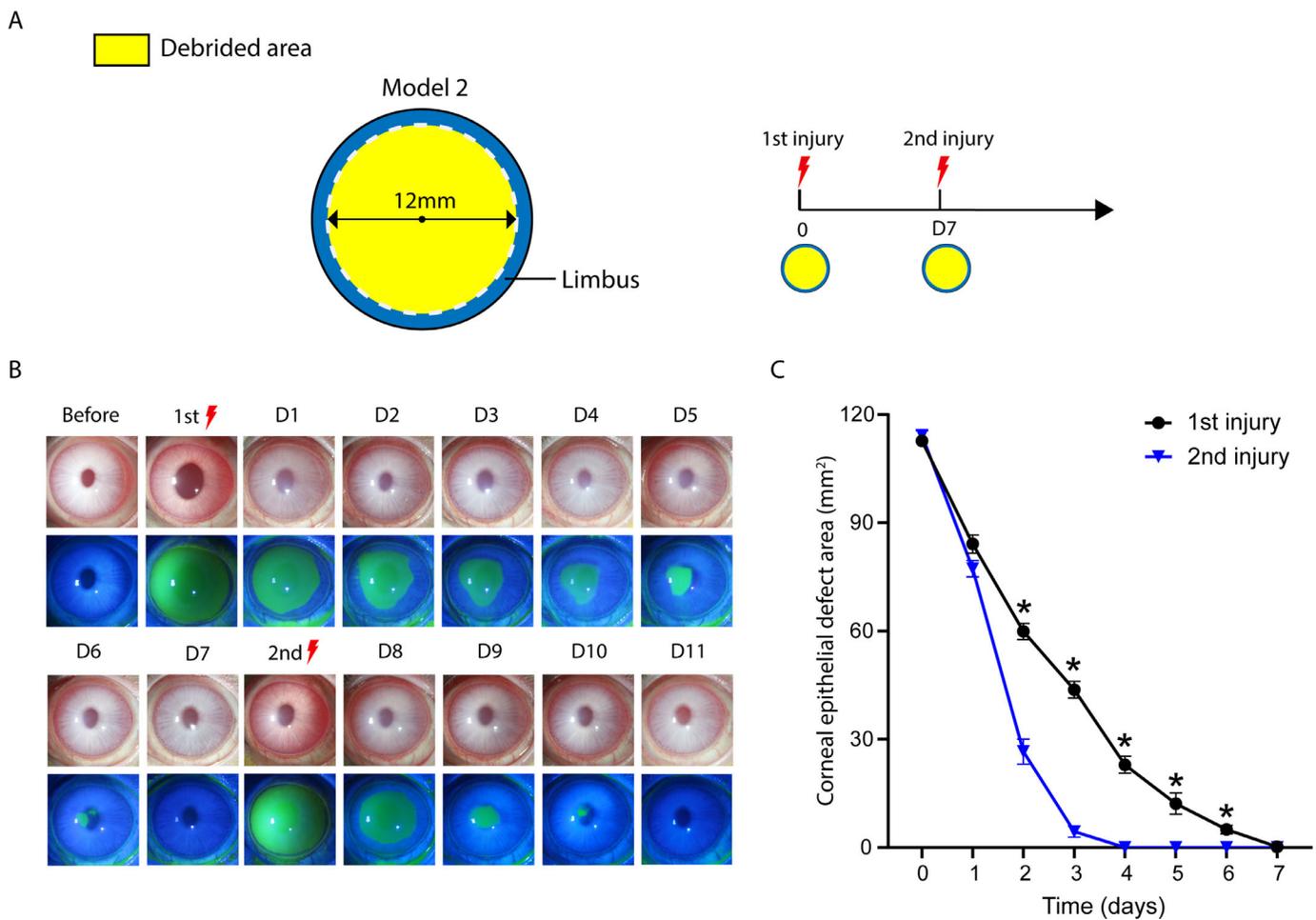


Figure 6. Evaluation of the healing ability of limbal corneal epithelium in whole central corneal epithelium injury rabbit model (model 2). (A) Schematic diagram of model 2 (right panel) and the repetitive injury time axis (left panel). (B) Representative slit-lamp images of eyes in model 2. At 7 days after the first injury, the corneal epithelium completely repaired and then the second injury was performed on the repaired cornea. (C) Quantitative comparison of the central epithelial defect area between the first- and second-time injuries in the model 2 (* $P < 0.05$, $n = 6$).

the limbus-derived cells after 2 days post-second injury (Fig. 5A, “H64”). Upon calculating the percentage of central epithelial repaired area at 16 hours, the repair area of the repetitive injuries (Fig. 5A, “H32”) was significantly lower than the one-time injuries (Fig. 5B). These results imply the limited replication capacity of CECs.

We also established a whole central corneal epithelium injury rabbit model (model 2) to test the replication capacity of LSCs so that only the limbal epithelium was retained (Fig. 6A). The second injury was performed at 7 days after the first injury when the defect epithelium of the first injury was completely repaired (Figs. 6A, 6B). Interestingly, it took 7 days to achieve complete repair during the first injury but only 4 days for the second injury (Fig. 6B, $n = 6$). To quantitatively compare the differences of wound healing between the first- and second-time injuries in model 2, we measured the central corneal defect areas of each sample until fully repaired and found that at the time points of day 2 to day 4 post-second injury (Fig. 6B, “D9” to “D11”), the healing of epithelium defect areas was significantly faster than the first time (Fig. 6B, “D2” to “D4”; Fig. 6C, $P < 0.05$, $n = 6$).

Discussion

Understanding the cellular and molecular changes during corneal wound healing will allow us to design better treatment strategies for corneal injury, even for regeneration. Accumulating evidence has shown that CECs also have the ability to repair corneal epithelial cells in addition to LSCs.^{15,16} In this study, we identified the wound-healing capacity of CECs and compared the differential gene expression after corneal injury to the normal state in CECs and LSCs. We found the CECs could resurface the epithelial defect area independent of LSCs, but the DEGs of the injured CECs were largely different from that of LSCs. The upregulated genes in LSCs were mostly enriched in the biological processes of cell division and mitotic nuclear division; however, the upregulated genes in CECs were mostly enriched in the biological processes of signal transduction and cell adhesion. These differences suggest that CECs repair the defective corneal epithelium with mechanisms different from those used by LSCs, which probably depends on cell immigration. These results also confirmed the view that LSCs, which are normally slow cycling but can be stimulated to proliferate in response to injury, are the main source of regenerating epithelial cells.^{30,31}

To further verify this finding, we designed two corneal epithelium injury models, a ring-like epithelium

residual model (model 1) and a central injury model (model 2), and performed injuries twice to explore the differences between the repairs. In model 1, noticeable central corneal epithelium healing was observed before the limbal epithelium migrated. After the second injury, the CECs performed repairs at a significantly slower rate than that for one-time injury at 16 hours in model 1 ($P < 0.05$). However, the limbal epithelium repaired completely regardless of one or two injuries. These results further verified the conclusions of previous studies that CECs only contributed to the closure of corneal small wounds in acute wound healing,^{15,16} but LSCs are the main source of regenerative epithelial cells.^{6,30,31}

Intriguingly, the epithelium repaired faster after the second injury than the first time in model 2. A similar phenomenon occurred in the epidermal stem cells (EpSCs) in which postinflamed mice closed their wounds ~ 2.5 times faster than naive mice and a prolonged memory to acute inflammation that enabled EpSCs to hasten barrier restoration following subsequent tissue damage.^{32,33} As shown in Figure 2C, the cell cycle-related genes were significantly upregulated in the LSCs after the first injury, indicating that these cells were actively proliferating. On another hand, the cell cycle time, defined as the period between one mitosis and the next, of the mouse corneal epithelium is about 3.4 to 8.9 days.³⁴ It indicates that there might be still some actively proliferating LECs at the seventh day after the first injury (the day of the second injury). In summary, the inflammatory memory and cell cycle time of the LSCs might be the reasons for the faster healing phenomenon in the second injury.

Furthermore, we examined the expression of selected wound-healing related genes. Keratins are a group of water-insoluble proteins that are specifically expressed in different types of epithelial cells as cell markers to reveal the different pathophysiological status.^{35,36} Among these upregulated DEGs in CECs, *KRT16* expression was altered most dramatically (with the highest log2FC), and it was also significantly upregulated in LSCs. *KRT16* belongs to type I cytokeratin, and mutations in *KRT16* can cause pachyonychia congenita or palmoplantar keratoderma.^{37,38} Overexpression of *KRT16* is associated with many cancers because of its positive correlation with cancer cell migration, invasion, proliferation, and epithelial-mesenchymal transition (EMT).^{39,40} As *KRT16* is synthesized only transiently when the cells undergo exponential growth, it is often recognized as a marker of activated proliferation in different types of keratinocytes, including the corneal epithelium.^{41–43} *KRT16* is different from stem cell markers, such as *KRT15* and *KRT14*, which are often located in the

basal layer and are often associated with the suprabasal compartment. Therefore, KRT16 is also recognized as a differentiation-related keratin.⁴³ We verified *KRT16* expression through qPCR and immunofluorescence staining, and the results were in accordance with the results of RNA-seq. KRT16 was expressed at low levels in normal corneal epithelium and was located only in the suprabasal layer, but it was significantly overexpressed in LSCs and CECs after 16 hours of injury. These results suggest that LSCs and CECs are both activated to proliferate after injury and contribute to corneal wound healing.

Integrins are essential for cell adhesion during migration and are essential for tissue repair. Although many different cell surface receptors are involved in the migration of different types of cells, integrins comprise a major family of migration-promoting receptors.^{44,45} ITGB6 is a heterodimeric cell surface receptor that is absent from normal epithelium but is expressed in wound-edge keratinocytes during reepithelialization.⁴⁶ ITGB6 is involved in wound healing and the pathogenesis of diseases, including fibrosis and cancer,⁴⁷ and was also significantly upregulated in both CECs and LSCs in the current study, indicating that the migration of both CECs and LSCs is activated after 16 hours of injury.

In summary, using these rabbit ocular surface injury models, this study provides the genetic framework of CECs and LSCs underlying corneal wound healing for the first time and expands our current understanding of the molecular pathways activated in the course of early corneal epithelium healing. These results suggest that both CECs and LSCs are activated after injury and contribute to wound healing. However, the proliferation ability of CECs is limited, and LSCs are the main source of regenerative epithelial cells. Finally, the therapeutic implications of these findings are currently unclear, but this study provided baseline data that could help facilitate future research in the identification and development of novel approaches to improve corneal wound healing. In addition, given that many other pathologic processes, such as infection and EMT, are also related to wound-healing processes, it is possible that elucidation of the molecular mechanism to modulate wound healing may lead to novel therapeutic approaches for other diseases.

Acknowledgments

Supported by Projects of Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010468).

Disclosure: **W. Zhang**, None; **X. Lan**, None; **J. Zhu**, None; **C. Zhang**, None; **Y. Huang**, None; **K. Mo**, None; **J. Tan**, None; **H. Guo**, None; **H. Huang**, None; **M. Li**, None; **H. Ouyang**, None; **L. Wang**, None

* WZ and XL contributed equally to this work.

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