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IFITM1/OVOL1 Axis Is a Novel Regulator of the Expansion of the Limbal Epithelial Stem/Early Transient Amplifying Cell Population

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ABSTRACT

Limbal epithelial stem cells (LESCs), located in the basal layer of the limbal epithelium, rarely proliferate under normal conditions. Upon proliferation, LESCs give rise to early transient amplifying (eTA) cells, which are thought to be morphologically and phenotypically indistinguishable from LESCs. Following corneal epithelial wounding, LESCs are activated to repair the corneal epithelium via expansion of eTA cells, a process crucial for maintaining corneal epithelial homeostasis and tissue transparency as well as essential for clear vision. To understand how this process is regulated, we conducted a single cell RNA sequencing assay of mouse corneal rims with and without injury and observed an expansion of the stem/eTA cell cluster after corneal injury. Interestingly, we found that Interferon Induced Transmembrane Protein 1 (IFITM1) was predominantly expressed in stem/eTA cells and was positively associated with such stem/eTA cell expansion after corneal wounding. In vivo knockdown of IFITM1 using an AAV (adeno-associated virus) vector significantly attenuated stem/eTA cell expansion and activation of stem/eTA cells to proliferate after mouse corneal wounding. In human limbal epithelial cell cultures, IFITM1 positively impacted the proliferation of stem/eTA cell-enriched limbal epithelial cells, contributing to expansion of the stem/eTA cell population. Such expansion was due, in part, to inhibition of OVOL1 (Ovo like zinc finger 1), a negative regulator of epithelial cell proliferation. These results provide key molecular insights into how stem cell activation and eTA cell expansion are regulated. Elucidating the IFITM1/OVOL1 pathway that governs stem/eTA cell proliferation not only deepens our knowledge of tissue homeostasis but also opens avenues for developing novel regenerative therapies.

Huimin Jiang and Parisa Foroozandeh are contributed equally to this work.

1 | Introduction

The corneal epithelium is a self-renewing stratified squamous epithelium [1–6]. A unique feature of the corneal epithelium is that the stem cells, which govern self-renewing tissues, are not located in the corneal epithelium; rather, they are preferentially situated in the basal layer of the limbal epithelium (called limbal epithelial stem cells (LESCs); see [1, 4, 5, 7–10] and references therein). LESCs possess properties such asquiescence, extensive proliferative capacity, relatively undifferentiated states, and the ability to generate committed progeny: transit amplifying (TA) cells [1, 4, 5, 7–16]. Early TA cells (eTA), which represent the immediate stem cell progeny, also reside in the limbal epithelial basal layer and have high proliferative capacity [3, 5]. It is believed that LESCs and eTA cells are phenotypically and functionally indistinguishable [17, 18]. Since LESCs are essential for maintaining corneal epithelial homeostasis, the loss or dysfunction of LESCs results in persistent epithelial defects and loss of vision [19–26]. Therefore, it is clinically important to understand the molecular signaling pathways that are key for regulating stem cell physiology, including stem cell proliferation.

In addition to corneal epithelial homeostasis, LESCs also play an important role in corneal wound healing. Recently, two discrete LESC populations have been identified by single cell RNA sequencing and lineage tracing [15, 27]. The LESCs located in the outer limbus divide at a significantly lower rate than the LESCs residing in the inner limbus [15, 27]. In response to corneal injury such as chemical burn or physical debridement of the central corneal epithelium, numerous outer and inner LESCs enter the cell cycle, and thus, the limbal basal layer produces more epithelial cells for re-epithelialization of the corneal epithelium [3, 15, 27–29]. For decades, it has been demonstrated that one of the strategies to produce more corneal epithelial cells is to activate LESCs to proliferate, thus expanding the eTA cell population [3]. Since the activation of LESCs [3, 15, 27] and expansion of the eTA cell population [3] are crucial for corneal epithelial wound healing, understanding the molecular mechanisms regulating such processes is critical.

IFITM1(Interferon Induced Transmembrane Protein 1) is an interferon-induced antiviral protein. Recently, evidence showed that IFITM1 exerts functions other than antiviral, such as involvement in the maintenance of stem cells. IFITM1 is highly expressed in human embryonic stem cells, and such expression progressively decreases when stem cells differentiate [30]. IFITM1 is also expressed in mouse primordial germ cells (PGCs) indicating a role in PGC development [31]. In addition, IFITM1 plays an important role in cancer stem cells. For example, IFITM1 is required for the progression of non-small cell lung cancer (NSCLC) in patients [32]. Loss of IFITM1 leads to a significant reduction in sphere formation, which is considered a unique feature of cancer stem cells [32]. In colorectal cancer, IFITM1^{high} cells form organoids with more proliferating cells [33]. Such a positive role of IFITM1 in cancer cell proliferation is also observed in cervical squamous cell carcinoma [34]. Overall, these reports suggest that IFITM1 expression positively correlates with the numbers of cancer stem cells, and such correlation may be due to the positive role of IFITM1 on proliferation.

Ovo like zinc finger 1 (OVOL1) and OVOL2, encoding zinc finger protein homologous to *Drosophila melanogaster* Ovo, are important in the development of various tissues including cornea and epidermis [35]. OVOL2 has a role in maintaining the transcriptional profile of corneal epithelial cells by repressing mesenchymal genes [36]. Mutations in OVOL2 have been associated with posterior polymorphous corneal dystrophy, an inherited disorder of the corneal endothelium [37]. Loss of OVOL1 leads to a defect in proliferation in the epidermis during development [38], indicating an inhibitory role of OVOL1 in cell proliferation. OVOL1 is downregulated in cutaneous squamous cell carcinoma [39] and mediates the suppression of proliferation in oral squamous cell carcinoma cells by inhibiting ZEB1(Zinc Finger E-Box Binding Homeobox 1) expression [40]. However, unlike OVOL2, the role of OVOL1 in the limbus/cornea remains unclear.

Here, we utilized single cell RNA sequencing (scRNA seq), which is an ideal approach to characterize rare cell populations such as stem cells, to identify novel regulators that may be responsible for the activation of stem cells and consequently the expansion of the eTA cell population after corneal injury. Our scRNA seq experiments demonstrated an expansion of the stem/eTA cell cluster following corneal injury. We observed that IFITM1 was upregulated in the stem/eTA cell population after mechanical and chemically induced corneal injury. AAV (adeno-associated virus) mediated delivery of shRNA-Ifitm1 reduced Ifitm1 expression in the limbal epithelium and attenuated the expansion of the stem/eTA cell cluster in response to corneal injury. Our findings demonstrate that IFITM1 is required for the activation of LESCs and the expansion of the TA cell population in response to corneal injury.

2 | Materials and Methods

2.1 | Animal Experiments

Animal procedures were approved by the Northwestern University Animal Care and Use Committee and adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wt/wt C57BL/6 mice were purchased from The Jackson Laboratory. Mice were housed with free access to food and water in a barrier facility or a confinement facility for AAV transduction. Both male and female mice at 6–8 weeks of age were used.

AAV vector with shRNA-IFITM1 was purchased from VectorBuilder and packaged as previously described [41]. The AAV serotype is AAV8. To topically apply AAV vector onto mouse corneas, mice were anesthetized and 5 μL of AAV vector ($1\times10^{\wedge9}$gc/μL) was applied topically once. Two shRNA sequences were used: AAV shRNA IFITM1 and AAV shRNA IFITM1 #3816.

To generate debridement wounds, as previously described [14], central corneal epithelium was removed using a rotating diamond burr while the peripheral corneal and limbal epithelia remained intact. For the alkali burn model, mouse central corneas were exposed to 1 M NaOH solution for 30 s. Three days post injury, mouse corneas were isolated as previously

described [18]. For the BrdU pulse labeling assay, mice were injected with BrdU (50 μg/kg) intraperitoneally 1 h prior to euthanasia [42].

2.2 | Single Cell RNA Sequencing Analysis

Single-cell isolation from corneal and limbal tissues. Eyeballs were cut about 1 mm behind the limbus toward the back of the eyeball using ophthalmological surgical scissors. This yields a corneal/limbal button including limbus and partial conjunctiva. The iris was scraped off using the round handle side of a forceps. In this way, a limbal/corneal rim containing primarily epithelium, stroma, and endothelium is obtained. Tissues were incubated with 0.7 mg/mL liberase TM (from Roche) in RPMI at 37°C for 1 h and then with 0.25%Trypsin for 15 min. Single-cell suspension was subjected to scRNA-seq analysis using the 10X genomics pipeline as previously described [14, 18, 43]. Raw sequencing data was processed using CellRanger. Data normalization was performed by R Package. Seurat was used to account for the differences in the cell number between samples during integration and clustering as previously described [14, 18, 43, 44]. For data alignment, we selected 4000 highly variable genes in each data matrix and performed "FindIntegrationAnchors" and "IntegrateData" functions for integration. Next, we performed the clustering using 'FindClusters' in Seurat to identify cell type clusters. UMAP of each data set were visualized. The Seurat FindConservedMarkers() function was used to obtain a list of marker genes conserved across conditions. These marker genes for each cluster were used to annotate these clusters as specific cell types. Dot Plot was used to visualize conserved cell type markers and show both the expression level and the percentage of cells in a cluster expressing those marker genes. "FindMarkers" was used to identify the differentially expressed genes (DEG) between each sample in each cluster. Functional Annotation Clustering of DEGs was performed in DAVID Functional Annotation Bioinformatics Resources. AddModuleScore analysis was conducted using a gene set that plays critical roles in regulating apoptosis from the MSigDB Collections. The scRNA-seq data that support the findings of this study are submitted to the NCBI GEO database (GSE275678 and GSE277598).

Raw scRNA-seq datasets of human corneas (GSE218123), corneal epithelium with dry eye disease (GSE182582), and diabetic corneal epithelium (GSE182477) were downloaded from the NCBI GEO database and analyzed as described above.

2.3 | Cell Culture

Limbal epithelial cells were isolated from human corneal/limbal rims obtained from the Eversight eye banks (Ann Arbor, MI, USA) as described before [18]. Briefly, to isolate limbal epithelial cells, the cornea and conjunctiva were cut out from the tissues and thus the limbal rim was dissected out. The limbal rim was digested in dispase to isolate limbal epithelial cells. Primary human limbal epithelial cells (HLECs) were cultured in keratinocyte SFM media (Life Technologies), and first passage cells were used for experiments. Immortalized limbal epithelial cells, hTCEpi, were cultured in keratinocyte serum free media (Life Technologies) as described previously [18]. To knock down gene expression, cells were transfected with a 5 nM siRNA pool against IFITM1, OVOL1 (Invitrogen, Carlsbad, CA, USA) or nontarget control (Invitrogen, Carlsbad, CA, USA) as previously described [42]. Cells incubated with BrdU (10 μM) for 1 h were used for the BrdU pulse labeling assay [42]. Total RNA was extracted for RT-qPCR as described below.

2.4 | Immunostaining

Sections with mouse eye tissues were subjected to immunostaining with primary antibodies: IFITM1, OVOL1, KRT15, Ki67, p63, cleaved caspase 3 antibodies at 1:50 dilution, or BrdU mouse monoclonal antibody (G3G4, Developmental Studies Hybridoma Bank) at 1:10 dilution as previously described [14]. For cell culture, coverslips with cells were fixed in cold methanol at −20°C for 10 min. Following antigen retrieval, sections were incubated overnight at 4°C with BrdU mouse monoclonal antibody (G3G4, Developmental Studies Hybridoma Bank) at 1:10 dilution, IFITM1 mouse monoclonal antibody at 1:50 dilution, or OVOL1 rabbit polyclonal antibody at 1:50 dilution. Slides were visualized with a Nikon Ti-2 (Nikon, Tokyo, Japan) microscope (Northwestern CAM core) [18]. ImageJ was used for cell counting and relative fluorescence intensity analysis.

2.5 | Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs were isolated from cells and mouse corneas and purified using an miRNeasy kit (Qiagen, Hilden, Germany). RT-qPCR was performed with a BioRad CFX System using the Roche FastStart Essential DNA Green Master (Roche, Branchburg, NJ, USA) according to the manufacturer's instructions. The primer pairs for RT-qPCR were designed using the IDT PrimerQuest Primer Design Tool (IDT, Coralville, IA, USA).

2.6 | Flow Cytometry

To determine cell death, 48 h after transfection with siRNAs, cells were subjected to staining with an antibody for the Annexin V-FITC. Apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's protocols. To determine the cell cycle, cells were fixed in 75% cold ethanol (1 mL) and then incubated in 500 μL propidium iodide (50 μg/mL) containing 200 μg/mL RNase A (Sigma; St. Louis, MO) at 37°C for 20 min. Analysis was conducted using the FACSCalibur Flow Cytometer (BD Biosciences).

2.7 | Bulk RNA Sequencing

HLECs isolated from 3 donors were transfected with a 5 nM siRNA pool against IFITM1 (Invitrogen, Carlsbad, CA, USA) or nontarget control (Invitrogen, Carlsbad, CA, USA) as previously described [42]. Three days later, cells were processed for total RNA isolation using miRNeasy kit (Qiagen, Hilden, Germany). Expression profiling was conducted using a Next-Generation Sequencing platform at Northwestern University Center for

Genetic Medicine core. The quality of reads, in FASTQ format, was evaluated using FastQC. Reads were trimmed to remove Illumina adapters from the 3′ ends using cutadapt (http://code.google.com/p/cutadapt/). Trimmed reads were aligned to the *Homo sapiens* genome (hg38) using STAR [45]. Read counts for each gene were calculated using htseq-count [46] in conjunction with a gene annotation file for hg38 obtained from Ensembl (http://useast.ensembl.org/index.html). Normalization and differential expression were calculated using DESeq2 that employs the Wald test [47]. The cutoff for determining significantly differentially expressed genes was an FDR-adjusted *p*-value less than 0.05 using the Benjamini-Hochberg method. Gene differential expression profiles obtained from RNA-seq analysis were subjected to gene ontology analysis using DAVID.

2.8 | Quantification and Statistical Analysis

Unpaired *t* test and paired *t* test were performed to determine statistical significance. The data are shown as means ± standard deviation (SD). Statistical significance was defined as * $p < 0.05$. All experiments were replicated at least three times.

3 | Results

3.1 | scRNA-Seq Analyses Reveal That Corneal Injury With NaOH Results in the Expansion of the Stem/eTA Cell Cluster

Since limbal epithelial stem cells reside in the limbal epithelial basal layer and are a rare cell population, it is difficult to isolate and characterize this discrete stem cell population. Thus, to investigate how the activation of stem cells and the expansion of the eTA cell population are regulated following a corneal injury, we conducted a scRNA-seq analysis of mouse corneas that were either exposed to a 1 M NaOH solution for 30 s or uninjured (controls). Seurat-based analysis of the scRNA-seq data was conducted, and we identified 15 distinct clusters (Figure 1A). Using well-known marker genes, the cell types of these clusters were identified and characterized (Figure 1B, Figure S1). Among the 15 clusters, 8 were mesenchymal cells, expressing the mesenchymal marker Vimentin (*Vim*). These 8 clusters are subdivided into 3 keratocyte clusters, a corneal Schwann cell cluster, a smooth muscle cell cluster, a vascular endothelial cell cluster, and 2 macrophage clusters based on additional specific marker expression.

The remaining 7 cell clusters were identified as epithelial in nature since they lacked mesenchymal markers (e.g., *Vim*). Cluster 4 and 13 were identified as conjunctival epithelial cells based on the high expression of conjunctival markers such as keratin 13 (*Krt13*), keratin 19 (*Krt19*) and mucin1 (*Muc1*) [15, 48, 49] (Figure 1B). Cluster 0 highly expressed glycoprotein hormone subunit alpha 2 (*Gpha2*), *Cd63*, interferon induced transmembrane protein 3 (*Ifitm3*), apolipoprotein E (*Apoe*), which are putative limbal epithelial stem/eTA cell markers [15, 16]. In addition, cluster 0 expressed low levels of differentiation markers such as desmocollin-3 (*Dsc3*) and desmoglein 3 (*Dsg3*) compared to the rest of the epithelial clusters [15, 50], confirming that cluster 0 is comprised predominately of limbal epithelial stem/eTA cells. Clusters 2 and 5 were identified as more mature TA cell populations since TA cell markers including the marker of proliferation Ki-67 (*Mki67*), histone cluster 1, H2ap (Hist1h2ap), stathmin 1 (*Stmn1*), deoxyuridine triphosphatase (*Dut*), and helicase, lymphoid specific (*Hells*) [18, 51–53] were enriched in these two clusters. Cluster 1 highly expressed basal cell markers (e.g., Integrin beta 4 (*Itgb4*)) [54, 55]; while cluster 3 highly expressed differentiation markers (e.g., *desmosome proteins, tight junction proteins*) [15, 50]. Thus, cluster 1 was designated as corneal epithelial basal cells and cluster 3 as differentiated corneal epithelial cells. Consistent with the idea that stem cells are activated to generate more eTA cells in response to injury [3, 5], the percentage of stem/eTA cells (cluster 0) increased in the NaOH injured WT cornea by an average of 25% compared to uninjured WT cornea (average 5%) (Figure 2A,B), indicating an expansion of the stem/eTA cell population.

3.2 | scRNA-Seq Analysis Identifies Ifitm1 as a Novel Regulator of the Expansion of the Stem/Early TA Cell Cluster in Response to Corneal Injury

Activation of quiescent stem cells to proliferate leads to the expansion of proliferating eTA cells [3]. Thus, to understand how such expansion is controlled, we searched for proliferation-related genes among the most highly differentially expressed genes (DEGs) in the stem/eTA cells (cluster 0) between uninjured and injured corneas. In uninjured corneas, of the top DEGs, *Ifitm1* was predominantly expressed in the stem/eTA cell population (Figure 2C). To validate this observation in the human limbus, we analyzed a scRNA-seq dataset of human corneas (GSE218123) [56] (Figure S2A) and found that IFITM1 was mainly expressed in the human limbal stem/eTA cell cluster (Figure S2B). This suggests that in both mouse and human, IFITM1 plays a role in limbal epithelial stem/eTA cells. Immunofluorescence staining revealed that IFITM1 was more highly expressed in the limbal epithelium than in the corneal epithelium. Furthermore, IFITM1 was detected in those limbal epithelial cells expressing p63 and Krt15, putative limbal epithelial stem cell markers (Figure S2C,D). Bulk RNA seq suggests that *Ifitm1* is significantly upregulated in label-retaining cells (putative stem cells) in the mouse limbal epithelium [57] and colocalized with C/EBPδ, a putative stem cell marker, in the human limbal epithelial basal layer [58]. It has been shown that *IFITM1* plays a positive role in cancer cell proliferation [33, 34]. Thus, we hypothesize that *Ifitm1* may play a role in the proliferation of limbal epithelial stem/eTA cells. Following corneal injury, our scRNA-seq analysis showed that *Ifitm1* expression was upregulated in the stem/eTA cell population compared with the uninjured control (Figure 2D). To validate this, RT-qPCR showed that Ifitm1 expression was markedly upregulated in mouse corneas 3 days after a NaOH burn (Figure S2E). Not surprisingly, immunostaining detected an increase in IFITM1 protein levels in the stem/eTA cell-enriched limbal epithelium of NaOH-injured mouse corneas compared with the uninjured controls (Figure 2E). Collectively, these observations indicate that *Ifitm1* expression is positively correlated with the expansion of the stem/eTA cell population.

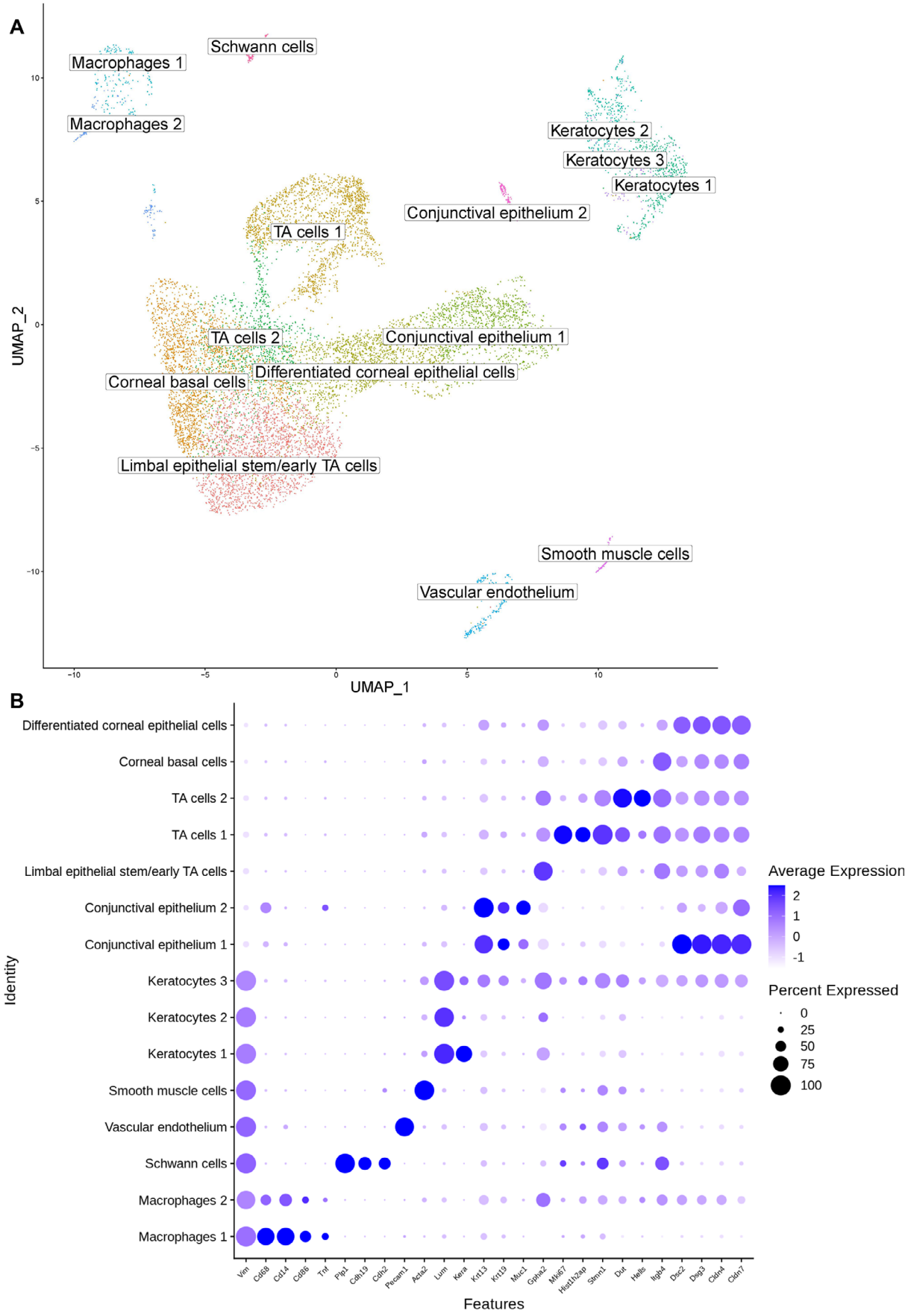

FIGURE 1 | scRNA-seq demonstrated 15 clusters in cells isolated from limbal/corneal tissues of uninjured and NaOH injured mice. (A) UMAP visualized 15 differential clusters of cells. (B) Dot plot was used to visualize conserved cell type markers and show both the expression level and the percentage of cells in a cluster expressing those marker genes.

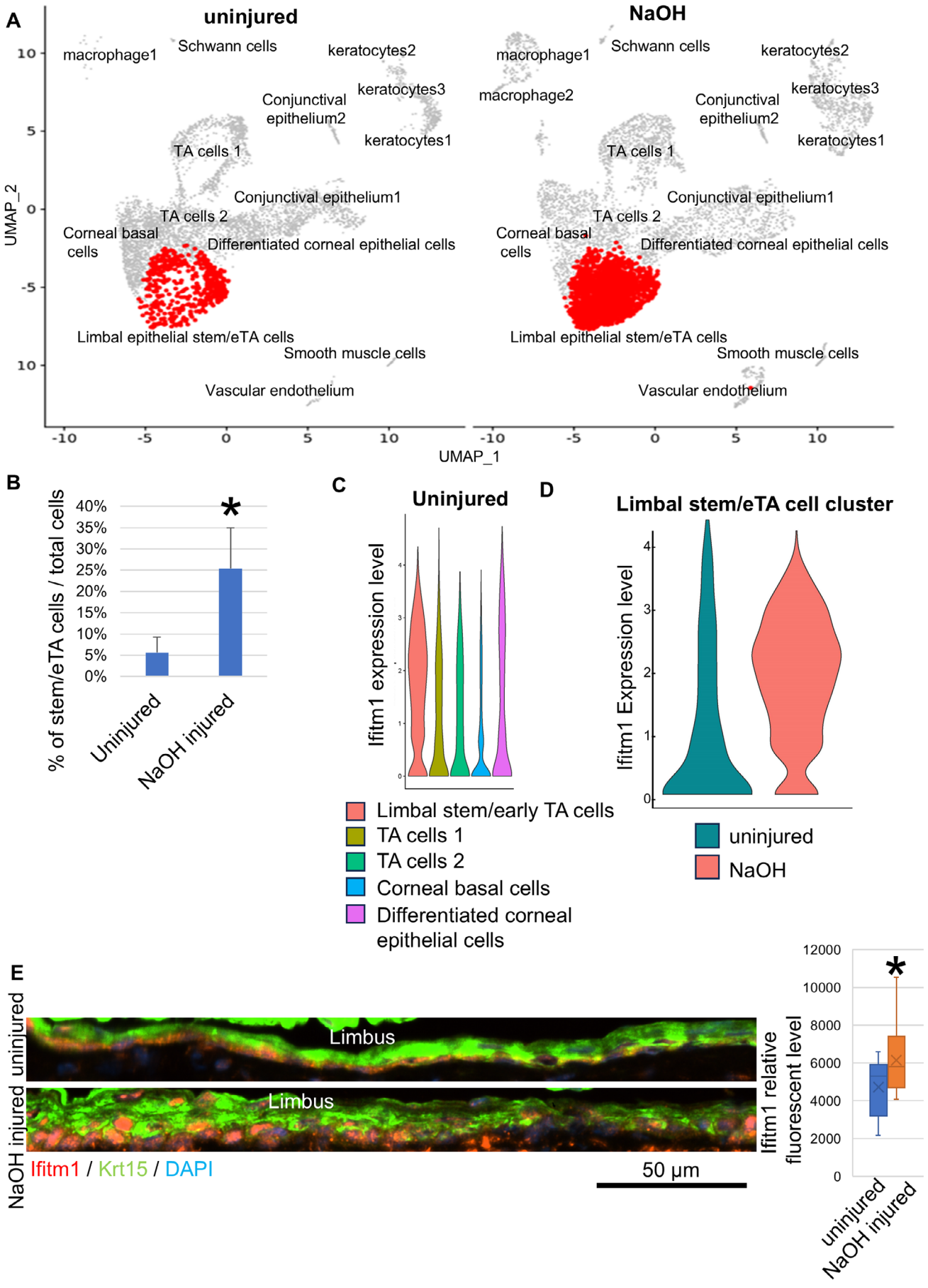

FIGURE 2 | Legend on next page.

FIGURE 2 | *Ifitm1* is predominately expressed in stem/early TA cells and its expression is positively associated with the expansion of the stem/early TA cell cluster after injury. (A) UMAP visualized 15 differential clusters of cells in each sample. The stem/early TA cell cluster was highlighted, which visualized that the cell number of stem/eTA cell population was increased in NaOH injured WT cornea compared to in uninjured WT cornea. (B) Counting the cell numbers of stem/eTA cell population showed that NaOH injury significantly increased the cell number within the stem/eTA population. $N=3$ for each group. (C) Violin plots show the expression of gene *Ifitm1* among epithelial clusters. Among epithelial cell clusters, *Ifitm1* was highly expressed in the limbal stem/early TA cell cluster. (D) Violin plots show that the expression of *Ifitm1* was markedly increased in limbal stem/early TA cells by NaOH injury compared to uninjured control. (E) Immunofluorescent images of IFITM1 in limbal epithelium (KRT15+ cells) showing that the relative fluorescent levels for IFITM1 were markedly increased in NaOH injured mouse limbal epithelium ($N=13$) compared to uninjured control ($N=11$). $^*p<0.05$.

3.3 | IFITM1 Is Required for the Expansion of the Stem/Early TA Cell Cluster After Corneal Injury

To examine whether the upregulation of *Ifitm1* is required for the expansion of the stem/eTA cell population in response to corneal injury, wild type mouse eyes were topically treated with AAV-shRNA-Ifitm1 or AAV-empty vector (control) once, 14 days before they were subjected to a corneal NaOH burn. AAV-shRNA-Ifitm1 treatment downregulated Ifitm1 expression in mouse limbal epithelium (Figure 3A). Three days after corneas were injured, a scRNA-seq assay was conducted using cells isolated from corneal/limbal tissues. The scRNA-seq assay identified the stem/eTA cell population using *Gpha2* expression (Figure S3), as a limbal epithelial stem/eTA cell marker [15, 16]. In AAV-empty vector (AAV-EV) treated mouse eyes (control), NaOH injury increased the numbers of stem/eTA cells compared to uninjured eyes as seen previously (Figure 3B,C). Such expansion of the stem/eTA cell population following corneal injury was markedly reduced in AAV-shRNA-Ifitm1 treated eyes (Figure 3B,C). This strongly suggests a positive role of *Ifitm1* in stem/eTA cell expansion after corneal injury. A possible interpretation of such a reduction in the cell number of the stem/eTA cell population is that cell viability is affected. To test this, we evaluated the strength of depletion of Ifitm1 associated with apoptosis by quantifying the overall activity of a gene set that plays critical roles in regulating apoptosis from the MSigDB Collections. This approach is more robust than individual gene expression as such overall activity of a gene set relies on multiple genes. Thus, we conducted the AddModuleScore analysis [59] and found that there was no significant difference in the overall activity of the gene set related to apoptosis in the stem/eTA cell cluster of uninjured, NaOH+AAV-EV, and NaOH+AAV-shIfitm1-treated mice(Figure 3D). Consistently, immunostaining for cleaved caspase 3 did not detect any positive cells in the limbal epithelium of both NaOH+AAV-EV and NaOH+AAV-shIfitm1-treated mice (Figure S4C). The AddModuleScore analysis as well as immunostaining for cleaved caspase 3 provides evidence that the effect of the depletion of Iftim1 is not due to alteration of cell viability.

In response to corneal injury, LESCs are activated to proliferate and thus generate more proliferating eTA cells, which results in an overall increase in the proliferative cell population in the limbal epithelium [3, 28, 29]. Thus, to further determine the role of *Ifitm1* in stem/early TA cell expansion, we investigated whether knockdown of *Ifitm1* will reduce the rate of proliferation in the limbal epithelium after injury in vivo using a BrdU labeling assay, which detects cells in the S phase of DNA synthesis. Wild type mouse eyes were topically treated with AAV-shRNA-Ifitm1 or AAV-EV (control) and subsequently subjected to either corneal epithelial debridement wounding or a NaOH burn. BrdU labeling showed that 24 h after corneal injury, knockdown of *Ifitm1* by AAV-shRNA-Ifitm1 diminished the BrdU+ cells in the limbal epithelium compared with control (Figure 4A,B). Cell proliferation was also evaluated by immunostaining for Ki67. The alteration of the proliferative status of the AAV-shRNA-Ifitm1 transduced limbal epithelium was evidenced by a significant decrease in the number of Ki67+ cells (Figure 4C,D). We also tested the effect of another shRNA for IFITM1 and found that similarly, the AAV shRNA IFITM1 #3816 can knockdown the expression of IFITM1 in limbal epithelium and reduce the BrdU+ cells in limbal epithelium after NaOH burn (Figure S4A,B). These observations suggest that *Ifitm1* is required for limbal epithelial cell proliferation in response to corneal injury in vivo and further confirm the positive role of *Ifitm1* in stem/eTA cell expansion following injury.

3.4 | IFITM1 Positively Regulates Proliferation of Human Limbal Epithelial Cells

To investigate whether IFITM1 induces stem/eTA cell proliferation in the human eye and consequently the expansion of stem/eTA cells, we utilized stem cell-enriched primary cultures of human limbal epithelial cells (HLECs). HLECs were transfected with an siRNA pool against *IFITM1* (siIFITM1) that resulted in a significant reduction of *IFITM1* in these cells (Figure S5A). To assess cell proliferation, a BrdU labeling assay was conducted. After a 72-h transfection period, we observed a marked increase in the percentage of BrdU+ cells in siIFITM1 transfected HLECs compared to the scrambled control siRNA (siControl) transfected cells (Figure 5A). Similar results were observed using hTCEpi cells, a human limbal epithelial cell line, with a reduction in BrdU+ cells with siIFITM1 transfection (Figure 5B). The alteration of the proliferative status of the siIFITM1 transfected cells was also evidenced by a significant increase in the number of hTCEpi cells in the S phase of the cell cycle detected by flow cytometry assays (Figure S2F). This suggests that IFITM1 promotes the stem cell-enriched human limbal epithelial to proliferate, which may contribute to the increased number of stem/eTA cells noted after injury.

To exclude the possibility that the reduction of proliferation in the cells with IFITM1 knockdown is due to a loss in cell viability, we determined the extent of cell death using flow cytometry. hTCEpi cells were stained with annexin V. As expected, there was no significant difference in annexin V staining between the

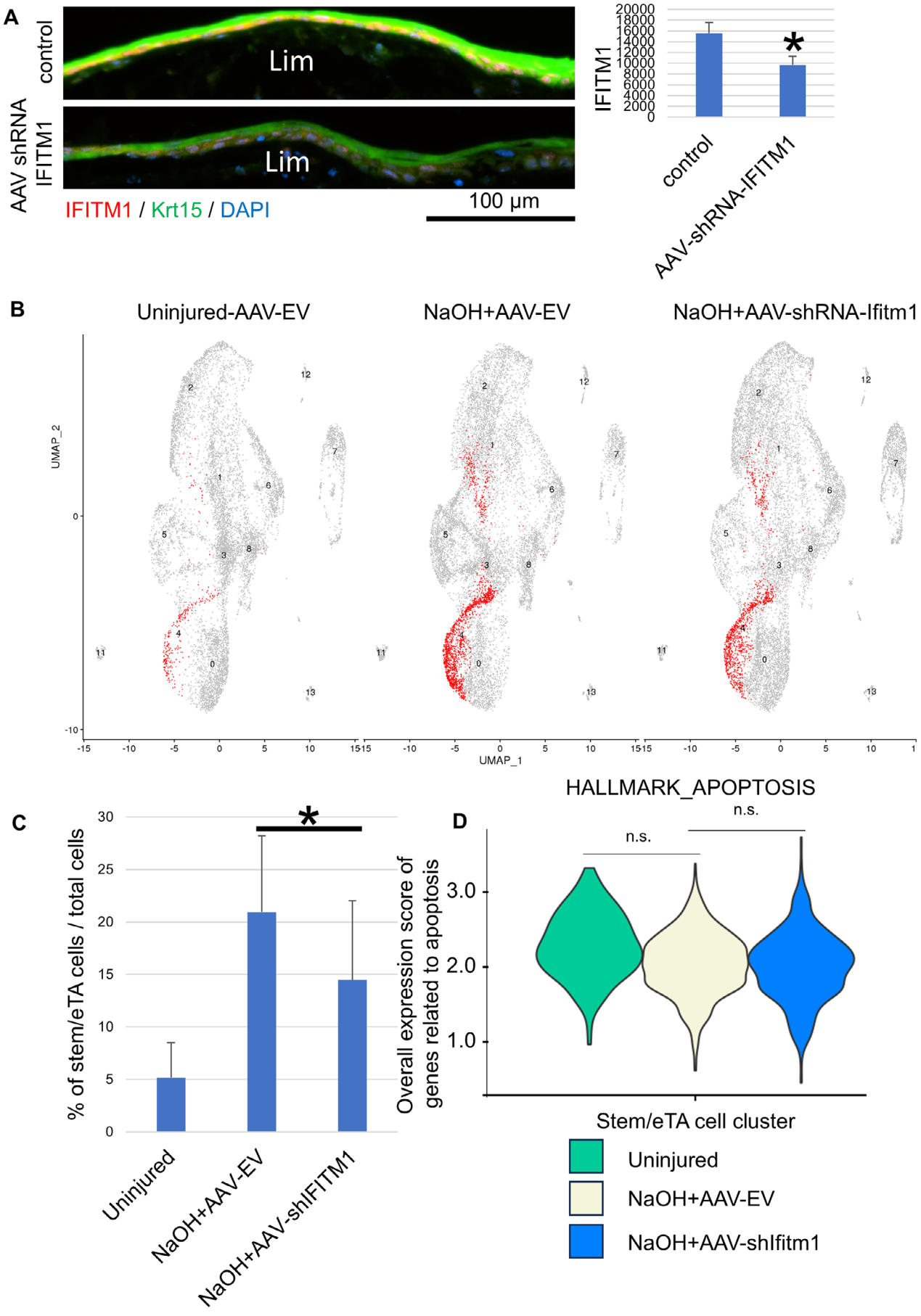

FIGURE 3 | Legend on next page.

FIGURE 3 | Knockdown of *Ifitm1* reduces the cell number in the stem/early TA cell population after NaOH injury. Wild type mouse eyes were topically treated with AAV-shRNA-Ifitm1 or AAV-empty vector (control) and subsequently were subjected to a corneal NaOH burn. (A) Immunofluorescent images visualized the expression of *Ifitm1* in limbal epithelium (Krt15+ cells) and showed that IFITM1 proteins were markedly decreased in limbal epithelium of mice topically applied with AAV-shRNA-Ifitm1 ($N=3$) compared to AAV empty vector (control; $N=3$). (B) Single cell RNA sequencing was conducted and UMAP visualized differential clusters of cells from mice with 3 different treatments: Uninjured, NaOH injury+AAV-empty vector, and NaOH injury+ AAV-shRNA-Ifitm1. Red dot highlights stem/early TA cell population. (C) Counting the cell numbers of stem/eTA cell population showed that NaOH injury increased the cell number of the stem/eTA population while such increase was reversed by topical treatment with AAV-shRNA-Ifitm1. $N=2$ for each treatment. $^*p<0.05$. (D) AddModuleScore analysis was conducted using a gene set that plays critical roles in regulating apoptosis from the MSigDB Collections. The overall expression score of genes related to apoptosis was calculated to indicate no difference in the expression of apoptosis-related genes in these 3 groups. n.s.: Not significance.

cells with IFITM1 knockdown and control cells (Figure S6G), indicative that cell survival is not affected by the depletion of IFITM1.

3.5 | IFITM1 Positively Regulates Proliferation via Negatively Regulating OVOL1

To explore the mechanism underlying the positive effect of IFITM1 on limbal epithelial cell proliferation, bulk RNA sequencing was conducted using total RNA isolated from HLECs transfected with siIFITM1 or siControl (Figure S5). RNA sequencing identified that 945 DEGs were significantly changed more than 2-fold between siIFITM1 and siControl treated HLECs (Figure S6A). Gene ontology (GO) analysis of these top DEGs (Figure S6B) indicated that the most enriched GO term was primarily involved in "cell division". Among genes involving "cell division", *OVOL1* plays an important role in regulating proliferation and cell fate commitment in epidermal keratinocytes [38]. Immunostaining detected that IFITM1 and OVOL1 were co-expressed in HLECs in vitro and in mouse limbal epithelium in vivo (Figure S6C,D), which suggests that IFITM1 may regulate OVOL1 expression. Our RNA sequencing and RT-qPCR validation showed that knockdown of IFITM1 significantly increased OVOL1 expression (Figure S6A,E). This observation confirmed the negative regulation of Ovol1 expression by Ifitm1. In support of such negative regulation, a NaOH injury to mouse corneas reduced OVOL1 (Figure 6A) in the limbal epithelium compared with the uninjured mice, while IFITM1 expression was increased (Figure 2E). In mice, OVOL1 expression was markedly enhanced in the limbal epithelium of mice topically applied with AAV-shRNA-Ifitm1 compared to AAV-EV control (Figure 6B), indicating IFITM1 negatively regulates OVOL1 expression in vivo. Thus, to explore whether the decreased proliferation in cells with knockdown of *IFITM1* is due to increased *OVOL1* expression, we conducted a rescue experiment by knocking down *OVOL1* in cells lacking *IFITM1*. RT-qPCR results confirmed a significant decrease of *OVOL1* or *IFITM1* expression after cells were transfected with an siRNA pool against *OVOL1* (siOVOL1) or an siRNA pool against *IFITM1* (siIFITM1), respectively (Figure S6E,F). Knockdown of *OVOL1* in HLECs resulted in a marked increase in the percentage of BrdU+ cells (Figure 6C). Knockdown of IFITM1 reduced the percentage of BrdU+ cells (Figure 6C) while such a reduction was reversed by knockdown of OVOL1 (Figure 6C). Furthermore, knockdown of *IFITM1* and *OVOL1* did not affect cell survival as evidenced by no significant difference in annexin V staining between the cells with siIFITM1, siOVOL1, and sicontrol (Figure S6G). These observations confirmed that *IFITM1* positively regulates cell proliferation, in part, via negatively regulating *OVOL1*.

3.6 | Downregulation of Ifitm1 Is Detected in Corneal Diseases Associated With Dysfunction of LESCs

It has been demonstrated that many corneal diseases (e.g., dry eye disease and diabetic keratopathy) are associated with dysfunction of LESCs, leading to impaired activation of stem cells in response to corneal injury. We examined scRNA-seq datasets of dry eye disease (GSE182582) and diabetic corneas (GSE182477) and found that *Ifitm1* expression was reduced in the limbal stem/eTA cell population of both dry eye disease and diabetic corneas compared to healthy controls, which was accompanied by an increase in Ovol1 expression (Figure S7). These observations suggest that downregulation of *Ifitm1* may contribute to defective stem cell activation in these diseases.

4 | Discussion

Following a corneal injury, proliferation in the limbal epithelium is induced to generate more epithelial cells, resulting in re-epithelialization of the corneal epithelium. The induced proliferation is attributed to three strategies: (i) activation of the rarely-cycling stem cells to divide, thus producing more early TA cells and expanding the early TA cell population; (ii) increasing the number of times the TA cells can replicate before they become postmitotic; and (iii) increasing the efficiency of stem/eTA cell replication by shortening the cell cycle time [3]. Even though these three strategies have been proposed for decades, direct evidence for how these processes are regulated is lacking due to the heterogeneity of the limbal epithelial basal layer, as well as the paucity of discrete stem cell markers, which makes it difficult to isolate and characterize the stem cell population in vivo. Here, we utilized scRNA seq, an ideal approach to characterize rare cell populations such as stem cells [18, 60]. We show that an upregulation of *Ifitm1* after corneal injury contributes to activation of LESCs and thus, the expansion of eTA cells, which is one of the three strategies. The activation of LESCs and expansion of eTA cells enhances proliferation in the limbal basal layer. The positive role of *Ifitm1* in activating the proliferation of LESCs is partially attributed to *Ifitm1* inhibiting *Ovol1*, a negative regulator of epithelial cell proliferation [38]. Interestingly, *Ovol1* expression is downregulated in limbal epithelium after corneal injury. Taken together, these observations indicate that *Ifitm1* plays a positive role in regulating stem

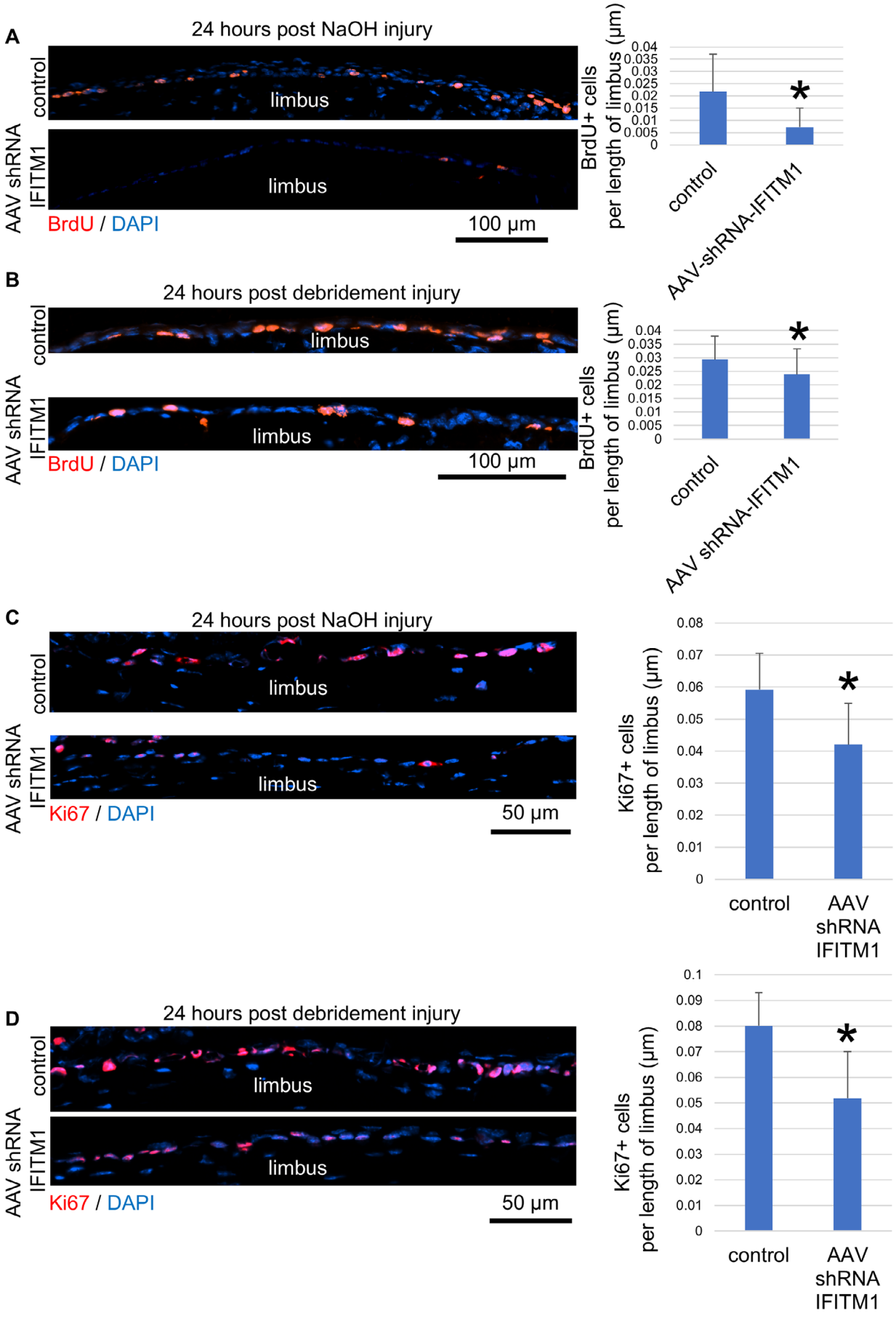

FIGURE 4 | Legend on next page.

FIGURE 4 | Knockdown of *Ifitm1* in vivo reduces limbal epithelial cell proliferation. Mouse ocular surfaces were topically treated once with AAV-shRNA-Ifitm1 or AAV empty vector (control). Fourteen days after AAV application, corneas were subjected to NaOH injury (A, C) or debridement wounding (B, D). (A) 24 h after NaOH injury, immunofluorescent images showed that BrdU+ cells in the limbal epithelium of mice with AAV-shRNA-Ifitm1 ($N=13$) were decreased compared to control ($N=10$). (B) 24 h after debridement wounding, immunofluorescent images showed that BrdU+ cells in the limbal epithelium of mice with AAV-shRNA-Ifitm1 ($N=9$) were decreased compared to control ($N=9$). The BrdU+ cells were counted using ImageJ. *$p<0.05$. (C) 24 h after NaOH injury, immunofluorescent images showed that Ki67+ cells in the limbal epithelium of eyes with AAV-shRNA-Ifitm1 ($N=3$) were decreased compared to control ($N=3$). (D) 24 h after debridement wounding, immunofluorescent images showed that Ki67+ cells in the limbal epithelium of mouse eyes treated with AAV-shRNA-Ifitm1 ($N=4$) were decreased compared to control ($N=4$). The Ki67+ cells were counted using ImageJ. *$p<0.05$.

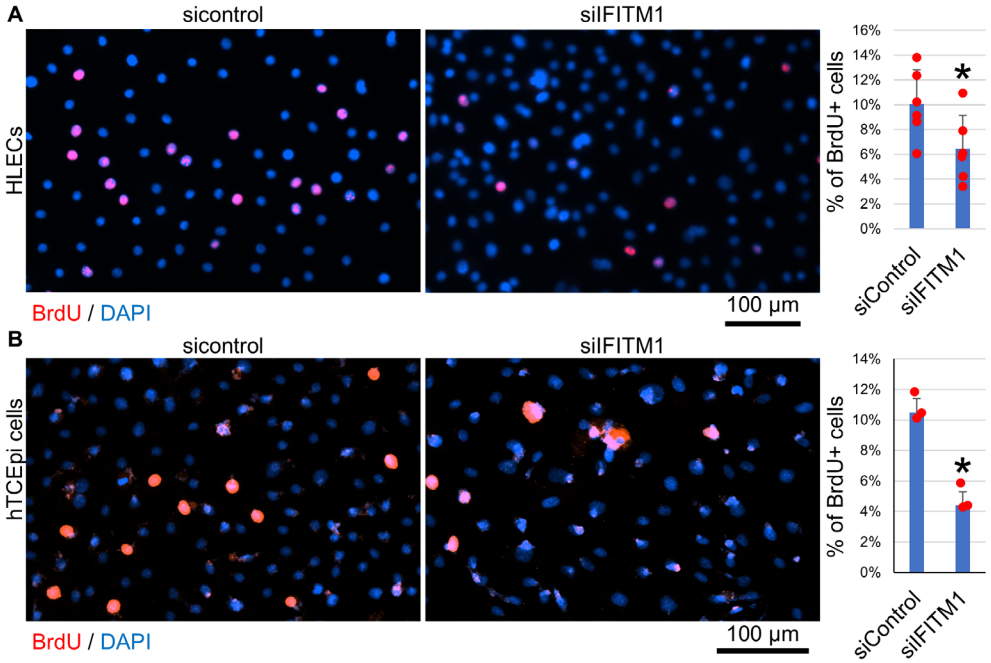

FIGURE 5 | Knockdown of *IFITM1* reduces limbal epithelial cell proliferation. HLECs (A, $N=6$) and cells from a limbal epithelial cell line hTCEpi (B, $N=3$) were transfected with siControl or siIFITM1. Immunofluorescent images visualized the BrdU+ cells at 72 h after transfection. The percentage of BrdU+ cells were quantified by Image J and the results showed a decreased percentage of BrdU+ cells after siIFITM1 transfection. *$p<0.05$.

cell activation and consequently eTA cell expansion in vivo. Our novel findings provide a rationale to develop an AAV vector delivering IFITM1 cDNA. Topical application of AAV-IFITM1 cDNA should directly promote the proliferation of LESCs in patients, which may have considerable therapeutic potential in treating diseases associated with the loss of LESCs, such as LSCD. It has been suggested that some of the putative human limbal epithelial stem cell markers are expressed in the basal layer of the entire mouse corneal epithelium [61], suggesting a difference in their expression pattern in human vs. mouse. Interestingly, we have demonstrated that in both human and mouse corneas, IFITM1 is predominantly expressed in limbal epithelial stem cells, which suggests that the findings from the mouse experiments in the present study should be translatable to human diseases. Interestingly, IFITM3 is also predominantly expressed in mouse outer limbal stem cells and depletion of IFITM3 results in a more differentiated state [15]. This confirms a connection between IFITM family proteins and stem cell maintenance and functions.

It has been shown that several genes and pathways can regulate the proliferation of stem/eTA cell-enriched limbal epithelial cells. For example, knockout of *Adrb2*, which is the predominant adrenergic receptor expressed in the limbal epithelium, enhances limbal epithelial cell proliferation and the expression of putative limbal stem cell markers via targeting SHH signaling [62]. We have shown that inhibition of autophagy in vivo suppresses proliferation in the limbal epithelium after wounding [29]. Overexpression of *Foxc1* promotes proliferation in the limbal epithelium of Pax6+/− mice following central corneal debridement injury [63]. However, in these studies, due to the lack of discrete markers for LESCs, it was impossible to determine the numbers of stem cells using immunostaining or flow cytometry with putative limbal stem cell markers. Thus, it remains unclear whether such alterations in limbal epithelial proliferation are due to changes in the number of stem/eTA cells or the frequency of quiescent stem/eTA cell cycling to generate their progeny. Here, we took advantage of scRNA seq, which is an ideal approach to interrogate rare cell populations such as stem/eTA cells. This is the first demonstration providing direct evidence that the stem/eTA cell number is regulated by *Ifitm1*. Our approach can be used to determine the roles of the other potential regulatory genes in stem cell expansion not only in the cornea but also in other tissues.

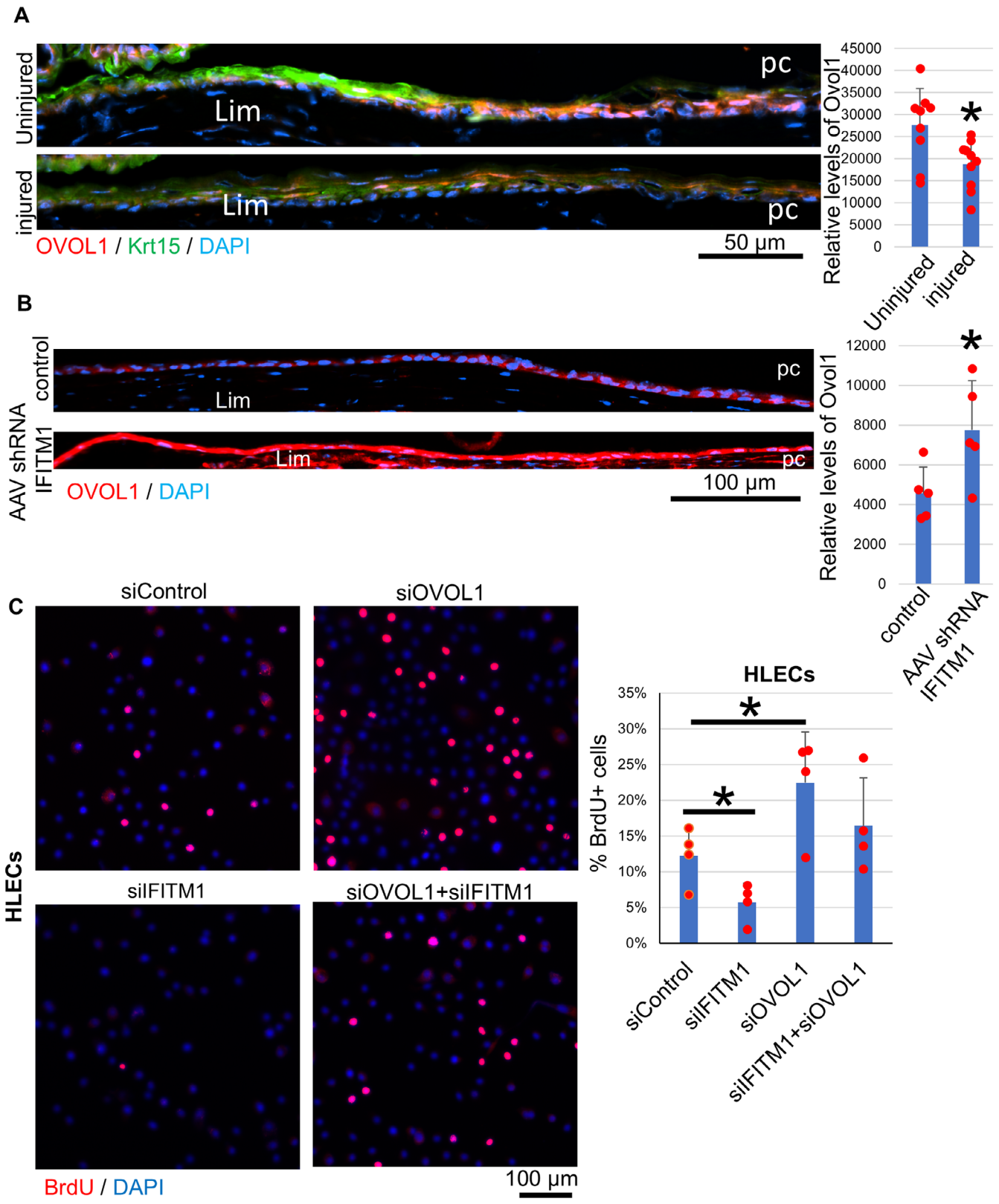

FIGURE 6 | IFITM1 positively regulates limbal epithelial proliferation via OVOL1. (A) Immunofluorescent staining indicated that a NaOH burn in mouse corneas reduced OVOL1 in the limbal epithelium compared with uninjured mice. Relative fluorescence intensity was analyzed using ImageJ. *$p < 0.05$. (B) Immunofluorescent staining showed an increase in Ovol1 expression in the limbal epithelium of mice treated with AAV-shRNA-Ifitm1 compared with AAV-EV control mice. Relative fluorescence intensity was analyzed using ImageJ. *$p < 0.05$. (C) HLECs were transfected with siControl, siIFIMT1, siOVOL1 or siIFIMT1+ siOVOL1 for 72 h. The percentage of BrdU+ cells were quantified by ImageJ. *$p < 0.05$. pc: Peripheral cornea.

OVOL1 and OVOL2 belong to a transcription factor family, encoding zinc finger proteins homologous to *Drosophila melanogaster* Ovo. OVOL2 expression increased in posterior polymorphous corneal dystrophy, an autosomal dominant inherited disorder of the corneal endothelium [37]. In the corneal epithelium, OVOL2 is an important regulator of the human corneal epithelial cell transcriptional program by repressing genes related to epithelial-to-mesenchymal transition [36], suggesting a role in regulating corneal epithelial commitment. In contrast to OVOL2, the function of OVOL1 in the cornea was unknown. Interestingly, OVOL1 negatively regulates cell proliferation in a variety of tissues. During epidermal development, the absence of Ovol1 markedly enhances keratinocyte proliferation [38]. During the induction of pluripotent stem cells (iPSCs), knockdown of OVOL1 enhances proliferation in cells with high KLF4 levels [64]. OVOL1 is downregulated in cutaneous squamous cell carcinoma [39] and mediates the suppression of proliferation in oral squamous cell carcinoma cells by inhibiting ZEB1 expression [40]. Here we show that the knockdown of Ovol1 induces limbal epithelial cell proliferation, which is crucial for the maintenance of corneal homeostasis.

Author Contributions

Huimin Jiang, Parisa Foroozandeh, Han Peng designed research; Huimin Jiang, Parisa Foroozandeh, Han Peng analyzed data; Huimin Jiang, Parisa Foroozandeh, Nihal Kaplan, Dan Xu, Wending Yang, Xiaolin Qi, Elif Kayaalp Nalbant, Elwin D. Clutter, Yongling Zhu, Jian Xu, Matthew John Schipma, Ziyou Ren performed research; Han Peng wrote the paper; Yongling Zhu, Jian Xu contributed new reagents or analytic tools.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Stored in repository.

References

1. A. Pajoohesh-Ganji and M. A. Stepp, "In Search of Markers for the Stem Cells of the Corneal Epithelium," *Biology of the Cell* 97 (2005): 265–276.

2. G. J. Parfitt, B. Kavianpour, K. L. Wu, Y. Xie, D. J. Brown, and J. V. Jester, "Immunofluorescence Tomography of Mouse Ocular Surface Epithelial Stem Cells and Their Niche Microenvironment," *Investigative Ophthalmology & Visual Science* 56 (2015): 7338–7344.

3. M. S. Lehrer, T. T. Sun, and R. M. Lavker, "Strategies of Epithelial Repair: Modulation of Stem Cell and Transit Amplifying Cell Proliferation," *Journal of Cell Science* 111, no. Pt 19 (1998): 2867–2875.

4. M. A. Stepp and J. D. Zieske, "The Corneal Epithelial Stem Cell Niche," *Ocular Surface* 3 (2005): 15–26.

5. G. Cotsarelis, S. Z. Cheng, G. Dong, T. T. Sun, and R. M. Lavker, "Existence of Slow-Cycling Limbal Epithelial Basal Cells That Can Be Preferentially Stimulated to Proliferate: Implications on Epithelial Stem Cells," *Cell* 57 (1989): 201–209.

6. H. Peng, N. Kaplan, R. B. Hamanaka, et al., "microRNA-31/Factor-Inhibiting Hypoxia-Inducible Factor 1 Nexus Regulates Keratinocyte Differentiation," *Proceedings of the National Academy of Sciences of the United States of America* 109 (2012): 14030–14034.

7. A. Schermer, S. Galvin, and T. T. Sun, "Differentiation-Related Expression of a Major 64K Corneal Keratin In Vivo and in Culture Suggests Limbal Location of Corneal Epithelial Stem Cells," *Journal of Cell Biology* 103 (1986): 49–62.

8. M. Notara, A. Alatza, J. Gilfillan, et al., "In Sickness and in Health: Corneal Epithelial Stem Cell Biology, Pathology and Therapy," *Experimental Eye Research* 90 (2010): 188–195.

9. J. M. Wolosin, M. T. Budak, and M. A. Akinci, "Ocular Surface Epithelial and Stem Cell Development," *International Journal of Developmental Biology* 48 (2004): 981–991.

10. T. T. Sun, S. C. Tseng, and R. M. Lavker, "Location of Corneal Epithelial Stem Cells," *Nature* 463 (2010): E10–E11.

11. R. Hayashi, M. Yamato, H. Sugiyama, et al., "N-Cadherin Is Expressed by Putative Stem/Progenitor Cells and Melanocytes in the Human Limbal Epithelial Stem Cell Niche," *Stem Cells* 25 (2007): 289–296.

12. R. M. Lavker and T. T. Sun, "Epidermal Stem Cells: Properties, Markers, and Location," *Proceedings of the National Academy of Sciences of the United States of America* 97 (2000): 13473–13475.

13. G. Pellegrini, E. Dellambra, O. Golisano, et al., "p63 Identifies Keratinocyte Stem Cells," *Proceedings of the National Academy of Sciences of the United States of America* 98 (2001): 3156–3161.

14. H. Jiang, M. Liu, W. Yang, et al., "Activation of Limbal Epithelial Proliferation Is Partly Controlled by the ACE2-LCN2 Pathway," *iScience* 27 (2024): 110534.

15. A. Altshuler, A. Amitai-Lange, N. Tarazi, et al., "Discrete Limbal Epithelial Stem Cell Populations Mediate Corneal Homeostasis and Wound Healing," *Cell Stem Cell* 28 (2021): 1248–1261.

16. J. Collin, R. Queen, D. Zerti, et al., "A Single Cell Atlas of Human Cornea That Defines Its Development, Limbal Progenitor Cells and Their Interactions With the Immune Cells," *Ocular Surface* 21 (2021): 279–298.

17. C. S. Potten and M. Loeffler, "Stem Cells: Attributes, Cycles, Spirals, Pitfalls and Uncertainties. Lessons for and From the Crypt," *Development* 110 (1990): 1001–1020.

18. N. Kaplan, J. Wang, B. Wray, et al., "Single-Cell RNA Transcriptome Helps Define the Limbal/Corneal Epithelial Stem/Early Transit Amplifying Cells and How Autophagy Affects This Population," *Investigative Ophthalmology & Visual Science* 60 (2019): 3570–3583.

19. Y. Dong, H. Peng, and R. M. Lavker, "Emerging Therapeutic Strategies for Limbal Stem Cell Deficiency," *Journal of Ophthalmology* 2018 (2018): 7894647.

20. G. Pellegrini, P. Rama, S. Matuska, et al., "Biological Parameters Determining the Clinical Outcome of Autologous Cultures of Limbal Stem Cells," *Regenerative Medicine* 8 (2013): 553–567.

21. P. Rama, S. Matuska, G. Paganoni, A. Spinelli, M. De Luca, and G. Pellegrini, "Limbal Stem-Cell Therapy and Long-Term Corneal Regeneration," *New England Journal of Medicine* 363 (2010): 147–155.

22. A. V. Ljubimov and M. Saghizadeh, "Progress in Corneal Wound Healing," *Progress in Retinal and Eye Research* 49 (2015): 17–45.

23. K. Xu and F. S. Yu, "Impaired Epithelial Wound Healing and EGFR Signaling Pathways in the Corneas of Diabetic Rats," *Investigative Ophthalmology & Visual Science* 52 (2011): 3301–3308.

24. A. Kabosova, A. A. Kramerov, A. M. Aoki, G. Murphy, J. D. Zieske, and A. V. Ljubimov, "Human Diabetic Corneas Preserve Wound Healing, Basement Membrane, Integrin and MMP-10 Differences From Normal Corneas in Organ Culture," *Experimental Eye Research* 77 (2003): 211–217.

25. K. P. Xu, Y. Li, A. V. Ljubimov, and F. S. Yu, "High Glucose Suppresses Epidermal Growth Factor Receptor/Phosphatidylinositol 3-Kinase/Akt Signaling Pathway and Attenuates Corneal Epithelial Wound Healing," *Diabetes* 58, no. 5 (2009): 1077–1085.

26. M. Saghizadeh, S. Soleymani, A. Harounian, et al., "Alterations of Epithelial Stem Cell Marker Patterns in Human Diabetic Corneas and Effects of c-Met Gene Therapy," *Molecular Vision* 17 (2011): 2177–2190.

27. O. Farrelly, Y. Suzuki-Horiuchi, M. Brewster, et al., "Two-Photon Live Imaging of Single Corneal Stem Cells Reveals Compartmentalized Organization of the Limbal Niche," *Cell Stem Cell* 28 (2021): 1233–1247.

28. R. M. Lavker and T. T. Sun, "Epithelial Stem Cells: The Eye Provides a Vision," *Eye (London, England)* 17 (2003): 937–942.

29. J. K. Park, H. Peng, J. Katsnelson, et al., "MicroRNAs-103/107 Coordinately Regulate Macropinocytosis and Autophagy," *Journal of Cell Biology* 215 (2016): 667–685.

30. X. Wu, J. M. Robotham, E. Lee, et al., "Productive Hepatitis C Virus Infection of Stem Cell-Derived Hepatocytes Reveals a Critical Transition to Viral Permissiveness During Differentiation," *PLoS Pathogens* 8 (2012): e1002617.

31. S. S. Tanaka, Y. L. Yamaguchi, B. Tsoi, H. Lickert, and P. P. Tam, "IFITM/mil/Fragilis Family Proteins IFITM1 and IFITM3 Play Distinct Roles in Mouse Primordial Germ Cell Homing and Repulsion," *Developmental Cell* 9, no. 6 (2005): 745–756.

32. Y. G. Yang, Y. W. Koh, I. N. Sari, et al., "Interferon-Induced Transmembrane Protein 1-Mediated EGFR/SOX2 Signaling Axis Is Essential for Progression of Non-Small Cell Lung Cancer," *International Journal of Cancer* 144 (2019): 2020–2032.

33. A. Kelemen, I. Carmi, A. Oszvald, et al., "IFITM1 Expression Determines Extracellular Vesicle Uptake in Colorectal Cancer," *Cellular and Molecular Life Sciences* 78 (2021): 7009–7024.

34. W. Zheng, Z. Zhao, X. Yi, et al., "Down-Regulation of IFITM1 and Its Growth Inhibitory Role in Cervical Squamous Cell Carcinoma," *Cancer Cell International* 17 (2017): 88.

35. K. Saxena, S. Srikrishnan, T. Celia-Terrassa, and M. K. Jolly, "OVOL1/2: Drivers of Epithelial Differentiation in Development, Disease, and Reprogramming," *Cells, Tissues, Organs* 211 (2022): 183–192.

36. K. Kitazawa, T. Hikichi, T. Nakamura, et al., "OVOL2 Maintains the Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-To-Mesenchymal Transition," *Cell Reports* 15 (2016): 1359–1368.

37. D. D. Chung, W. Zhang, K. Jatavallabhula, A. Barrington, J. Jung, and A. J. Aldave, "Alterations in GRHL2-OVOL2-ZEB1 Axis and Aberrant Activation of Wnt Signaling Lead to Altered Gene Transcription in Posterior Polymorphous Corneal Dystrophy," *Experimental Eye Research* 188 (2019): 107696.

38. M. Nair, A. Teng, V. Bilanchone, A. Agrawal, B. Li, and X. Dai, "Ovol1 Regulates the Growth Arrest of Embryonic Epidermal Progenitor Cells and Represses c-Myc Transcription," *Journal of Cell Biology* 173 (2006): 253–264.

39. T. Ito, G. Tsuji, F. Ohno, T. Nakahara, H. Uchi, and M. Furue, "Potential Role of the OVOL1-OVOL2 Axis and c-Myc in the Progression of Cutaneous Squamous Cell Carcinoma," *Modern Pathology* 30 (2017): 919–927.

40. C. Xu, T. Yan, and J. Yang, "OVOL1 Inhibits Oral Squamous Cell Carcinoma Growth and Metastasis by Suppressing Zinc Finger E-Box Binding Homeobox 1," *International Journal of Clinical and Experimental Pathology* 12 (2019): 2801–2808.

41. J. Xu, A. Contractor, and Y. Zhu, "Protocol to Map Multi-Transmitter Neurons in the Mouse Brain Using Intersectional Strategy," *STAR Protocols* 3 (2022): 101907.

42. N. Kaplan, S. Wang, J. Wang, et al., "Ciliogenesis and Autophagy Are Coordinately Regulated by EphA2 in the Cornea to Maintain Proper Epithelial Architecture," *Ocular Surface* 21 (2021): 193–205.

43. H. Peng, N. Kaplan, M. Liu, H. Jiang, and R. M. Lavker, "Keeping an Eye out for Autophagy in the Cornea: Sample Preparation for Single-Cell RNA-Sequencing," *Methods in Molecular Biology* 2879 (2023): 113–122.

44. R. Satija, J. A. Farrell, D. Gennert, A. F. Schier, and A. Regev, "Spatial Reconstruction of Single-Cell Gene Expression Data," *Nature Biotechnology* 33 (2015): 495–502.

45. A. Dobin, C. A. Davis, F. Schlesinger, et al., "STAR: Ultrafast Universal RNA-seq Aligner," *Bioinformatics* 29 (2013): 15–21.

46. S. Anders, P. T. Pyl, and W. Huber, "HTSeq—A Python Framework to Work With High-Throughput Sequencing Data," *Bioinformatics* 31, no. 2 (2015): 166–169.

47. M. I. Love, W. Huber, and S. Anders, "Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data With DESeq2," *Genome Biology* 15 (2014): 550.

48. I. K. Gipson, "Distribution of Mucins at the Ocular Surface," *Experimental Eye Research* 78 (2004): 379–388.

49. S. C. Tseng, M. J. Jarvinen, W. G. Nelson, J. W. Huang, J. Woodcock-Mitchell, and T. T. Sun, "Correlation of Specific Keratins With Different Types of Epithelial Differentiation: Monoclonal Antibody Studies," *Cell* 30, no. 2 (1982): 361–372.

50. C. L. Loughner, A. Tiwari, D. Kenchegowda, S. Swamynathan, and S. K. Swamynathan, "Spatiotemporally Controlled Ablation of Klf5 Results in Dysregulated Epithelial Homeostasis in Adult Mouse Corneas," *Investigative Ophthalmology & Visual Science* 58 (2017): 4683–4693.

51. J. M. Li, S. Kim, Y. Zhang, et al., "Single-Cell Transcriptomics Identifies a Unique Entity and Signature Markers of Transit-Amplifying Cells in Human Corneal Limbus," *Investigative Ophthalmology & Visual Science* 62 (2021): 36.

52. P. Català, N. Groen, J. A. Dehnen, et al., "Single Cell Transcriptomics Reveals the Heterogeneity of the Human Cornea to Identify Novel Markers of the Limbus and Stroma," *Scientific Reports* 11 (2021): 21727.

53. J. B. Lin, X. Shen, C. W. Pfeifer, et al., "Dry Eye Disease in Mice Activates Adaptive Corneal Epithelial Regeneration Distinct From Constitutive Renewal in Homeostasis," *Proceedings of the National Academy of Sciences of the United States of America* 120 (2023): e2204134120.

54. M. A. Stepp, "Corneal Integrins and Their Functions," *Experimental Eye Research* 83 (2006): 3–15.

55. M. A. Kurpakus, M. T. Maniaci, and M. Esco, "Expression of Keratins K12, K4 and K14 During Development of Ocular Surface Epithelium," *Current Eye Research* 13 (1994): 805–814.

56. G. Maiti, M. R. Monteiro de Barros, N. Hu, et al., "Single Cell RNA-Seq of Human Cornea Organoids Identifies Cell Fates of a Developing Immature Cornea," *PNAS Nexus* 1 (2022): pgac246.

57. R. Sartaj, C. Zhang, P. Wan, et al., "Characterization of Slow Cycling Corneal Limbal Epithelial Cells Identifies Putative Stem Cell Markers," *Scientific Reports* 7 (2017): 3793.

58. L. Takacs, E. Toth, G. Losonczy, et al., "Differentially Expressed Genes Associated With Human Limbal Epithelial Phenotypes: New Molecules That Potentially Facilitate Selection of Stem Cell-Enriched Populations," *Investigative Ophthalmology & Visual Science* 52 (2011): 1252–1260.

59. I. Tirosh, B. Izar, S. M. Prakadan, et al., "Dissecting the Multicellular Ecosystem of Metastatic Melanoma by Single-Cell RNA-Seq," *Science* 352 (2016): 189–196.

60. X. Zhang and L. Liu, "Applications of Single Cell RNA Sequencing to Research of Stem Cells," *World Journal of Stem Cells* 11 (2019): 722–728.

61. J. Li, Y. Xiao, T. G. Coursey, et al., "Identification for Differential Localization of Putative Corneal Epithelial Stem Cells in Mouse and Human," *Scientific Reports* 7 (2017): 5169.

62. Z. Zhang, L. Yang, Y. Li, et al., "Interference of Sympathetic Overactivation Restores Limbal Stem/Progenitor Cells Function and Accelerates Corneal Epithelial Wound Healing in Diabetic Mice," *Biomedicine & Pharmacotherapy* 161 (2023): 114523.

63. W. Yang, S. K. Lee, O. J. Lehmann, et al., "FoxC1 Activates Limbal Epithelial Stem Cells Following Corneal Epithelial Debridement," *Experimental Eye Research* 234 (2023): 109599.

64. H. Kagawa, R. Shimamoto, S. I. Kim, et al., "OVOL1 Influences the Determination and Expansion of iPSC Reprogramming Intermediates," *Stem Cell Reports* 12 (2019): 319–332.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.