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A Single-Cell Atlas of Diabetes-Associated Remodeling in the Human Corneal Limbus

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Key words: Human cornea, Diabetes, Single-cell RNA-seq, diabetic cornea, limbal stem cells, limbus, Single-cell transcriptomics.

Abstract

Purpose: Diabetes mellitus (DM) disrupts epithelial integrity, sensory function, basement membrane composition, wound healing, and stem cell function. Corneal epithelial homeostasis and wound healing involve a complex coordination of various sub-populations of limbal cells. However, the transcriptomic changes at the single cell level in these cell-populations associated with diabetes have never been addressed. This study aims to generate a high-resolution single-cell transcriptomic atlas of human DM corneal limbal tissue to advance our conceptual understanding of human diabetes-associated changes in cellular types and composition, transcriptional states, cellular trajectories and intercellular communication.

Methods: Single-cell sequencing (scRNA-seq) was performed on isolated corneolimbal epithelial tissues from a total of six age-matched human donors, including three non-diabetic (N) and three DM donors using 10X Genomics. Selected transcriptomic findings were assessed at the protein level by immunofluorescence staining of corneal sections.

Results: Thirteen distinct cell clusters were identified based on top expressed gene markers in both N and DM samples. Analyses of differentially expressed genes (DEGs), biological pathways, pseudotime trajectories, and subclustering identified DM-associated transcriptional changes, cell-cycle differences, and altered inferred intercellular signaling patterns. Pathways analysis highlighted extracellular matrix organization, adhesion, inflammation, and ligand–receptor interactions that may be associated with diabetes-impaired corneal homeostasis. Immunofluorescence showed corresponding differences in protein staining patterns for selected markers, including decreased IFITM3, MMP-1, periostin, LGI4, and eNOS, and increased ADAMTS-4.

Conclusions: We provide the first high-resolution single-cell transcriptome atlas of DM-associated alterations in the human corneolimbal niche and identify molecular pathways and cellular populations that may contribute to compromised corneal homeostasis and regenerative

capacity in DM.

1. Introduction

Diabetes mellitus (DM) leading to visual impairment poses a major challenge facing the health system. In 2022, an estimated 828 million individuals worldwide were living with diabetes [1]. DM affects all parts of the eye, with diabetic retinopathy (DR) being the most severe ocular complication [2]. However, the cornea is also frequently affected by DM with various alterations observed in 45-70% of diabetic patients [3]. Corneal manifestations of DM include epithelial defects and fragility, recurrent epithelial erosions, decreased sensitivity, ulcers, edema, abnormal wound repair, and the dysfunction of limbal epithelial stem cells (LESC), collectively referred to as diabetic keratopathy, as well as sub-basal nerve abnormalities, manifested by progressive loss of corneal sensation, known as diabetic neuropathy [4].

The corneal limbus, located at the corneoscleral junction, contains a specialized microenvironment that harbors LESC. These stem cells maintain corneal epithelial homeostasis and regeneration throughout life by continuously generating transit amplifying cells (TAC) that migrate centripetally to replenish the corneal epithelium. The limbal niche consists of a heterogeneous population of epithelial, stromal, vascular, immune, and neural cells whose coordinated interactions regulate stem cell quiescence, activation, and differentiation [5]. Disruption of this niche or dysfunction of LESC can impair corneal epithelial renewal and contribute to pathological conditions such as limbal stem cell deficiency and defective wound healing [6]. Emerging evidence suggests that DM alters limbal stem cell function and niche regulation, contributing to their impaired epithelial repair [7-8]. Chronic hyperglycemia exposes corneolimbal tissues to persistent metabolic stress through the accumulation of advanced glycation end products (AGEs), oxidative stress, and chronic low-grade inflammation [4,8]. These diabetes-associated changes disrupt the extracellular matrix (ECM), alter cell-cell and cell-matrix signaling, and impair communication between LESC and supporting niche cells [4,7,8]. Because

the integrity of the limbal niche depends on coordinated interactions among epithelial, stromal, immune, vascular, and neural components, disruptions of this microenvironment can affect LESC maintenance and regenerative function [5,6]. In diabetes, LESC abnormalities and impaired corneal epithelial regeneration and wound healing have been documented and recognized features of diabetic keratopathy [7,8]. Despite increasing evidence supporting these pathogenic mechanisms, the cellular populations and molecular pathways underlying diabetes-associated limbal niche dysfunction remain incompletely understood.

In recent years, single-cell RNA sequencing (scRNA-seq) has emerged as a powerful tool enabling comprehensive cell mapping leading to major findings based on single cell transcriptome profiling [9-11]. This approach has allowed to assess cellular heterogeneity, identify rare or novel cell subpopulations, and facilitate the cellular trajectory analysis to reveal dynamic cellular transitions during development, stem cell differentiation, wound healing, and disease *vs*. healthy state at single cell resolution [12-16]. In the past decade, scRNA-seq has shown profound effects on many research areas including the ocular surface field [11-12, 17-22]. It allowed the generation of cell atlases of many tissues and revealed mechanisms involved in development and stem cell differentiation. In the mouse cornea, scRNA-seq revealed two LESC populations, one maintaining corneal epithelial homeostasis and renewal, and the other participating in wound healing and boundary formation with conjunctiva [18]. It has also helped uncover several new putative LESC markers [18-22]. These studies showed the complexity of limbal LESC-harboring compartment and the importance of examining various cell populations in corneal wound healing and disease. Despite these advances, transcriptomic alterations of limbal epithelial populations associated with systemic diseases such as DM remain poorly understood, particularly at single-cell resolution. Several single-cell transcriptomic studies have generated detailed cellular atlases of the normal human and mouse cornea and limbus, identifying major epithelial, stromal, immune, vascular, and neural cell populations as well as candidate LESC markers [12,17-21]. However, these studies focused on healthy tissues or developmental biology and did not investigate how diabetes

alters the cellular composition, transcriptional programs, cell-cell communication, and ECM remodeling of the human corneolimbal niche. Thus, a comprehensive single-cell characterization of diabetes-associated changes across multiple limbal cell populations has remained lacking. Therefore, we examined global altered gene expressions across heterogeneous corneolimbal cell populations, their cellular diversity, and their interactions within the complex limbal niche in non-diabetic (N) versus DM human corneolimbal donor tissues. To address the existing knowledge gap, we performed the first examination of scRNA-seq of human corneolimbal tissues from DM and N donors to comprehensively define diabetes-associated changes in cellular composition and transcription across distinct cell populations of limbal niche. Our analysis revealed that, particularly within LESC/VEC and limbal progenitor cell (LPC) clusters, DEGs are enriched in pathways related to ECM organization, cell adhesion, inflammatory signaling, and tissue remodeling, indicating DM-associated niche remodeling and impaired regenerative homeostasis. Immunofluorescence assessment showed DM-associated alterations in select markers including decreased IFITM3, MMP-1, periostin, LGI4, and eNOS, and increased ADAMTS-4, while also revealing transcript-protein discordance for targets such as GPHA2. Collectively, this study provides a comprehensive single-cell transcriptomic atlas of DM human limbal niche and identifies candidate molecular pathways and cellular populations through which DM may compromise corneal homeostasis and regenerative capacity, offering potential targets for therapeutic intervention in diabetic keratopathy.

2. Materials and Methods

2.1. Human specimens

Age-matched and research-consented human N and DM donor autopsy globes and corneas (Supplementary Material Table 1) were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) or the Center for Vision and Eye Banking Research, Eversight

(Cleveland, OH) in Optisol (Chiron Vision, Claremont, CA) or similar corneal preservation medium within 72 hours after death and used in accordance with the approved IRB protocol Pro00019393 from Cedars-Sinai Medical Center.

2.2. Single cell isolation and sequencing

Corneolimbal epithelial tissues, including residual peripheral cornea and conjunctiva, were dissected from a total of six total age-matched human donors, including three DM and three N donors using 8.5-mm trephine. Mean age was 66.3 ± 13.6 years in the N group and 76.7 ± 2.5 years in the DM group, with no statistically significant difference between groups (Welch's t-test, $p = 0.318$). The standardized trephine-based dissection was used to minimize variability in tissue dissection and, therefore, reduced potential differences in the relative abundance of conjunctival, limbal, and corneal epithelial populations among samples. Epithelial cells were then isolated by sequential enzymatic digestion using Dispase II (2.4 U/ml) followed by trypsin-EDTA to generate single-cell suspensions, as previously described [23]. An average of 4,893 single cells (range: 2,060-7,854) were collected from each sample and subsequently sequenced on an Illumina NovaSeq 6000 platform according to the manufacturer's instructions (10X Genomics, Pleasanton, CA) at the Genomics Core at Cedars-Sinai Medical Center. Cellular suspensions were loaded on a Chromium Controller instrument to generate single-cell Gel Bead-In-Emulsions. Sequencing libraries were generated and loaded on a NovaSeq 6000 (Illumina, San Diego, CA) with a target depth of approximately 200,000 reads per cell. The achieved mean sequencing depth varied from 52,808 to 143,007 reads per cell across donors.

2.3. Bioinformatics analysis

Cell Ranger (v9.0.1;10x Genomics) was used to generate raw gene–barcode expression matrices for each sample using the GRCh38-2020-A reference provided by 10x Genomics. Ambient RNA contamination and empty droplets were reduced using CellBender [24] (v0.3.2; remove-background mode) with default parameters, and the corrected matrices were imported into R

(v4.2.1). DoubletFinder [25] (v2.0.6) was applied independently to each sample. The optimal pK was selected by parameter sweeping based on the maximum BCmetric, and the expected doublet number was estimated based on the 10x Genomics multiplet-rate relationship (~0.8% per 1,000 recovered cells) and adjusted for homotypic doublets. Only cells classified as singlets were retained for downstream analysis. Seurat objects were generated using Seurat (v5.3.0) [26]. Quality control (QC) filtering retained cells with more than 400 and fewer than 10,000 detected genes, more than 300 and fewer than 100,000 UMIs, and less than 10% mitochondrial RNA content. QC metrics are detailed in Supplementary Dataset 2. Following doublet removal and QC, the six-donor datasets were merged and normalized using SCTransform with 4,000 variable features. Data integration was performed using the CCAIntegration method implemented in Seurat v5. Based on the PCA variance profile and elbow plot, the first 41 dimensions were selected for downstream analysis. Neighborhood graphs were constructed using FindNeighbors, and clustering was performed using FindClusters at a resolution of 0.25. UMAP was used for dimensionality reduction and visualization. Cell-type annotation was performed based on previously reported marker genes, together with DotPlot, VlnPlot, and FeaturePlot visualizations generated in Seurat. Cell-type composition was calculated separately for each donor as the proportion of retained cells assigned to each annotated population. Donor-level differential-abundance analysis between N and DM samples was performed using Propeller [27], with individual donors treated as biological replicates. P values were adjusted for multiple testing using the Benjamini–Hochberg method, with FDR <0.05 considered statistically significant. Cell-level exploratory differential expression analysis between DM and N samples within each annotated population was performed using the FindMarkers function with MAST [28]. Genes with a nominal P value < 0.05 and a |log2 fold change| > 1 were considered candidate DEGs. The fold-change threshold was applied to focus the analysis on genes showing substantial expression differences. Benjamini–Hochberg-adjusted P values were also calculated and reported. Donor-aware differential expression was additionally performed using a pseudobulk approach. Raw RNA UMI

counts were aggregated for each donor and cell population, with the three N and three DM donors treated as biological replicates. Genes with at least 10 pseudobulk counts in at least three donors were retained. Differential expression between DM and N groups was performed using DESeq2 [29], with FDR < 0.05 was used to define statistically significant pseudobulk DEGs. An age-adjusted analysis was additionally performed as a sensitivity analysis. Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted using clusterProfiler (v4.14.6) [30]. Over-representation analysis was performed using MAST-defined candidate DEGs. Ranked-list gene-set enrichment analysis (GSEA) was additionally performed using all tested genes ranked according to average log2 fold change between DM and N cells. Pathways with FDR < 0.05 were considered significantly enriched. Trajectory analysis was performed using Monocle3 (v1.3.7) [31] on selected epithelial clusters. The Seurat-derived UMAP embedding was used for principal-graph learning, with branching permitted across the analyzed populations. The pseudotime root was manually assigned to the LESC/VEC population based on its stem/progenitor identity. Sensitivity analyses were additionally performed using alternative root states and by reconstructing the trajectory after exclusion of canonical S-phase and G2/M-phase cell-cycle genes. Cell-cell communication analysis was performed using CellChat (v2.2.0) [32]. Log-normalized RNA expression data from selected cell populations were analyzed separately for pooled DM and N samples using the complete human CellChat database, including Secreted Signaling, ECM Receptor, Non-protein Signaling, and Cell-Cell Contact interactions. Communication probabilities were calculated using the trimean method without population-size weighting, and interactions involving cell populations with fewer than 10 cells were excluded. Pathway-level communication networks were subsequently aggregated and compared between DM and N groups. To assess robustness to differences in cell number and composition, matched-cell-number subsampling was performed within each cell population, followed by repeated CellChat analysis across 50 iterations.

2.4. Immunostaining

Transverse 5-µm thick sections of *ex vivo* corneas from DM and N donors were fixed in 1% formalin for 5 min, permeabilized in 0.5% Triton X-100 (MilliporeSigma, St. Louis, MO), blocked in 5% bovine serum albumin (BSA) (MilliporeSigma) or normal goat serum (NGS) (MilliporeSigma), incubated overnight at 4ºC with primary antibodies and then with AlexaFluor 488 or 594 conjugated secondary antibodies (Thermo Fisher Scientific, Carlsbad, CA) for 1 hour at room temperature. Slides were mounted using ProLong™ Gold Antifade mounting medium with DAPI (Thermo Fisher Scientific). Images were acquired using identical exposure times for each marker across all conditions. The images shown are representative of at least three independent donor samples, with a minimum of three sections analyzed per donor. Detailed antibody information and donor sample sizes are provided in Supplementary Material Table 3.

3. Results

3.1. Single-cell transcriptomics profile reveals the presence of heterogeneous cell types in the human limbus.

Single-cell partitioning and library preparation were performed using the 10x Genomics Chromium platform, and the resulting libraries were sequenced on the Illumina NovaSeq 6000. After quality-control filtering, doublet exclusion, batch correction, and dataset integration, a total of 20240 out of 29360 cells were retained for downstream analysis. Study workflow, including collection of human N and DM donor limbal tissues, tissue dissociation, scRNA-seq, bioinformatic integration, clustering, and downstream analyses, is illustrated in Figure 1A. Unsupervised clustering and UMAP visualization identified 13 transcriptionally distinct cell clusters representing epithelial and niche-associated cell populations within the human corneolimbal samples (Figure 1B). The number of cells retained from each donor and the contribution of each donor to each annotated

cell population are summarized in Supplementary Dataset 4.

Cluster annotation was performed using established marker genes together with the top DEGs shown in the heatmap and violin plots (Figure 1C, D). Three clusters expressed high levels of *KRT13, KRT19, KRT7, MUC1,* and *MUC4,* consistent with conjunctival epithelial cell (CjEC) identity, and were therefore annotated as CjEC1, CjEC2, and CjEC3 [33-36]. Although CjEC1 and CjEC2 clusters shared a common conjunctival epithelial profile, they exhibited distinct transcriptional signatures, suggesting heterogeneity within the conjunctival compartment. A separate epithelial cluster expressing *BCAM* and *NTRK2* was annotated as basal corneal epithelial cells (B-CEC) [37-38]. The remaining clusters represented diverse cell populations of the limbal epithelial and stromal niche. A prominent cluster expressing *GPHA2, SLC6A6, MMP10,* and *IFITM3* was identified as limbal progenitor cells (LPC), consistent with previously reported limbal stem/progenitor-associated markers [19-20]. A highly proliferative cluster enriched for *MKI67* and *BIRC5* was classified as transit amplifying cells (TAC) [19]. A distinct epithelial population characterized by expression of the differentiation-associated markers *KRT3* and *KRT12*, together with *CXCL14*, was annotated as superficial limbal epithelial cells (S-LEC) [20,21]. Interestingly, putative LESC markers *ABCG2, SOX17,* and *CD200* were highly expressed in the same cluster as vascular endothelial cell (VEC) markers *CLDN5* and *PECAM1* [19,39-42]. To further interrogate this heterogeneity, we performed additional subclustering analysis; however, VEC and LESC-associated markers remained incompletely resolved across subclusters (Supplementary Figure 1). Specifically, *CD34* and *PECAM1* co-localized with *ABCG2*, *SOX17*, and *CD200* within the same subcluster, while *SOX17* and *SOX18* showed differential distribution across subclusters. Because these transcriptomic profiles did not permit definitive assignment of this population as either LESC or VEC, we conservatively designated this cluster as limbal epithelial stem cell/vascular endothelial cell (LESC/VEC). DEGs including *p* value and multiple-testing-adjusted *p* values for LESC/VEC subclusters are listed in Supplementary Dataset 5. Additional niche-associated populations included neural crest progenitor cells (NCPC) marked by

NES and *SOX2,* limbal stromal cells/mesenchymal stem cells (LSC/MSC) expressing *LUM, PDGFRB,* and *THY1,* an immune cell (IC) cluster expressing *CCL5*, a distinct immune/Langerhans cell (IC/LC) cluster characterized by *C1QC* expression, and melanocytes (MEL) expressing *MLANA.* Notably, the MEL cluster also showed strong and selective expression of *ABCB5,* which has been previously considered to be a LESC marker [43-44]. The complete list of DEGs across all annotated cell clusters is provided in Supplementary Dataset 6.

To further explore transcriptional relationship among epithelial populations, we performed pseudotime trajectory analysis using the LESC/VEC cluster as the literature-informed root based on the established model of limbal stem/progenitor–driven corneal epithelial renewal [19,20] (Figure 1E, F). Under this root assignment, LESC/VEC, LPC, B-CEC, TAC, and S-LEC occupied distinct positions along the inferred pseudotime trajectory. Consistent with this ordering, LPC predominantly occupied intermediate pseudotime positions, whereas B-CEC and TAC extended across intermediate-to-later pseudotime and S-LEC were mainly enriched at later pseudotime values. However, the relative ordering of B-CEC and TAC was sensitive to the exclusion of cell-cycle genes and was therefore not interpreted as a fixed sequential relationship. These findings illustrate transcriptional relationships among the epithelial populations that are consistent with a limbal stem/progenitor to differentiated epithelial framework.

3.2. Subclustering and pseudotime analysis reveals transcriptional heterogeneity within the LPC population.

To further resolve the heterogeneity within the LPC population, we performed subclustering analysis on pooled DM and N LPC cells, which identified three transcriptionally distinct LPC subclusters (0–2) (Figure 2A). Donor-specific cell counts and proportions as well as the DEGs defining each subcluster are provided in Supplementary Dataset 7. The percentage contribution of each donor is shown in Supplementary Figure 2. These subclusters occupied partially overlapping yet separable regions in UMAP space, indicating that the LPC compartment is not

uniform but instead consists of multiple cellular states.

Subcluster 0 exhibited elevated expression of limbal progenitor–associated genes, including *KRT14, KRT15, SLC6A6* and *GPHA2,* supporting its designation as a relatively undifferentiated basal-like subpopulation (Figure 2B) [20, 45-46]. This interpretation was further supported by the heatmap of top DEGs (Figure 2C), which showed enrichment of *SPARCL1, GPHA2,* and *DKK3* in subcluster 0. *SPARCL1* and *GPHA2* have been linked to limbal progenitor identity, whereas *DKK3* is associated with Wnt-related regulation of progenitor cell maintenance [46-48]. Subcluster 1 exhibited reduced expression of the progenitor-associated genes *KRT15* and *GPHA2* relative to subcluster 0. Also, this subcluster showed increased expression of genes such as *FOS, JUN, JUNB,* and *EGR1* (Early Growth Response 1). The *FOS, JUN,* and *JUNB* genes are components of the AP-1 transcriptional program and *EGR1* is a well-established immediate-early response gene induced during epithelial activation, stress signaling, and wound repair [49-50]. This expression pattern may reflect a transcriptional shift away from a more quiescent progenitor-like state. Subcluster 2 displayed elevated expression of *KRT17* relative to subclusters 0 and 1, together with upregulation of *TNFRSF12A, AREG,* and *LAMC2,* consistent with an activated, stress-responsive, and migratory progenitor-like phenotype. TNFRSF12A (Fn14) has been implicated in epithelial survival, regeneration, and wound responses following injury or stress [51-52]. Notably, AREG is an EGFR ligand that promotes downstream ERK signaling and has been implicated in cell survival, proliferation, differentiation, and matrix remodeling [53-54]. LAMC2 encodes the laminin γ2 chain of laminin-332, which is increased at the leading edge of migrating epithelia and contributes to basement membrane interaction and tissue remodeling [55]. Pseudotime analysis further showed inferred transcriptional relationships among the three LPC subclusters (Figure 2D), with distinct distributions of pseudotime values across subclusters (Figure 2E). LPCs comprise multiple dynamic states and demonstrate transcriptional heterogeneity within the LPC population, with subclusters exhibiting progenitor-associated, immediate-early/stress responsive, and activation/migration-associated expression profiles.

3.3. Differential gene expression analysis identifies new diabetes-altered markers.

After defining and annotating distinct cell clusters within the limbal epithelial landscape, DEGs between DM and N samples were first assessed at the cell level using MAST for exploratory analysis. We first examined UMAP visualizations of corneolimbal cell clusters under DM and N conditions separately, which identified a total of 13 clusters without evident condition-specific clustering patterns (Figure 3A). A circular UMAP integrating cell cycle states, cell populations, and experimental conditions is presented in Figure 3B, providing a comprehensive overview to facilitate comparative analysis across cell types. Despite the absence of distinct clustering differences between conditions, DEG analysis across the 13 cell clusters revealed widespread transcriptional alterations between DM and N samples (Figure 3C). The number of candidate DEGs per cluster was based on the specified thresholds ($p < 0.05$ and |log2 fold change| > 1) and the number of upregulated and downregulated genes within each cluster is summarized in Supplementary Dataset 8. The complete list of DEGs is provided in Supplementary Dataset 9. Specifically, key representative DM-associated genes and their cluster-specific expression patterns, with a focus on LESC/VEC and LPC clusters, are summarized in Table 1 [56-68].

In addition to transcriptional changes, we assessed cell-type composition at the donor level (Figure 3D). Donor-level differential abundance analysis showed that none of the annotated cell populations differed significantly in relative abundance between N and DM donors after multiple-testing correction (Supplementary Dataset 10). Although the LESC/VEC and LSC/MSC populations showed lower mean proportions in DM donors, these differences did not reach statistical significance. The numerical trend observed for the LESC/VEC population is directionally consistent with previous studies reporting diabetes-associated alterations and dysfunction of limbal epithelial stem/progenitor cells [4,69,70]. We next assessed cell-cycle phase distributions across major cell populations under DM and N conditions (Figure 3E). TACs showed the strongest proliferative profile in both groups, with cells almost entirely enriched in G2/M and virtually no G1

fraction. In DM samples, LESC/VEC shifted toward G1 with a corresponding reduction in G2/M, whereas LPC and LSC/MSC showed only minor differences between conditions. In contrast, B-CEC, S-LEC, and NCPC exhibited a relative increase in G2/M in DM, most prominently in NCPC. MEL showed only modest changes.

To assess the consistency of the transcriptional findings at the donor level, we additionally performed donor-aware pseudobulk differential-expression analysis. The donor-aware pseudobulk analysis identified FDR-significant diabetes-associated genes in several cell populations, although several preselected genes of interest did not reach FDR-adjusted significance. (Supplementary Dataset 11). Accordingly, the cell-level MAST findings are interpreted as exploratory candidate transcriptional patterns.

Table 1. Selected diabetes-altered genes and their cluster-specific expression patterns in limbal cell populations

Gene names	Cluster	*p* value	Avg log2FC	Gene function	Reference
ADAMTS4	LESC/VEC	1.56e-02	1.8668	*ADAMTS4* encodes a secreted extracellular matrix protease that cleaves proteoglycans such as aggrecan and brevican, thereby contributing to matrix remodeling and tissue degradation. It is increased in the vitreous of patients with proliferative DR.	[56]
	LPC	4.00e-04	3.0061		
POSTN	LESC/VEC	2.00e-04	-1.9787	*POSTN* encodes periostin, a secreted extracellular matrix protein that supports cell adhesion, tissue remodeling, and wound repair by interacting with matrix components and integrin-mediated signaling. It plays a role in LESC maintenance and is increased upon wounding of human LESC cultures.	[57, 58]
	LPC	5.98e-12	1.7307		
LGALS7B	LESC/VEC	3.55e-04	-5.9422	*LGALS7B* encodes galectin-7 that belongs to a family of β-galactoside-binding proteins that regulate cell–cell and cell–matrix interactions and plays a role during skin wounding. Its expression is reduced in keratinocytes under high glucose environment in a streptozotocin-induced diabetic mouse model.	[59, 60]
	LPC	1.28e-70	-2.6787		
IGF1	LESC/VEC	1.20e-06	1.6297	*IGF1* encodes insulin-like growth factor 1, a secreted growth factor that promotes cell proliferation, survival,	[61, 62]

				differentiation, and tissue growth through activation of IGF signaling pathways. It plays a role in maintenance of corneal epithelial homeostasis and enhances corneal epithelial cell migration.	
SERPINE1	LESC/VEC	1.37e-06	-1.1889	*SERPINE1* is a serine protease inhibitor that functions as the inhibitor of tissue plasminogen activator. It is suppressed in diabetic mice and plays a role in epithelial wound healing.	[63]
	LPC	9.18e-14	1.2641		
GPHA2	LESC/VEC	6.18e-01	3.8309	*GPHA2* encodes glycoprotein hormone subunit alpha 2, a secreted cystine-knot hormone subunit that can heterodimerize with GPB5 to form thyrostimulin and mediate cell-surface receptor signaling. In the cornea, it is expressed in the limbal progenitor cell-population.	[18, 20, 46, 64]
	LPC	2.72e-43	3.0030		
IFITM3	LPC	1.07e-10	-1.3124	*IFITM3* encodes an interferon-induced transmembrane protein that restricts viral entry and infection by altering endosomal and plasma membrane properties as part of the innate immune response. It supports the undifferentiated state of limbal progenitor cell population.	[18, 65]
MMP1	LPC	2.46e-05	-0.7777	*MMP1* encodes interstitial collagenase, a zinc-dependent matrix metalloproteinase that degrades extracellular matrix components, especially fibrillar collagens, and thereby contributes to tissue remodeling and wound repair. It plays a role in cell migration in human corneal epithelial cell cultures.	[66]
NOS3	LESC/VEC	3.78e-02	0.8609	*NOS3* encodes nitric oxide synthase 3 (eNOS), which generates nitric oxide from L-arginine as a biologic mediator involved in multiple physiological processes, and genetic variation in this gene has been associated with susceptibility to coronary spasm. It plays an important role in stimulating wound healing and growth factor-stimulated angiogenesis.	[67]
MYCT1	LESC/VEC	9.07e-03	-0.3227	*MYCT1* encodes MYC target 1, a MYC-responsive protein thought to participate in MYC-regulated cellular processes such as growth, proliferation, and differentiation.	[68]

3.4. Altered inferred cell–cell communication in the diabetic limbal niche

To characterize how DM affects intercellular communication within the limbal epithelial niche, we

performed ligand–receptor interaction analysis across all identified cell clusters. Overall, DM samples exhibited an increased number of interactions in terms of number and strength compared to N group (Figure 4A). Specifically, the total number of interactions increased from 4,156 in N to 5,608 in DM samples, accompanied by a substantial rise in overall interaction strength (56.6 vs. 90.5, respectively). Differential interaction analysis revealed increased signaling interactions among multiple limbal cell populations under DM conditions, particularly involving B-CEC and LSC/MSC cells (Figure 4B). In terms of interaction number, signaling from LSC/MSC to NCPC (dark red square) showed the greatest increase under DM conditions. In contrast, S-LEC, when acting as a ligand sender, exhibited reduced interactions with S-LEC and TAC populations (blue squares). Regarding interaction strength, B-CEC displayed the strongest signaling toward B-CEC and TAC (dark red square), whereas NCPC showed the lowest interaction strength toward LSC/MSC (blue square). To further characterize pathway-level alterations in intercellular communication, we analyzed global signaling patterns across limbal cell populations under DM and N conditions (Figure 4C–D). Comparative analysis of signaling information flow revealed marked differences between the two conditions. Several signaling pathways, including *NRXN* (neurexin signaling), *SPP1* (osteopontin signaling), *CALCR* (calcitonin receptor signaling), and *CADM* (cell adhesion molecule signaling), exhibited higher activity in N samples, indicating enrichment of pathways associated with cell–cell communication, adhesion, and tissue homeostasis [71-76]. In contrast, DM samples showed increased activity in pathways such as NRG (neuregulin signaling), PTN (pleiotrophin signaling), PLAU, NOTCH, and VEGF, which are broadly associated with epithelial activation, ECM remodeling, and angiogenic or progenitor-related signaling [77-85]. These shifts suggest a transition from homeostatic signaling in the N limbal niche toward remodeling- and activation-associated signaling under DM conditions. As a sensitivity analysis, we repeated CellChat inference after matching cell numbers within each included population across N and DM conditions. The higher total interaction number and strength in DM were retained in all 50 subsampling iterations, and all nine highlighted pathways maintained

the same direction of condition-associated difference.

3.5. Differential gene expression and functional enrichment analyses reveal DM-associated alteration in LESC/VEC and LPC populations.

To further define the biological impact of DM on limbal stem and progenitor cells, we performed differential expression and functional enrichment analyses, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), in clusters of LESC/VECs and LPCs. In LESC/VEC cluster, the heatmap revealed a distinct DM-associated transcriptional profile, with increased expression of genes such as *ADAMTS4, ALOX5AP, CADM2, MFGE8, FLRT4,* and *PAX3,* whereas genes including *PTPN5, LGALS7B, SERPINE1, COL6A1, PLPP2, POSTN,* and *FAM107A* were reduced in DM samples (Figure 5A). GO analysis showed that these DM-altered genes were enriched in pathways related to extracellular matrix organization, extracellular structure organization, collagen-containing extracellular matrix, glycosaminoglycan biosynthetic processes, and cell adhesion (Figure 5B). KEGG pathway analysis further highlighted enrichment of MAPK signaling, focal adhesion, Ras signaling, IL-17 signaling, NF-κB signaling, ECM-receptor interaction, and additional inflammatory and matrix-remodeling pathways (Figure 5C). In the LPC cluster, a similarly distinct transcriptional change was observed (Figure 5D). DM LPCs showed increased expression of *GPHA2, GPHB5, C2CD4A, CNN3, REPS1, SERPINE1, POSTN, ADAMTS4, KCNA1, MUC7, CCDC180,* and *SLC14A1,* whereas *MGP, LGALS7B,* and *IFITM3* decreased relative to N controls. GO enrichment analysis indicated that DM-associated genes in LPCs were mainly linked to collagen-containing extracellular matrix, cell–cell adhesion via plasma membrane adhesion molecules, extracellular matrix structural constituents, basement membrane, and cadherin-related binding functions (Figure 5E). KEGG analysis showed significant enrichment in pathways involved in cell adhesion molecule interactions, cytokine-related signaling, ECM–receptor interaction, hematopoietic cell lineage, tight junctions, and type 1 diabetes mellitus (Figure 5F). These pathway-level changes are consistent with impaired limbal niche homeostasis

and altered regenerative capacity in the DM cornea. Additional volcano plots and GO and KEGG enrichment analyses for the remaining 11 clusters are provided in the Supplementary Figure 3-5.

To further assess the directionality of pathway-level changes without applying a DEG significance threshold, we performed ranked-list GSEA using all protein-coding genes tested in the MAST analysis. In LPC cluster, pathways related to cell adhesion, ECM organization, ECM–receptor interaction, focal adhesion, cadherin signaling, and cytokine–receptor interactions were significantly enriched toward the diabetic condition, whereas oxidative phosphorylation, mitochondrial respiration, and ribosomal programs were enriched toward the non-diabetic condition. LESC/VEC population also exhibited directional changes in ECM-, adhesion-, and stress/inflammatory signaling-related programs, further supporting diabetes-associated remodeling of this niche-associated population. Complete GSEA results across all annotated cell populations are provided for KEGG pathways and GO Biological Process terms in Supplementary Dataset 12 and 13, respectively. The corresponding subsets meeting an FDR-adjusted *P* value < 0.05 are provided in Supplementary Dataset 14 and 15.

3.6. Protein-level assessment of selected DM-associated markers identified by scRNA-seq.

To assess selected transcriptional alterations identified by scRNA-seq at protein level, we performed immunofluorescence staining on *ex vivo* corneal sections from DM and N donors (Supplementary Table 1 and 3) and compared protein expression patterns with single-cell transcriptomic data (Figures 6, 7). Donor-specific UMAP visualization of gene expression was also performed to further assess consistency across each donor, as shown in Supplementary Figure 6. The selected proteins were chosen to represent key diabetes-associated transcriptional changes across relevant corneolimbal populations, prior biological evidence, and relevance to processes implicated in diabetic corneal pathology. By scRNA-seq, *IFITM3/Fragilis3* expression was broadly reduced in DM samples across multiple cell populations (Figure 6A). Immunostaining showed strong IFITM3 signal in the basal limbal epithelium of N corneas, whereas markedly

weaker staining was observed in DM tissues. Similarly, *MMP1* transcript abundance was reduced in DM samples, while immunofluorescence showed reduced MMP-1 protein staining in DM corneas (Figure 6B). In N corneas, MMP-1 showed prominent epithelial localization, with consistent membrane staining throughout the epithelium, in keeping with the expression pattern reported by Collin et al. [20], whereas DM tissues exhibited markedly reduced staining intensity. Among the additional diabetes-associated candidates, *LGI4* was of particular interest because of its reported roles in niche-associated signaling and its notable transcriptomic change within the NCPC population. LGI4 transcript abundance was reduced in DM samples, most prominently within NCPC. Immunofluorescence showed lower LGI4 protein staining in the limbal epithelium and stroma of DM compared with N tissues (Figure 6C). Likewise, *POSTN* transcript abundance was reduced in the LESC/VEC population, while immunofluorescence showed reduced staining of its encoded protein, periostin, in DM corneas. Periostin staining was strong in the basal epithelium of N tissues but markedly attenuated in DM tissues (Figure 6D). These findings indicate coordinated suppression of ECM–associated and niche-supportive proteins in DM. In contrast, several markers were upregulated in DM samples in the scRNA-seq data. *ADAMTS4* transcript abundance was increased in DM samples, while immunofluorescence showed increased ADAMTS-4 protein staining predominantly in the basal and suprabasal epithelial layers (Figure 7A). *GPHA2,* a limbal progenitor–associated marker, was upregulated at the transcript level in DM samples, especially in LPC and NCPC clusters (Figure 7B). However, protein-level analysis revealed slightly stronger GPHA2 staining in the basal epithelium of N corneas. This basal epithelial staining pattern is consistent with that previously reported in the human limbus [46]. We next examined *NOS3* transcript abundance and its encoded protein, eNOS. At the transcript level, *NOS3* showed a modest increase in DM samples, particularly within the LESC/VEC cluster (Figure 7C). In contrast, immunostaining revealed a reduced eNOS protein signal in the DM limbal epithelium compared to N controls. However, donor-level analysis revealed populational heterogeneity among DM samples: one DM donor exhibited markedly higher *NOS3* expression,

whereas the other two donors showed lower levels. In contrast, N donors displayed relatively consistent *NOS3* expression within the LESC/VEC cluster (Supplementary Figure 6G). Overall, the immunofluorescence findings provided spatial protein-level observations that were generally consistent with transcriptomic findings, while also revealing some discrepancies between transcriptional and protein-level changes.

4. Discussion

In this study, we applied scRNA-seq to provide the first characterization of the DM impact on the cellular composition, transcriptional landscape, and intercellular communication networks of the human limbal niche. Building upon prior corneal scRNA-seq studies, our analysis identified distinct cell clusters within the limbal niche including LPC, LESC/VEC, MEL, IC and NCPC, revealing the cellular heterogeneity of the limbal epithelium [19-21]. Pseudotime and subclustering analyses revealed that LPC exist in multiple transcriptionally distinct states. Notably, LPC subcluster 2 enriched for *KRT17*, *TNFRSF12A*, *AREG*, and *LAMC2* exhibited transcriptional features consistent with epithelial activation, stress response, and migration [51-55,86]. These findings suggest the transcriptional heterogeneity of LPC within the human limbal epithelium.

An unexpected finding in the scRNA-seq was the co-expression of vascular endothelial cell (VEC) markers *(CLDN5, PECAM1)* and LESC markers *(ABCG2, SOX17, CD200)* in a shared cluster. Li et al. identified a LESC population characterized by *TSPAN7, SOX17, SELE, ECSCR, RAMP3,* and *IGFBP4* and further reported *PECAM1/PECAM1* signaling involving LESC [87]. Although *CLDN5* is classically associated with VEC, it has also been detected in selected epithelial populations and has been shown to localize to tight junctions in human colonic epithelial cells [19, 39-42]. This observation suggests that the mixed transcriptional profile observed in our dataset may reflect biological heterogeneity or shared gene-expression patterns among cell populations within the limbal niche. The precise cellular identity and biological significance of this cluster need further investigation. A surprising finding in the scRNA-seq data was the expression of *ABCB5* in

the MEL cluster. ABCB5 has been considered to be a LESC marker, known for its role in corneal epithelial homeostasis and regeneration [43-44]. A loss of function study with *Abcb5* knockout mouse showed depletion of quiescent LESC and the subsequent defects in corneal differentiation and wound healing [43]. At the same time, several reports documented the expression of *ABCB5* in melanocytes and melanoma cells [88-90]. The appearance of *ABCB5* in the MEL cluster might indicate a close proximity of the limbal melanocytes to LESC in the limbal niche or a limitation of the scRNA-seq analysis. However, *ABCB5* may be indeed a marker of limbal melanocytes, suggesting their importance as supporting cells in the maintenance of stem cell niche and consequently in corneal differentiation and wound healing and warrants further investigation [91]. For the first time, we identified DM-associated alterations across multiple biological levels by scRNA-seq, including gene expression, predicted intercellular signaling, cell-type composition, and selected protein-level change. In terms of cell-cycle dynamics, a shift toward a greater representation of cells in G1 arrest in DM LESC/VEC clusters was characterized. These findings are consistent with the LESC dysfunction previously documented by us in diabetic keratopathy and suggest a link between these alterations and the impaired regenerative capacity and chronic corneal wound healing deficits observed in DM [4, 69, 70].

At the molecular level, our differential expression analysis identified broad diabetes-associated transcriptional changes across corneolimbal cell populations, including genes involved in ECM organization, cell adhesion, and epithelial homeostasis. Within the LESC/VEC and LPC populations, *LGALS7B* expression was reduced at the transcript level. Galectin-7B, encoded by *LGALS7B*, has been implicated in epithelial re-epithelialization and wound repair, and reduced galectin-7 expression has been reported under high-glucose conditions [59,60,92]. Further protein-level validation is needed to confirm the *LGALS7B* transcriptomic finding. Downregulation of *POSTN, LGI4, LGALS7B,* and *IFITM3* suggests compromised epithelial barrier function and weakened cell–matrix interactions, which are hallmarks of DM corneal pathology [19, 46, 92-95]. Specifically, the observed reduction in IFITM3 protein staining, a protein crucial for maintaining

epithelial barrier integrity and immune modulation, may exacerbate epithelial vulnerability to infections and inflammation, consistent with prior findings on DM epithelial dysfunction [93-95]. In contrast, transcriptional upregulation of *ADAMTS4* and *SERPINE1* (in LPC cluster) indicates enhanced matrix remodeling and proteolytic activity, consistent with disrupted ECM turnover. We have previously shown that *MMP10* gene and protein expression was upregulated in DM corneas [96]. In this study we also observed an upregulation in *MMP10* in DM samples. In contrast, MMP-1 protein staining was reduced in DM tissue. This imbalance in MMP activity could lead to a disrupted ECM turnover, which could impair tissue repair processes. These results corroborate prior studies indicating the essential roles of MMPs in maintaining corneal integrity during wound healing [11, 22, 96-99]. Further, reduced periostin and LGI4 protein staining, both associated with cell-matrix interactions, correlates well with weakened epithelial adhesion and increased fragility in DM corneas. These changes are consistent with the structural disruptions observed clinically in diabetic keratopathy [4, 7, 8]. A particularly notable observation is some discordance between transcriptomic and protein-level expression of select markers. For instance, *GPHA2,* an LPC marker implicated in corneal epithelial cell survival and proliferation [20, 45], was upregulated at the transcript level in DM samples, particularly within LESC/VEC and LPC clusters, despite prior reports indicating minimal transcriptional alterations in the DM corneal epithelium [100]. However, protein analysis revealed somewhat stronger GPHA2 expression in N tissues. Our findings reveal a divergence between mRNA and protein expression. Such discrepancies may reflect multiple regulatory mechanisms, including microRNA-mediated regulation, translational control, and altered protein stability or turnover under diabetic conditions. Epigenetic mechanisms may also contribute indirectly by influencing gene expression, phenomena that are increasingly recognized in gene expression regulation [8, 23, 101-102]. However, to establish causes of the observed mRNA–protein discordance requires further investigation. Similarly, we found that *NOS3* transcript abundance showed a modest increase in DM samples, whereas eNOS protein staining was reduced in DM limbal epithelium. Aligned with the findings of Yanagiya et al., eNOS activity

has been demonstrated in both the corneal epithelium and endothelium [103]. It is a key enzyme responsible for the generation of nitric oxide (NO), a critical mediator of ocular physiology [104]. Consistent with its functional importance, exogenous NO donors acting through the NOS3 pathway have been shown to enhance the viability and migration of human corneal epithelial cells, thereby promoting more rapid wound healing [105].

Beyond transcriptional changes, our study revealed that DM may reshape inferred cell-cell communication networks within the limbal niche. We observed an overall increase in both the number and strength of ligand–receptor interactions in DM samples, indicating heightened but potentially dysregulated signaling activity. At the pathway level, DM tissues exhibited loss of activity in NRXN and SPP1 predicted signaling, which is associated with tissue maintenance and inflammatory responses. Consistent with reports of decreased neurexin family members, particularly NRXN3, in DM retinal tissue linked to early neuronal dysfunction, reduced *NRXN* signaling in our data suggests impaired synapse-like and adhesion-mediated intercellular communication within the limbal niche [71]. Interestingly, SPP1 predicted signaling was also reduced in our dataset, despite prior studies showing increased SPP1 expression under hyperglycemic conditions and in DM tissues, where it contributes to calcification, inflammation, and disease progression [106-107]. In contrast, our DM samples exhibited enriched predicted signaling pathways associated with tissue remodeling, stress response, and pathological activation, including NRG*,* PTN*,* PLAU*,* NOTCH*,* and VEGF signaling. Notably, within LPC clusters, NRG signaling was reduced in DM samples, consistent with reportedly decreased levels of NRG4, a neuregulin family ligand that activates NRG signaling, in DM and its complications, suggesting impaired NRG-mediated protective and metabolic signaling [108-109]. The enrichment of these other pathways suggests activation of wound-like or regenerative programs, potentially reflecting a compensatory response to chronic metabolic stress and microenvironmental damage [110-111]. From a translational perspective, our data highlight DM-

associated transcriptional alterations and enrichment in LESC/VEC and LPC populations. LESC/VEC in DM samples exhibited transcriptional changes associated with ECM organization, collagen-related processes, focal adhesion, and stress-responsive signaling pathways, including *MAPK, Ras,* and *NF-κB.* These changes are consistent with a stem-like population residing within a remodeled and stress-exposed niche, rather than a fully preserved homeostatic microenvironment [70, 112-115]. LPCs exhibited DM-associated in cell–matrix interactions, adhesion-related signaling, and inflammatory or immune-linked pathway enrichment, together with altered expression of matrix-remodeling genes such as *ADAMTS4, SERPINE1,* and *POSTN.* Importantly, this LPC signature was accompanied by reduced expression of factors linked to epithelial protection and progenitor-associated function, including *IFITM3, MMP1,* and *LGALS7B,* supporting the interpretation that DM LPCs adopt an activated but functionally compromised state rather than an efficiently regenerative one. At the intercellular level, these findings are also consistent with broader disruption of niche communication in DM, including reduced homeostatic signaling and relative enrichment of remodeling- and activation-associated pathways [116-117]. These findings may inform future studies evaluating whether modulation of cell population–specific signaling, particularly LPC-associated pathways while preserving limbal stem/progenitor-associated functions, could help restore corneolimbal homeostasis.

Several limitations should be acknowledged. The sample size, although consistent with current single-cell studies of human tissues, remains limited, and inter-donor variability may influence observed patterns. In addition, the scRNA-seq cohorts were not perfectly age-matched, and potential age-related effects cannot be excluded. Given the limited number of biological replicates, age could not be reliably adjusted for the in donor-level analysis. Because the dissected specimens included residual peripheral corneal and conjunctival components, minor variation in the extent of these tissues may also have influenced the observed cell-type proportions. Moreover, while scRNA-seq provides high-resolution transcriptomic data, it does not fully capture post-transcriptional regulation or protein activity, as evidenced by some discrepancies observed in this

study. Future studies with larger, better age-matched cohorts and integrated multi-omics approaches, including proteomics and spatial transcriptomics, will be important to further elucidate the mechanisms underlying DM alterations in the corneolimbal niche.

5. Conclusions

In conclusion, our study provides a comprehensive single-cell atlas of DM-associated changes in the human limbal epithelium and niche. Our findings identify coordinated DM-associated differences in stem/progenitor cell transcriptional states, extracellular matrix regulation, and inferred intercellular communication, and selected proteins involved in epithelial maintenance and cell–matrix signaling, which together may affect limbal niche homeostasis. These findings not only advance our understanding of diabetic keratopathy but also highlight broader principles of how systemic metabolic diseases can perturb epithelial stem cell niches, with implications for regenerative medicine and precision therapeutics.

Future research should focus on elucidating the differentiation trajectories of limbal progenitor cells under DM conditions and exploring the interplay between systemic DM factors, such as hyperglycemia, oxidative stress, epigenetic changes, and inflammation, and the limbal niche.

Investigating the crosstalk between immune cells and progenitor populations may also uncover new therapeutic targets for mitigating diabetic keratopathy. Importantly, these findings extend beyond the cornea, providing a model for understanding how systemic diseases disrupt epithelial stem cell dynamics in other tissues. The methodologies employed in this study, particularly scRNA-seq, underscore its potential for uncovering disease-specific molecular alterations and informing precision medicine approaches.

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Author contributions

DZ, RS, YW contributed to design and methodology, supervision, investigation and analyses, and writing, reviewing, editing and approving the manuscript. SME contributed to design and methodology, investigation and analysis, and reviewing, editing and approving the manuscript. AJP, CA, JI, AF, OBS, AAK, ZPW contributed to investigation and data collection, and reviewing and approving the manuscript. AVL contributed to conceptualization, design and methodology, supervision, funding acquisition, reviewing, editing and approving the manuscript. MS contributed to conceptualization, design and methodology, supervision, investigation and analyses, funding acquisition, project administration and writing, reviewing, editing and approving the manuscript. MS is the guarantor of this work.

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Data Sharing Statement

The Gene Expression Omnibus (GEO) accession number is GSE326016. The reviewers access link below and the token is cxubacaodzstncn:

(https://urldefense.com/v3/__https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE326016_;!!KOmnBZxC8_2BBQ!21s8NBHWFmxeh5RhVOFykEWMN3uJTxnxBHX4lYKev-YdpbIuESE_YTW47I9owAAnMhb-QKPnVYNW3V-XqOOp$)

Conflict of interest statement

The authors have declared that no conflict of interest exists.

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