Genome-Wide Transcriptional Analysis of Differentially Expressed Genes in Diabetic, Healing Corneal Epithelial Cells: Hyperglycemia-Suppressed TGFβ3 Expression Contributes to the Delay of Epithelial Wound Healing in Diabetic Corneas

Patients with diabetes mellitus (DM) may develop corneal complications and delayed wound healing. The aims of this study are to characterize the molecular signatures and biological pathways leading to delayed epithelial wound healing and to delineate the involvement of TGFβ3 therein. Genome-wide cDNA microarray analysis revealed 1,888 differentially expressed genes in the healing epithelia of normal (NL) versus type 1 DM rat corneas. Gene ontology and enrichment analyses indicated TGFβ signaling as a major altered pathway. Among three TGFβ isoforms, TGF-β1 and β3 were upregulated in response to wounding in NL corneal epithelial cells (CECs), whereas the latter was greatly suppressed by hyperglycemia in rat type 1 and 2 and mouse type 1 DM models. Functional analysis indicated that TGF-β3 contributed to wound healing in NL corneas. Moreover, exogenously added TGF-β3 accelerated epithelial wound closure in type 2 rat and type 1 mouse DM corneas via Smad and PI3K-AKT signaling pathways, autoregulation, and/or upregulation of Serpine1, a well-known TGFβ target gene. Taken together, our study for the first time provides a comprehensive list of genes differentially expressed in the healing CECs of NL versus diabetic corneas and suggests the therapeutic potential of TGF-β3 for treating corneal and skin wounds in diabetic patients.

Diabetes 2014;63:715–727 | DOI: 10.2337/db13-1260

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Received 15 August 2013 and accepted 25 October 2013.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-1260/-/DC1.

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With the rapid increase in the prevalence of diabetes mellitus (DM), ocular complications have become a leading cause of blindness around the world. In addition to abnormalities of the retina (retinopathy) and the lens (cataracts), various types of corneal disorders are also relatively common in DM patients (1). Hyperglycemia significantly alters epithelial structure and function, resulting in basal cell degeneration (2), decreased cell proliferation (3,4), superficial punctate keratitis (5), breakdown of barrier function, fragility (6,7), recurrent erosions, and persistent epithelial defects (8), depending on the duration of DM and on the serum concentration of glycated hemoglobin HbA1c. The epithelial abnormalities, termed keratopathy/epitheliopathy, are likely the results of these pathological changes and are resistant to conventional treatment regimens (9). Hence, a better understanding of the pathogenesis of diabetic keratopathy should lead to a better management of the disease.

Similar to other mucosal linings, the corneal epithelium is under constant environmental insults, often resulting in tissue injury. Prompt healing of the injured epithelium is vital to maintaining a clear, healthy cornea and for preserving vision (10). Healing involves a number of processes, including cell migration, proliferation, differentiation, apoptosis, and tissue remodeling (11). Hyperglycemia has profound effects on these biological processes. Unlike diabetic retinopathy, diabetic keratopathy does not cause many detectable clinical symptoms unless corneal epithelial cells (CECs) are removed or an eye is injured (12). Delayed epithelial wound healing may lead to sight-threatening complications such as stromal opacification, surface irregularity, and microbial keratitis (9). Hyperglycemia is likely to execute its adverse effects on corneal wound healing by modifying the expression of a host of wound response genes. To date, a genome-wide screen for genes, their associated pathways, and the networks affected by DM in CECs in vivo and their roles in wound closure have not been reported for the cornea.

Recently, we developed/adapted several diabetic models and demonstrated that diabetic rat corneas exhibit a similar pathology of human diabetic keratopathy, including decreased corneal sensitivity, reduced tear secretion, and most important, delayed epithelial wound healing, indicating that these are useful models to study impaired wound healing in diabetic corneas (4,6,7). In this study, we took advantage of an easily procurable epithelial cell population during epithelial debridement and from migrating epithelial sheets that have moved into the original wound bed. Using a genome-wide cDNA microarray, we profiled gene expression in DM and normal (NL) rat CECs. We identified 1,888 probe sets with more than 1.5-fold changes in the healing CECs of DM compared with NL corneas and found transforming growth factor β (TGFβ) signaling as a major pathway affected by hyperglycemia in DM CECs. We further demonstrated for the first time that wound-induced upregulation of TGFβ3 is dampened by hyperglycemia and that exogenously added TGFβ3 accelerated delayed epithelial wound closure in three rodent diabetic models. We proposed that TGF-β3 is a suitable therapeutic for treating delayed diabetic wound healing in peripheral tissues such as the cornea and skin.

**RESEARCH DESIGN AND METHODS**

**Animals and Induction of Diabetes**

All investigations conformed to the regulations of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health. Streptozotocin (STZ) induction of type 1 DM Sprague-Dawley (SD) rats was as described (4,7), and type 2 DM Goto-Kakizaki (GK) rats were both maintained in the Kresge Eye Institute animal facility under standard conditions. C57BL/6 (B6) mice were induced to develop type 1 DM according to a low-dose STZ induction protocol (mouse). Glucose levels and body weight were monitored weekly. Animals with blood glucose levels higher than 400 mg/dL (STZ-SD rats), 220 mg/dL (GK rats), and 350 mg/dL (B6 mice) were considered diabetic and were used, with age-matched animals as the control, at 8 weeks after STZ treatment for SD rats, 6 months old for GK rats, and 10 weeks after STZ for B6 mice. These are times when epithelial wound closure is significantly delayed and many pathologies can be observed in the DM animals (6,7,13).

**Corneal Epithelial Debridement Wound**

Anesthetized rats and mice were first demarcated with a trephine in the central cornea (circular wound of 5 mm for rats and 2 mm for mice), and CECs were removed with a blunt scalpel blade under a dissecting microscope (4,6,7). The blade with scraped CECs was immediately immersed in liquid nitrogen, and the visible ice was collected into a test tube, placed on dry ice, and stored at −80°C. The sample was marked as unwounded (UW). Bacitracin opthalmic ointment was applied to the cornea after surgery to prevent infection. At 42 h (rats) or 24 h (mice) postwounding (hpw), the same size trephine was used to mark the original wound, and CECs within the circle were removed, collected as described for the original wounding, and marked as healing CECs.

**RNA Extraction and Real-Time PCR**

For RNA isolation, CECs were scraped off of corneas, with two corneas pooled into one tube as one sample. RNA was extracted from the collected CECs using the RNaseasy Mini Kit (QIAGEN). cDNA was generated with an oligo (dT) primer (Invitrogen), followed by analysis using real-time PCR with the Power SYBR Green PCR Master Mix (AB Applied Biosystems), based on the expression of β-actin.

**Gene Array and Functional Analysis**

cDNAs were synthesized and hybridized to Affymetrix GeneChip Rat Genome 230, and 2.0 array was performed by the Microarray Core Facility at Wayne State University according to the manufacturer’s protocols. The
microarray data were evaluated by the Genomic Core Laboratory of the National Institute of Diabetes and Digestive and Kidney Diseases. The ANOVA analysis for the robust multichip average normalized data sets was completed by the commercial Partek Genomic Suite microarray data analysis software (http://www.partek.com/partekgs/). Signaling pathway and functional network analyses were performed using Genomatrix Pathways System software.

Subconjunctival Injection of Small Interfering RNA and Recombinant TGF-β Polypeptides

For mice, 5 μL solution was injected into the subconjunctival space at one site at the superior part of the cornea and for rats, 20 μL at two sites, and 10 μL each at the superior and inferior quadrants. TGF-β3 was injected 4–6 h and short interfering RNA (siRNA) 24 h before wounding.

Immunohistochemistry of Rat, Mouse, and Human Corneas

Rat eyes were enucleated, embedded in Tissue-Tek O.C.T. compound, and frozen in liquid nitrogen. Sections (6 μm thick) were cut and mounted to polylysine-coated glass slides. NL and diseased human corneas obtained from the Midwest Eye Bank were embedded in Tissue-Tek O.C.T. compound immediately upon arrival. The cryostat sections of wounded and healed organ cultured human corneas of diabetic and age-controlled groups were a gift from Dr. Alexander V. Ljubimov, Cedars-Sinai Medical Center (2). After drying in air, followed by a 10-min fixation in 4% paraformaldehyde, slides were blocked with 2% BSA in PBS for 1 h at room temperature. Sections were then incubated with rabbit primary antibody (TGF-β1, TGF-β2, and TGF-β3 1:50, Abcam). This was followed by fluorescein isothiocyanate anti-rabbit antibody (1:100; Jackson ImmunoResearch Laboratories). Slides were mounted with Vectashield mounting medium containing DAPI and examined under a Carl Zeiss fluorescence microscope Axioplan 2 equipped with an ApoTome digital camera or using confocal microscopy (TCSSP2; Leica). Controls were similarly treated, but the primary antibody was replaced with rat or rabbit IgG.

Determination of Protein Expression and Phosphorylation by Western Blot Analysis

The CECs removed during corneal debridement (the control) and PW, but before wound closure, were collected from NL and diabetic rat corneas and lysed with radioimmunoprecipitation assay buffer. Protein concentrations were determined using a Micro BCA kit. Levels of various proteins or their phosphorylation were determined using specific antibodies. For each condition, two samples were shown with β-actin levels as the internal controls.

Statistical Analysis

Data are presented as means ± SE. Statistical differences among three or more groups were first identified using one-way ANOVA, followed by the Student t test for pairwise comparison. Differences were considered statistically significant at P < 0.05.

RESULTS

We previously showed that epithelial wound healing, as seen in DM patients, was significantly delayed in the corneas of STZ-induced type 1 and GK type 2 DM rats compared with the control rats (4,7,13,14). We reasoned
Table 1—Top up- and downregulated genes

| Gene symbol | Gene title                          | Fold change | P value   |
|-------------|-------------------------------------|-------------|-----------|
| **The top 10 most up- and downregulated genes in healing/UW CECs of NL rats** |
| S100a9*     | S100 calcium binding protein A9     | 705.415     | 1.64E-10  |
| Serpinb2*   | Serine (or cysteine) peptidase inhibitor, clade B, member 2 | 563.839     | 2.14E-10  |
| Defb4*      | Defensin β 4                        | 372.452     | 1.03E-09  |
| Car12*      | Carbonic anhydrase 12               | 316.135     | 1.02E-11  |
| Serpinb10*  | Serine/cysteine peptidase inhibitor, clade B, member 10 | 305.695     | 2.38E-11  |
| ALV472      | Similar to ALV472                   | 272.388     | 1.45E-10  |
| Tnc*        | Tenascin C                          | 266.987     | 5.27E-08  |
| S100a8*     | S100 calcium binding protein A8     | 249.303     | 9.58E-09  |
| Kr17*       | Keratin 17                          | 202.958     | 2.20E-11  |
| Sprr1b      | Small proline-rich protein 1B (cornifin) | 202.042    | 1.23E-06  |
| Tril        | TLR4 interactor with leucine-rich repeats | 27.792      | 7.17E-07  |
| Dync1i1     | Dynein cytoplasmic 1 intermediate chain 1 | 30.563     | 4.65E-11  |
| Igfbp5      | Insulin-like growth factor binding protein 5 | 32.459    | 5.81E-08  |
| Lm3         | Leucine rich repeat neuronal 3      | 34.154      | 1.48E-07  |
| Gpm6a       | Glycoprotein m6a                     | 35.599      | 0.000105  |
| Igfbp5      | Insulin-like growth factor binding protein 5 | 21.836     | 1.50E-07  |
| Wnt2*       | Wingless-type MMTV integration site family member 2 | 21.992     | 0.000251  |
| Gad1        | Glutamate decarboxylase 1           | 21.548      | 2.44E-08  |
| Tacr2       | Tachykinin receptor 2               | 42.053      | 1.54E-08  |
| Cxcl14*     | Chemokine (C-X-C motif) ligand 14    | 45.971      | 1.54E-08  |
| Ptpro*      | Protein tyrosine phosphatase, receptor type, O | 56.52      | 1.38E-08  |
| **The top 10 most up- and downregulated genes in healing/UW CECs of DM rats** |
| S100a9*     | S100 calcium binding protein A9     | 458.928     | 2.81E-10  |
| Serpinb10*  | Serine peptidase inhibitor, clade B, member 10 | 311.583    | 2.32E-11  |
| Car12*      | Carbonic anhydrase 12               | 273.911     | 1.25E-11  |
| Defb4*      | Defensin β 4                        | 243.856     | 1.85E-09  |
| Kr17*       | Keratin 17                          | 217.086     | 1.99E-11  |
| Sprr1b      | Small proline-rich protein 1B (cornifin) | 201.148    | 1.24E-06  |
| S100a8*     | S100 calcium binding protein A8     | 166.442     | 1.75E-08  |
| AVLV472     | Similar to AVLV472                  | 165.464     | 2.54E-10  |
| Serpinb10*  | Serine peptidase inhibitor, clade B, member 2 | 122.676    | 6.71E-10  |
| Pdpn*       | Podoplanin                          | 122.676     | 6.71E-10  |
| Igfbp5      | Insulin-like growth factor binding protein 5 | 21.992     | 0.000251  |
| Wnt2*       | Wingless-type MMTV integration site family member 2 | 21.992     | 0.000251  |
| Dync1i1     | Dynein cytoplasmic 1 intermediate chain 1 | 32.555     | 4.02E-11  |
| Igfbp5      | Insulin-like growth factor binding protein 5 | 21.836     | 1.50E-07  |
| Wnt2*       | Wingless-type MMTV integration site family member 2 | 21.992     | 0.000251  |
| Gad1        | Glutamate decarboxylase 1           | 21.548      | 2.44E-08  |
| Cxcl14*     | Chemokine (C-X-C motif) ligand 14    | 30.087      | 3.88E-08  |
| Ptpro*      | Protein tyrosine phosphatase, receptor type, O | 51.929     | 1.63E-08  |
| **The top 10 most up- and downregulated genes in UW DM/NL rat CECs** |
| Ephx2*      | Epoxide hydrolase 2, cytoplastic     | 14.8351     | 4.45E-08  |
| Akr1b8      | Aldo-keto reductase family 1, member B8 | 3.35444    | 0.033642  |
| Elv4        | Elongation of very long chain fatty acids-like 4 | 3.18504    | 0.030089  |
| Ctdspl      | CTD (carboxy-terminal domain) small phosphatase-like | 2.89265    | 0.019859  |
| Pdyn*       | Prodynorphin                        | 2.88355     | 0.024604  |
| Col12a1     | Collagen, type XII, a 1             | 2.69369     | 0.047762  |
| CAMK1D      | Similar to calcium/calmodulin-dependent protein kinase 1D | 2.66033    | 0.000102  |
| Lect1       | Leukocyte cell derived chemotaxin 1 | 2.50341     | 0.000163  |
| Phb2        | Prohibitin 2                        | 2.42591     | 0.014843  |
| Dda1h       | Dimethylarginine dimethyl aminohydrolyase 1 | 2.34997    | 0.004703  |
| Ggt1        | γ-Glutamyltransferase 1             | 3.13265     | 0.023773  |
| Mtmr1       | Myotubularin related protein 1      | 3.38125     | 0.003785  |
| GBP6        | Similar to guanylate binding protein family, member 6 | 3.3911      | 0.002009  |
| Aqp2        | Aquaporin 2 (collecting duct)       | 3.777       | 0.025883  |
| Krt8        | Keratin 8                          | 3.7889      | 0.000127  |
| Wdfc5       | WAP four-disulfide core domain 5    | 4.4483      | 0.002422  |
| Gpm6a       | Glycoprotein m6a                     | 4.6711      | 0.015797  |
| SNX10       | Sorting nexin 10                   | 5.5743      | 2.27E-05  |
| Fam111a     | Family with sequence similarity 111, member A | -8.8546    | 0.037588  |
| C1q3*       | Complement component 1, q subcomponent-like 3 | -10.238    | 2.65E-06  |

Continued on p. 719
that genes induced in response to wounding and affected by hyperglycemia are responsible for the delay of epithelial wound closure in the cornea, and we performed a genome-wide microarray expression analysis to compare the gene expression profiles of CECs collected during the creation of epithelial debridement wounds at 0 h and at 42 h postwound from the control, NL, and DM rat corneas (Fig. 1A). The rats used were 14 weeks old, with 8 weeks of hyperglycemia, the duration sufficient to affect the rate of epithelial wound closure. Using the Rat Genome GeneChip, we detected 5,426 probe sets differentially expressed at least in one of four paired comparisons (Fig. 1B). When healing was compared with UW CECs, a two-fold change cutoff was used, resulting in more than 3,000 probe sets differentially expressed in NL and DM corneas. When DM CECs were compared with NL CECs, a 1.5-fold change cutoff was used because a much smaller number of differentially expressed genes was detected: 772 (397 increase and 377 decrease) in UW CECs and 1,888 probe sets (636 increase and 1,253 decrease) in healing CECs. Supplementary Table 1 lists the differentially expressed genes (probe sets). Table 1 lists the top 10 most up- or downregulated genes in four paired comparisons.

| Gene symbol | Gene title                              | Fold change | P value |
|-------------|-----------------------------------------|-------------|---------|
| Ephx2*      | Epoxide hydrolase 2, cytoplasmic         | 11.9119     | 8.65E-08|
| CD99I2      | Similar to MIC2 like 1                   | 3.57749     | 0.001322|
| Arid2       | AT rich interactive domain 2 (Arid-rfx like) | 3.03913     | 0.007696|
| Ctdsp1      | CTD (carboxy-terminal domain) small phosphatase-like | 2.66501     | 0.020035|
| Gsta4       | Glutathione S-transferase α 4            | 2.57045     | 0.005536|
| Phgdh       | Phosphoglycerate dehydrogenase           | 2.50389     | 0.001145|
| Adcd1       | AT hook, DNA binding motif, containing 1 (Rattus norvegicus) | 2.46515     | 0.002914|
| Phb2        | Prohibitin 2                             | 2.37832     | 0.016485|
| CD99L2      | Similar to MIC2 like 1                   | 2.26117     | 0.003917|
| Auts2l      | Autism susceptibility candidate 2-like    | 2.24651     | 0.007632|
| Wnt2*       | Wingless-type MMTV integration site family member 2 | −4.2398     | 0.019534|
| Plod2       | Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2 | −4.2793     | 2.67E-06|
| Adm*        | Adrenomedullin                           | −4.5972     | 0.007971|
| Fam3b*      | Family with sequence similarity 3, member B | −5.1147     | 0.000102|
| Postn       | Periostin, osteoblast specific factor     | −5.4259     | 0.002689|
| Ceacam1*    | Carcinoembryonic antigen-related cell adhesion molecule 1 | −6.4485     | 0.010465|
| Cald1       | Caldesmon 1                              | −6.7921     | 0.002262|
| Tmem35      | Transmembrane protein 35                 | −7.5325     | 0.000227|
| Atf3*       | Activating transcription factor 3         | −7.5617     | 0.006267|
| Vim*        | Vimentin                                 | −8.6761     | 0.000222|

*The expression pattern of the genes was confirmed at least by real-time PCR in one or more animal DM models.

Pair 2: Healing Versus UW CECs in DM Corneas

Compared with NL, 288 more probe sets were differentially expressed in the healing CECs of DM corneas, and 2,497 probe sets showed altered expression in both NL and DM CECs. Among the 10 most highly upregulated genes, 9 were also found in DM corneas with comparable fold changes. Tenascin C (Tnc) was replaced by podoplanin (Pdpn), a marker of lung injury (19), in DM CECs. On the downregulated gene list, Wnt2 was worthy of mention because it was not expressed in UW CECs. The downregulation of Wnt2 in DM compared with NL healing CECs was verified by real-time PCR (Fig. 2C).

Pair 3: DM Versus NL UW CECs

Compared with healing versus UW CECs in pairs 1 and 2, there were fewer differentially expressed genes in DM versus NL CECs, with much lower fold changes. Thus, we lowered the cutoff value to 1.5-fold changes. The most highly up- and downregulated genes were Ephx2, known to contribute to renal injury during diabetes (20), and C1ql3, a gene only found in the central nerve system to regulate synapse formation (21), respectively. Upregulation of Ephx2 in DM CECs was verified by real time PCR (Fig. 2D).

Pair 4: DM Versus NL Healing CECs

Compared with homeostatic cells, a relatively large number of genes were differentially expressed: 1,888 probe sets in DM versus NL healing CECs. Among the
105 highly induced probe sets, only 17 probe sets represent known genes, including Ephx2. On the other hand, many probe sets with downregulated expression in DM, compared with in NL healing CECs, contained gene identities. These include vimentin, ATF3, Wnt2, Tnc, peroxidasin (a gene associated with anterior segment dysgenesis (22) and MMP12. Figure 2E and F show the verifications of the expression patterns of vimentin and MMP12.

Among the 56 genes listed in Table 1, the expression patterns of 17 genes were confirmed in one or more DM models by real-time PCR and/or immunohistochemistry.

**Figure 2**—Real-time PCR verification of the expression patterns of six selected genes in healing vs. homeostatic CECs in NL rat corneas. The RNA samples were obtained as described in Fig. 1. The scraped CECs from NL and STZ-DM rat corneas for creating a wound were marked as 0 h and from the wound bed at 42 hpw and subjected to real-time PCR with primers specific for S100A9 (A), CXCL14 (B), Wnt2 (C), Ephx2 (D), Vim (E), and MMP12 (F). The data are presented as fold changes over non-DM, homeostatic CECs (1). Three samples were collected from three rats for each condition, and two independent experiments were performed. *P < 0.05; **P < 0.01.

Decreased Expression of TGF-β3 in Healing CECs of Diabetic Corneas

Gene ontology (GO) analysis of 1,888 probe sets revealed many signaling pathways were altered, including pathways mediated by IL-1 (1.02e-3, 22/132, also see Fig. 3), HGF (2.8e-314/75), MMP (1.58e-4, 23/124), and TGFβ (7.39e-3, 43/360). Similar to GO Analysis, enrichment analysis of genes differentially expressed in DM versus NL healing CECs revealed TGFβ-dependent epithelial-mesenchymal transition to be a major event altered therein. Hence, we focused on TGFβ signaling pathways.
To assess the expression patterns of the TGF-β family, real-time RT-PCR was performed (Fig. 3A) using STZ-SD rats. All three isoforms were detected, whereas mRNA for IL-1β (as a control) was undetectable in UW NL and DM CECs. Wounding induced IL-1β expression at the mRNA levels, and this upregulation was more apparent in DM healing CECs. The levels of TGF-β1 were also elevated in healing CECs; however, no difference was detected between DM and NL corneas (Fig. 3B). The levels of TGF-β2 were consistent under four conditions. TGF-β3 was greatly upregulated in the healing CECs of NL, but the levels of TGF-β3 in DM CECs remained at a level comparable to that of UW CECs. We have established three rodent diabetic models: STZ-SD rats (4,7), GK rats (6), and STZ-B6 mice. The expression of TGF-β3 was further assessed by real-time PCR, which showed significant upregulation in the healing CECs of NL, but not DM, corneas in all three rodent models (Fig. 3B–D).

The expression and distribution of TGF-β3 in were also assessed using immunohistochemistry (Fig. 4). Although TGF-β3 abundantly stained the entire healing corneal epithelial sheet, from the leading edge to the border region between the cornea and limbus in SD rat corneas, only a few TGF-β3 positive cells were found at the leading edge of the migratory epithelial sheet in type 1 DM rat corneas; the rest of the epithelial sheet stained low or negative.

**TGF-β3 is Necessary for Proper Wound Healing**

We next investigated whether TGF-β3 is required for wound healing in NL rats. We used subconjunctival injection of siRNA in Wistar rats and observed a significant delay in TGF-β3-specific but not the control siRNAs compared with the control rats (Fig. 5A). Western blotting analysis of CECs revealed a basal expression of TGF-β3 in UW CECs and elevated levels in healing CECs in the Wistar rat, and this wounding-induced TGF-β3

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**Figure 3**—mRNA Expression of TGFβ isoforms detected by RT-PCR and verification of TGFβ3 expression by real-time PCR. The RNA samples were obtained as described in Fig. 1. The scraped CECs from NL and STZ-DM rat and mouse corneas for creating a wound were marked as UW and from the wound bed at 42 hpw (rats) or 24 hpw (mice) and were subjected to RT-PCR with GAPDH as the internal control and IL-1β as a positive control. The data were presented as fold changes over non-DM, homeostatic CECs (1). For each condition three samples were collected from three rats/mice. Four independent experiments were performed: two with SD and STZ-SD, one with Wistar and GK rats, and one with B6 and STZ-B6 mice. *P < 0.05; **P < 0.01.

**Figure 4**—Immunohistochemistry of TGF-β3 distribution in healing and UW corneas. The corneas of SD and STZ-SD were wounded and O.C.T. snap frozen at 42 hpw, followed by sectioning and immunostaining with antibodies against TGF-β3; DAPI was used to stain nuclei. Low magnification (×5) images of the entire cornea were taken and images stitched to present the whole from limbus to wound center. Inserts are high magnification (×20) images of the leading edge. The figure is representative of three corneas per condition from two independent experiments.
Western blotting. Although TGF-β1 was upregulated in healing DM CECs, exogenously added TGF-β3 appeared to have no effects on its expression. The levels of TGF-β2 were low and unchanged in all the samples. Interestingly, exogenously applied TGF-β3 increased TGF-β3 expression. Although some elevated active TGF-β3 molecules could be from exogenously added TGF-β3 (15 kDa), the latent form (47 kDa) can only be from endogenous sources, indicating an autoregulation of TGF-β3 expression in rat corneas.

In STZ-B6 mice, the addition of TGF-β significantly increased the rate of epithelial closure at 16 and 24 hpw (Fig. 7A and B). When Serpine1, a common target of TGF-β (23), as an example, was used, our data showed that its mRNA levels were correlated to the levels of TGF-β3 activity and to that of the rates of wound closure in NL and DM mice (Fig. 7C).

TGF-β3 Expression in Cultured Human UW and PW Corneas

Delayed epithelial wound healing has also been shown in organ-cultured human corneas (2). We obtained NL and DM corneal cryostat sections without (direct embedded in O.C.T.) or with epithelial wounding, which were allowed to heal in an organ culture setting (2), and assessed TGF-β3 expression in these corneas using confocal microscopy (Fig. 8). In UW corneas, there were detectable levels of TGF-β3 in the epithelial layer. In the PW cornea with healed epithelial wound (2), all cell layers were positive with a strong TGF-β3 immunoreactivity in NL corneas, whereas weak staining was detected in DM CECs.

DISCUSSION

In this study, we used genome-wide cDNA microarrays to compare the gene expression profiles of healing versus UW and DM versus NL CECs. Our results revealed that wounding dramatically altered the expression of a large number of genes in NL and DM corneas. Bioinformatics revealed many TGF-β-mediated signaling pathways, and biological processes were altered. Using three diabetic mouse and rat models, we showed that TGF-β3 activity is required for proper wound healing in NL corneas, whereas exogenously added TGF-β3 promotes epithelial wound healing in the corneas of type 1 DM rats and mice and type 2 DM rats. The effects of TGF-βs on epithelial wound closure are related to its ability to activate Smad-dependent and -independent pathways, to modulate its own expression, and to mediate target gene expression. Taken together, our study suggests that the hyperglycemia-suppressed genes, such as TGF-β3 and Serpine1, might be used as therapeutic reagents to accelerate delayed epithelial wound healing in diabetic corneas.

The genome-wide cDNA array analyses revealed a greatly altered expression of genes in response to wounding and illustrated genes affected by hyperglycemia at a much larger scale in CECs. Unlike previous
studies using limited arrays and RNA isolated from the cornea (24,25), our approach used CECs and thus avoided false-positive results from infiltration of immune cells in the stroma. Although most of the genes detected with differential expression are likely from epithelial cells, a small portion of differentially expressed genes might be from dendritic cells because they migrate with the healing epithelial sheets (26). Moreover, 8 weeks of hyperglycemia represented an early stage of DM when a significant delay of epithelial wound closure has begun (4,7). This allows us to characterize early events of diabetes complication, which can be effectively intervened with or even reversed. With these advantages, our genome-wide cDNA array revealed a large number of genes (probe sets) differentially expressed, and the expression of many genes was verified by other means and/or consistent with their known function, further validating the value of the genome-wide microarray data.

Comparison of healing DM with healing NL CECs revealed that 1,888 probe sets had altered expression. Intriguingly, of the 36 probe sets with more than threefold increases in DM healing versus NL healing, only 4 are identified genes; the rest were derived from DNA sequence tags without gene identities. Among four genes, the upregulation of Ephx2, which was linked to subclinical cardiovascular disease in the Diabetes Heart Study (27), is most interesting because it was shown to increase manifestations of diabetic nephropathy in mice (20). There were 17 genes with fourfold decreases, and among them, ATF3 and CEACAM1 have been shown to be associated with DM (28,29). The role of Ephx2, ATF3, and Ceacam1 or the lack thereof in diabetic corneas remains to be determined.

GO analyses revealed that the signaling pathway mediated by TGF-β-Smad was impaired in DM healing CECs. Because TGF-β has been known to play a key role in corneal wound healing and fibrosis and the major cellular source of TGF-β in corneal wounds is the epithelium (30), we assessed the expression of three TGFβ isoforms and observed that hyperglycemia had no effects on TGFβ1 but greatly suppressed TGFβ3 in DM healing CECs. Earlier studies reported that TGF-β1 and β2 are both seen within the corneal epithelium in the cornea and the stroma in the injured cornea, whereas TGF-β3 is not found in the anterior eye (for review see (31)). More recent studies reported that TGF-β3 was found in the
basal cells of regenerating areas as well as in uninjured regions of the cornea after corneal injury (32). We presented strong evidence of upregulation of TGF-β3 expression in three animal models as well as in cultured human corneas. Hence, we conclude that TGF-β3 is expressed in CECs and its expression is upregulated in response to wounding in NL corneas. More strikingly, we showed almost total suppression of wound-induced TGF-β3 upregulation in diabetic corneas, suggesting a unique role of TGF-β3 in mediating epithelial wound closure. To our knowledge, our study is the first to unambiguously demonstrate the suppressive effects of hyperglycemia on the expression of TGFβ3 but not TGFβ1 at mRNA and protein levels in response to wounding.

Although TGFβ1, β2, and β3 share 60–80% identity, they are encoded by distinct genes, have different sequences in promoters, and exhibit different physiological and pathological activities in vivo (33). The importance of TGF-β in maintaining tissue homeostasis and in regulating wound healing in the cornea has been

![Image](image1.png)

**Figure 7**—Effects of TGF-β3 on epithelial wound closure in B6 and STZ-B6 mouse corneas. A: Recombinant TGF-β3 (14 ng), with PBS as the control, was injected into subconjunctival space at inferior side of B6 and STZ-B6 mice 4 h before wounding. A 2-mm epithelial wound was made and allowed to heal for 16 h in B6 mice and for 24 h in STZ-B6 mice, and then fluorescence stained, and photographed. NL cornea injected with PBS (1), NL cornea injected with TGF-β3 (2), DM (STZ) cornea injected with PBS (3), and DM cornea injected with TGF-β3 (4). B: Changes in the mean of the remaining wound areas in pixels were calculated by Adobe Photoshop software (n = 5) with P value. C: CECs collected during wounding (first two lanes) and at 16 hpw (1 and 2) or 24 hpw (3 and 4) in PBS (1 and 3) and TGF-b3 (2 and 4) treated corneas were extracted and subjected to real-time PCR analysis for Serpine1 expression (n = 3). **P < 0.01.

![Image](image2.png)

**Figure 8**—Expression and distribution of TGF-β3 in cultured human NL and DM corneas with or without wounding. Human diabetic corneas (B and D) and age-matched controls (A and C) were directly processed for (A and B) or were wounded (6 mm) before organ culture, as described by Saghizadeh et al. (50). At 10 days after wounding, corneas were frozen in O.C.T. C: The cryostat sections were stained with rabbit anti-human TGF-β3 antibody with nonspecific rabbit IgG as the negative control (insert c' in NL healed cornea). Photos show merged images of confocal microscopy and are the representative of two animals in each group.
reported (for review see [31]). The response of TGF-β to wounding can be mediated through Smad-dependent and -independent signaling pathways in a manner dependent on cell type and context (34–37). Among Smad-independent pathways, the PI3K/AKT pathway plays a significant role in regulating TGF-β-mediated responses (35). Demonstration that wound-induced TGF-β3 expression in CECs is sensitive to hyperglycemia suggests a distinctive regulation and a unique role of the isoform in modulating epithelial wound healing.

To define the role of identified genes, we administered siRNA or recombinant TGFβ3 to the corneas using subconjunctival injection, which is a common procedure performed at ophthalmologists’ office to deliver drugs to treat corneal diseases and glaucoma and has been shown to allow bevacizumab to penetrate the intact cornea (38–40). Using subconjunctival injection to deliver TGF-β3 siRNA in NL corneas and recombinant TGF-β3 in NL and DM corneas, we demonstrated that TGF-β3 upregulation in response to epithelial wounding contributes to epithelial wound healing and activates canonical and noncanonical signaling pathways. The altered expression of TGF-β3 was also confirmed in cultured human corneas after wound healing, indicating the relevance of the study. Moreover, we demonstrated that exogenous TGF-β3 partially restored the healing rate of epithelial wounds in diabetic corneas of three rodent models, suggesting an important role for the isoform in mediating epithelial response to injury. Strikingly, we discovered that exogenously applied TGF-β3 elevated the levels of latent TGF-β3 but not TGF-β1 or β2 at the protein levels, suggesting an autoregulated expression of TGF-β3 in the healing CECs such as that observed in the mouse skin carcinogenesis model (41). Although the mechanisms underlying hyperglycemia-suppressed wound-induced TGF-β3 expression are unclear, early inhibitory effects of hyperglycemia on TGF-β3 expression, which differs from that of TGF-β1 (42), may exacerbate the suppression of TGF-β3 upregulation in response to wounding. Finally, we showed that the expression of Serpine1, a well-known TGFβ target gene (43,44), is correlated to the activity of TGF-β3 in healing CECs of NL and DM corneas.

The discovery that TGF-β has a unique role in mediating corneal epithelial wound healing is of great significance because 1) the major cellular source of TGF-β in corneal wounds is the epithelium (30); 2) TGF-β3, in contrast to TGF-β1, is considered an antifibrotic (45); 3) TGF-β3 halts fibroblast migration and selectively promotes reepithelialization in the skin wounds (46), indicating a critical role in skin wound healing; and 4) recombinant TGF-β3 has been undergoing phase 3 clinical trials as an antiscarring agent for adult skin wounds (45,47,48). The trial was considered a failure due to increased angiogenesis in postsurgery skin (45). However, unlike the pathogenesis of diabetic retinopathy, nephroropathy, and atherosclerotic plaque where there is excessive angiogenesis, blood vessel growth is impaired in diabetic wound healing (49). Hence, TGF-β3 may potentially be used as a useful therapy to treat delayed wound healing in the cornea and in the skin for the benefit of accelerating wound healing and reducing inflammation that should outweigh adverse effects caused by neovascularization, which may not occur during diabetic wound healing (49). Further study of the mechanism of the action and target gene expression of TGF-β3 in the corneal and skin may lead to clinical trials of TGF-β3 for its unique function in epithelial cells under hyperglycemia conditions.

Acknowledgments. The authors thank Drs. Ashok Kumar, Jia Yin, and Patrick Lee of Wayne State University School of Medicine for critical reading of the manuscript and are grateful to Dr. Alexander V. Ljubimov, Regenerative Medicine Institute, Cedars-Sinai Medical Center, for providing human corneal cryostat sections.

Funding. The authors received support from National Institutes of Health/National Eye Institute R01-EY-10869, R01-EY-17960 (to F.-S.X.Y.), P30-EY-04078 (National Eye Institute core to Wayne State University), and Research to Prevent Blindness (to Kresge Eye Institute).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. I.B., H.S., and N.G. performed laboratory testing, and edited and checked the accuracy of the manuscript. F.W. performed RNA preparation for microarray and part of the immunohistochemistry analysis. X.M. performed laboratory testing and data analysis. W.C. performed bioinformatics analysis of cDNA array. Z.L. contributed to discussion and reviewed the manuscript. F.-S.X.Y. was responsible for study design and recruitment, contributed to sample collection and data analysis, and reviewed and edited the manuscript. F.-S.X.Y. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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