Identification of Wnt-5a Receptors Important in Diabetic and Non-Diabetic Corneal Epithelial Wound Healing

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Purpose. Persistent epithelial alterations such as delayed wound healing are a key feature of diabetic corneal disease. Previously, we reported that epigenetic changes in the diabetic cornea led to the suppression of Wnt-5a, and that addition of Wnt-5a accelerated wound healing. In this study, we set to determine which Wnt receptor(s) mediated Wnt-5a induced stimulation of diabetic corneal epithelial wound healing.

Methods. Human limbal epithelial cells (LECs) were isolated from postmortem diabetic and non-diabetic donor eyes for single-cell RNA sequencing (scRNA-seq) and DNA methylation analysis. These analyses were validated by qRT-PCR, western blot, or immunostaining of corneal tissue sections. Cultured primary LECs were transfected with small interfering RNA (siRNA) to specific Wnt receptors to evaluate their role in scratch wound healing in the presence or absence of 200 ng/mL Wnt-5a.

Results. Single-cell RNA sequencing analysis revealed differential gene expression of Wnt receptors, *ROR2*, *MCAM*, *FZD5*, *FZD6*, and *FZD7*. DNA methylation arrays showed hypomethylation of *ROR2* gene promoter in diabetic versus non-diabetic LECs by 41.3% (**$P < 0.01$) resulting in increased ROR2 protein expression. Non-diabetic cells transfected with siRNA to knockdown *ROR2* but not *FZD5*, *FZD6*, *FZD7*, *MCAM*, and *RYK* showed significantly decreased wound healing by approximately 50% (*$P < 0.05$) versus control siRNA. In diabetic LECs, knockdown of *ROR2* significantly inhibited wound healing by 40% (*$P < 0.05$) and of *FZD5* partially blocked wound healing that could not be restored by the addition of Wnt-5a.

Conclusions. Wnt-5a seems to mediate wound healing in diabetic LECs mainly through receptor tyrosine kinase like orphan receptor 2 with Frizzled-5 serving as a possible co-receptor with a smaller effect.

Keywords: Wnt-5a, ROR2, diabetes, frizzled receptors, corneal epithelial wound healing

Diabetes-associated visual impairment is one of the leading causes of preventable blindness in working-age adults worldwide.[1,2] The most severe ocular complication due to diabetes is retinopathy, affecting approximately 26% to 33% of patients.[3] However, 45% to 70% of patients with diabetes present with corneal abnormalities such as epithelial defects (keratopathy), decreased sensitivity, a decrease in subbasal corneal nerves (neuropathy), abnormal stromal changes, endothelial dysfunction, and biomechanical alterations.[4] Delayed epithelial wound healing due to the dysfunction of limbal epithelial stem cell (LESC)[4,5] is a hallmark of diabetic keratopathy, which may underlie the increased risk of developing postoperative complications from vitrectomy for nonclearing hemorrhage, corneal transplantation, cataract removal, and vision correcting refractive surgeries.[6,7] Although the number of diabetic patients with corneal abnormalities is greater than those with retinal changes, the diabetic corneal disease remains underdiagnosed and current treatments are mostly symptomatic.[4]

Abnormalities in Wnt signaling constitute an important mechanism of diabetic corneal alterations.[8–9] The human Wnt family of highly conserved, secreted signaling lipoproteins comprises 19 members that play a critical role in development, homeostasis, and disease governing processes like cell fate determination, cell proliferation and migration, cell differentiation, stem cell maintenance and regeneration.[10–12] Wnts belong to two classes defined by the ability to act as oncogenes. Transforming/canonical Wnts, such as Wnt-1 and Wnt-3, activate canonical Wnt/β-catenin pathway.[12] Without canonical Wnts, cytoplasmic β-catenin is phosphorylated in the destruction complex with Axin 1, adenomatous polyposis coli, casein kinase 1, and glycogen synthase kinase-3 β, ubiquitinated by β-transducin repeats-containing proteins, and degraded. Wnt binding to the receptors Frizzled and low-density lipoprotein-related receptors 5 and 6 leads to β-transducin repeats-containing proteins disassociation from destruction complex and inhibits β-catenin phosphorylation. Then, cytoplasmic β-catenin can travel to the nucleus, associate with lymphoid enhancer-binding factor 1/T-cell factor transcription factors and activate the expression of genes such as *MYC*, *JUN*, *cyclin D 1* (*CCND1*), and *AXIN1*, thus regulating a number of cellular processes.[11] Nontransforming/noncanonical Wnts, for example, Wnt-4, Wnt-5a, and Wnt-11, activate β-catenin–independent calcium ($Ca2^{+}$)-dependent and planar cell polarity (PCP) pathways. PCP pathway regulates cell polarity in development and cell migration through Rho guanosine triphosphate hydrolases (GTPases) and c-Jun N-terminal kinase. Ca^{2+}-dependent pathway activates phospholipase C (PLC), leading to Ca^{2+} mobilization and activation of Ca^{2+} sensing enzymes (e.g., protein kinase C [PKC]) that mediate cell migration and axon guidance, among other cellular processes.[12–13] Wnts bind to various receptors that often complex with each other. Canonical pathway mainly uses low-density lipoprotein-related receptors 5 and 6 complex with Frizzled family receptors. Noncanonical Wnts may use various Frizzled co-receptors with receptor tyrosine kinase like orphan receptor, receptor-like tyrosine kinase (RYK), melanoma cell adhesion molecule (MCAM), or cell surface glycoprotein Muc18, and tyrosine-protein kinase-like 7, depending on the cell type.[14]

Stable diabetic complications seem to be mainly due to epigenetic alterations with DNA methylation, histone modifications, and microRNA changes.[15–17] We previously reported DNA hypermethylation of *WNT5A* gene promoter as well as upregulation of miR-203a that inhibits Wnt-5a in diabetic corneal epithelium, both resulting in suppression of Wnt-5a protein expression.[9] Addition of exogenous recombinant Wnt-5a to diabetic limbal epithelial cell (LEC) cultures enriched in LESC and to organ-cultured corneas stimulated wound healing and normalized LESC phenotype. Wnt-5a exerts its effects in diabetic LECs through the activation of Ca^{+2}-dependent pathway by phosphorylating PLCγ1 and PKCβ.[9] In this study, we identified Wnt-5a receptor(s) mediating its effects on diabetic limbal epithelial wound healing.

Materials and Methods

Human Specimens

Seventeen age-matched diabetic (from 9 males and 8 females) and 21 non-diabetic (from 13 males and 8 females) human donor whole globes or corneas (Supplementary Table S1) were obtained from the National Disease Research Interchange (Philadelphia, PA, USA) and from the Center for Vision and Eye Banking Research, Eversight (Cleveland, OH, USA) in Optisol (Chiron Vision, Claremont, CA) or similar medium within 48 hours after death. Non-diabetic donor corneoscleral rims discarded after corneal transplantation were obtained from Dr. E. Maguen. All donor tissues were used under the approved Cedars-Sinai Medical Center Institutional Review Board protocol Pro00019393. The human tissue experiments complied with the guidelines of the ARVO Best Practices for Using Human Eye Tissue in Research (Nov2021). The donor age was not statistically different between the two groups (non-diabetic median age, 72.00 $\pm$ 17.48 years; diabetic median age; 74.00 $\pm$ 10.36 years; $P = 0.14$). Of all tissues, 22 (57.9%) were from male donors and 16 (42.1%) were from female donors.

Single-Cell RNA Sequencing (scRNA-seq)

Cells were isolated from three diabetic (all with non–insulin-dependent diabetes) and three non-diabetic human donor limbal tissues with residual peripheral cornea and conjunctiva using dispase II digestion as previously described.[18] Approximately 7000 single cells were captured for sequencing on a 10X Genomics Chromium Controller according to manufacturer's instructions (10X Genomics, Pleasanton, CA, USA) at the Applied Genomics, Computation & Translational Core at Cedars-Sinai Medical Center. Expression matrices were obtained using CellRanger v7.2. After performing quality control and removal of batch effect using Seurat, the data were integrated using FindIntegrationAnchors. The data were scaled, and clustering was performed using FindNeighbors and FindClusters to obtain 13 clusters with a resolution of 0.2. After conducting t-distributed stochastic neighbor embedding, dot plots were generated to annotate the cell populations and relative expression of individual genes of interest in specific clusters. Differential expression analysis between diabetic and non-diabetic genes for each cluster was performed using the FindMarkers function.

DNA Methylation Analysis

DNA was extracted from LEC cultures enriched in LESCs. Cells were obtained from five diabetic and six non-diabetic donors. Bisulfite conversion was carried out according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Genome-wide DNA methylation profiling using Illumina Infinium 850K MethylationEPIC BeadChip (Illumina, San Diego, CA, USA) was performed as described by us previously.[9] This DNA methylation dataset is available from the public GEO repository under accession no. GSE229328.

qRT-PCR

Total RNA was extracted from three diabetic and three non-diabetic limbal donor tissue using PureLink RNA mini kit (Thermo Fisher Scientific, Carlsbad, CA, USA). RNA was quantified and quality (A260/280) was checked using Nanodrop 2000 (Thermo Fisher Scientific). cDNA was prepared using reverse transcription mastermix purchased from Fluidigm (Standard BioTools, South San Francisco, CA, USA) and high-throughput qRT-PCR was performed using Fluidigm dynamic arrays on BioMark HD System according to the manufacturer's instructions (Standard BioTools, South San Francisco, CA, USA) at the Applied Genomics, Computation & Translational Core at Cedars-Sinai Medical Center.

β-actin was used as a housekeeping control. The primer sequences are listed in Supplementary Table S2.

LEC Isolation and Maintenance

Primary diabetic and non-diabetic LECs were dissociated from donor eyes using dispase II digestion and cultured in serum-free EpiLife medium containing 10 ng/mL human EGF as described previously.[18]

Western Blot

Protein was extracted from flash frozen and powdered limbal tissue and quantified using detergent compatible Bradford assay (Thermo Fisher Scientific). Gradient 4–20% tris-glycine SDS polyacrylamide gels (Thermo Fisher Scientific) were loaded with equal amounts of protein and subsequently transferred to 0.2 µm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Nonspecific binding was eliminated by blocking the membranes with 5% milk in tris-buffered saline with 0.1% Tween-20. Gel loading was normalized by β-actin. Primary antibodies were incubated overnight at 4°C (Supplementary Table S3). IRDye 800CW or 680RD donkey anti-rabbit or anti-mouse secondary antibodies (LI-COR Biotechnology, Lincoln, NE, USA) were used at 1:15,000 dilution. Protein bands were visualized, and results quantified on Odyssey Clx System (LI-COR Biotechnology).

Fluorescent Immunostaining

We fixed 5-µm-thick transverse ex vivo corneal tissue sections with 1% formalin for 5 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes and blocked in 5% normal goat serum for 1 hour at room temperature. The sections were incubated in antibodies against ROR2, RYK, Muc18, Frizzled-5, Frizzled-6, and Frizzled-7 (antibodies are listed in Supplementary Table S3) at 4°C overnight. The following day, the slides were washed, incubated in AlexaFluor 488- or 594-conjugated secondary antibodies and mounted using ProLong Gold Antifade mounting medium with DAPI (Thermo Fisher Scientific) as described previously.[9]

Small Interfering RNA (siRNA) Transfection

We transfected 60% to 80% confluent diabetic and non-diabetic LEC cultures seeded at the same density with 50 nM of siRNA to *ROR2*, *FZD5*, *FZD6*, *FZD7*, *MCAM*, and *RYK* or negative control siRNA (Dharmacon, Lafayette, CO, USA), using Lipofectamine RNAiMAX in OptiMEM serum-free medium (Thermo Fisher Scientific) for 24 hours according to the manufacturer's instructions, after which the medium was replaced with EpiLife medium for LEC cultures. Cells were used for further processing 72 hours after transfection.

Scratch Wound Healing

Scratch wounds were created by mechanically removing the monolayer of cultured LECs transfected with siRNA using a P200 micropipette tip as described previously.[9] The cultures were monitored, and the open area of the wounds were photographed every 6 hours for 24 hours using the Incucyte live cell analysis system (Essen Bioscience, Ann Arbor, MI, USA). The cultures were healing in the presence or absence of human recombinant Wnt-5a (200 ng/mL) (R&D Biosystems, Minneapolis, MN, USA). The closure of wounds was calculated using Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was performed using Student's t test for two-group comparison or ANOVA for multiple group comparison in Prism10 (GraphPad Software, San Diego, CA, USA), with a P value of less than 0.05 considered significant. Experiments were performed in triplicate using cells from three to seven individual donors (detailed in Supplementary Table S1) and repeated twice. Values were expressed as mean ± SEM, except for age analysis, which is expressed as mean ± SD.

Results

Differential Gene Expression and Methylation of Wnt Receptors in Diabetic and Non-Diabetic Limbus

To identify the differentially expressed Wnt receptor genes between diabetic and non-diabetic cornea, we performed droplet-based scRNA-seq with cells from limbal tissue and residual cornea and conjunctiva. After quality assessment filtering and removal of batch effects, 13 distinct clusters of cells were identified based on the expression of known marker genes (to be published elsewhere). These clusters comprised conjunctival cells, terminally differentiated corneal epithelial cells, LECs at various stages of differentiation including putative LESCs, limbal progenitor cells (LPCs), and transit amplifying cells, as well as other supporting cells such as neural crest progenitor cells (NCPCs), melanocytes, immune cells, and limbal stromal cells/mesenchymal stem cells (Fig. 1A). Gene expression analysis revealed differentially expressed Wnt receptor genes in diabetic versus non-diabetic cell clusters. As illustrated in Figs. 1B and 1C, *ROR2* was noticeably upregulated in several diabetic versus non-diabetic cell clusters including transit amplifying cells, superficial LEC, LPCs, and basal corneal epithelial cells. Similarly, *FZD6* was also slightly upregulated in diabetic LESC, superficial LEC, and LPC clusters. In contrast, *FZD5* and *MCAM* were only upregulated in the diabetic LESC cluster but not in other LEC clusters. Moreover, several *FZD* genes (*FZD1*, *FZD2*, *FZD4*, *FZD5*, *FZD6*, *FZD7*, and *FZD8*) were downregulated in the diabetic NCPC cluster, especially *FZD1*, *FZD5*, *FZD7*, and *FZD8*.

To confirm the scRNA-seq findings, we first validated the differential gene expression of various Wnt receptors by qRT-PCR. *FZD2* and *FZD9* were omitted from this analysis due to very low expression of these receptors in the LEC clusters. As shown in Fig. 2A, *ROR2* and *FZD6* were upregulated in diabetic limbal tissue by approximately 1.2-fold and 1.3-fold, respectively, compatible with the scRNA-seq results. It should be noted that by qRT-PCR *MCAM*, *FZD1*, *FZD5*, *FZD7*, and *FZD8* genes were downregulated in the diabetic group by 36%, 31%, 35%, 28%, and 43%, respectively, somewhat diverging from the scRNA-seq data.

Because stable diabetic complications may be largely due to epigenetic changes, we also examined our previously published dataset[9] comparing global DNA methylation profiles between diabetic and non-diabetic LEC enriched in LESC. Whereas *WNT5A* gene promoter was

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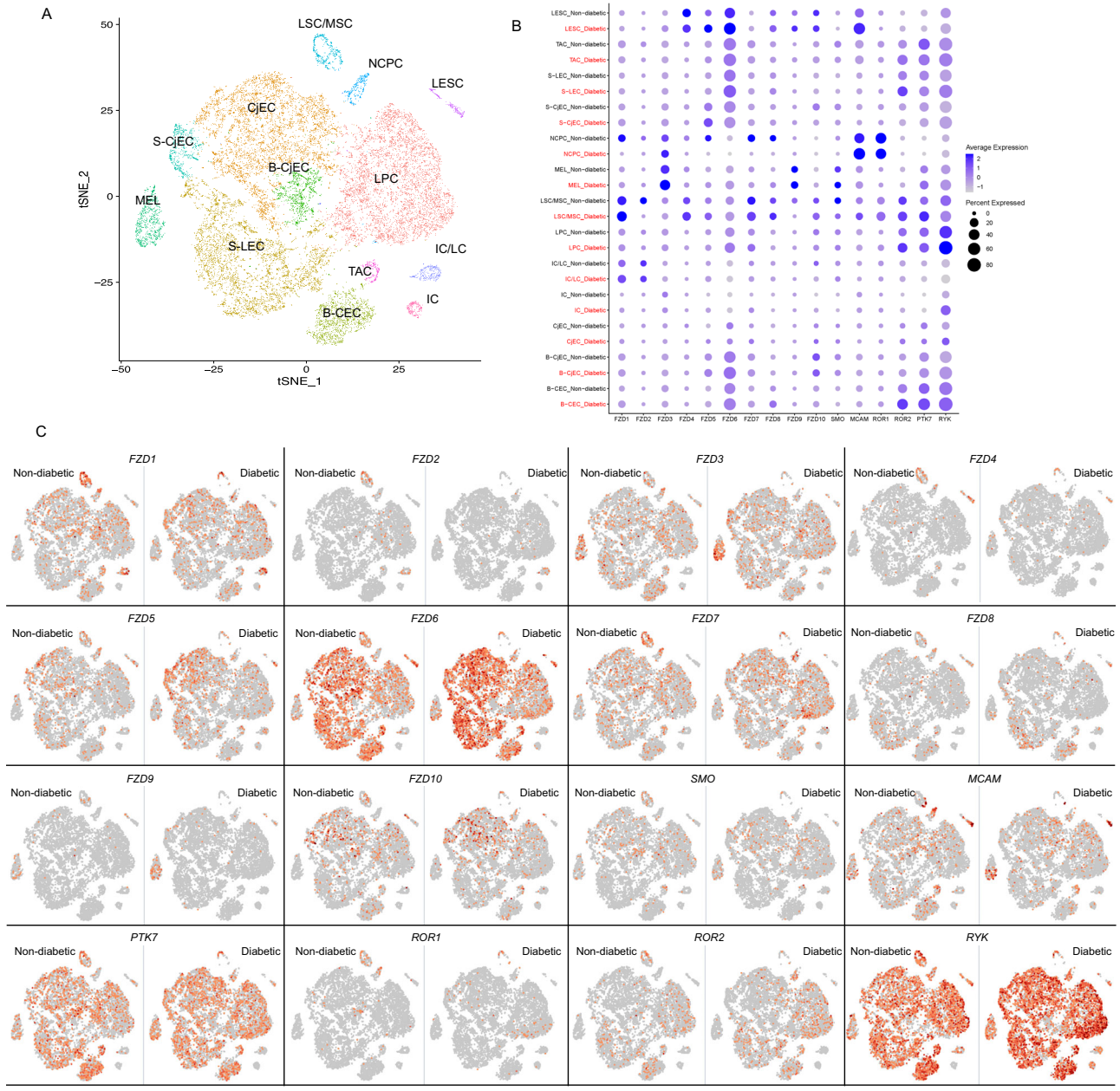

Figure 1. scRNA-seq analysis of human diabetic and non-diabetic corneas showing differential expression of Wnt receptor genes. (**A**) t-distributed stochastic neighbor embedding (t-SNE) plot showing 13 distinct cell clusters from diabetic ($n = 3$) and non-diabetic ($n = 3$) corneoscleral rims consisting of the limbus and residual cornea and conjunctiva. (**B**) Dotplot and (**C**) t-SNEs show the differential expression of various Wnt receptor genes in specific cell clusters. Note changes in individual Wnt receptor expression in specific cell clusters, especially in NCPC. B-CEC, basal corneal epithelial cell; B-CjEC, basal conjunctival epithelial cell; CjEC, conjunctival epithelial cell; IC, immune cell; IC/LC, immune cell/Langerhans cell; LSC/MSC, limbal stromal cell/mesenchymal stem cell; MEL, melanocyte; S-CjEC, superficial conjunctival epithelial cell; S-LEC, superficial LEC; TAC, transit amplifying cell.

hypermethylated in diabetic LEC, *ROR2* promoter was significantly hypomethylated in diabetic LEC compared with non-diabetic LEC (Fig. 2B). None of the other Wnt receptors were differentially methylated between diabetic and non-diabetic LECs. Because DNA hypomethylation usually results in increased gene expression, a higher expression of the *ROR2* gene was anticipated in diabetic LEC, which was in fact revealed by qRT-PCR and scRNA-seq.

Validation of Differentially Expressed Wnt Receptor Proteins

To further validate the differential gene expression of Wnt receptors (Figs. 1 and 2) at the protein level, we performed Western blot analysis and immunostaining in diabetic and non-diabetic ex vivo limbal tissue. As seen in Figures 3A and 3B, there was no significant difference in the expression of Frizzled-1 and Frizzled-8 between the two groups by

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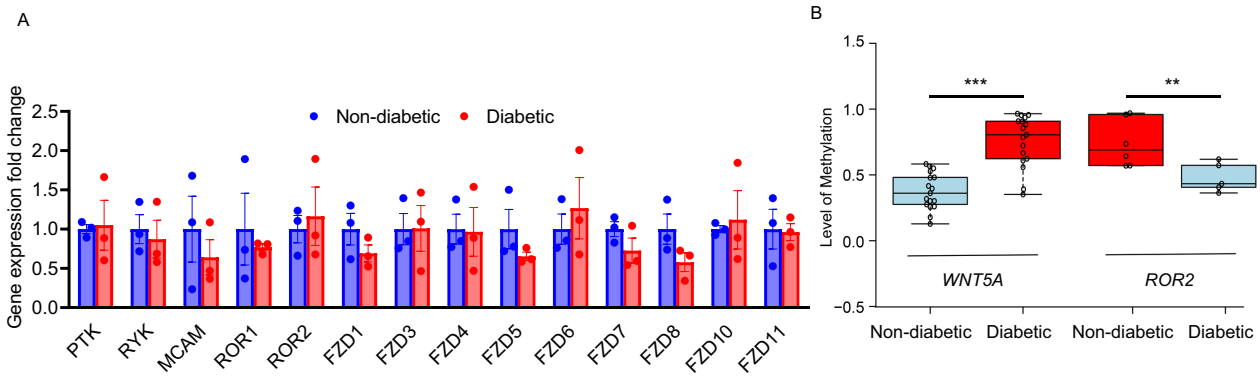

Figure 2. Differential gene expression and DNA methylation analysis of human diabetic and non-diabetic limbus and LEC enriched in LESC. (**A**) High throughput qRT-PCR analysis comparing gene expression levels of various Wnt receptor genes between diabetic ($n = 3$) and non-diabetic ($n = 3$) limbal tissue. (**B**) Global DNA methylation analysis of diabetic ($n = 5$) versus non-diabetic ($n = 6$) primary human LEC enriched in LESC showing differential DNA methylation levels in probes corresponding to 1000 bp across the transcription start site of *WNT5A* and *ROR2* gene promoter sites. Each circle represents a probe, and line in box indicates median of methylation levels. $^{**}P < 0.01$; $^{***}P < 0.001$. Note DNA hypomethylation of *ROR2* promoter in diabetic cells, as opposed to Wnt-5a DNA hypermethylation. Part of the figure related to Wnt-5a was reproduced for comparison from our previous publication.[9]

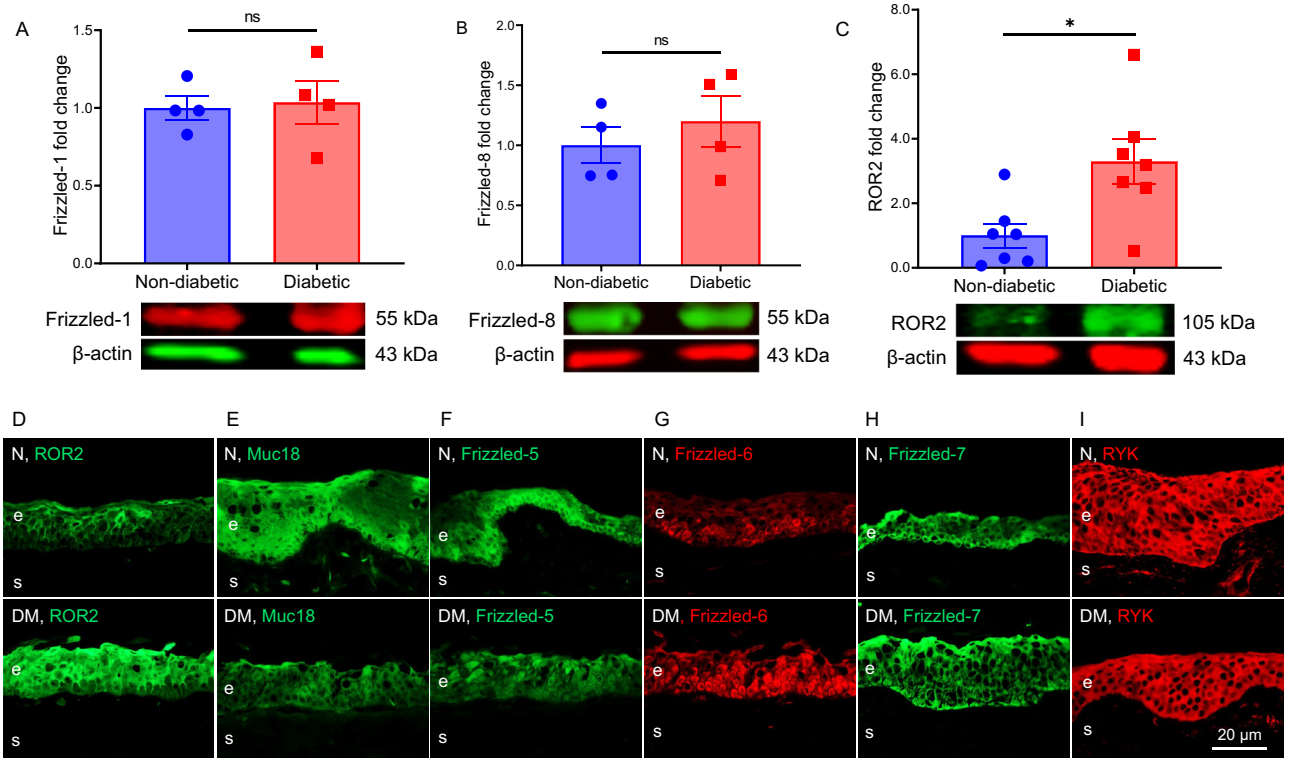

Figure 3. Validation of differentially expressed Wnt receptor proteins in diabetic and non-diabetic ex vivo limbal tissue. Western blot analysis of (**A**) Frizzled-1, (**B**) Frizzled-8, and (**C**) ROR2 in diabetic versus non-diabetic ex vivo limbal tissue ($n = 4$–7). Immunostaining of ex vivo corneal sections showing differential expression of (**D**) ROR2, (**E**) Muc18, (**F**) Frizzled-5, (**G**) Frizzled-6, (**H**) Frizzled-7, and (**I**) RYK in diabetic and non-diabetic limbal epithelium ($n = 3$). Values are mean ± SEM. Note protein expression changes in diabetic limbus for ROR2, Muc18, Frizzled-5, and Frizzled 6, with no significant changes for Frizzled-1, Frizzled-8, and RYK. $^{*}P < 0.05$; ns, nonsignificant. DM, diabetic; e, epithelium; N, non-diabetic; s, stroma.

western blot. However, the expression of ROR2 increased in the diabetic limbal sections by 3.2-fold ($^{*}P < 0.05$) by Western blot (Fig. 3C) as well as by immunostaining (Fig. 3D), confirming the scRNA-seq and qRT-PCR data, as well as the DNA promoter hypomethylation. Furthermore, the expression of Muc18 (protein encoded by *MCAM*) and Frizzled-5 was decreased and of Frizzled-6 and Frizzled-7 was increased in diabetic versus non-diabetic limbal epithelium (Figs. 3E–H). As a control, tissue sections were immunostained for RYK (Fig. 3I) that did not change between diabetic and non-diabetic groups, in line with the scRNA-seq and qRT-PCR data.

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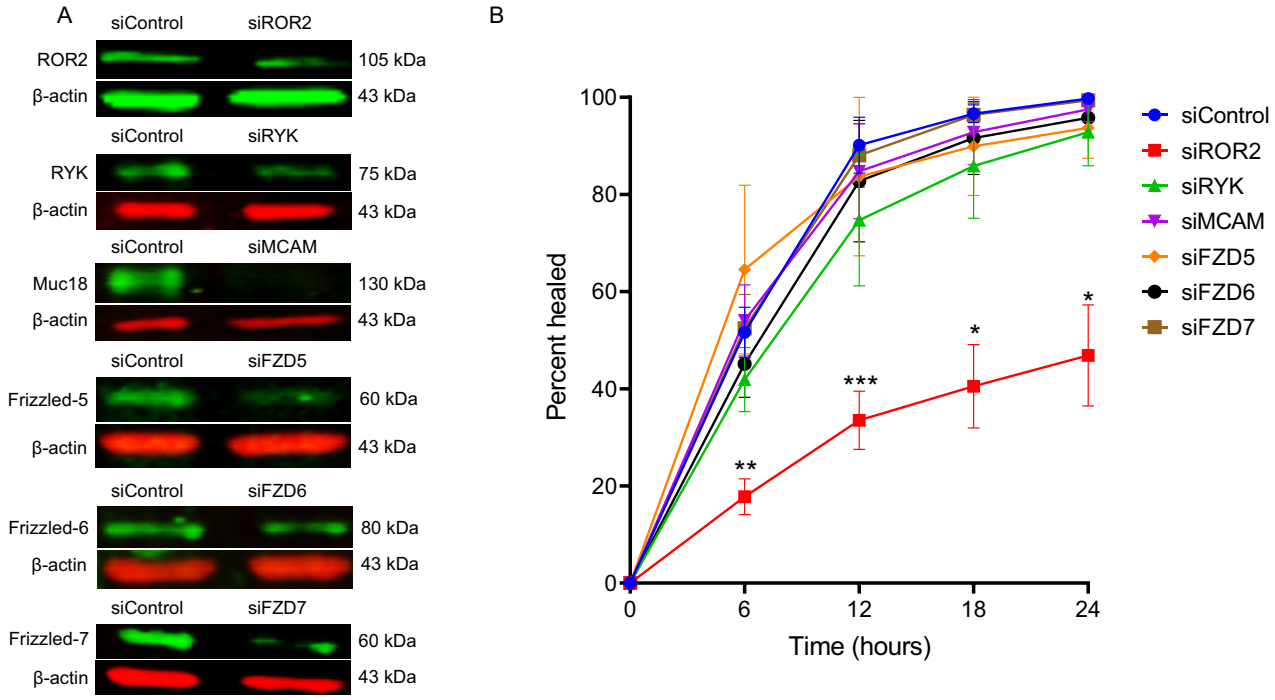

Figure 4. Wound healing of non-diabetic primary LEC transfected with Wnt receptor siRNAs. (**A**) Western blot analysis of non-diabetic LEC ($n = 3$) transfected with specific Wnt receptor siRNAs showing successful knockdown of receptor expression. (**B**) Scratch wound healing analysis showing healing time of non-diabetic LEC transfected with specific Wnt receptor siRNAs. Values are mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ versus siControl. Note marked efficiency of siRNAs in downregulating all studied receptors and significant decrease in wound healing only with ROR2 siRNA.

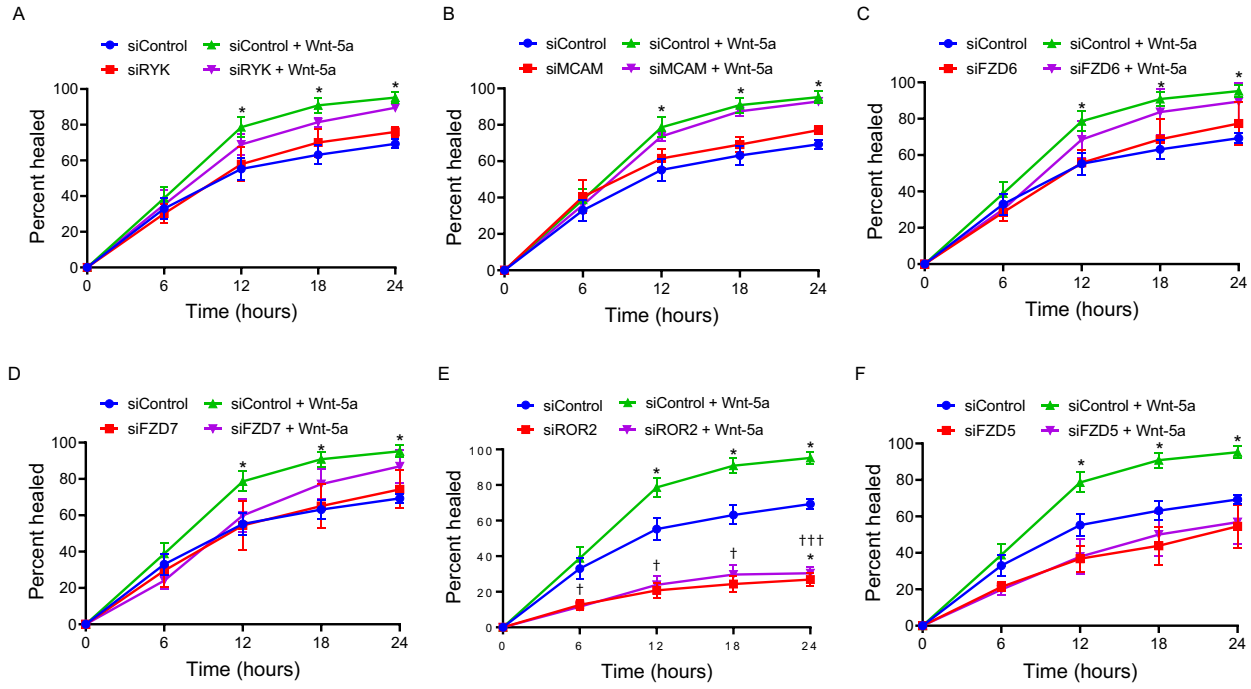

Figure 5. Wound healing of diabetic primary LEC transfected with Wnt receptor siRNAs. Scratch wound healing analysis of diabetic LEC ($n = 3$) transfected with (**A**) RYK, (**B**) MCAM, (**C**) FZD6, (**D**) FZD7, (**E**) ROR2, and (**F**) FZD5 siRNA and treated with or without Wnt-5a (200 ng/mL). Values are mean ± SEM. $^{*}P < 0.05$ versus siControl; $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ versus siControl + Wnt-5a. Note significant delay in wound healing by ROR2 siRNA that could not be rescued by exogenous Wnt-5a and partial effect of FZD-5 siRNA.

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Wound Healing of Non-Diabetic LEC Transfected With Wnt Receptor siRNAs

To examine the functional role of the receptor(s) responsible for the Wnt-5a induced stimulation of wound healing, we transfected non-diabetic LEC with specific siRNAs to knockdown *ROR2*, *MCAM*, *FZD5*, *FZD6*, *FZD7*, and *RYK*. As shown in Figure 4A, all siRNAs successfully decreased the expression of their respective protein targets as compared with the control siRNA. Furthermore, non-diabetic LEC transfected with *ROR2* siRNA showed significantly slower scratch wound healing (by approximately 50%; $*P < 0.05$) as compared with the control siRNA at 24 hours, whereas other receptors had no effect on wound healing (Fig. 4B). These data suggest that ROR2 mediates Wnt5a-dependent wound healing in non-diabetic LEC.

Wound Healing of Diabetic LEC Transfected With Wnt Receptor siRNAs

We then transfected diabetic LECs with specific receptor siRNAs with and without the addition of 200 ng/mL human recombinant Wnt-5a. As expected, when Wnt-5a was added to diabetic LEC transfected with control siRNA, there was a significant, 1.37-fold ($*P < 0.05$) increase in wound healing versus control siRNA alone (Fig. 5). Diabetic LEC transfected with siRNA to *RYK*, *MCAM*, *FZD6*, and *FZD7* alone or in the presence of Wnt-5a showed no significant difference in wound healing as compared with control siRNA alone or with Wnt-5a, respectively (Figs. 5A–D). However, when these cultures were transfected with *ROR2* siRNA, there was a significant 40% decrease in wound healing as compared with control siRNA by 24 hours. Moreover, the addition of Wnt-5a to these cells did not abrogate this effect, indicating that ROR2 was required for Wnt-5a to increase wound healing in diabetic LEC (Fig. 5E). Interestingly, these cells transfected with *FZD5* siRNA (but not others), showed a partial nonsignificant decrease in wound healing as compared with control siRNA, and the addition of Wnt-5a could not increase the healing (Fig. 5F), suggesting that Frizzled-5 may act as a co-receptor to ROR2 in diabetic LEC. This effect of *FZD5* siRNA was not observed in non-diabetic LEC (Fig. 4B). Importantly, when organ-cultured diabetic corneas were transfected with *ROR2* siRNA and then wounded with n-heptanol, no healing was observed, further confirming that ROR2 was the main Wnt-5a receptor mediating its wound healing effects (not shown here).

Discussion

Traditionally, Wnt signaling has been associated with the regulation of embryonic development and cancer. However, since its discovery more than three decades ago, it has become one of the most widely studied signaling systems for a range of other human conditions including, but not limited to, aging,[19–20] Alzheimer's disease,[21] cardiovascular disease,[22] and metabolic diseases.[23] In patients with type 2 diabetes, it is a key contributor of microvascular complications, such as retinopathy, nephropathy, and neuropathy, and macrovascular complications such as coronary artery disease, cerebrovascular disease, and peripheral arterial disease.[24] Recent work from our laboratory[9] showed that diabetes caused epigenetic alterations, namely, DNA methylation and microRNA changes that resulted in the suppression of noncanonical Wnt-5a in LEC. Furthermore, we also showed that in the diabetic LEC, Wnt-5a activated the Ca^{+2} signaling pathway via PLCγ1 and PKCβ phosphorylation.[9]

In this study, we applied scRNA-seq technology to compare gene expression of Wnt receptors in diabetic versus non-diabetic human corneas and found differential expression of several Wnt receptors. This method revealed that the diabetic changes in the expression of specific receptor genes were not uniform in the epithelial cells but in several instances were pronounced in specific cell clusters. A general trend was a decrease in Wnt receptor gene expression, especially in the NCPC cluster with noticeable downregulation of *FZD1*, *FZD5*, *FZD7*, and *FZD8*. A notable exception was *ROR2* gene upregulated in several clusters, which correlated with DNA hypomethylation at the promoter region.

Several studies have reported that Wnt-5a recruits ROR2 to activate noncanonical Wnt signaling pathways.[25–27] Indeed, in both diabetic and non-diabetic LECs, we found that the silencing of *ROR2* by siRNA impaired wound healing significantly. Furthermore, in diabetic LECs, suppression of *ROR2* blocked Wnt-5a–induced wound healing, suggesting that it is the main receptor by which Wnt-5a exerts its effects. This finding is in line with the above-mentioned studies in other tissues showing that Wnt-5a signals through ROR2.[25–27] The importance of ROR2 as the main corneal Wnt-5a receptor was also corroborated in diabetic organ-cultured corneas, which became unable to heal epithelial wounds after *ROR2* siRNA transfection (data not shown here).

Surprisingly, the *ROR2* gene promoter DNA was found to be hypomethylated in diabetic LECs, resulting in an increase in its protein expression. This may be a feedback attempt of the cells to compensate for significant decrease of Wnt-5a ligand, and further determines its critical role as the primary receptor of Wnt-5a in diabetic LEC. However, this partial compensation by the receptor was not enough to counteract low levels of Wnt-5a and normalize pertinent signaling, evidenced by decreased wound healing and low expression of various putative LESC markers in cultured diabetic epithelial cells and organ-cultured corneas. As we have recently published, these common features of the diabetic corneas seem to depend on Wnt-5a, because its addition brought back these parameters to nearly normal levels.[9]

Wnt-5a is also known to use the Frizzled receptors for signaling in several tissues.[28–34] These receptors often act together with other Wnt receptors in binding to the Wnt ligands and engaging different pathways.[10] Indeed, in diabetic LECs, siRNA silencing of *FZD5* partially blocked wound healing. This finding suggests that diabetic cells, in the absence of enough Wnt-5a[9] may additionally recruit Frizzled-5 as a co-receptor with ROR2 to amplify the ligand binding, internalization, and signaling. In contrast, non-diabetic cells have enough Wnt-5a and possibly do not need to recruit another receptor for ligand binding.

Although some other Wnt receptors also showed differential expression between diabetic and non-diabetic corneas by scRNA-seq and qRT-PCR, they did not have any functional effect on Wnt-5a mediated wound healing in diabetic or non-diabetic LECs. This result may be because these changes were observed only in certain subpopulations of cells, whereas the wound healing analysis was performed in LEC cultures presumably consisting of several subtypes of LECs. Similarly, we observed a discrepancy between some scRNA-seq and qRT-PCR results in terms of the upregula-

tion or downregulation of gene expression in diabetic versus non-diabetic cells. For instance, *FZD5* and *MCAM* seemed to be upregulated in diabetic LESC by scRNA-seq, but their expression by qRT-PCR did not reflect this change. This may be due to the use for qRT-PCR analysis of whole limbal tissue containing several cell types in addition to LESC that make up only a very small subset of limbal cells.

A limitation of this study is that the downstream signaling of Wnt-5a through ROR2 in non-diabetic corneas and through ROR2 + Frizzled-5 in diabetic corneas remains unexplored. It is known that ROR2 can homodimerize to activate Ca^{2+} signaling pathway by itself.[35] It may be suggested that, in non-diabetic corneas where there is enough Wnt-5a, ROR2 through homodimer formation may activate signaling fully, without the need of co-receptor Frizzled-5. If Wnt-5a is repressed like in diabetic corneas,[9] Frizzled-5 may need to be complexed with ROR2 to attempt signaling activation. There is also evidence that ROR2 via its cysteine-rich domain augments the function of a receptor super-complex with Frizzled to transduce Wnt-5a signals.[36] It may be suggested that complexation of Frizzled-5 with ROR2 drives the Wnt-5a signaling to the Ca^{2+} rather than to the PCP pathway that entails the formation of ROR2 complex with Vangl2.[37] In fact, activation of Wnt-5a Ca^{2+} signaling by ROR2–Frizzled-5 has been observed in other systems including renal tubular epithelial cells injury,[38] and interleukin-1β stimulated corneal endothelial cells where this complex can also inhibit the RhoA-mediated PCP pathway.[39] Unraveling these receptor interactions and diabetes influence on regulation of downstream pathways merits further investigation.

In conclusion, we found that Wnt-5a action occurs through ROR2 that is its main signaling receptor mediating wound healing in diabetic LECs. Frizzled-5 may also have an effect on Wnt-5a stimulation of wound healing, although only partially as a co-receptor with ROR2 and only in diabetic LECs.

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