

Long Noncoding RNA 3632454L22RiK Contributes to Corneal Epithelial Wound Healing by Sponging miR-181a-5p in Diabetic Mice

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PURPOSE. This work explores the abnormal expression of long noncoding RNAs (lncRNAs), microRNAs (miRNAs) and messenger RNAs (mRNAs) in diabetic corneal epithelial cells (CECs) and constructs an associated competitive endogenous RNA (ceRNA) network. Moreover, we revealed that Rik may exert advantageous effects on diabetic corneal epithelial wound closure by sponging miR-181a-5p.

METHODS. We obtained the profiles of differentially expressed lncRNAs (DElncRNAs) of CECs of type 1 diabetic versus control corneas by microarray and summarized the differentially expressed miRNAs (DEmiRNAs) and differentially expressed genes (DEGs) data by published literature. Subsequently, the ceRNA network was constructed using bioinformatics analyses. The levels of lncRNA ENSMUST00000153610/3632454L22Rik (Rik) and miR-181a-5p were verified. The localization of Rik was identified with fluorescence in situ hybridization (FISH), and dual-luciferase assays proved the targeted relationship between Rik and miR-181a-5p. Furthermore, we validated the functional impact of Rik in vitro.

RESULTS. Overall, 111 upregulated and 117 downregulated DElncRNAs were detected in diabetic versus control CECs. The level of Rik located in both the cytoplasm and the nucleus was clearly downregulated, whereas miR-181a-5p was upregulated in vitro and in vivo in the diabetic group versus the control group. Rik can act as a ceRNA to bind to miR-181a-5p, thus promoting diabetic corneal epithelial wound healing in vitro.

CONCLUSIONS. This work investigated the expression profile of DElncRNAs and constructed ceRNA networks of diabetic CECs for the first time. Furthermore, we revealed that Rik may positively impact diabetic corneal epithelial wound healing by sponging miR-181a-5p, providing a novel potential therapeutic target of diabetic keratopathy (DK).

Keywords: competing endogenous RNA (ceRNA), long noncoding RNAs (lncRNA), microRNAs (miRNA), diabetic keratopathy (DK), corneal epithelial wound healing

Diabetes mellitus (DM), a common metabolic disease leading to numerous complications, including ocular complications, has rapidly increased in prevalence over the years.¹ Chronic hyperglycemia significantly interferes with epithelial structure and function, leading to diabetic keratopathy (DK)/epitheliopathy.^{2,3} Although often ignored by both scientists and physicians alike, it can cause delayed wound healing, persistent epithelial defects, impaired corneal sensitivity, corneal ulcers, neurotrophic keratopathy, and even visual loss.⁴⁻⁶ The focus of most previous studies has mainly been on single factor receptors.⁷⁻⁹ Presently, systematic analysis of genetic alterations has attracted growing attention.¹⁰ The microarray results showed that hyperglycemia significantly alters the expression of messenger RNAs (mRNAs) and microRNAs (miRNAs) in corneal epithelial cells (CECs).¹¹⁻¹³ However, to date, people still lack a cure for DK, and a full understanding of the pathobiology remains elusive.¹⁴

Long noncoding RNAs (lncRNAs), a class of noncoding RNA molecules greater than 200 nucleotides in length, play key roles in diverse ranges of biological processes and diseases.^{15,16} Specifically, lncRNAs can regulate cis or trans

gene expression at the epigenetic level, transcriptional level, and posttranscriptional level by regulating histone modification, chromatin modification, protein functional activity, RNA metabolism, and others.¹⁷ In these multiple mechanisms, lncRNAs, containing abundant miRNA response elements (MREs), can communicate with target genes and coregulate each other by competitively binding shared miRNAs.¹⁸ Moreover, various miRNAs have been reported to regulate corneal nerve regeneration and damage repair in diabetic mice.¹⁹⁻²¹ Nine miRNAs were reported to be significantly dysregulated in CECs from type 1 DM mice, and miR-204-5p was proven to impact corneal epithelial wound closure.²² As reported in previous studies, various lncRNAs were found to play critical roles in diabetic complications²³ and multiple ocular diseases, such as retinopathy and pterygium.²⁴⁻²⁶ However, the roles of lncRNAs and the lncRNA-associated competitive endogenous RNA (ceRNA) network in corneal epithelial wound closure in DM mice remain unknown.

In this work, we determined the lncRNA profiles of CECs from control (Ctrl) and type 1 DM mice for the first time and explored the interaction networks

among lncRNAs, miRNAs, and mRNAs. Furthermore, lncRNA ENSMUST00000153610/3632454L22Rik (Rik) was revealed to act as a ceRNA to sponge miR-181a-5p and promote diabetic corneal epithelial wound healing in vitro.

MATERIALS AND METHODS

Experimental Animals

C57BL/6 male mice (8 weeks old), purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Streptozotocin (STZ) induction of type 1 DM mice was performed as previously described.^{27,28} We prepared low-dose STZ (50 mg/kg; Sigma–Aldrich, St. Louis, MO, USA) mixed with ice-cold citrate-citric acid buffer (pH = 4.5) and then injected the mice intraperitoneally for 5 consecutive days in low light immediately. Sixteen weeks after injection, DM mice with blood glucose levels (Ascencia Contour Glucometer, Bayer Diabetes Care, Elkhart, IN, USA) higher than 16.67 mmol/L were used in this work.

Corneal Sensitivity

We used a Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres Cedex, France) to assess the corneal sensitivity of Ctrl and DM mice without anesthesia as previously described.²⁹ The longest filament length (each measurement shortened from 6 cm by 0.5 cm) causing a positive result was considered the threshold of corneal sensitivity.

Corneal Epithelial Debridement Wounds

All mice were anesthetized through intraperitoneal injection of 0.6% sodium pentobarbital, and then 2% lidocaine was applied to the ocular surface. CECs were scraped off using an Algerbrush II corneal rust ring remover (Alger Co., Lago Vista, TX, USA) after demarcation with a trephine (2.5 mm). Subsequently, ofloxacin eye ointment was applied to the cornea to avoid infection. At 0, 24, and 48 hours after debridement, fluorescein sodium was used for staining, and photographs were taken using a Haag–Streit Slit Lamp BQ 900 (Haag–Streit AG, Bern, Switzerland). The stained areas were measured with ImageJ software to evaluate the degree of residual epithelial defects.

lncRNA Expression Microarray Analysis

We selected CECs that were scraped off from the surface of central corneas from four DM and four age-matched normal mice using a corneal epithelium spatula. CECs of individual mice were collected in EP tubes and stored at -80°C . The lncRNA expression microarray was performed by KangCheng Biotechnology Co. Total RNA was extracted from CECs with TRIzol reagent (Invitrogen). The quantity and quality of total RNAs were valued by a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA), and samples with an OD260/OD280 ratio between 1.8 and 2.1 were used. Moreover, the integrity of RNAs was also analyzed by denaturing agarose gel electrophoresis. Briefly, each RNA sample was amplified and transcribed into fluorescent cRNA. Then, cRNAs were hybridized to the microarray (Arraystar Mouse lncRNA Microarray version 4.0). Next, the array was washed

and fixed, and then the array was scanned with an Agilent Scanner G2505C system. GeneSpring GX version 12.1 software was used for quantile normalization and further data processing. Differentially expressed lncRNAs (DElncRNAs) were identified using fold change ≥ 1.2 and $P < 0.05$ as the cutoff criteria.

Construction of miRNA/Targets and ceRNA Network

Bioinformatics analyses were used to construct the network among DElncRNAs, differentially expressed miRNAs (DEmiRs), and differentially expressed genes (DEGs). DEmiR and DEG data were acquired from published literature. The prediction of miRNA targets was carried out using the miRanda³⁰ and TargetScan³¹ algorithms. Cytoscape 3.8.2 was used for network visualization.

Quantitative real-time-PCR Verification of Differential lncRNAs and miRNAs

Total RNA was isolated from CECs as described previously. Then, cDNA was synthesized with a Thermo Scientific Rever-taid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA) and amplified by quantitative real-time PCR (qRT-PCR) with ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using β -actin and U6 as internal references. Primer sequences of the selected lncRNAs and miRNAs are listed in Table 1.

Luciferase Gene Reporter Assay

The psiCHECK vector (Promega Corporation, Madison, WI, USA) constructs with wild-type or mutant Rik were generated. The miR-181a-5p mimics or negative controls were transfected into cells using Lipofectamine 3000 (Invitrogen). After 48 hours of cell lysis, firefly and Renilla luciferase activities were detected by a Promega Dual-Luciferase system E2920 (Promega Corporation, USA).

Cell Culture and Treatment

The mouse TKE2 corneal epithelial cell line (Public Health England; Cat No. 11033107) was provided by Dr. Tetsuya Kawakita of Keio University.^{32,33} Cells were cultured in keratinocyte serum-free medium (KFSM; Life Technologies Corporation, Grand Island, NY, USA) supplemented with 2.5 ng/mL human recombinant epidermal growth factor and 25 $\mu\text{g}/\text{mL}$ bovine pituitary extract. Then, the cell lines were starved overnight in KFSM medium without adding any components. The following day, the medium was replaced with complete medium, and then 1 M D-glucose solution or 1 M mannitol solution was added. Nothing was added to the cells in the no glucose (NG) control group. The final concentration for the high-glucose (HG) group and the high-mannitol (HM) group was 30 mM.^{34–36}

Fluorescence In Situ Hybridization of lncRNA

A fluorescence in situ hybridization (FISH) kit (RiboBio) was used in this work according to the manufacturer's protocol. In short, cells were fixed in 4% paraformaldehyde solution

TABLE 1. Primers Used in This Work

Gene	Forward	Reverse
ENSMUST00000144705	TGTGCCATGTTGGCTGGTAT	CATTCCCCAACTCAGGCTTTTC
ENSMUST00000197415	GCATTGGATTCTTGGAACTG	CAAACCAGGGCTAGAGAAATGG
ENSMUST00000162370	TCCGAACCAACCAGAACACAA	TCCCCAACAGATGCAAGAT
ENSMUST00000233842	CCAAGTTCTCGGTGTGCGTT	ATACGTGGGATCTGCCAGCTT
ENSMUST00000227433	TGCCTGTGGCACTGTCACAT	GTGTGACCCCTTTGTAGGCTAT
AK008372	TCGCTCAGCAGACCTGAAAA	AACAGCAAGGCCAGGACATC
ENSMUST00000139005	GGTGTGAGCCTCTTAGCCTTTTC	GGTGTCAAACATGGAGGATGTAG
ENSMUST00000010224	CCCCCTCTTCTTACACGTGC	CGTTGGCCAGCAGGTTCT
ENSMUST00000133488	GGAGACTCCACGCTAATGTAAGGA	CACGATCACTCTGGGTCCCTT
ENSMUST00000228460	GCTGGGAATTTGAGGCTTCTT	CAGTCTCCATGCCAGAAAAACC
ENSMUST00000177010	GAGGCTGAAAGCAGGGATTG	AGTAATCCGAAGATGCCTTAGAC
ENSMUST00000208069	AAGAGGATAGTGTGGGTTCCCTT	CCCGTTTCTAGGCAGTTGAC
ENSMUST00000153610	GGCTGCGGACTTCATCTATGTAA	TTCATCGAATCAACACAAAGGAA
miR-181a-5p	CATTCAACGCTGTGCGGTGAGT	GATCGCCCTTCTACGTCGTAT
β -actin	ACTGCCGATCCTCTTCTCT	TCAACGTACACTTCATGATGGA
U6	CGCTTCGGCAGCACATATACTA	GGAACGCTTCACGAATTTGC

for 10 minutes at room temperature and permeabilized with 0.1% Triton X-100 for 5 minutes at 4°C. Then, we rinsed the cells with PBS. The RNA was hybridized with a Cy3-labeled probe for 18S, U6 and Rik overnight at 37°C and protected from light. Cells were rinsed with SSC (0.3 M NaCl and 0.03 M Na₃ citrate) at 42°C, and the nuclei were then stained with DAPI. Fluorescence images were acquired with an LSM880 Zeiss inverted microscope (CarlZeiss, Germany).

Cell Transfection

We used a plasmid to overexpress lncRNA Rik, and the corresponding negative control (NC) was also prepared. The plasmid constructs and the NC were built by RiboBio Co., Ltd. Cells inoculated at 70% confluence were transfected with Lipofectamine 3000 following the manufacturer's protocol and cultured for 72 hours.

Cell Wound-Scratch Assay

After transfection, the cells were cultured in NG, HM, and HG medium. We used 200- μ L pipette tips to create a scratch injury. Images were taken at 0 and 48 hours after scratching. The percentage of covered area was analyzed with ImageJ software. All scratch injury models were compared with control cells transfected with NC.

Statistics

All experimental results are presented as the mean values \pm SD. Each experiment was conducted at least three times independently. The statistical analysis was performed by Student's *t*-test or 1-way ANOVA using GraphPad Prism version 8.0.1. A *P* value < 0.05 (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001) was considered statistically significant.

RESULTS

Successful Establishment of Experimental DK in Mice

In this work, type 1 DM mice were generated by intraperitoneal injection of STZ and measured in a random-feeding

state. Four months later, STZ-treated mice exhibited hyperglycemia and a significant loss of body weight (Figs. 1A, 1B), which met the criteria for diabetes diagnosis. Diabetes induced a significant decrease in corneal sensitivity, as previously reported (Fig. 1C).^{12,36} Moreover, we evaluated the effect of hyperglycemia on the rate of corneal epithelial wound healing. As previously reported, it was clearly delayed in the corneas of DM mice (Figs. 1D, 1E).³⁵

lncRNA Profiles in Diabetic CECs

To gain insight into the biological roles of lncRNAs in diabetic CECs, we determined the expression profile of lncRNAs in DM versus Ctrl mice. Mouse lncRNA Array version 4.0 was applied (Ctrl group, 4 replicates and DM group, 4 replicates), and a total of 18,855 lncRNA candidates were detected. Then, a 1.2-fold change cutoff was used. Among these, 111 and 117 lncRNAs were clearly upregulated and downregulated, respectively (Fig. 2A). The hierarchical cluster analysis revealed a striking difference in lncRNA expression patterns between the two groups (Fig. 2B). Furthermore, according to their genomic locations, these lncRNAs were aligned to exon sense-overlapping (42%), intergenic (42%), natural antisense (12%), intronic antisense (4%), intron sense-overlapping (2%), and bidirectional (1%; see Figs. 1, 2C). The length of lncRNA transcripts largely fluctuated within 1000 nt (Fig. 2D). We also characterized the chromosomal distributions of all lncRNAs (*P* < 0.05) and DELs (Fig. 2E). Moreover, to validate the accuracy of the microarray, 12 of the top DELs were randomly selected for qPCR determination (Figs. 3A, 3B). The qPCR results corroborated the microarray results. Overall, we determined the dysregulated lncRNAs in diabetic CECs.

lncRNA-miRNA-mRNA Network Construction

MiRNAs have been implicated in the pathophysiological processes of numerous diseases,³⁷ including delayed diabetic corneal epithelial wound closure.²² However, their upstream regulation varies across diseases and tissues. Cytoplasm lncRNAs also function as ceRNAs by sponging miRNAs.^{15,18} Hence, we excluded lncRNAs that localized to the nucleus exclusively from the 228 DELs identified by the lncAtlas database for subsequent studies. From the

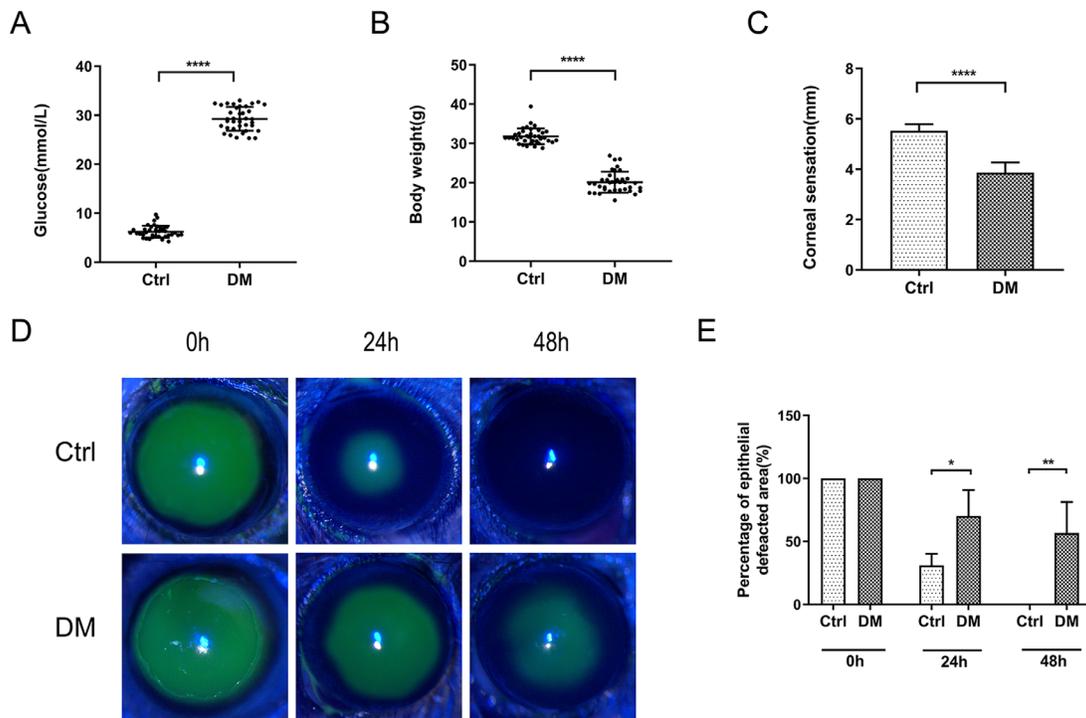


FIGURE 1. Diabetic keratopathy in STZ-induced diabetic mice. (A) Blood glucose values and (B) body weight values on 4 months after STZ-injection. (C) Corneal sensitivity values were clearly decreased in DM mice. (D, E) Corneas stained with fluorescein at 0, 24, and 48 hours after injury.

research of Gao, nine DEMiRs that were significantly dysregulated in CECs of type 1 DM mice were acquired.²² Information of DEMiRs is listed in Table 2. By searching the results of 8 studies, 17 DEGs were found to be affected by

hyperglycemia on CECs (Table 3).^{7,33,34,38–42} In light of the lncRNA-miRNA and miRNA-mRNA interactive pairs, a potential ceRNA network was constructed using the TargetScan and miRanda algorithms (Fig. 4).

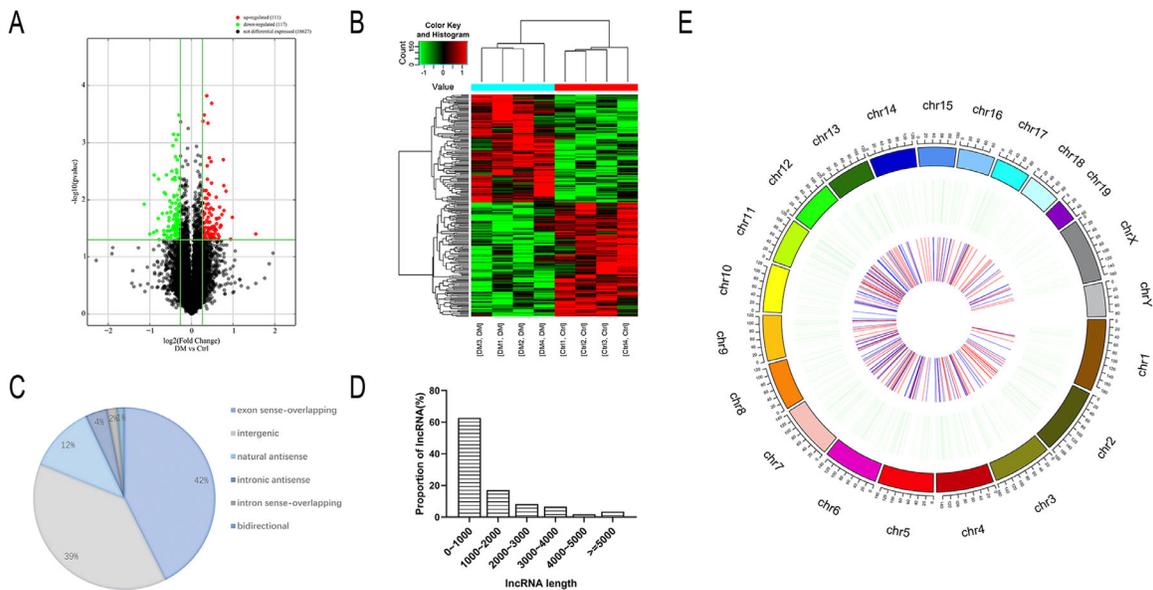


FIGURE 2. Total lncRNAs detected by microarray in DM and control groups. (A) Volcano plot and (B) cluster analysis of DELs (fold change ≥ 1.2) in corneal epithelial tissues. Red indicates relative high expression; green, low expression. There were four in the control group and four in the STZ-treated group. (C) The genomic location of lncRNAs. (D) lncRNAs length distribution. (E) Chromosomal distribution of expressed lncRNAs ($P < 0.05$). The outermost circle is the mouse chromosomes map. The inner layers from the outside to the inside indicate the expression distribution of all lncRNAs ($P < 0.05$) and the DELs.

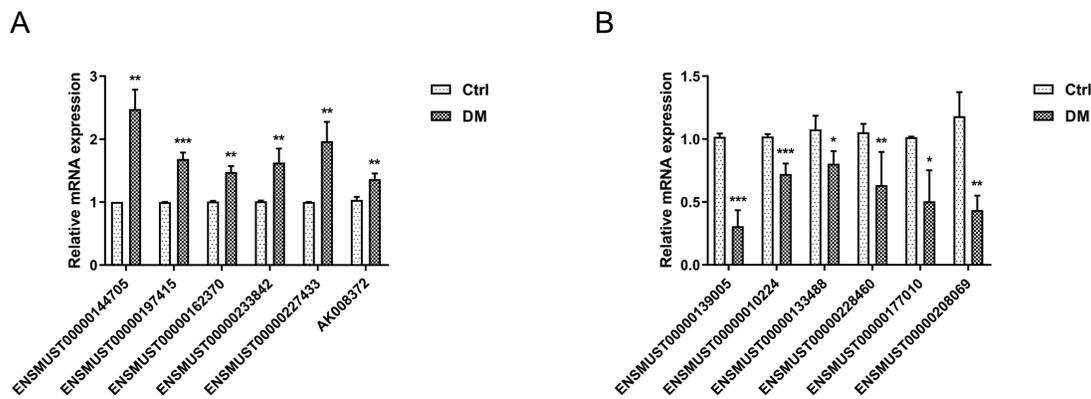


FIGURE 3. Validation of DELs detected by microarray. (A) Upregulated EDLs. (B) Downregulated DELs ($n = 4/\text{group}$).

TABLE 2. DE miRNAs of 1 Research

	Annotation	Reference
Gao 2014	mmu-miR-22-3p mmu-miR-122-5p mmu-miR-181a-5p mmu-miR-181b-5p mmu-miR-200a-3p mmu-miR-204-5p mmu-miR-138-5p mmu-miR-181c-5p mmu-miR-181d-5p	MicroRNA-204-5p-Mediated Regulation of SIRT1 Contributes to the Delay of Epithelial Cell Cycle Traversal in Diabetic Corneas.

Rik Functions Via Sponging miR-181a-5p

In our previous research, we found a protective effect of inhibiting miR-181a-5p on corneal epithelial wound healing in type 1 DM mice.²¹ Herein, to elucidate the function of DELs, among the numerous targets, the lncRNA Rik with high expression abundance and included in the GENCODE database was selected for further studies. By sponging miR-181a-5p, Rik regulates 5 genes related to diabetic CECs: Plau, Serpine1, Sirt1, Sema3c, and Hmgb1 (Fig. 5A). We validated the Rik and miR-181a-5p expression levels in both CECs (Figs. 5B, 5C) and TKE2 cell lines (Figs. 5D, 5E). The results indicated that the expression levels of Rik and miR-181a-5p were clearly downregulated and upregulated compared with those of the controls, respectively, in vivo and in vitro. The FISH array showed that Rik was located in both the cytoplasm and nucleus, further suggesting that Rik might exert an important function during the development of corneal epithelial wound closure by acting as a ceRNA of miR-181a-5p (Fig. 5F). Bioinformatics prediction tools further demonstrated that miR-181a-5p targeted the 3'UTRs of Rik with complementary seed regions. Luciferase reporter plasmids of mutant-type (MUT) Rik and wild-type (WT) Rik were constructed. Clearly, the luciferase activity of plasmids cotransfected with WT-Rik and miR-181a-5p mimics was significantly decreased (Fig. 5G).

Rik Overexpression Promotes Migration of TKE2 Under High Glucose

To determine the function of Rik, a plasmid targeting Rik was used to transfect TKE2 cells for the cell scratch assay. In TKE2 cells, the scratch injury assay indicated an apparent

lower rate in the healing of cells in the HG treatment ($41.74 \pm 3.45\%$) compared with the NG group ($72.56 \pm 2.45\%$) or the HM group ($64.39 \pm 6.44\%$), and the healing rate in the AD-NC treatment group was lower than that in the AD-Rik group ($59.76 \pm 5.44\%$) under high-glucose conditions (Figs. 6A, 6B). Overall, we speculated that Rik can specifically sponge miR-181a-5p to act as a ceRNA and promote diabetic corneal epithelial wound healing.

DISCUSSION

Diabetes incidence is rising worldwide year by year, causing multiorgan damage. Unlike diabetic retinopathy, in patients with DK, hyperglycemia does not cause detectable and obvious clinical symptoms unless CECs are damaged or eyes are injured.⁴³ Patients with DK present with delayed wound healing of the corneal epithelium and decreased corneal sensitivity.³⁸ Our limited knowledge of the pathogenesis of delayed epithelial wound closure leads to the importance of identifying its pathogenesis. lncRNAs are important and pervasive genes that can act as ceRNAs and are involved in the development of diseases.^{15,18} However, the role of lncRNAs and lncRNA-associated ceRNAs in diabetic CECs remains unclear. Over the years, this is the first work to explain the lncRNA-miRNA-mRNA regulatory network in diabetic CECs. Buried in other kinds of RNAs, lncRNAs generally exhibit low abundance.⁴⁴ There are many errors and clear limitations in RNA-Seq, exon detection, or RNA quantification.^{45,46} The microarray is rich, precise, and comprehensive and therefore remains the preferred platform for lncRNA expression profiling.⁴⁷ In this work, an lncRNA microarray was performed to detect lncRNA expression. A total of 228 (111 lncRNAs, upregulated; and 117 lncRNAs, downregulated) significantly DELs were detected in the DM versus Ctrl groups. The qRT-PCR analyses of some lncRNAs demonstrated the high credibility of the microarray in this study. Moreover, subcellular localization of lncRNAs is the basis of the ceRNA mechanism,⁴⁸ but it has rarely been mentioned in the previous literature. In this work, lncRNAs localized to the nucleus exclusively identified by the lncATLAS database were expelled. The subcellular localization of lncRNAs must be demonstrated in further research. Further in-depth studies would likely advance our knowledge on the ceRNA regulatory networks in corneal epithelial wound closure of DM mice.

TABLE 3. DEGs of Eight Studies

	Annotation	Reference
Zhu 2019	Egfr/Igf1r/Serpine1/Sirt1//Aldh1a1/Igf1	The impact of hyperglycemia on the corneal epithelium: Molecular mechanisms and insight.
Lee 2019	Sema3a/Sema3c/Nrp2	Opposing Effects of Neuropilin-1 and -2 on Sensory Nerve Regeneration in Wounded Corneas: Role of Sema3C in Ameliorating Diabetic Neurotrophic Keratopathy.
Yang 2020	Ctnnb1/Tcf4/Ccnd1	Insulin Promotes Corneal Nerve Repair and Wound Healing in Type 1 Diabetic Mice by Enhancing Wnt/ β -Catenin Signaling.
Li 2020	Mmp13	Leucine-rich α -2-glycoprotein-1 promotes diabetic corneal epithelial wound healing and nerve regeneration via regulation of matrix metalloproteinases.
Sun 2015	Plau	Hyperglycemia-suppressed expression of Serpine1 contributes to delayed epithelial wound healing in diabetic mouse corneas.
Zhang 2018	Ntn1	Netrin-1 promotes diabetic corneal wound healing through molecular mechanisms mediated via the adenosine 2B receptor.
Li 2021	Nampt	Hyperglycemia-reduced NAD (+) biosynthesis impairs corneal epithelial wound healing in diabetic mice.
Hou 2021	Hmgb1	Expression profiles and potential corneal epithelial wound healing regulation targets of high-mobility group box 1 in diabetic mice.

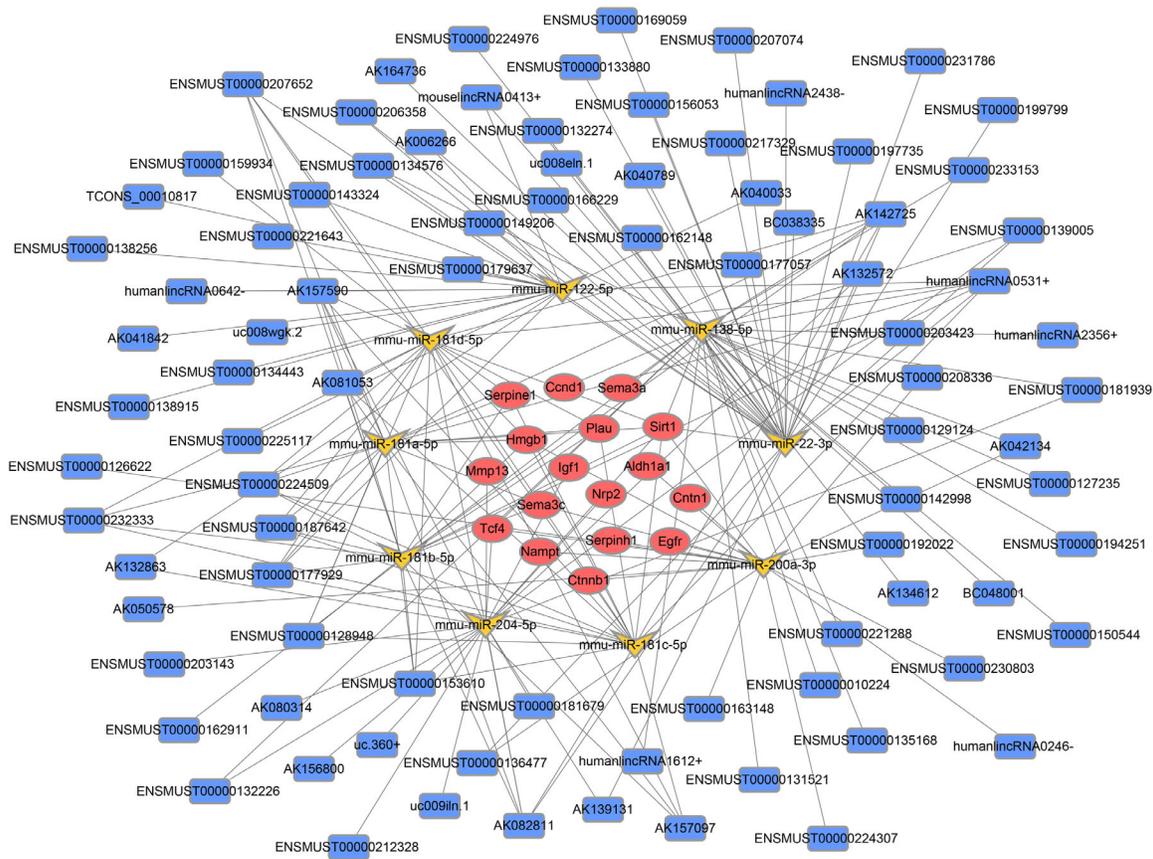


FIGURE 4. A ceRNA map. Blue arrowheads represent lncRNAs; yellow ovals, miRNAs; red hexagons, the target genes related to diabetic CECs.

In research on noncoding RNAs (ncRNAs) in diabetic CECs, miRNAs were most widely studied. Nine miRNAs were found to be significantly dysregulated in diabetic CECs, six miRNAs were upregulated, and three miRNAs were down-regulated.²² Among them, miR-204-5p-targeted SIRT delayed TKE2 cell cycle traversal and then postponed corneal epithelial wound healing upon high-glucose challenge. Addi-

tionally, our previous research found that an miR-181a-5p antagomir clearly contributed to corneal epithelium healing in type 1 DM mice.²¹ Apart from these DEMiRs, miR-146a, miR-424, and miR-10b have also been found to be altered and perform a function in human diabetic corneas.^{13,49} To further improve the accuracy of this study, only experimentally verified miRNAs in CECs of type 1 DM mice were

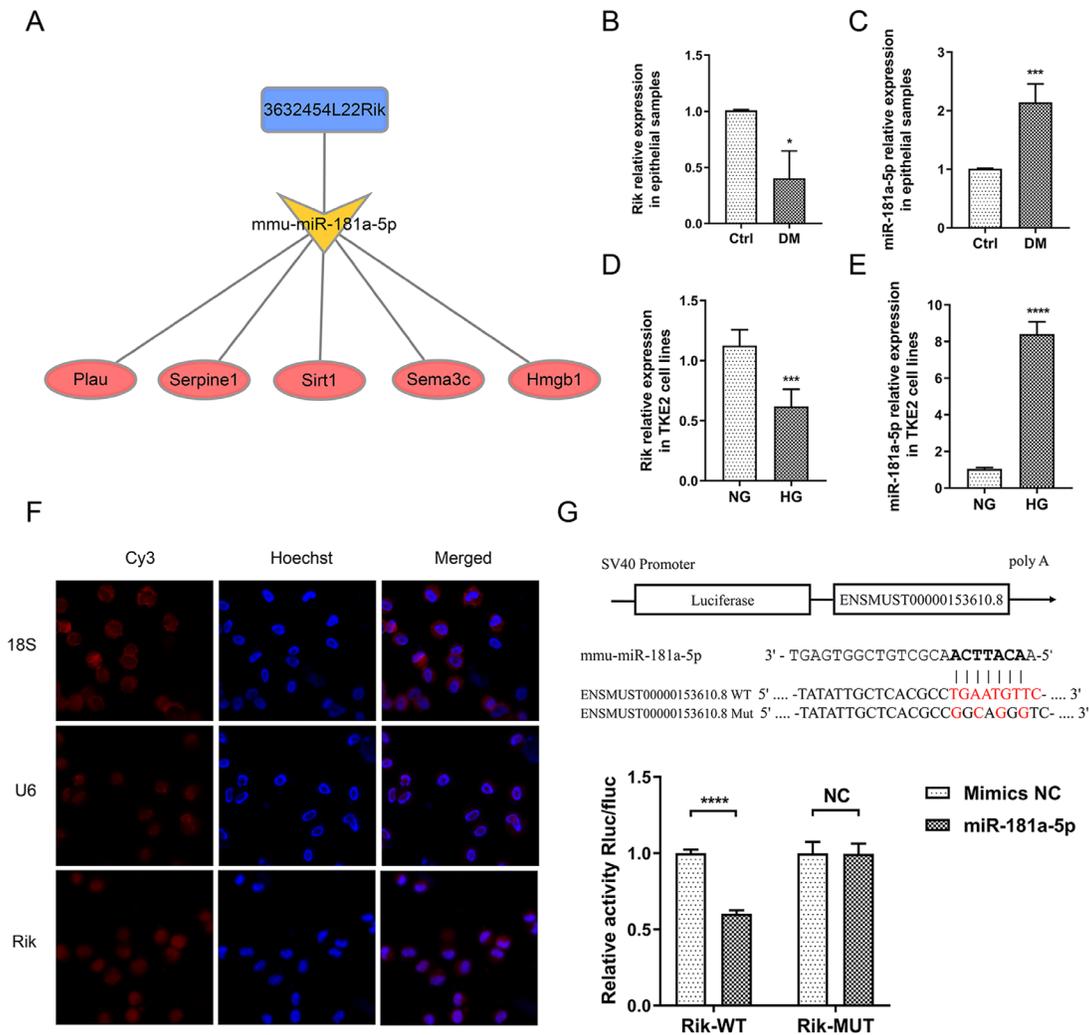


FIGURE 5. Rik functions via sponging miR-181a-5p. (A) A subnetwork of ceRNA network. (B–E) Validation of selected lncRNA and miRNA by using qRT-PCR in both epithelial samples ($n = 4/\text{group}$) and TKE2 cell lines ($n = 4/\text{group}$). (F) Representative images of fluorescent in situ hybridizations of Rik (red) co-labeled with DAPI (blue) in cultured TKE2 18S rRNA as the cytoplasmic control, and U6 snRNA as the nuclear control. (G) Dual-luciferase reporter assay between Rik and miR-181a-5p.

included. To date, there have been no reports about other ncRNAs in diabetic CECs.

According to previous reports, 17 DEGs were regarded as regulating epithelial homeostasis in type 1 DM mice. Then, the TargetScan and miRanda algorithms were used to build the ceRNA network. Nevertheless, some genes (Bcl-2, Hgf, and Smad2, etc.),³⁸ which have not been validated in CECs of type 1 DM mice, were also not included in this work.

To further elucidate the ceRNA network of diabetic CECs, a subnetwork was extracted from the global ceRNA network. Our previous study showed that the inhibition of miR-181a-5p expression has a protective effect on corneal epithelium healing in type 1 DM mice. To further explore the ceRNA mechanism in the pathogenesis of delayed corneal epithelial wound healing, we focused on lncRNAs that were included in the GENCODE database and capable of binding miR-181a-5p based on databases (TargetScan and miRanda). Then, a novel lncRNA, Rik, with high expression abundance was selected as a biomarker for further investigation. Using the TargetScan and miRanda algorithms,

five DEGs were found to be regulated by miR-181a-5p. For these genes, their differential expression changes and mechanisms have been well elaborated in previous studies. For example, Serpine1 was correlated with Plau levels and the rates of diabetic epithelial wound closure.⁴⁰ Reduced expression of Sirt1 could delay diabetic corneal epithelial wound healing through the IGFBP3/IGF-1R/AKT pathway via P53.⁸ Hyperglycemia-suppressed Sema3c postponed diabetic corneal wound healing.³⁹ Hmgb1 is highly involved in diabetic CECs, and its blockade contributes to diabetic corneal epithelial wound healing.⁴² Given the classic role of lncRNA as a ceRNA, we hypothesize that Rik may accelerate epithelial wound closure in diabetic corneas by sponging miR-181a-5p. Figure 7 briefly displays how Rik functions.

To further elucidate the ceRNA mechanism, the potential mechanism and function of Rik were explored. The TKE2 cell line has been widely used in the research of corneal epithelial lesions.^{7,22,34–36,41,50,51} Naturally, the expression of miR-181a-5p and Rik was verified in both CECs and TKE2 cell lines by qRT-PCR, and the luciferase reporter assay confirmed the interaction between Rik and

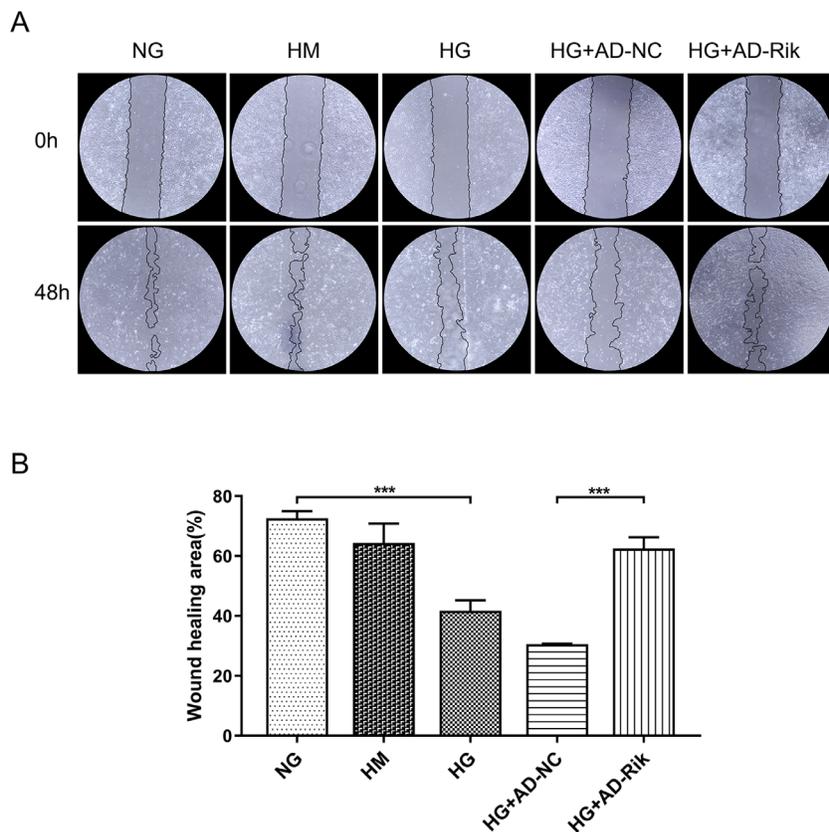


FIGURE 6. Overexpression of Rik promotes the TKE2 cell wound closure under hyperglycemic conditions. (A) Microscopy images of wounded TKE2 cells with Rik overexpression ($n = 3/\text{group}$). (B) Percentage of gap area and migration rate were calculated with Image J software.

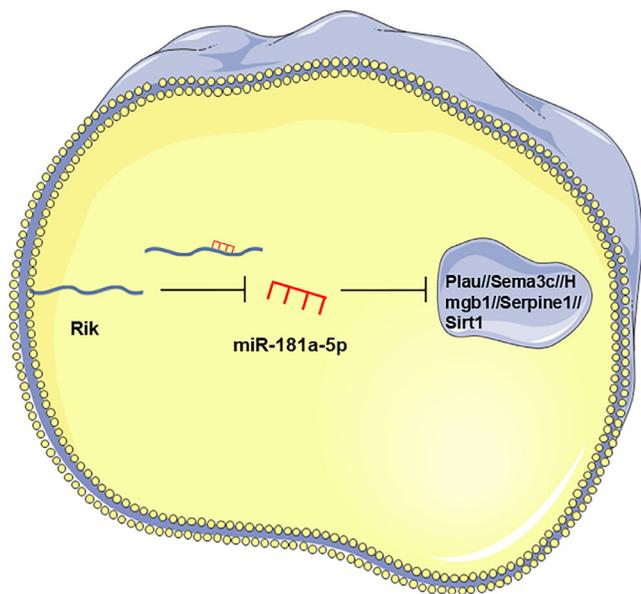


FIGURE 7. Function schematic model of Rik. This pattern outlines the process of Rik acting as a ceRNA in mediating corneal epithelial wound closure.

miR-181a-5p. RNA FISH experiments targeting Rik showed both cytoplasmic and nuclear dots, further suggesting that Rik might perform important functions through the ceRNA mechanism. In addition, the scratch assay experiment revealed that overexpression of Rik could significantly promote TKE2 cell migration in a hyperglycemic environment. It is worth noting that there was a significant migration of TKE2 cells after scratching at 24 hours, and the healing area of the cells was larger at 48 hours in our study, consistent with studies in numerous diabetic corneal epithelial lesions.³⁴⁻³⁶ TKE2 cells were cultured in keratinocyte serum-free medium (KSFM) supplemented with human recombinant epidermal growth factor and bovine pituitary extract.^{22,34} The complete medium contains no serum, which may result in cell growth not as fast as expected, warranting our further investigation.

Our study also presents some limitations. The sample size of the gene expression profile was modest, and no microarray or RNA-Seq data of DEMiRs or DEGs in CECs of type 1 DM mice were found in the GEO and Array Express databases. Moreover, some genes or miRNAs related to the physiopathology of diabetic CECs have not been verified by experiments in type 1 DM mice, although with high conservation across several animal species, none of them has been included in our study. All of these factors might hinder us from establishing a relevant and comprehensive regulatory ceRNA network. Given the low conservation of Rik (76%), its role in humans deserves further investigation. Finally,

the function and mechanism of a large number of lncRNAs located in the nucleus deserve further investigation.

To our knowledge, this is the first work revealing lncRNA profiling and the ceRNA network of diabetic corneal epithelial wound closure. A significantly downregulated lncRNA, Rik, was revealed to potentially activate diabetic corneal epithelial wound healing by sponging miR-181a-5p. Other lncRNAs aside from Rik might also rescue DM mice from corneal injury, warranting further studies.

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