MicroRNA-184 Regulates Corneal Lymphangiogenesis

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PURPOSE. MicroRNAs are a class of small noncoding RNAs that negatively regulate gene expression by binding to complementary sequences of target messenger RNA. Their roles in corneal lymphangiogenesis are largely unknown. This study was to investigate the specific role of microRNA-184 (mir-184) in corneal lymphangiogenesis (LG) in vivo and lymphatic endothelial cells (LECs) in vitro.

METHODS. Standard murine suture placement model was used to study the expressional change of mir-184 in corneal inflammatory LG and the effect of synthetic mir-184 mimic on this process. Additionally, a human LEC culture system was used to assess the effect of mir-184 overexpression on cell functions in vitro.

RESULTS. Expression of mir-184 was significantly downregulated in corneal LG and, accordingly, its synthetic mimic suppressed corneal lymphatic growth in vivo. Furthermore, mir-184 overexpression in LECs inhibited their functions of adhesion, migration, and tube formation in vitro.

CONCLUSIONS. These novel findings indicate that mir-184 is involved critically in LG and potentially could be used as an inhibitor of the process. Further investigation holds the promise for divulging new therapies for LG disorders, which occur inside and outside the eye.

Keywords: lymphangiogenesis, cornea, microRNA, lymphatic endothelial cell

The lymphatic network penetrates most tissues and its dysfunction is associated with a broad spectrum of disorders, such as cancer metastasis, inflammation, transplant rejection, hypertension, obesity, and lymphedema.^{1,2} After being neglected for centuries due to historical reasons and technical limitations, lymphatic research has gained significant attention and great progress in recent years. However, to date, few effective treatments are available for lymphatic disorders. Therefore, it is imperative to identify new regulators of lymphangiogenesis (LG; the formation of lymphatic vessels) in the hope of developing novel therapeutic strategies.

The cornea offers an ideal site for LG research. Due to its accessible location, transparent nature, and alymphatic feature under normal condition, this tissue provides a favorable model to study inducible lymphatic growth without having to distinguish from preexisting or background vessels.² Corneal LG can be induced by a number of pathologic insults, such as inflammation, infection, trauma, and chemical burns, and it is a primary mediator of transplant rejection.^{2–4}

MicroRNAs are a class of small noncoding RNAs that regulate gene expression by RNA silencing and posttranscriptional regulation.^{5,6} Their specific roles in the eye and eye-related diseases remain largely unknown. A recent study using microRNA arrays to compare mouse cornea to epithelial-rich footpads has identified microRNA-184 (mir-184) as the most abundantly expressed microRNA in the mouse cornea.⁷ The restrictive expression profile of mir-184 in the normal and alymphatic cornea^{7,8} has prompted us to evaluate its potential role in corneal LG and whether it can be used as an antilymphangiogenic factor.

We report the novel finding that mir-184 is significantly downregulated in corneal inflammatory LG and, accordingly, its synthetic mimic inhibits corneal lymphatic growth in vivo.

Moreover, mir-184 overexpression in human lymphatic endothelial cells (LECs) in vitro suppresses their functions of adhesion, migration, and tube formation. These results together reveal that mir-184 is a negative regulator of the lymphangiogenic process. Further investigation on this natural regulator holds the great promise for developing new and effective treatment for LG-related diseases in the body.

METHODS

Animals, LECs, and Reagents

Normal adult 6- to 8-week-old male BALB/c mice were purchased from Taconic Farms (Germantown, NY, USA). All mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the protocols approved by the Animal Care and Use Committee of the institute. Mice were anesthetized using a mixture of ketamine, xylazine, and acepromazine (50, 10, and 1 mg/kg body weight, respectively) for each surgical procedure. Human neonatal primary microdermal LECs were purchased from Lonza (Walkersville, MD, USA) and maintained in EGM-2MV cell culture medium (Lonza) according to manufacturer's instructions. Matrigel, collagen type I, and calcein AM were purchased from BD Biosciences (San Jose, CA, USA). Mir-184 mimic and control RNA were purchased from Dharmacon, Inc. (Lafayette, CO, USA) and Ambion (Austin, TX, USA).

Induction of Corneal LG and Pharmaceutical Intervention

The experiments were performed as described previously.^{9,10} A standard suture placement model was used to induce corneal

FIGURE 1. Mir-184 expression is downregulated in corneal inflammatory LG. (A) Real-time PCR results showing mir-184 is significantly downregulated in the inflamed cornea 2 weeks after suture placement. (B) Representative images of immunofluorescent microscopic analysis showing significantly reduced lymphatic vessels in the inflamed cornea after mir-184 mimic treatment. White dashed line: demarcation of the limbus between the cornea and conjunctiva. Scale bars: 200 μ m. Summarized data in terms of lymphatic invasion area are presented in (C). Data are expressed as mean $\pm$ SEM, * $P < 0.05$.

inflammatory LG. Briefly, three 11-0 nylon sutures (AROSurgical, Newport Beach, CA, USA) were placed into the stroma of central corneas without penetrating into the anterior chamber. Mice were randomized to receive subconjunctival injections of either mir-184 mimic (10 μ g; Dharmacon, Inc.) or control on days 0 and 3 after suture placement. Experiments were repeated twice with a total of six mice in each group.

Immunofluorescent Microscopic Assay and Lymphatic Quantification

The experiments were performed as reported previously.^{9,10} Briefly, whole-mount corneas were sampled at 1 week after suture placement and fixed in acetone for immunofluorescent staining. Lymphatic vessels were recognized by purified rabbit-anti-mouse LYVE-1 antibody, which was visualized by Cy3-conjugated donkey-anti-rabbit secondary antibody. Samples were covered with Vector Shield mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA).

Digital images were taken with an epifluorescence microscope (AxioImager M1; Carl Zeiss AG, Göttingen, Germany) and analyzed using the National Institutes of Health (NIH; Bethesda, MD, USA) ImageJ software (available in the public domain at <http://imagej.nih.gov/ij/>). The percentage scores of LG coverage areas were obtained by normalizing to control groups defined as being 100%. The differences were analyzed using the Mann-Whitney U test with $P < 0.05$ as significant.

Reverse Transcription and Real-Time PCR

Total RNA from LECs or central corneal epithelium was extracted using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using the miScript II RT Kit (Qiagen). Real-time PCR was performed using miScript SYBR Green PCR Kit with specific primers to mature mir-184 using the mir-184_1 miScript Primer Assay (Qiagen) and measured by the CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA). Relative expression of the mir-184 was calculated from the δ -Ct (threshold cycle) of the targeted gene normalized to the δ -Ct of the *RNU6B* reference gene.¹¹ Traditional PCR products also were run on a 2% agarose gel.

Mir-184 Ectopic Expression in LECs

Transfections were done with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. As reported previously, the transfection of either mir-184 mimic or control RNA was done overnight at 37°C in a 5% CO₂ humidified air incubator.⁹

Adhesion Assay

The experiment was performed as described previously.⁹ At 72 hours following the transfection, 100 μ L cells (3×10^5 cells/mL) were added to collagen type I-coated 96 plate wells and incubated for 30 minutes at 37°C. The plates then were washed several times and incubated with calcein (1 μ g/mL) in Hank's buffered salt solution (HBSS) for 30 minutes at room temperature. Plates were washed with PBS and fluorescent intensity from bound cells was measured with a Spectramax M5e microplate reader (Molecular Devices, Sunnyvale, CA, USA). Assays were performed in triplicate and repeated at least three times.

Migration Assay

At 72 hours following the transfection, a 200- μ L pipette tip was used to create linear wounds within LEC monolayers. Phase images of the scratches were taken at time 0 and 29 hours using a Zeiss Axio Observer A1 inverted microscope (Carl Zeiss AG). For better visualization of the scratch area by end of the study at 29 hours, the cells were stained with crystal violet. The TScratch program (Tobias Gebäck and Martin Michael Peter Schulz, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland) was used to determine the percent of the open area.¹²

Tube Formation Assay

As previously reported,^{9,10} 72 hours following transfection, LECs were seeded (2×10^4 cells/well) onto 96-well plates containing solidified Matrigel and monitored for 24 hours under a Zeiss Axio Observer A1 inverted microscope (Carl Zeiss AG). Phase images of tubes were taken and total tube

FIGURE 2. Overexpression of mir-184 in LECs in vitro. **(A)** Gel images of mir-184 PCR product 48 hours after transfection with mir-184 synthetic mimic. **(B)** Quantitative real-time PCR result showing significant increase of mir-184 in the LECs after transfection. Data are expressed as mean $\pm$ SEM, * $P < 0.05$.

lengths were analyzed by NIH ImageJ software. Assays were performed in triplicate and repeated at least three times.

Statistical Analysis

The mean difference was analyzed by Student's *t*-test using Prism software (GraphPad, La Jolla, CA, USA) unless otherwise indicated. The differences between the treatment and control groups were considered statistically significant when $P < 0.05$.

RESULTS

Mir-184 is Downregulated in Corneal LG

To investigate the role of mir-184 in corneal inflammatory LG, we first assessed the expressional change of mir-184 in the inflamed cornea following suture placement and lymphatic ingrowth. As shown in Figure 1A by real-time PCR analysis, the expression level of mir-184 in the inflamed cornea was significantly downregulated, compared to the normal condi-

FIGURE 3. Mir-184 overexpression inhibits LEC functions of adhesion and migration. **(A)** Summarized data showing significant suppression of LEC adhesion to collagen type I after transfection with mir-184 mimic. **(B, C)** Summarized data **(B)** and representative images **(C)** showing significant inhibition of LEC migration in a wound healing assay after transfection with mir-184 mimic. Scale bars: 500 μ m. Data are expressed as mean $\pm$ SD, * $P < 0.05$.

FIGURE 4. Mir-184 overexpression inhibits LEC tube formation. (A) Representative micrographs showing significant inhibition of LEC capillary tube formation on Matrigel after transfection with mir-184 mimic. Scale bars: 200 μ m. (B) Summarized data on total tubule length measurement. Data are expressed as mean $\pm$ SD, * P < 0.05.

tion (* P < 0.05). This result indicated that mir-184 may function as a natural inhibitor of corneal LG.

Synthetic Mimic of Mir-184 Suppresses Corneal LG In Vivo

To further explore whether mir-184 can be used as an inhibitor of corneal LG, we next assessed the effect of mir-184 administration on inflammatory LG using synthesized mir-184 mimic, which acts to emulate the effect of mir-184. As shown in Figures 1B and 1C, our results from whole-mount corneal immunofluorescent microscopic analysis demonstrated that subconjunctival delivery of mir-184 mimic significantly reduced the lymphatic invasion area in the inflamed corneas (* P < 0.05).

Ectopic Expression of Mir-184 in LECs In Vitro

We next used a human LEC culture system to study gain-of-function of mir-184 in vitro. To approach this, we first transfected LECs with mir-184 mimic, and confirmed enhanced expression of mir-184 in these cells by traditional and real-time PCR analysis (Fig. 2). As revealed by the agarose gel images in Figure 2A, transfected LECs with mir-184 mimic showed an abundant PCR product corresponding to mature mir-184. Figure 2B depicts the real-time PCR analysis confirming a significant fold increase of mir-184 expression in LECs after the transfection (* P < 0.05). These results indicated that our approach is suitable to ectopically express mir-184 in LECs for further gain-of-function studies, as presented below.

Mir-184 Overexpression Inhibits LEC Adhesion

Adhesion is an important function of LECs in the lymphangiogenic process. We next determined whether mir-184 regulates

LEC adhesion in vitro. At 72 hours following the transfection with mir-184 mimic or control RNA, LECs were subjected to a collagen I adhesion assay, as reported previously.⁹ Our results showed that mir-184 overexpression in LECs led to a significant reduction in cell adhesion (Fig. 3A, * P < 0.05), suggesting a negative inhibitory role of mir-184 in this function.

Mir-184 Overexpression Reduces LEC Migration

To assess whether mir-184 also is involved in LEC migration in vitro, we performed the wound healing scratch assay 72 hours following the transfection with mir-184 mimic or control RNA. As presented in Figures 3B and 3C, mir-184 transfected cells showed a significant decrease in the rate of wound closing with larger open area (* P < 0.05). These results indicated that mir-184 negatively regulates LEC migration as well.

Mir-184 Overexpression Suppresses LEC Tube Formation

We also examined the effect of mir-184 overexpression on the ability of LECs to organize into capillary-type tubes using a three-dimensional (3D) culture system. At 72 hours following the transfection with either mir-184 mimic or control RNA, LECs were seeded on Matrigel, a basement membrane matrix, and observed for 24 hours. As shown in Figures 4A and 4B, mir-184 ectopic expression revealed a significant reduction in total tubule length (* P < 0.05), confirming an inhibitory role of mir-184 in this important LEC function in vitro.

DISCUSSION

In summary, to our knowledge we provided the first evidence that mir-184 negatively regulates the LG process. We reported two important findings: Mir-184 expression is significantly downregulated in corneal inflammatory LG, and mir-184 mimic can be used to suppress corneal LG in vivo and LEC functions in vitro. Taken together, this study not only divulges mir-184 as a natural suppressor of LG, but also puts forth the use of mir-184 mimics as a novel strategy for LG therapy.

Mir-184 has a restrictive expression profile in the cornea, brain, and testes.^{7,8,13} Previously reported microarray analysis revealed that it is one of the most abundantly expressed microRNAs in the corneal epithelium.⁷ In the current study, we showed that mir-184 is significantly downregulated during corneal inflammatory LG, and it is highly indicated this microRNA may act to maintain the alymphatic status of the cornea under normal condition. Allied to this speculation is our additional data showing that reintroduction of mir-184 into the cornea suppresses lymphatic formation. As an immune privileged tissue, the cornea has developed mechanisms that actively maintain its lymphatic-free status under normal condition, which are yet to be fully explored and understood. Recently, it was reported that a soluble VEGFR-2 is secreted by the corneal epithelium and acts to suppress LG.¹⁴ This current study not only divulges mir-184 as a new and natural inhibitor of LG, but also indicates that multiple mechanisms and factors are involved in maintaining the alymphatic status of the cornea, which warrants further investigation.

It is known that mir-184 mutation results in corneal pathologies in keratoconus and the endothelial dystrophy, iris hypoplasia, congenital cataract and stromal thinning (EDICT) syndrome.^{15,16} To date, there has been no study linking mir-184 to corneal pathologic LG. Previously, it was reported that mir-184 is involved in ischemia-induced retinal angiogenesis, and the downstream targets of mir-184 mediated angiogenesis are Wnt-receptor Frizzled 7 (Fzd7) and VEGFA.^{17,18} In our

study, we did not detect a significant change in corneal angiogenesis as LG with mir-184 mimic treatment. It is yet to be determined whether factors of the Wnt family and/or VEGF family, such as VEGF-C, are the regulatory components of mir-184 for corneal LG.

Research on mechanisms of corneal LG has broad implications. The use of synthetic microRNA mimic as a treatment, also known as “microRNA replacement therapy,” has emerged as a promising approach for disease treatment and has gained significant attention in cancer therapy.¹⁹ MicroRNA replacement therapy focuses to reintroduce the microRNA to restore loss of function. In this study, using in vivo murine LG model and in vitro human primary LEC culture system, we put forth a similar therapeutic approach for LG interference and suggest mir-184 could be used for the treatment of lymphatic-related diseases, which occur widely inside and outside the eye.

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