A Potential Research Target for Scleral Remodeling: Effect of MiR-29a on Scleral Fibroblasts

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Keywords
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Abstract
Introduction: The purpose of this study was to determine whether miR-29a regulates cell survival and apoptosis and the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), MMP-2, and collagen I in scleral fibroblasts. Methods: We transfected scleral fibroblasts with the miR-29a mimic and inhibitor. The effects of miR-29a on cell proliferation and apoptosis were determined using the CCK-8 assay and flow cytometry, respectively. Quantitative polymerase chain reaction (qPCR) was used to determine whether miR-29a regulates the mRNA levels of PTEN, MMP-2, and collagen I. The protein expression of PTEN, MMP-2, and collagen I was also assessed by western blot analysis. Results: The results of CCK-8 showed that, at 0, 24, 48, and 72 h after transfection, the relative optical density values in the mimic group were 0.233 ± 0.005, 0.380 ± 0.008, 0.650 ± 0.040, and 0.906 ± 0.032, and in the inhibitor group were 0.272 ± 0.011, 0.393 ± 0.029, 0.597 ± 0.059, and 0.950 ± 0.101, respectively. The flow cytometry results showed that the apoptosis rates of each group were as follows: the mimic group (0.043 ± 0.007), the NC group (0.040 ± 0.006), the inhibitor group (0.032 ± 0.003), the inhibitor NC group (0.027 ± 0.010), the lipofectamine group (0.027 ± 0.005), and the blank group (0.031 ± 0.009). The qPCR results indicated that in the mimic group, PTEN (0.795 ± 0.182, \( p = 0.2783 \)), MMP-2 (0.621 ± 0.105, \( p = 0.0033 \)), and COL1A1 (0.271 ± 0.100, \( p = 0.0002 \)) expression decreased, whereas in the inhibitor group, PTEN (1.211 ± 0.100, \( p = 0.2614 \)), MMP-2 (1.161 ± 0.053, \( p = 0.1190 \)), and COL1A1 (1.7040 ± 0.093, \( p = 0.0003 \)) increased. Western blot analysis showed that in the mimic group, the expression of PTEN (0.392 ± 0.039, \( p < 0.0001 \)), MMP-2 (0.577 ± 0.017, \( p < 0.0001 \)), and COL1A1 (0.072 ± 0.006, \( p < 0.0001 \)) protein decreased, whereas in the inhibitor group, PTEN (1.043 ± 0.042, \( p = 0.9413 \)), MMP-2 (1.397 ± 0.075, \( p = 0.0002 \)), and COL1A1 (1.935 ± 0.081, \( p < 0.0001 \)) expression increased. Conclusion: MiR-29a inhibits the expression of PTEN, MMP-2, and collagen I on scleral fibroblasts, which may provide a basis studies in sclera.

Introduction
Myopia has gradually become one of the main ocular diseases causing vision impairment over the last 20 years throughout the world [1]. The incidence of myopia and high myopia, accompanied by ocular complications, is expected to increase simultaneously in the future [2].

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High myopia is generally defined as a spherical equivalent lens $<-6.0$ diopters or an ocular axial length $\geq 26.0$ mm [3, 4]. With a sharp rise in prevalence, myopia imparts a heavy economic burden to public health systems [5] and significant challenges to patients’ eyesight because it aggravates the risk of other pathologic changes in the eyes, such as cataracts, glaucoma, retinal detachment, and macular degeneration, which may lead to irreversible vision loss [6]. Therefore, there is an urgent need to understand the underlying molecular pathology of myopia.

While the exact causes of the increasing prevalence of myopia are unclear, accelerated scleral remodeling is considered one common factor that leads to myopia. The scleral thickness of the myopic eye [7, 8] decreases significantly with excessive ocular axial elongation to the extent that images of distant objects are focused in front of the retina, which results in blurred vision [9, 10]. These changes are related to decelerated synthesis and accelerated degradation of the scleral extracellular matrix (ECM) [11–13] and the decrease in collagen diameter [8, 14]. In mammals, the dry weight of collagen accounts for nearly 90% of the dry weight of the sclera [15, 16]. Collagen I is the major component of the extracellular matrix [17, 18] and a central part of the biomechanics of the sclera. Its dynamic change is particularly important for the progression of myopia and scleral collagen I is significantly decreased in myopic models [19]. The factors influencing scleral collagen I remain to be defined.

Matrix metalloproteinase-2 (MMP-2), a member of the MMP family, is involved in degrading the extracellular matrix. Increased MMP-2 levels generate weakened sclera as well as ECM remodeling in animal models of myopia [20, 21]. Furthermore, after the scleral fibroblasts were transfected by miR-29a, expression of MMP-2 in the cells would be inhibited, which may represent a therapeutic target for the prevention and treatment of myopia [22].

MicroRNAs (miRNAs) are a class of small noncoding RNAs containing approximately 22 nucleotides in length. They can bind to the complementary 3′-untranslated region of a targeted mRNA [23] to regulate a variety of pathophysiological events, such as differentiation, proliferation, and apoptosis. Disorders of miRNAs are closely related to a variety of diseases [24–26]. MiRNA plays an important role in ocular development and associated diseases. Previous studies have indicated that miRNA may serve as a biomarker for the diagnosis and treatment of myopia. The miR-29 clusters, which include miR-29a, miR-29b, and miR-29c, influence the development of myopia, and variations of miR-29a are associated with high myopia [27–29]. In addition, it was demonstrated that miR-29 regulates phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression in different cells including human retinal pigment epithelial cells [30–32]. TargetScan (http://www.targetscan.org), along with earlier studies, revealed that PTEN may be a target of miR-29; however, it remains to be determined whether PTEN is regulated by miR-29a on scleral fibroblasts.

PTEN normally acts as a tumor suppressor gene [33] and negatively regulates the PI3K/Akt signaling pathway to modulate various cellular activities, such as proliferation, apoptosis, migration, and ECM metabolism [34–36]. As previously described, scleral ECM remodeling plays an important role in myopia development and the PI3K/Akt signaling pathway is involved in the development of myopia [37, 38]. Consequently, PTEN may be involved in scleral fibroblast extracellular matrix remodeling leading to myopia.

Although the effects of miRNAs on the extracellular matrix and PTEN have been well established, their role in scleral fibroblasts has not been clarified. In the present study, we determined the effects of miR-29a on cell survival and apoptosis and the expression of PTEN, MMP-2, and collagen I on scleral fibroblasts, which provide insight into the molecular pathology of myopia.

Materials and Methods

Ethics

The study was approved by the Ethics Committee of Yongchuan Hospital of Chongqing Medical University.

Cell Culture

The human scleral fibroblasts were incubated in Dulbecco’s Modified Eagle Medium containing 4.5 g/L D-glucose (Gibco, USA) and supplemented with 10% fetal bovine serum (Biological Industries, Israel) and 1% penicillin-streptomycin solution (Beyotime, China) and incubated in a humidified atmosphere of 5% CO$_2$ at 37°C. The medium was changed every 2–3 days. When the cells reached 70–80% confluence, they were passaged using 0.25% trypsin-EDTA (Gibco).

Cell Transfection

Cell transfection in a 6-well plate was used as an example. The scleral fibroblasts were seeded into 6-well plates to obtain a cell density of approximately 50% at transfection. The medium was changed before transfection at 1.5 mL per well. SiRNAs, including miR-29a mimic, miR-29a inhibitor, NC, and inhibitor NC, were synthesized by GenePharma (Shanghai, China). The NC and inhibitor NC were negative controls with no homology to the target gene sequences. Five microliters of lipofectamine 2000 transfection reagent (Invitrogen, USA) was added into 245 µL of Opti-MEM reduced serum medium (Gibco) without antibiotics, and 5 µL siRNA was added into another 245 µL of the same type of me-
medium. After 5 min, they were mixed together and placed at room temperature for 20 min to form a transfection complex. Subsequently, the complex was immediately and evenly added to the 6-well plate containing the cells so that the final concentration of each siRNA was 50 nM. After 24–72 h, the cells from different groups were used for the subsequent experiments. The lipofectamine group only contained lipofectamine 2000 transfection reagent without siRNA, whereas the blank group did not contain either.

Cell Proliferation Assays
The scleral fibroblasts were seeded into 96-well plates at a density of 5,000 cells per well. After 24 h, the cells were transfected with siRNA and then incubated for 0–3 days. At a specific time each day, 10 µL of Cell Counting Kit-8 (CCK-8) reagent (DOJINDO, Japan) was added to each well along with 100 µL of medium. After 2 h of incubation, the optical density (OD) values at 450 nm were measured using a Varioskan Flash (Thermo Scientific, USA) plate reader.

Cell Apoptosis Assays
After 48 h of transfection, the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) was used to detect apoptosis. Briefly, cells were washed twice with ice-cold PBS after collection, resuspended using 100 µL of 1× binding buffer, and followed by incubation with 5 µL of Annexin V FITC and 5 µL PI for 15 min.
at room temperature in the dark. The detection of all samples was performed on a CytoFLEX flow cytometer (Beckman Coulter, USA), and the data were analyzed using FlowJo software version 10.4.0.

**Total RNA Isolation and Reverse Transcription**

RNA from the cells was extracted using Trizol reagent (Invitrogen) after 48 h of transfection. The RNA concentration and purity were assessed. The isolated RNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara, Japan). For the reverse transcription of miR-29a along with its reference gene, U6, gene-specific primers were synthesized (Sangon Biotech, China) and the sequences of miR-29a was 5′-GTCGTATCAGTG-CAGGGTCGAGGTATTTCGACTGGATACTAAGCCG-3′ and U6 was 5′-CTCAACTGGTGTCGAGTGCAATTTCAGTTGAGAAAAATATGG-3′.

**Quantitative Polymerase Chain Reaction**

Quantitative PCR (qPCR) was done in a 25 μL reaction containing 12.5 μL TB Green Premix Ex Taq II (Takara), 2 μL of cDNA, 1 μL of forward primer, and 1 μL of reverse primer, and supplemented with nuclease-free water using a CFX96 Real-Time System (BIO-RAD, USA). The reaction conditions were as follows: 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing/extension at 60°C for 30 s. The primers were used to amplify miR-29a, U6, PTEN, MMP2, collagen I, and GAPDH. Relative expression levels were determined using the 2−ΔΔCt method.

**Western Blot Analysis**

After cells were transfected for 48 h, total protein extracts were prepared in RIPA lysis buffer containing PMSF. The enhanced BCA Protein Assay Kit (Beyotime) was used to determine the protein concentrations. Next, 30 μg of protein was subjected to 10%
sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes. The membranes were blocked in 5% nonfat milk at room temperature for 1 h, followed by incubation with primary antibodies to PTEN, MMP2, and COL1A1 (Abcam, UK) overnight at 4°C. GAPDH was used as a control. The membranes were incubated with HRP-conjugated secondary antibodies (Abcam) at room temperature for 1 h and the blots were visualized with chemiluminescent HRP Substrate (Immobilon, USA) on a ChemiDoc XRS+ system (BIO-RAD). Gray values of the proteins were analyzed using Image J software.

Statistical Analysis
The above experiments were performed in triplicate. Data were analyzed by SPSS 25.0 as well as Prism 9.0. A one-way analysis of variance and unpaired t tests were used to compare differences among the groups. p values <0.05 were considered statistically significant. The results are expressed as the mean ± standard deviation.

Results

Expression of miR-29a on Scleral Fibroblasts after Transfection with Mimic and Inhibitor
After 48 h of transfection, the miR-29a levels in the different groups were measured using qPCR. The level of miR-29a in the mimic group was statistically significant (p = 0.0003; Fig. 1a). The level of miR-29a in the inhibitor group was 10-fold lower compared with that in the blank group and was statistically significant (p < 0.0001; Fig. 1b).

Effect of miR-29a on Proliferation and Apoptosis
To determine the effect of miR-29a on cell growth and apoptosis on scleral fibroblasts, we measured the OD value of cells at 0, 24, 48, and 72 h and the apoptosis rate at 48 h after transfection. At 0, 24, 48, and 72 h after transfection, the relative OD values for the mimic group (0.233 ± 0.005, 0.380 ± 0.008, 0.650 ± 0.040, 0.906 ± 0.032) and inhibitor group (0.272 ± 0.011, 0.393 ± 0.029, 0.597 ± 0.059, 0.950 ± 0.101) showed no significant difference compared with that of the blank group (p > 0.9999; Fig. 2). At 48 h after transfection, there was no significant difference in apoptosis rate among the mimic group (0.043 ± 0.007), inhibitor group (0.032 ± 0.003), or the blank group (0.031 ± 0.009) (p > 0.05; Fig. 3).

MiR-29a Influences the Expression of PTEN, MMP-2, and COL1A1 mRNA after Transfection
qPCR analysis was used to determine whether miR-29a regulates the expression of PTEN, MMP-2, and CO-
L1A1 on scleral fibroblasts. Compared with the blank group, the expression of PTEN was downregulated in the mimic group (0.795 ± 0.182, \( p = 0.2783 \)) and upregulated in the inhibitor group (1.211 ± 0.100, \( p = 0.2614 \)), with no significant difference. The expression of MMP-2 was significantly downregulated in the mimic group (0.621 ± 0.105, \( p = 0.0033 \)) while upregulated in the inhibitor group (1.161 ± 0.053, \( p = 0.1190 \)) without significance. The expression of COL1A1 was significantly downregulated in the mimic group (0.271 ± 0.100, \( p = 0.0002 \)) and significantly upregulated in the inhibitor group (1.704 ± 0.093, \( p = 0.0003 \)) as shown in Figure 4.

**Different Effects on the Expression of PTEN, MMP-2, and COL1A1 Protein following Transfection with miR-29a Mimic and miR-29a Inhibitor**

Western blot analysis was used to measure the intracellular expression of PTEN, MMP-2, and COL1A1 proteins. The analysis indicated that the expression of PTEN (0.392 ± 0.039, \( p < 0.0001 \)), MMP-2 (0.577 ± 0.017, \( p < 0.0001 \)), and COL1A1 (0.072 ± 0.006, \( p < 0.0001 \)) in the mimic group was significantly lower compared with that in the blank group. The expression of PTEN (1.043 ± 0.042, \( p = 0.9413 \)), MMP-2 (1.397 ± 0.075, \( p = 0.0002 \)), and COL1A1 (1.935 ± 0.081, \( p < 0.0001 \)) in the inhibitor group was significantly increased compared with that in the blank group.

![Fig. 5. Expression of PTEN, MMP-2, and COL1A1 protein was detected by western blot analysis. The expression of PTEN (0.392 ± 0.039, \( p < 0.0001 \)), MMP-2 (0.577 ± 0.017, \( p < 0.0001 \)), and COL1A1 (0.072 ± 0.006, \( p < 0.0001 \)) protein was lower in the mimic group than that in the blank group. The expression of PTEN (1.043 ± 0.042, \( p = 0.9413 \)), MMP-2 (1.397 ± 0.075, \( p = 0.0002 \)), and COL1A1 (1.935 ± 0.081, \( p < 0.0001 \)) protein was higher than that in the blank group, but there was no significant difference in PTEN expression.](image-url)
Discussion

In this study, we determined whether miR-29a influences cell survival and regulates the expression of PTEN, MMP-2, and collagen I on scleral fibroblasts. The results indicated that cell growth and apoptosis may not be affected by miR-29a, whereas it appears to suppress the expression of PTEN, MMP-2, and collagen I on scleral fibroblasts, which provides a novel finding for sclera research.

Numerous studies have revealed that scleral remodeling is an important factor in the development of myopia. Collagen I is the main component of the sclera [18]. The thinner scleral wall along with a longer eye axial causes myopia [10, 13]; hence, the metabolism of scleral collagen I is considered to be a key factor in myopia development. Some studies have indicated that miR-29 is closely associated with ocular diseases, including myopia. Zhang et al. [22] demonstrated that miR-29a suppresses the expression and secretion of MMP-2 in human scleral fibroblasts and retinal pigment epithelial cells. Furthermore, Xie et al. [28] demonstrated that the miR-29a rs157907 A/G polymorphism is associated with the reduced risk of high myopia in the Chinese population. Genipin may contribute to the expression of COL1A1 by downregulating the miR-29 cluster [20], and was shown to be a safe and effective treatment for myopia [39]. In the present study, we found that the downregulation of miR-29a promoted the expression of collagen I in cells, which was consistent with that of previous studies. This result suggested that miR-29a regulated the expression of collagen I, which provided insight into the molecular pathology of myopia.

The expression and activity of PTEN are regulated by several factors including miRNAs, phosphorylation, acetylation, oxidation, and ubiquitination [40]. MiR-29 upregulates the expression of PTEN and downregulates collagen I simultaneously in hepatic stellate cells, thus ameliorating liver fibrosis [41]. Zhao et al. [42] used a luciferase reporter assay to show that miR-29a negatively regulates the expression of PTEN through direct targeting. At the mRNA level, results indicated that the upregulation of miR-29a significantly inhibited the expression of MMP-2 and collagen I and downregulation of miR-29a only increased the expression of collagen I. In addition, while the results were not statistically significant, expression of PTEN was lower in the mimic group compared with that in the blank group, and the levels of PTEN and MMP-2 were higher in the inhibitor group compared with that in the blank group. At the protein level, upregulation of miR-29a suppressed the expression of PTEN, MMP-2, and collagen I and downregulation of miR-29a only significantly increased the expression of MMP-2 and collagen I, whereas it had no significant effect on PTEN expression. It is well-known that eukaryotic regulation of gene expression occurs at different times and places, and these events do not take place simultaneously, as it is a very sophisticated and complex process. The regulation of gene expression plays an important role in the development of myopia. Therefore, if any factor is affected, many cellular events may be influenced, which can lead to the development of disease. Based on our results, the expression of PTEN mRNA and protein was not completely synchronized, whereas the effects of mimic and inhibitor did not occur simultaneously.

Previous studies have indicated that MMP-2 could degrade scleral collagen I, leading to scleral remodeling during the development of myopia. For example, genipin upregulated collagen I expression by downregulating miR-29 cluster and MMP-2 expression [20]; hence, the expression of MMP-2 and collagen I showed opposite trends. Interestingly, in our research, we found that upregulation of miR-29a inhibited both the expression of MMP-2 and the expression of collagen I, which was inconsistent with the abovementioned results. Because gene expression regulation complex, perhaps the inhibition of miR-29a on collagen I was superior to that of MMP-2, so the increase of scleral collagen I after MMP-2 downregulation by higher levels of miR-29a was offset by the direct inhibition of miR-29a on collagen I, which ultimately led to the downregulation of collagen I.

Zhang et al. [22] found that miR-29a had no significant effect on the growth of scleral fibroblasts. The growth, differentiation, and proliferation of scleral fibroblasts are affected by many factors including hypoxia, TGF-β1, and mechanical stress. In the present study, we found that miR-29a did not significantly influence the survival of scleral fibroblasts. The influence of miR-29a on cell number may be more indicative of the effect of miR-29a on intracellular proteins rather than changes in the number of cells affecting the expression of these proteins.

This study only focused on the regulation of miR-29a on PTEN, MMP-2, and collagen I, as well as the growth of scleral fibroblasts in vitro. It also lacked animal models for vivo experiments and an analysis of clinical specimens. Moreover, we only confirmed that miR-29a down-
regulated PTEN and collagen I on scleral fibroblasts but did not specify the regulatory mechanism. Moreover, we did not determine whether PTEN affected proliferation, apoptosis, and the expression of collagen I on scleral fibroblasts. Further studies are needed to analyze differential expression associated with miR-29a in the aqueous humor of high myopic patients and their counterparts. Establishing myopia animal models will also enable us to determine whether miR-29a affects collagen I via targeting PTEN on scleral fibroblasts and will clarify the underlying mechanism. In conclusion, we demonstrated that miR-29 regulated the expression of PTEN, MMP-2, and collagen I on scleral fibroblasts, which would provide a method to study scleral remodeling.

**Statement of Ethics**

The cell lines were purchased from Shanghai GuanDao Biological Engineering Co., Ltd. This study protocol followed the Declaration of Helsinki and it was reviewed and approved by the Ethics Committee of Yongchuan Hospital of ChongQing Medical University, and the approval number was Ethics Review No. 112, 2021.

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**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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**Author Contributions**

Qiaying Yang conducted experiments and completed the paper. Sha Lv, Huirong Zhu, and Liming Zhang participated in the analysis as well as interpretation of experimental data. Hua Li and Shengfang Song revised the work critically.

**Data Availability Statement**

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.
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