Original Paper

Genome-Wide Identification of Circular RNAs as a Novel Class of Putative Biomarkers for an Ocular Surface Disease

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Key Words

Pterygium • Circular RNA • Pterygium fibroblast • Pterygium epithelium

Abstract

Background/Aims: Pterygium is a common ocular surface disease with an unknown etiology and threatens vision as it invades into the cornea. Circular RNAs (circRNAs) are a novel class of RNA transcripts that participate in several physiological and pathological processes. However, the role of circRNAs in pathogenesis of pterygium remains largely unknown.**Methods:** Genome-wide circRNA expression profiling was performed to identify pterygium-related circRNAs. GO analysis, pathway analysis, and miRNA response elements analysis was performed to predict the function of differentially expressed circRNAs in pterygium. MTT assays, Ki67 staining, Transwell assay, Hoechst 33342 staining, and Calcein-AM/PI staining were performed to determine the effect of circRNA silencing on pterygium fibroblast and epithelial cell function. **Results:** Approximately 669 circRNAs were identified to be abnormally expressed in pterygium tissues. GO analysis demonstrated that the host genes of differentially expressed circRNAs were targeted to extracellular matrix organization (ontology: biological process), cytoplasm (ontology: cellular component), and protein binding (ontology: molecular function). Pathway analysis showed that dysregulated circRNAs-mediated regulatory networks were mostly enriched in focal adhesion signaling pathway. Notably, circ_0085020 (circ-LAPTM4B) was shown as a potential biomarker for pterygium. circ_0085020 (circ-LAPTM4B) silencing affected the viability, proliferation, migration, and apoptosis of pterygium fibroblast and epithelial cells in vitro. **Conclusions:** This study provides evidence that circRNAs are involved in the pathogenesis of pterygium and might constitute promising targets for the therapeutic intervention of pterygium.© 2018 The Author(s)
Published by S. Karger AG, Basel

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Introduction

Pterygium is a common ocular surface disease only observed in humans. Pterygium is characterized by the overgrowth of conjunctiva into the cornea. It affects visual function in the advanced cases, leading to redness and irritation [1]. Although the pathogenesis of pterygium is still uncertain, recent studies have provided some insights into the pathogenesis. Aberrant cell proliferation, defects in wound healing, cell transformation, abnormal matrix remodeling, angiogenesis, oxidative stress, and genetic susceptibility has been implicated in the formation and development of pterygium [2].

Current methods for pterygium treatment mainly include surgical resection combined with conjunctival autograft, mitomycin C administration, or other adjunctive forms of treatment [3-5]. However, the high recurrence of surgical management means that that nonsurgical method for pterygia treatment might be beneficial, particularly in these countries where medical resources are not well developed. Thus, further studies are still required to clarify the mechanism of the formation and development of pterygium.

More importantly, pterygium research has important and widespread clinical significance. The pathogenesis of pterygium is usually associated with abnormal fibroblast proliferation and matrix deposition. Its clinical severity is determined by the amount of fibrous tissues, implying that pterygium is a type of fibrotic disease [6, 7]. Epidemiological data, ray tracing models and histopathological changes have shown that chronic UV exposure is a widely accepted aetiological factor in the pathogenesis of pterygium. Pterygium shares many common features with UV damaged skin. Compared to other organs affected by fibrosis or photo-damage, it is accessible to obtain human pterygium tissues through surgical excision [8, 9]. Thus, pterygium is also a very suitable and relevant model for dissecting the molecular mechanism of fibrosis and photo-damage. Clarifying the mechanism of pterygium formation would also have important implications for the pathogenesis of other diseases, such as skin photo-damage, liver, kidney, and lung fibrotic diseases.

Circular RNAs (circRNAs) is a class of non-coding RNAs that are widely expressed in mammals. They are generated by the joining of the 5' splice site with the 3' splice site via back-splicing [10]. circRNA expression is often highly conserved across species and appear highly stable comparing to their linear parental transcripts [11, 12]. Circular RNAs participate in a wide range of developmental and physiological processes, including fibrosis, cell proliferation, apoptosis, differentiation, and angiogenesis [13, 14]. Abnormal circRNA expression has been shown in several human disorders, such as cancers, inflammatory diseases, and neurological diseases [15-17]. However, the characterization and function of circRNAs in pterygium is still unknown.

In this study, we collected the paired pterygium and normal conjunctiva to determine the role of circRNAs in the pathogenesis of pterygium. Approximately 669 circRNAs were found to be abnormally expressed in pterygium tissues. We characterized one circular, circ_0085020 (circ-LAPTM4B), which was significantly up-regulated in pterygium tissues. Functional assays revealed that circ_0085020 (circ-LAPTM4B) could regulate pterygium fibroblast and epithelial cell function *in vitro*. This study shows that circRNAs are potential targets for the therapeutic intervention of pterygium.

Materials and Methods

Specimen collection

This study was approved by Nanjing Medical University Committee for Research Ethics. All patients were given the informed consent before inclusion. The inclusion criteria were the presence of untreated primary pterygium, clinical indication for conventional excision of pterygium with auto-transplantation of conjunctiva. The corneal ingrowth in patients was ranging from 2 mm to 4 mm. A small piece of normal conjunctival tissue was excised as control tissue. The collected samples were transferred to 1.5 ml tubes and stored at -80 °C for further analysis.

RNA isolation

Total RNA was isolated from the clinical samples using TRIzol reagent (Ambion, Austin, TX, US) according to the manufacturer's protocol. The integration of RNAs for microarray analysis was estimated by the Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). The purity and quantity of other RNAs was estimated by the NanoDrop ND-1000 (NanoDrop, Wilmington, USA). The integrity of total RNAs was also estimated by the electrophoresis on denaturing agarose gel.

Microarray analysis

Total RNAs were extracted from 3 paired pterygium and normal conjunctiva. The corneal ingrowth in patients was 4 mm, 3 mm, and 4.5 mm, respectively. The patients include two males (age: 48 and 54) and one female (age: 46). Total RNAs were treated with RNase R to remove the linear RNAs. The enriched circRNAs were amplified and transcribed into fluorescent cRNA, and then purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The Cy3-labeled cRNAs were hybridized onto the SBS Human Circular RNA Microarray using Gene Expression Hybridization Kit (Agilent technologies, Santa Clara, CA, US). The slides were washed after hybridization, and scanned by Agilent Microarray Scanner (Agilent technologies, Santa Clara, CA, US). All data were extracted using the Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US).

Bioinformatics analysis

Circular RNA expression profiles were compared by two-way ANOVA. The expression level was given as average normalized (log transformed) signal intensity. Correction for multiple testing was conducted using the false discovery rate (FDR) controlling procedure. GO analysis and pathway analysis of the host genes of circRNAs was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov>). The binding pattern between miRNAs and circRNAs were predicted based on the miRanda and TargetScan algorithm.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNAs from the pterygium and normal conjunctival tissues were reversely transcribed using PrimeScript RT reagent Kit (Takara). qPCRs were conducted using SYBR Premix Ex Taq (Takara) in the PikoReal Real-Time PCR System (Thermo Scientific). The divergent primers for circRNA quantification were designed based on the backsplice junction sequence of circRNAs. The relative expression change of each circRNA was calculated by the cycle threshold method ($\Delta\Delta Ct$ method).

Isolation and culture of pterygium epithelium and fibroblast

Primary epithelial and fibroblasts were isolated using the explants methods with slight modifications [18, 19]. In brief, the fresh pterygium sample was cut into small pieces (1-2 mm in diameter), washed in Hank's solution, and placed in a culture dish as explants in DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Epithelial cells migrated from explants between 3 and 5 days and showed cobblestone morphology. Fibroblasts migrated from the pterygium body between 7 to 14 days and showed elongated spindle-shaped morphology.

Cell viability assay

Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium-bromide assay (MTT) [20]. Briefly, pterygium epithelium or fibroblasts were plated at a density of about 1.5×10^4 cells per well in 96-well plates. After the required treatments, these cells were incubated with MTT (0.5 mg/ml) for 3 h at 37°C, and then DMSO solution was added to dissolve formazan crystals. The absorbance was detected at absorbance of 570 nm by a microplate reader (Molecular Devices).

Hoechst staining

After the required treatments, pterygium epithelium or fibroblast were fixed in 4% formaldehyde for 10 min, and then permeabilized using Triton-X 100 for 15 min. These cells were stained with Hoechst 33342 (150 µg/ml) for 10 min, and then washed with PBS buffer. The staining result was observed using an Olympus IX-73 microscope [21].

Propidium iodide (PI)/Calcein-AM double staining

After the required treatments, pterygium epithelium or fibroblasts were fixed with 4% formaldehyde for 10 min, stained with Calcein-AM solution (10 $\mu\text{mol/L}$) for 10 min, and then stained with PI solution (10 $\mu\text{mol/L}$) for additional 10 min. The live cells were observed at the excitation filter of 490 nm. Apoptotic cells were observed at the excitation filter of 545 nm [22].

Cell migration assay

Pterygium epithelium or fibroblasts were placed in the upper chamber of a Transwell (5×10^4 cells per chamber, Corning) coated with fibronectin (1 $\mu\text{g/ml}$). After the required treatment, pterygium epithelium or fibroblasts were allowed for migrating for 8 h at 37°C. The non-migrated cells were scraped off the upper surface of the membrane. The migrated cells remaining on the bottom surface were stained with the crystal violet, and then observed using an Olympus IX-73 microscope [23].

Statistical analysis

The statistical difference was determined by Student's *t* test (when 2 groups were compared) or one-way ANOVA test (when >2 groups were compared). *Post hoc* Tukey analyses were performed when $P < 0.05$. $P < 0.05$ was considered statistically significant.

Results

Identification of differentially expressed circRNAs between normal conjunctiva and pterygium

To identify pterygium-related circular RNAs, we performed circRNA expression profiles between normal conjunctiva and pterygium tissues using the SBC Human ($4 \times 180\text{K}$) Circular RNA Microarray (Shanghai Biotechnology Co., Ltd.). The box plots show the distribution of expression levels for all samples in the circRNA microarray dataset (Fig. 1A). We used the volcano plot to filter differentially expressed circRNAs between normal conjunctiva and pterygium tissues (Fig. 1B). We set a threshold as fold change greater than 2.0, and identified 669 differentially expressed circRNAs, including 341 up-regulated circRNAs and 328 down-regulated circRNAs (Table 1, conjunctiva versus pterygium). We then used the Top 20 dysregulated circRNAs to perform hierarchical clustering analysis. The up-regulated or down-regulated circRNAs were clustered into the same samples (Fig. 1C). To verify the results of microarray data, we randomly selected 20 dysregulated

Fig. 1. Identification of differentially expressed circRNAs between normal conjunctiva and pterygium (A) The box plot shows the distribution of circular RNA expression levels for all samples in the microarray dataset. The box plot consists of boxes with a central line and two tails. The central line represents the median of the data, whereas the tail represents the upper and lower quartiles. (B) The volcano plot shows differentially expressed circRNAs between normal conjunctiva and pterygium samples. (C) Heatmaps were generated through the hierarchical cluster analysis to show the top 20 dysregulated circRNAs between normal conjunctiva and pterygium samples. Red indicates up-regulated circular RNAs, and green indicates down-regulated circular RNAs. (D) qRT-PCRs were used to detect the expression pattern of the top 20 dysregulated circRNAs using the normal conjunctiva samples and pterygium samples for microarray.

Table 1. Differentially expressed circRNAs between paired conjunctiva and pterygium

circRNA	Fold change	circRNA	Fold change	circRNA	Fold change	circRNA	Fold change
Up-regulated							
hsa_circ_0085019	17.01	hsa_circ_0014715	3.22	hsa_circ_0060059	2.57	hsa_circ_0016486	2.30
hsa_circ_0010170	12.01	hsa_circ_0091055	3.19	hsa_circ_0069843	2.57	hsa_circ_0042594	2.30
hsa_circ_0087653	10.71	hsa_circ_0035918	3.19	hsa_circ_0045762	2.56	hsa_circ_0014613	2.29
hsa_circ_0085013	8.90	hsa_circ_0042563	3.18	hsa_circ_0031778	2.56	hsa_circ_0023763	2.29
hsa_circ_0085020	8.88	hsa_circ_0082590	3.18	hsa_circ_0031031	2.55	hsa_circ_0043325	2.29
hsa_circ_0031978	8.05	hsa_circ_0060231	3.17	hsa_circ_0070498	2.55	hsa_circ_0001145	2.28
hsa_circ_0069798	7.98	hsa_circ_0015173	3.17	hsa_circ_0031013	2.54	hsa_circ_0081998	2.28
hsa_circ_0015295	7.05	hsa_circ_0076144	3.12	hsa_circ_0033397	2.54	hsa_circ_0058178	2.28
hsa_circ_0015288	7.03	hsa_circ_0082378	3.11	hsa_circ_0059979	2.54	hsa_circ_0057143	2.27
hsa_circ_0015310	6.90	hsa_circ_0022450	3.10	hsa_circ_0031265	2.54	hsa_circ_0010098	2.27
hsa_circ_0071473	6.27	hsa_circ_0007473	3.09	hsa_circ_0052770	2.54	hsa_circ_0063171	2.26
hsa_circ_0027279	6.19	hsa_circ_0001448	3.08	hsa_circ_0052775	2.53	hsa_circ_0042404	2.26
hsa_circ_0088312	6.19	hsa_circ_0083345	3.07	hsa_circ_0019062	2.53	hsa_circ_0015001	2.25
hsa_circ_0015306	6.09	hsa_circ_0059911	3.07	hsa_circ_0030778	2.52	hsa_circ_0030629	2.25
hsa_circ_0084390	5.97	hsa_circ_0036939	3.05	hsa_circ_0002679	2.52	hsa_circ_0042699	2.25
hsa_circ_0015305	5.88	hsa_circ_0059987	3.05	hsa_circ_0061373	2.52	hsa_circ_0083287	2.25
hsa_circ_0008786	5.60	hsa_circ_0046913	3.05	hsa_circ_0030666	2.51	hsa_circ_0052604	2.25
hsa_circ_0039233	5.48	hsa_circ_0015860	3.04	hsa_circ_0084446	2.51	hsa_circ_0052570	2.24
hsa_circ_0030352	5.46	hsa_circ_0009755	3.04	hsa_circ_0074493	2.51	hsa_circ_0052741	2.23
hsa_circ_0041603	5.45	hsa_circ_0042252	3.03	hsa_circ_0061372	2.50	hsa_circ_0023688	2.23
hsa_circ_0056937	5.42	hsa_circ_0017303	3.03	hsa_circ_0054135	2.49	hsa_circ_0083799	2.23
hsa_circ_0000142	5.17	hsa_circ_0008019	2.99	hsa_circ_0059505	2.48	hsa_circ_0083560	2.22
hsa_circ_0076138	5.11	hsa_circ_0070608	2.98	hsa_circ_0003538	2.48	hsa_circ_0057471	2.22
hsa_circ_0029427	5.08	hsa_circ_0036384	2.95	hsa_circ_0012617	2.48	hsa_circ_0052878	2.21
hsa_circ_0087883	4.60	hsa_circ_0014717	2.95	hsa_circ_0072097	2.47	hsa_circ_0035994	2.21
hsa_circ_0068028	4.55	hsa_circ_0060636	2.92	hsa_circ_0060275	2.47	hsa_circ_0076987	2.21
hsa_circ_0021414	4.51	hsa_circ_0008988	2.90	hsa_circ_0009620	2.47	hsa_circ_0088711	2.21
hsa_circ_0056875	4.47	hsa_circ_0022437	2.90	hsa_circ_0021902	2.46	hsa_circ_0078241	2.20
hsa_circ_0004930	4.44	hsa_circ_0057730	2.90	hsa_circ_0068787	2.45	hsa_circ_0010413	2.20
hsa_circ_0016479	4.37	hsa_circ_0024717	2.89	hsa_circ_0061201	2.45	hsa_circ_0030036	2.20
hsa_circ_0056156	4.33	hsa_circ_0017800	2.89	hsa_circ_0032891	2.44	hsa_circ_0047418	2.20
hsa_circ_0059375	4.33	hsa_circ_0002014	2.89	hsa_circ_0056614	2.44	hsa_circ_0058420	2.20
hsa_circ_0087880	4.31	hsa_circ_0070268	2.89	hsa_circ_0061881	2.43	hsa_circ_0063056	2.20
hsa_circ_0057237	4.27	hsa_circ_0015435	2.88	hsa_circ_0031856	2.43	hsa_circ_0009700	2.19
hsa_circ_0031698	4.20	hsa_circ_0008807	2.86	hsa_circ_0013908	2.43	hsa_circ_0028884	2.19
hsa_circ_0063794	4.17	hsa_circ_0006336	2.85	hsa_circ_0029423	2.43	hsa_circ_0024788	2.19
hsa_circ_0087881	4.17	hsa_circ_0046147	2.85	hsa_circ_0021564	2.42	hsa_circ_0032776	2.19
hsa_circ_0016062	4.11	hsa_circ_0021916	2.84	hsa_circ_0011218	2.42	hsa_circ_0046932	2.19
hsa_circ_0002999	4.10	hsa_circ_0070024	2.83	hsa_circ_0045602	2.41	hsa_circ_0034955	2.19
hsa_circ_0047466	4.09	hsa_circ_0029618	2.83	hsa_circ_0024180	2.41	hsa_circ_0084274	2.18
hsa_circ_0014540	4.08	hsa_circ_0008539	2.82	hsa_circ_0059985	2.40	hsa_circ_0054278	2.18
hsa_circ_0029908	4.05	hsa_circ_0017285	2.82	hsa_circ_0014260	2.40	hsa_circ_0035916	2.17
hsa_circ_0083394	4.00	hsa_circ_0080270	2.82	hsa_circ_0013512	2.39	hsa_circ_0083810	2.17
hsa_circ_0076145	3.95	hsa_circ_0085501	2.82	hsa_circ_0010811	2.38	hsa_circ_0033384	2.16
hsa_circ_0036594	3.95	hsa_circ_0014135	2.81	hsa_circ_0052767	2.38	hsa_circ_0016435	2.16
hsa_circ_0063421	3.86	hsa_circ_0020299	2.81	hsa_circ_0059655	2.38	hsa_circ_0074887	2.16
hsa_circ_0059373	3.83	hsa_circ_0056606	2.81	hsa_circ_0063262	2.38	hsa_circ_0043861	2.16
hsa_circ_0084770	3.79	hsa_circ_0022436	2.81	hsa_circ_0076995	2.38	hsa_circ_0083317	2.15
hsa_circ_0065564	3.76	hsa_circ_0005565	2.80	hsa_circ_0054668	2.38	hsa_circ_0072621	2.15
hsa_circ_0016118	3.70	hsa_circ_0072256	2.80	hsa_circ_0013157	2.37	hsa_circ_0025852	2.15
hsa_circ_0000410	3.66	hsa_circ_0022446	2.79	hsa_circ_0091220	2.37	hsa_circ_0056417	2.15
hsa_circ_0016187	3.66	hsa_circ_0014723	2.77	hsa_circ_0053883	2.36	hsa_circ_0025062	2.15
hsa_circ_0021565	3.62	hsa_circ_0012611	2.77	hsa_circ_0000466	2.36	hsa_circ_0084042	2.15
hsa_circ_0077876	3.60	hsa_circ_0054903	2.76	hsa_circ_0036495	2.35	hsa_circ_0054753	2.14
hsa_circ_0044683	3.58	hsa_circ_0051045	2.76	hsa_circ_0013510	2.35	hsa_circ_0001646	2.14
hsa_circ_0035980	3.56	hsa_circ_0057628	2.74	hsa_circ_0008763	2.35	hsa_circ_0050220	2.13
hsa_circ_0082595	3.54	hsa_circ_0014277	2.72	hsa_circ_0034578	2.35	hsa_circ_0002107	2.13
hsa_circ_0061884	3.52	hsa_circ_0015141	2.72	hsa_circ_0008758	2.34	hsa_circ_0026138	2.13
hsa_circ_0035012	3.51	hsa_circ_0013906	2.71	hsa_circ_0047419	2.34	hsa_circ_0031101	2.13
hsa_circ_0057923	3.48	hsa_circ_0086754	2.71	hsa_circ_0041929	2.34	hsa_circ_0084425	2.12
hsa_circ_0035991	3.47	hsa_circ_0011975	2.69	hsa_circ_0026663	2.33	hsa_circ_0004168	2.12
hsa_circ_0059908	3.46	hsa_circ_0090981	2.69	hsa_circ_0060254	2.33	hsa_circ_0063590	2.12
hsa_circ_0070617	3.43	hsa_circ_0014134	2.68	hsa_circ_0060095	2.33	hsa_circ_0078502	2.12
hsa_circ_0014270	3.42	hsa_circ_0054277	2.66	hsa_circ_0083290	2.33	hsa_circ_0011408	2.12
hsa_circ_0039483	3.41	hsa_circ_0013583	2.66	hsa_circ_0021897	2.33	hsa_circ_0028019	2.12
hsa_circ_0029718	3.33	hsa_circ_0061678	2.65	hsa_circ_0031428	2.33	hsa_circ_0053274	2.12
hsa_circ_0003959	3.33	hsa_circ_0033385	2.65	hsa_circ_0008836	2.32	hsa_circ_0012799	2.10
hsa_circ_0089340	3.32	hsa_circ_0005957	2.64	hsa_circ_0059664	2.32	hsa_circ_0085324	2.10
hsa_circ_0008595	3.32	hsa_circ_0077869	2.63	hsa_circ_0083804	2.32	hsa_circ_0052029	2.10
hsa_circ_0002604	3.31	hsa_circ_0062773	2.63	hsa_circ_0084109	2.32	hsa_circ_0078399	2.10
hsa_circ_0057728	3.30	hsa_circ_0019541	2.62	hsa_circ_0052713	2.32	hsa_circ_0084451	2.10
hsa_circ_0023585	3.29	hsa_circ_0000535	2.62	hsa_circ_0025214	2.32	hsa_circ_0069750	2.09
hsa_circ_0023762	3.27	hsa_circ_0020360	2.61	hsa_circ_0084936	2.31	hsa_circ_0020705	2.09
hsa_circ_0034691	3.27	hsa_circ_0022664	2.60	hsa_circ_0056410	2.31	hsa_circ_0059158	2.09
hsa_circ_0000101	3.27	hsa_circ_0013910	2.60	hsa_circ_0083288	2.31	hsa_circ_0077381	2.08
hsa_circ_0017288	3.25	hsa_circ_0072592	2.59	hsa_circ_0079489	2.31	hsa_circ_0008820	2.08
hsa_circ_0067780	3.24	hsa_circ_0072265	2.58	hsa_circ_0077728	2.30	hsa_circ_0039258	2.07
hsa_circ_0000412	3.24	hsa_circ_0013911	2.58	hsa_circ_0026128	2.30	hsa_circ_0064110	2.06
hsa_circ_0029711	3.23	hsa_circ_0060053	2.58	hsa_circ_0034758	2.30	hsa_circ_0031429	2.06
hsa_circ_0050051	3.23	hsa_circ_0076990	2.57	hsa_circ_0029623	2.30	hsa_circ_0036063	2.06
hsa_circ_0063626	2.05	hsa_circ_0006979	2.02	hsa_circ_0052727	2.05	hsa_circ_0016799	2.06
hsa_circ_0070908	2.04	hsa_circ_0027524	2.01	hsa_circ_0078266	2.05	hsa_circ_0022908	2.06
hsa_circ_0084444	2.03	hsa_circ_0023708	2.01	hsa_circ_0043192	2.05	hsa_circ_0081427	2.06
hsa_circ_0022933	2.03	hsa_circ_0072788	2.01	hsa_circ_0087052	2.05	hsa_circ_0069496	2.00
hsa_circ_0023518	2.03	hsa_circ_0043211	2.00	hsa_circ_0030686	2.00	hsa_circ_0030167	2.00
hsa_circ_0049078	2.00						

continued

circRNAs, including 10 up-regulated circRNAs and 10 down-regulated circRNAs for expression verification. 16 of 20 circRNA expression pattern were verified by qRT-PCRs, which showed similar expression pattern as previously detected by microarrays (Fig. 1D).

GO analysis and pathway analysis of the host genes of dysregulated circRNAs

The majority of circRNAs are generated from the exonic or intronic sequences of their host genes. Several circRNAs have shown as important regulators of the expression of their

cicRNA	Fold change	cicRNA	Fold change	cicRNA	Fold change	cicRNA	Fold change
Down-regulated							
hsa_circ_0087198	79.74	hsa_circ_0058201	2.54	hsa_circ_0023867	3.52	hsa_circ_0021876	2.12
hsa_circ_0025454	26.11	hsa_circ_0045686	2.54	hsa_circ_0089548	3.49	hsa_circ_0005937	2.11
hsa_circ_0002141	12.13	hsa_circ_0048089	2.54	hsa_circ_0067022	3.49	hsa_circ_0008331	2.11
hsa_circ_0056159	11.10	hsa_circ_0038727	2.53	hsa_circ_0023276	3.48	hsa_circ_0089246	2.11
hsa_circ_0064840	9.40	hsa_circ_0028128	2.52	hsa_circ_0058138	3.46	hsa_circ_0026337	2.10
hsa_circ_0034472	9.38	hsa_circ_0035165	2.52	hsa_circ_0075368	3.43	hsa_circ_0021409	2.10
hsa_circ_0009558	9.08	hsa_circ_0043819	2.52	hsa_circ_0082048	3.41	hsa_circ_0021300	2.10
hsa_circ_0062162	8.87	hsa_circ_0001555	2.51	hsa_circ_0058153	3.41	hsa_circ_0022618	2.09
hsa_circ_0088344	8.20	hsa_circ_0029345	2.50	hsa_circ_0005712	3.39	hsa_circ_0010591	2.08
hsa_circ_0086363	7.98	hsa_circ_0065742	2.50	hsa_circ_0074637	3.38	hsa_circ_0021283	2.08
hsa_circ_0078718	7.77	hsa_circ_0087408	2.50	hsa_circ_0085788	3.33	hsa_circ_0017452	2.08
hsa_circ_0054657	7.70	hsa_circ_0066378	2.49	hsa_circ_0030864	3.32	hsa_circ_0049396	2.08
hsa_circ_0056351	7.60	hsa_circ_0074324	2.47	hsa_circ_0035614	3.29	hsa_circ_0072068	2.08
hsa_circ_0054662	7.58	hsa_circ_0029338	2.47	hsa_circ_0054437	3.28	hsa_circ_0021866	2.08
hsa_circ_0079555	7.39	hsa_circ_0089694	2.47	hsa_circ_0059361	3.26	hsa_circ_0017491	2.08
hsa_circ_0090172	6.98	hsa_circ_0035522	2.47	hsa_circ_0025722	3.24	hsa_circ_0067057	2.08
hsa_circ_0006200	6.89	hsa_circ_0034277	2.47	hsa_circ_0069259	3.23	hsa_circ_0054324	2.08
hsa_circ_0084805	6.69	hsa_circ_0057402	2.47	hsa_circ_0049386	3.21	hsa_circ_0021084	2.08
hsa_circ_0079554	6.36	hsa_circ_0064831	2.46	hsa_circ_0081217	3.20	hsa_circ_0051925	2.08
hsa_circ_0002240	6.32	hsa_circ_0063935	2.46	hsa_circ_0001964	3.19	hsa_circ_0029324	2.08
hsa_circ_0085305	6.19	hsa_circ_0050038	2.45	hsa_circ_0062048	3.18	hsa_circ_0054580	2.07
hsa_circ_0057390	5.90	hsa_circ_0071886	2.44	hsa_circ_0089436	3.16	hsa_circ_0034935	2.07
hsa_circ_0000199	5.83	hsa_circ_0062764	2.44	hsa_circ_0057547	3.15	hsa_circ_0041675	2.07
hsa_circ_0064530	5.78	hsa_circ_0064577	2.44	hsa_circ_0023087	3.12	hsa_circ_0085920	2.07
hsa_circ_0087204	5.70	hsa_circ_0010505	2.43	hsa_circ_0074647	3.10	hsa_circ_0066825	2.07
hsa_circ_0049854	5.46	hsa_circ_0072744	2.42	hsa_circ_0058206	3.09	hsa_circ_0038009	2.07
hsa_circ_0066604	5.40	hsa_circ_0020205	2.42	hsa_circ_0030862	3.08	hsa_circ_0025898	2.07
hsa_circ_0012896	5.26	hsa_circ_0031925	2.42	hsa_circ_0082066	3.08	hsa_circ_0071382	2.07
hsa_circ_0088722	5.20	hsa_circ_0076628	2.42	hsa_circ_0013043	3.07	hsa_circ_0071821	2.07
hsa_circ_0027791	5.07	hsa_circ_0027007	2.41	hsa_circ_0057445	3.06	hsa_circ_0076610	2.07
hsa_circ_0021491	4.99	hsa_circ_0074034	2.41	hsa_circ_0026358	3.06	hsa_circ_0058788	2.06
hsa_circ_0045275	4.92	hsa_circ_0044837	2.40	hsa_circ_0078639	3.05	hsa_circ_0059268	2.06
hsa_circ_0054444	4.86	hsa_circ_0023450	2.40	hsa_circ_0013239	3.03	hsa_circ_0032021	2.06
hsa_circ_0090097	4.75	hsa_circ_0066676	2.40	hsa_circ_0089437	3.02	hsa_circ_0050636	2.06
hsa_circ_0069415	4.66	hsa_circ_0071409	2.39	hsa_circ_0026539	3.00	hsa_circ_0090811	2.05
hsa_circ_0028116	4.63	hsa_circ_0010800	2.39	hsa_circ_0044566	2.98	hsa_circ_0081975	2.05
hsa_circ_0019256	4.55	hsa_circ_0041954	2.39	hsa_circ_0086587	2.97	hsa_circ_0082296	2.05
hsa_circ_0023258	4.44	hsa_circ_0049461	2.37	hsa_circ_0016072	2.96	hsa_circ_0018529	2.05
hsa_circ_0074783	4.44	hsa_circ_0002588	2.36	hsa_circ_0021493	2.95	hsa_circ_0075248	2.05
hsa_circ_0056946	4.44	hsa_circ_0081148	2.36	hsa_circ_0077048	2.94	hsa_circ_0047176	2.05
hsa_circ_0028450	4.18	hsa_circ_0022846	2.36	hsa_circ_0013042	2.93	hsa_circ_0000298	2.05
hsa_circ_0080225	4.09	hsa_circ_0087813	2.36	hsa_circ_0005446	2.91	hsa_circ_0054586	2.05
hsa_circ_0043816	4.08	hsa_circ_0054919	2.36	hsa_circ_0075038	2.89	hsa_circ_0040430	2.04
hsa_circ_0027793	3.99	hsa_circ_0030867	2.35	hsa_circ_0049379	2.87	hsa_circ_0050434	2.04
hsa_circ_0054585	3.92	hsa_circ_0044346	2.35	hsa_circ_0023871	2.87	hsa_circ_0026013	2.04
hsa_circ_0006919	3.87	hsa_circ_0024745	2.35	hsa_circ_0039224	2.87	hsa_circ_0017079	2.04
hsa_circ_0002292	3.86	hsa_circ_0046087	2.34	hsa_circ_0031931	2.86	hsa_circ_0006782	2.04
hsa_circ_0014848	3.79	hsa_circ_0020073	2.33	hsa_circ_0056286	2.86	hsa_circ_0074306	2.03
hsa_circ_0091746	3.72	hsa_circ_0033841	2.33	hsa_circ_0051190	2.85	hsa_circ_0026538	2.03
hsa_circ_0003756	3.70	hsa_circ_0039053	2.32	hsa_circ_0060837	2.85	hsa_circ_0027021	2.03
hsa_circ_0039991	3.68	hsa_circ_0047223	2.31	hsa_circ_0085783	2.84	hsa_circ_0058208	2.03
hsa_circ_0043112	3.68	hsa_circ_0028512	2.31	hsa_circ_0027127	2.82	hsa_circ_0051506	2.03
hsa_circ_0074793	3.67	hsa_circ_0076212	2.30	hsa_circ_0086926	2.82	hsa_circ_0090466	2.02
hsa_circ_0016097	3.67	hsa_circ_0085965	2.29	hsa_circ_0039135	2.81	hsa_circ_0090810	2.02
hsa_circ_0089442	3.64	hsa_circ_0006836	2.28	hsa_circ_0007821	2.81	hsa_circ_0041521	2.02
hsa_circ_0091740	3.63	hsa_circ_0040008	2.26	hsa_circ_0063090	2.79	hsa_circ_0063349	2.02
hsa_circ_0054559	3.63	hsa_circ_0022735	2.26	hsa_circ_0045283	2.78	hsa_circ_0038133	2.02
hsa_circ_0091395	3.62	hsa_circ_0037191	2.26	hsa_circ_0026544	2.77	hsa_circ_0080498	2.01
hsa_circ_0003859	3.59	hsa_circ_0030853	2.25	hsa_circ_0029327	2.76	hsa_circ_0066386	2.01
hsa_circ_0028501	3.57	hsa_circ_0049004	2.25	hsa_circ_0023307	2.75	hsa_circ_0083607	2.01
hsa_circ_0045270	3.57	hsa_circ_0040046	2.25	hsa_circ_0048966	2.75	hsa_circ_0058209	2.00
hsa_circ_0017784	3.54	hsa_circ_0038718	2.25	hsa_circ_0029337	2.75	hsa_circ_0087806	2.00
hsa_circ_0018965	2.16	hsa_circ_0082215	2.25	hsa_circ_0058599	2.75	hsa_circ_0072904	2.21
hsa_circ_0073166	2.16	hsa_circ_0027307	2.24	hsa_circ_0057418	2.74	hsa_circ_0080982	2.21
hsa_circ_0085620	2.16	hsa_circ_0058883	2.24	hsa_circ_0019196	2.72	hsa_circ_0036610	2.19
hsa_circ_0010096	2.16	hsa_circ_0044462	2.24	hsa_circ_0030860	2.70	hsa_circ_0085922	2.19
hsa_circ_0083694	2.16	hsa_circ_0071759	2.24	hsa_circ_0003044	2.69	hsa_circ_0050674	2.19
hsa_circ_0025983	2.16	hsa_circ_0051784	2.24	hsa_circ_0001225	2.68	hsa_circ_0061143	2.19
hsa_circ_0079100	2.15	hsa_circ_0017865	2.23	hsa_circ_0067068	2.68	hsa_circ_0040112	2.19
hsa_circ_0006559	2.15	hsa_circ_0032326	2.23	hsa_circ_0026408	2.67	hsa_circ_0029322	2.18
hsa_circ_0008900	2.15	hsa_circ_0018702	2.23	hsa_circ_0024365	2.67	hsa_circ_0051193	2.18
hsa_circ_0007778	2.15	hsa_circ_0080497	2.22	hsa_circ_0067085	2.66	hsa_circ_0076797	2.18
hsa_circ_0065419	2.14	hsa_circ_0005366	2.22	hsa_circ_0037664	2.66	hsa_circ_0008675	2.18
hsa_circ_0055400	2.14	hsa_circ_0027033	2.22	hsa_circ_0029348	2.65	hsa_circ_0080834	2.17
hsa_circ_0036613	2.14	hsa_circ_0046970	2.21	hsa_circ_0023452	2.65	hsa_circ_0057010	2.17
hsa_circ_0049342	2.14	hsa_circ_0067573	2.21	hsa_circ_0064826	2.65	hsa_circ_0074307	2.17
hsa_circ_0051516	2.14	hsa_circ_0063118	2.21	hsa_circ_0089428	2.63	hsa_circ_0087713	2.17
hsa_circ_0052131	2.14	hsa_circ_0078356	2.13	hsa_circ_0013333	2.63	hsa_circ_0049390	2.58
hsa_circ_0084064	2.14	hsa_circ_0046756	2.13	hsa_circ_0017635	2.62	hsa_circ_0074649	2.58
hsa_circ_0072128	2.13	hsa_circ_0044524	2.12	hsa_circ_0070517	2.61	hsa_circ_0062146	2.57
hsa_circ_0002684	2.13	hsa_circ_0076099	2.12	hsa_circ_0088705	2.59	hsa_circ_0031928	2.56
hsa_circ_0084126	2.12	hsa_circ_0013063	2.12	hsa_circ_0009925	2.59	hsa_circ_0081827	2.56

host genes [14]. Therefore, GO analysis and pathway analysis of the host genes of circRNAs could provide some insights into the function of circRNAs. In this study, we showed that the significant enriched GO term of host genes of dysregulated circRNAs in biological process was extracellular matrix organization (Fig. 2A). The most significant enriched GO term in cellular component was cytoplasm (Fig. 2B). The most significant enriched GO term in molecular function was protein binding (Fig. 2C). Pathway analysis revealed the top 10 signaling pathway potentially involved in dysregulated circRNAs-mediated regulatory network. Of them, focal adhesion signaling pathway was ranked the top 1 (Fig. 2D).

miRNA response elements analysis of dysregulated expressed circRNAs in pterygium

Accumulating evidence has shown that several circRNAs contains conserved seed matches for miRNAs, and may function as microRNA (miRNA) sponges to play their biological roles [11]. We predicted whether dysregulated circRNAs may play their biological roles by acting as miRNA sponges in pterygium. circRNA/miRNA interaction was predicted based on TargetScan and miRana. We predicted whether the top 10 up-regulated circRNAs and the top 10 down-regulated circRNAs could bind to miRNAs. The results demonstrated that 16 circRNAs could potentially bind to at least five different miRNAs (Table 2), suggesting that most of dysregulated circRNAs could function as miRNA sponges to regulate the expression of target genes in pterygium.

circ_0085020 is shown as a potential biomarker for pterygium

The above-mentioned result showed that circ_0085020 was ranked among the top up-regulated circRNA in pterygium samples. We then collected 50 paired pterygium and normal conjunctiva to further evaluate the clinical relevance of circ_0085020. All of the patients had the untreated primary pterygium. They have been accepted conventional excision of pterygium with auto-transplantation of conjunctiva. The patients included 22 female (Age: 41.2±6.6 year) and 28 males (Age: 39.5±4.3 year). The corneal ingrowth in patients was ranging from 2 mm to 4 mm. The results showed that circ_0085020 expression was significantly up-regulated in pterygium compared with normal conjunctiva sample (Fig. 3).

circ_0085020 regulates fibroblast function in pterygium

Pterygium is an ocular surface condition characterized by abnormal fibroblast proliferation and matrix deposition [8]. We thus investigated the role of circ_0085020 in pterygium fibroblast function. circ_0085020 siRNA transfection significantly down-regulated circ_0085020 expression but did not affect the expression of host gene, LAPTM4B (Fig. 4A and 4B). MTT assays showed that circ_0085020 silencing significantly decreased pterygium fibroblast cell viability (Fig. 4C). Ki67 staining assays showed that circ_0085020 silencing significantly decreased pterygium fibroblast proliferation (Fig. 4D). Transwell assays revealed that circ_0085020 silencing inhibited pterygium fibroblast migration (Fig. 4E). Ultra-violet radiation exposure is an important risk factor for pterygium [24]. We also determined whether circ_0085020 regulated the development of UV-induced apoptosis using Hoechst

Fig. 2. GO enrichment and pathway analysis of the host genes of dysregulated circRNAs (A-C) GO enrichment analysis shows the vocabulary to describe differentially expressed circRNAs between paired conjunctiva and pterygium samples. The ontology covers three different domains: biological process (A), cellular component (B), and molecular function (C). (D) The bar plot displays the result of signaling pathway analysis, which demonstrates the top 10 signaling pathways involved in circRNA-mediated regulatory network in pterygium.

Table 2. Identification of potential miRNAs which can bind to dysregulated circRNAs

circRNA	Gene symbol	Fold Change	MRE1	MRE2	MRE3	MRE4	MRE5
Up-regulated							
hsa_circ_0085019	LAPTM4B	17.01	miR-625-5p	miR-1275	miR-1910-3p	miR-6511a-5p	miR-4665-5p
hsa_circ_0010170	MFAP2	12.01	miR-6893-5p	miR-6808-5p	miR-940	miR-873-5p.2	miR-1291
hsa_circ_0087653	CTSL2	10.71	miR-296-5p	miR-3918	miR-6772-3p	miR-4448	miR-4786-3p
hsa_circ_0085013	LAPTM4B	8.90	miR-6816-3p	miR-3127-5p	miR-4695-5p	miR-4266	miR-5187-5p
hsa_circ_0085020	LAPTM4B	8.88	miR-625-5p	miR-1275	miR-1910-3p	miR-6511a-5p	miR-4665-5p
hsa_circ_0031978	CDKN3	8.05	miR-4659a-3p	miR-4659b-3p	miR-548q	miR-4520-2-3p	miR-4519
hsa_circ_0069798	NMU	7.98	miR-4778-3p	miR-4480	miR-5000-3p	miR-4436a	miR-663b
hsa_circ_0015295	GAS5	7.05	miR-3620-3p	miR-6851-3p	miR-597-3p	miR-1205	miR-7160-5p
hsa_circ_0015288	GAS5	7.03	miR-766-3p	miR-3925-3p	miR-515-5p	miR-519e-5p	miR-6875-3p
hsa_circ_0015310	GAS5	6.90	miR-6810-5p	miR-6870-3p	miR-3117-3p	miR-18a-5p	miR-18b-5p
Down-regulated							
hsa_circ_0087198	GDA	79.74	—	—	—	—	—
hsa_circ_0025454	CD69	26.11	miR-6881-3p	miR-7111-3p	miR-6780a-3p	miR-3130-5p	miR-4482-5p
hsa_circ_0002141	ZDBF2	12.13	miR-2861	miR-3654	miR-124-3p.1	miR-506-3p	miR-124-3p.2
hsa_circ_0056159	IL1RN	11.10	—	—	—	—	—
hsa_circ_0064840	ITGA9	9.40	miR-4675	miR-4741	miR-376a-3p	miR-376b-3p	miR-6875-3p
hsa_circ_0034472	THBS1	9.38	miR-4736	miR-3064-3p	miR-6869-3p	miR-3975	miR-1908-3p
hsa_circ_0009558	ERRF11	9.08	miR-93-3p	miR-6071	miR-6515-3p	miR-3912-5p	miR-5693
hsa_circ_0062162	CECR2	8.87	miR-7111-3p	miR-877-3p	miR-6881-3p	miR-6875-3p	miR-1231
hsa_circ_0088344	STOM	8.20	—	—	—	—	—
hsa_circ_0087198	C9orf150	79.74	—	—	—	—	—

33342 and Calcein-AM/PI staining. circ_0085020 silencing further increased UV-induced pterygium fibroblast cell apoptosis as shown by increased apoptotic nuclei (condensed or fragmented) and PI-positive cells (dying or dead cells) (Fig. 4F and 4G).

circ_0085020 regulates epithelial function in pterygium

The epithelial cells also play an important role in the progression of pterygium, which is associated with the invasive and recurrent behavior [2]. circ_0085020 siRNA transfection down-regulated circ_0085020 expression but did not affect the expression of the host gene, LAPTM4B, in pterygium epithelial cells (Fig. 5A and 5B). circ_0085020 silencing significantly decreased pterygium epithelial viability (Fig. 5C), decreased pterygium epithelial proliferation (Fig. 5D), and inhibited pterygium epithelial migration (Fig. 5E). Moreover, circ_0085020 was involved in the regulation of epithelial apoptosis. circ_0085020 silencing further increased UV-induced pterygium epithelial apoptosis as shown by increased apoptotic nuclei (condensed or fragmented) and PI-positive cells (dying or dead cells) (Fig. 5F and 5G).

Discussion

Pterygium has very complicated pathogenesis, including extracellular matrix remodeling, inflammation, angiogenesis, aberrant proliferation, and apoptosis [1, 8]. However, the precise mechanism of pterygium formation and development is not fully understood. In this study, we determined circRNA expression profiling between paired conjunctiva and pterygium. Approximately 669 circRNAs were found to be abnormally expressed in pterygium. circ_0085020 (circ-LAPTM4B) was further verified to significantly be up-regulated in pterygium. circ_0085020 (circ-LAPTM4B) was involved in the pathogenesis of pterygium through regulating pterygium fibroblast and epithelial cell function.

circRNAs are usually generated from the exonic or intronic sequences. They can play their roles by acting as regulators of parental gene expression, regulators of splicing and transcription, or miRNA sponges [11]. Thus, it is possible to predict the functions of several

Fig. 3. circ_0085020 is shown as a potential biomarker for pterygium qRT-PCR assays were conducted to compare the expression difference of circ_0085020 between 50 paired pterygium and 50 normal conjunctiva (* $P < 0.05$ versus conjunctiva).

circRNAs via their parental genes. GO enrichment analysis showed that the most enriched GO term in biological process was extracellular matrix organization. The most enriched GO term in cellular component was cytoplasm. The most enriched GO term in molecular function was protein binding. Extracellular matrix remodeling via the actions of matrix metalloproteinases has been implicated in the pathogenesis of pterygia [8, 25]. After biogenesis, circRNAs can be efficiently transported to the cytosol since the endogenous circRNAs are usually localized

Fig. 4. circ_0085020 regulates fibroblast function in pterygium(A and B) Pterygium fibroblasts were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of circ_0085020, or left untreated (Ctrl) for 24 h. qRT-PCRs were conducted to detect circ_0085020 (A, n=4, * $P<0.05$ versus Ctrl group) and LPTM4B mRNA expression (B, n=4, * $P<0.05$ versus Ctrl group). (C) MTT assays were conducted to detect cell viability (n=4, * $P<0.05$ versus Ctrl group). (D) Cell proliferation was detected by Ki67 staining (n=4, * $P<0.05$ versus Ctrl group). Scale bar, 10 μ m. (E) Transwell assay and quantification analysis was conducted to detect cell migration (n=4, * $P<0.05$ versus Ctrl group). Scale bar, 50 μ m. (F) Apoptotic cells were analyzed using Hoechst staining. The data was shown as means $\pm$ SEM, and represented four individual experiments in which >300 cells were counted. Scale bar: 20 μ m. (G) Apoptotic cells were detected by PI/Calcein-AM double staining. Scale bar: 50 μ m. The significant difference was evaluated by one-way ANOVA followed by post-hoc Bonferroni's test comparison test. # indicated significance differences between the marked group.

Fig. 5. circ_0085020 regulates epithelial cell function in pterygium(A and B) Pterygium epithelium was transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of circ_0085020, or left untreated (Ctrl) for 24 h. qRT-PCRs were conducted to detect circ_0085020 (A, $n=4$, $*P<0.05$ versus Ctrl group) and LAPTMB4B mRNA expression (B, $n=4$, $*P<0.05$ versus Ctrl group). (C) MTT assays were conducted to detect cell viability ($n=4$, $*P<0.05$ versus Ctrl group). (D) Cell proliferation was detected by Ki67 staining ($n=4$, $*P<0.05$ versus Ctrl group). Scale bar, 10 μm . (E) Transwell assay and quantification analysis was conducted to detect cell migration ($n=4$, $*P<0.05$ versus Ctrl group). Scale bar, 50 μm . (F) Apoptotic cells were analyzed using Hoechst staining. The data was shown as means $\pm$ SEM, and represented four individual experiments in which >300 cells were counted. Scale bar: 20 μm . (G) Apoptotic cells were detected by PI/ Calcein-AM double staining. Scale bar: 50 μm . The significant difference was evaluated by one-way ANOVA followed by post-hoc Bonferroni's test comparison test. # indicated significance differences between the marked group.

to cytoplasm [26, 27] circRNA-mediated regulatory network would occur in cytoplasm, and affect the formation of pterygia via regulating extracellular matrix remodeling. Pathway analysis showed that focal adhesion signaling pathway was ranked the top 1 signaling pathway affected by circRNA-mediated network. Focal adhesion kinase (FAK) is a non-

receptor tyrosine kinase, which is known for its role in adhesion-mediated signaling. It plays important roles in matrix remodeling, and potentially regulates fibroblast cell and epithelial cell function [28, 29]. Based on the above-mentioned evidence, we speculated that circRNA-mediated regulatory network is involved in the pathogenesis of pterygium.

The majority of circRNAs possesses miRNA response elements (MREs), and have miRNA-binding potentials, which increases the complexity of RNA regulatory network [30]. Previous studies have shown that CDR1as harbors more than 70 conventional miR-7 binding sites [31]. circ-ITCH functions as miRNA (miR-7, miR-17, and miR-214) sponges and plays an inhibitory role in esophageal squamous cell carcinoma [32]. circHIPK3 is observed to sponge to 9 miRNAs with 18 potential binding sites [33]. We show that most of differentially expressed circRNAs in pterygium contain more than five different miRNA binding sites. miRNAs play important role in many cellular processes including cell growth, proliferation, differentiation, death, and apoptosis [34]. The role of miRNAs in pterygium has also been widely accepted [6, 35, 36]. circRNAs could compete with endogenous miRNAs by direct binding, resulting in increased levels of miRNA targets. Thus, it is not surprising that circRNA-miRNA-mRNA network is involved in the pathogenesis of pterygium.

circRNAs are specifically expressed in a developmental stage- and tissue-specific manner [37]. Unlike linear RNAs, circRNAs are very stable and resistant to RNase R. They are more suitable for serving as diagnostic or predictive biomarkers of human diseases [38]. Emerging evidence shows that circRNAs exhibit aberrant expression in atherosclerotic vascular disease, neurological disorders, and cancers, and serve as diagnostic or predictive biomarkers of these diseases [39, 40]. circRNAs can be detected in plasma, saliva, and blood [41-43]. Abnormal circRNA expression in disease condition may reflect either a condition-specific transcriptome change or a direct consequence of circRNA release from the diseased tissue. circ_0085020 (circ-LAPTM4B) expression is significantly up-regulated in pterygium compared with paired conjunctiva. We speculated that circ_0085020 (circ-LAPTM4B) silencing may be beneficial for the therapeutic intervention of pterygium.

Pterygium is usually characterized by abnormal fibroblast proliferation and matrix deposition. Its clinical severity is tightly associated with the fleshiness or amount of fibrous tissue [35]. Previous studies have shown that pterygium fibroblasts display characteristics of transformed cells, including loss of heterozygosity and microsatellite instability [44]. circ_0085020 (circ-LAPTM4B) up-regulation could significantly increase pterygium fibroblast cell proliferation and migration ability. Thus, we speculated that compared to normal conjunctival epithelial cells, circ_0085020 (circ-LAPTM4B) up-regulation epithelium would take longer to reach senescence. Fibroblast proliferation in pterygium would lead to increased matrix production, resulting in increased fleshy lesion. The epithelial cells play a crucial role in the formation of pterygium, which are responsible for the invasive and recurrent behavior of pterygium via regulating the secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) [8]. Moreover, abnormal apoptosis is shown in pterygium compared with the normal conjunctiva. Chronic UV exposure plays a major role in the pathogenesis of pterygium [45]. circ_0085020 (circ-LAPTM4B) up-regulation could prevent pterygium epithelium against UV-induced apoptosis. Previous study has revealed the presence of an anti-apoptotic protein Bcl-2 in pterygium epithelium but not conjunctiva, which counteracts the pro-apoptotic protein Bax [46, 47]. We speculated that circ_0085020 (circ-LAPTM4B) might play an anti-apoptotic role in the pathogenesis of pterygium similar as Bcl-2.

In conclusion, this study provides important evidence that circular RNAs are involved in the pathogenesis of pterygium. circ_0085020 (circ-LAPTM4B) up-regulation leads to abnormal pterygium fibroblast and epithelial function. Our finding may be applied for the treatment of pterygium. It may be possible to arrest pterygium formation by non-surgical treatment using the inhibitor of circ_0085020 (circ-LAPTM4B). In the fleshy pterygium lesions, these strategies may even play a role in the prevention of post-surgical recurrence after excision.

Acknowledgements

This work was generously supported by the grants from the National Natural Science Foundation of China (Grant No. 81371055 and 81570859 to Q.J., and Grant No. 81670878 to J.Y.) and Grants from innovation team Project Fund of Jiangsu Province (No. CXTDB2017010 to Q.J.).

Disclosure Statement

The authors declare that they have no conflict of interests.

References

- Chui J, Di Girolamo N, Wakefield D, Coroneo MT: The pathogenesis of pterygium: current concepts and their therapeutic implications. *Ocul Surf* 2008;6:24-43.
- Liu T, Liu Y, Xie L, He X, Bai J: Progress in the pathogenesis of pterygium. *Curr Eye Res* 2013;38:1191-1197.
- Hirst LW: The treatment of pterygium. *Surv Ophthalmol* 2003;48:145-180.
- Gris O, Güell JL, del Campo Z: Limbal-conjunctival autograft transplantation for the treatment of recurrent pterygium. *Ophthalmology* 2000;107:270-273.
- Hacıoğlu D, Erdöl H: Developments and current approaches in the treatment of pterygium. *Int Ophthalmol* 2017;37:1073-1081.
- Lan W, Chen S, Tong L: MicroRNA-215 regulates fibroblast function: insights from a human fibrotic disease. *Cell Cycle* 2015; 14:1973-1984.
- Ebbesen KK, Kjems J, Hansen TB: Circular RNAs: identification, biogenesis and function. *Biochim Biophys Acta* 2016;1859:163-168.
- Di Girolamo N, Chui J, Coroneo MT, Wakefield D: Pathogenesis of pterygia: role of cytokines, growth factors, and matrix metalloproteinases. *Prog Retin Eye Res* 2004;23:195-228.
- Yazar S, Cuellar-Partida G, McKnight CM, Quach-Thaniassorn P, Mountain JA, Coroneo MT, Pennell CE, Hewitt AW, MacGregor S, Mackey DA: Genetic and environmental factors in conjunctival UV autofluorescence. *JAMA Ophthalmol* 2015;133:406-412.
- Vicens Q, Westhof E: Biogenesis of circular RNAs. *Cell* 2014;159:13-14.
- Salzman J: Circular RNA expression: its potential regulation and function. *Trends Genet* 2016;32:309-316.
- Rybák-Wolf A, Stottmeister C, Glazar P, Jens M, Pino N, Giusti S, Hanan M, Behm M, Bartok O, Ashwal-Fluss R, Herzog M, Schreyer L, Papavasileiou P, Ivanov A, Ohman M, Refojo D, Kadener S, Rajewsky N: Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol Cell* 2015;58:870-885.
- Boeckel JN, Jaé N, Heumüller AW, Chen W, Boon RA, Stellos K, Zeiher AM, John D, Uchida S, Dimmeler S: Identification and characterization of hypoxia-regulated endothelial circular RNA. *Circ Res* 2015; 117:884-890.
- Barrett SP, Wang PL, Salzman J: Circular RNA biogenesis can proceed through an exon-containing lariat precursor. *Elife* 2015; 4:e07540.
- Li H, Hao X, Wang H, Liu Z, He Y, Pu M, Zhang H, Yu H, Duan J, Qu S: Circular RNA expression profile of pancreatic ductal adenocarcinoma revealed by microarray. *Cell Physiol Biochem* 2016; 40:1334-1344.
- Fu L, Yao T, Chen Q, Mo X, Hu Y, Guo J: Screening differential circular RNA expression profiles reveals hsa_circ_0004018 is associated with hepatocellular carcinoma. *Oncotarget* 2017;8:58405-58416.
- Lukiw WJ: Circular RNA (circRNA) in Alzheimer's disease (AD). *Front Genet* 2013;4:307.
- Di Girolamo N, Tedla N, Kumar RK, McCluskey P, Lloyd A, Coroneo MT, Wakefield D: Culture and characterisation of epithelial cells from human pterygia. *Br J Ophthalmol* 1999;83:1077-1082.
- Solomon A, Grueterich M, Li DQ, Meller D, Lee SB, Tseng SC: Overexpression of insulin-like growth factor-binding protein-2 in pterygium body fibroblasts. *Invest Ophthalmol Vis Sci* 2003;44:573-580.
- Shan K, Jiang Q, Wang XQ, Wang YN, Yang H, Yao MD, Liu C, Li XM, Yao J, Liu B, Zhang YY, J Y, Yan B: Role of long non-coding RNA-RNCR3 in atherosclerosis-related vascular dysfunction. *Cell Death Dis* 2016;7:e2248.
- Yan B, Yao J, Liu JY, Li XM, Wang XQ, Li YJ, Tao ZF, Song YC, Chen Q, Jiang Q: LncRNA-MIAT regulates microvascular dysfunction by functioning as a competing endogenous RNA. *Circ Res* 2015;116:1143-1156.

- 22 Yao J, Wang XQ, Li YJ, Shan K, Yang H, Wang YN, Yao MD, Liu C, Li XM, Shen Y, Liu JY, Cheng H, Yuan J, Zhang YY, Jiang Q, Yan B: Long non-coding RNA MALAT1 regulates retinal neurodegeneration through CREB signaling. *EMBO Mol Med* 2016;8:346-362.
- 23 Wang YN, Shan K, Yao MD, Yao J, Wang JJ, Li X, Liu B, Zhang YY, Ji Y, Jiang Q, Yan B: Long noncoding RNA-GASS: a novel regulator of hypertension-induced vascular remodeling. *Hypertension* 2016;68:736-748.
- 24 Kim KW, Park SH, Kim JC: Fibroblast biology in pterygia. *Exp Eye Res* 2016;142:32-39.
- 25 Riau AK, Wong TT, Lan W, Finger SN, Chaurasia SS, Hou AH, Chen S, Yu SJ, Tong L: Aberrant DNA methylation of matrix remodeling and cell adhesion related genes in pterygium. *PLoS One* 2011;6:e14687.
- 26 Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J: Natural RNA circles function as efficient microRNA sponges. *Nature* 2013;495:384-388.
- 27 Hansen TB, Wiklund ED, Bramsen JB, Villadsen SB, Statham AL, Clark SJ, Kjems J: MiRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *EMBO J* 2011;30:4414-4422.
- 28 Larsen M, Artym VV, Green JA, Yamada KM: The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr Opin Cell Biol* 2006;18:463-471.
- 29 Thiery JP, Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006;7:131-142.
- 30 Tay Y, Rinn J, Pandolfi PP: The multilayered complexity of ceRNA crosstalk and competition. *Nature* 2014;505:344-352.
- 31 Xu H, Guo S, Li W, Yu P: The circular RNA Cdr1as, via miR-7 and its targets, regulates insulin transcription and secretion in islet cells. *Sci Rep* 2015;5:12453.
- 32 Li F, Zhang L, Li W, Deng J, Zheng J, An M, Lu J, Zhou Y: Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/ β -catenin pathway. *Oncotarget* 2015;6:6001-6013.
- 33 Zheng Q, Bao C, Guo W, Li S, Chen J, Chen B, Luo Y, Lyu D, Li Y, Shi G, Liang L, Gu J, He X, Huang S: Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. *Nat Commun* 2016;7:11215.
- 34 Winter J, Jung S, Keller S, Gregory RI, Diederichs S: Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 2009;11:228-234.
- 35 Chien KH, Chen SJ, Liu JH, Woung LC, Chen JT, Liang CM, Chiou SH, Tsai CY, Cheng CK, Hu CC, Peng CH: Correlation of microRNA-145 levels and clinical severity of pterygia. *Ocul Surf* 2013;11:133-138.
- 36 Engelsvold DH, Utheim TP, Olstad OK, Gonzalez P, Eidet JR, Lyberg T, Trøseid AM, Dartt DA, Raeder S: miRNA and mRNA expression profiling identifies members of the miR-200 family as potential regulators of epithelial-mesenchymal transition in pterygium. *Exp Eye Res* 2013;115:189-198.
- 37 Chen LL: The biogenesis and emerging roles of circular RNAs. *Nat Rev Mol Cell Biol* 2016;17:205-211.
- 38 Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K, Li H: Circular RNA: A new star of noncoding RNAs. *Cancer Lett* 2015;365:141-148.
- 39 Lasda E, Parker R: Circular RNAs: diversity of form and function. *RNA* 2014;20:1829-1842.
- 40 Zhang SJ, Chen X, Li CP, Li XM, Liu C, Liu BH, Shan K, Jiang Q, Zhao C, Yan B: Identification and characterization of circular RNAs as a new class of putative biomarkers in diabetes retinopathy. *Invest Ophthalmol Vis Sci* 2017;58:6500-6509.
- 41 Memczak S, Papavasileiou P, Peters O, Rajewsky N: Identification and characterization of circular RNAs as a new class of putative biomarkers in human blood. *PLoS One* 2015;10:e0141214.
- 42 Koh W, Pan W, Gawad C, Fan HC, Kerchner GA, Wyss-Coray T, Blumenfeld YJ, El-Sayed YY, Quake SR: Noninvasive *in vivo* monitoring of tissue-specific global gene expression in humans. *Proc Natl Acad Sci USA* 2014;111:7361-7366.
- 43 Bahn JH, Zhang Q, Li F, Chan TM, Lin X, Kim Y, Wong DT, Xiao X: The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. *Clin Chem* 2015;61:221-230.
- 44 Spandidos DA, Sourvinos G, Kiaris H, Tsampanlakis J: Microsatellite instability and loss of heterozygosity in human pterygia. *Br J Ophthalmol* 1997;81:493-496.
- 45 Kwok LS, Coroneo MT: A model for pterygium formation. *Cornea* 1994;13:219-224.
- 46 Tan DT, Tang WY, Liu YP, Goh HS, Smith DR: Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. *Br J Ophthalmol* 2000;84:212-216.
- 47 Liang K, Jiang Z, Ding BQ, Cheng P, Huang DK, Tao LM: Expression of cell proliferation and apoptosis biomarkers in pterygia and normal conjunctiva. *Mol Vis* 2011;17:1687-1693.