• Basic Research •

Role of hsa_circ_0007482 in pterygium development: insights into proliferation, apoptosis, and clinical correlations

Li-Yun Zhang, Xin Liu, Shuo Zheng, Wen-Qun Xi, Xue-Ping Wu, Dan-Yao Nie, Hui-Ling Hu, Jian-Tao Wang, Xin-Hua Liu, Jing Zhang

Shenzhen Eye Hospital; Jinan University; Shenzhen Eye Institute, Shenzhen 518000, Guangdong Province, China

Correspondence to: Jing Zhang. Shenzhen Eye Hospital, 18# Zetian Road, Shenzhen 518000, Guangdong Province, China. jingzhyanke@163.com

Received: 2024-01-10 Accepted: 2024-05-17

Abstract

• **AIM:** To investigate the impact of hsa_circ_0007482 on the proliferation and apoptosis of human pterygium fibroblasts (HPFs) and its correlation with the severity grades of pterygium.

• **METHODS:** Pterygium and normal conjunctival tissues were collected from the superior area of the same patient's eye (*n*=33). The correlation between pterygium severity and hsa_circ_0007482 expression using quantitative reverse-transcription polymerase chain reaction (RT-qPCR) were analyzed. Three distinct siRNA sequences targeting hsa_circ_0007482, along with a negative control sequence, were transfected into HPFs. Cell proliferation was assessed using the cell counting kit-8. Expression levels of Ki67, proliferating cell nuclear antigen (PCNA), Cyclin D1, Bax, B-cell lymphoma-2 (Bcl-2), and Caspase-3 were measured *via* RT-qPCR. Immunofluorescence staining was employed to detect Ki67 and vimentin expressions. Apoptosis was evaluated using flow cytometry.

• **RESULTS:** Hsa_circ_0007482 expression was significantly higher in pterygium tissues compared to normal conjunctival tissues (*P*<0.001). Positive correlations were observed between hsa_circ_0007482 expression and pterygium severity, thickness, and vascular density. Knockdown of hsa_circ_0007482 inhibited cell proliferation, reducing the mRNA expression of Ki67, PCNA, and Cyclin D1 in HPFs. Hsa_circ_0007482 knockdown induced apoptosis, increasing mRNA expression levels of Bax and Caspase-3, while decreasing Bcl-2 expression in HPFs. Additionally, hsa_circ_0007482 knockdown attenuated vimentin expression in HPFs.

• **CONCLUSION:** The downregulation of hsa_circ_0007482 effectively hampers cell proliferation and triggers apoptosis in HPFs. There are discernible positive correlations detected between the expression of hsa_circ_0007482 and the severity of pterygium.

• **KEYWORDS:** hsa_circ_0007482; circular RNA; pterygium; cell proliferation; apoptosis

DOI:10.18240/ijo.2024.08.01

Citation: Zhang LY, Liu X, Zheng S, Xi WQ, Wu XP, Nie DY, Hu HL, Wang JT, Liu XH, Zhang J. Role of hsa_circ_0007482 in pterygium development: insights into proliferation, apoptosis, and clinical correlations. *Int J Ophthalmol* 2024;17(8):1387-1395

INTRODUCTION

Pterygium is a prevalent ocular surface disease characterized by abnormal proliferation of the bulbar conjunctiva and fibrovascular tissue, extending into the cornea within the palpebral fissure area. The overall prevalence of pterygium in China is approximately 9.84%, aligning closely with the global prevalence of 12%[1-2]. Pterygium can lead to various clinical challenges, such as eye irritation. Additionally, it has the potential to impact aesthetics, worsen corneal astigmatism, and may even result in visual impairment and ocular motility disorders[3-4]. Surgical excision stands as the primary treatment for pterygium. Even when combined with conjunctival autograft, amniotic membrane transplantation, or the use of antiproliferative drugs like mitomycin C during surgery, there remains a recurrence rate of 0.57%–27.27% post-surgery[5-6]. Surgery also carries the risk of serious complications, including corneal scarring, symblepharon, ocular motility disorder, and corneal ulcers[7]. Consequently, further studies are imperative to identify novel nonsurgical treatments that can inhibit pterygium growth or prevent recurrence.

The pathological characteristics of pterygium encompass cell proliferation, angiogenesis, inflammation, and epithelial-mesenchymal transition (EMT), which are very similar to

those of tumoral tissues[8-9]. A shared trait between pterygium and other fibrous diseases, both ocular and systemic (such as liver fibrosis, renal fibrosis, and pulmonary fibrosis), is the excessive proliferation of fibrous tissue[10]. Notably, pterygium tissue is easily accessible, and delving into its pathogenesis could yield significant insights into other fibrous diseases.

CircRNAs, known for their associations with physiological conditions and diseases, exhibit dysregulated expression in various disorders, including cancer[11-12]. They play pivotal roles in cell differentiation, proliferation, angiogenesis, migration, and carcinogenesis[13]. Specific circRNAs have been implicated in influencing EMT-related cellular functions during human EMT[14]. Extensive research has identified circRNAs as biomarkers and therapeutic targets across diverse diseases, ranging from cancer to cardiovascular, neurodegenerative, and ocular conditions[15-16]. For instance, circ_0043144 serves as a prognostic and diagnostic indicator for proliferative vitreoretinopathy[17]. Another study highlights the significantly increased expression of circ_0085020 in pterygium tissues, suggesting its potential as a biomarker for this condition[18]. Given the multifaceted biological functions of circRNAs, investigating the effects of hsa_circ_0007482 on pterygium becomes imperative.

In our previous research, several circRNAs, including hsa_circ_0007482, hsa_circ_0023988, hsa_circ_0004846, novel_circ_0000151, and novel_circ_0012935, were identified as players in pterygium development[19]. Existing studies have reported upregulated expression of hsa_circ_0007482 in keloid tissues, where it may promote fibroblast proliferation[20]. However, the specific role of hsa_circ_0007482 in pterygium development remains unclear. Consequently, this study aims to unravel the biological functions of hsa_circ_0007482 in the context of pterygium development.

MATERIALS AND METHODS

Ethical Approval This study followed the tenets of the Declaration of Helsinki and received approval from the Ethics Committee of Shenzhen Eye Hospital (approval code: 2022KYPJ005). All patients signed an informed consent form and volunteered to participate in the study.

Clinical Participants This study included patients with primary pterygium who underwent excision with a conjunctival autograft at Shenzhen Eye Hospital. The inclusion criteria were as follows: age>18y, presence of pterygium tissue growth located at the nasal palpebral fissure area, and the head of the pterygium invading the cornea by no less than 3 mm. Exclusion criteria encompassed pseudopterygium, conjunctival cyst, keratoconjunctival tumor, history of ocular trauma or other ophthalmic diseases, surgical history of other ophthalmic diseases, and a history of systemic diseases such as hyperthyroidism and tumors.

The severity of primary pterygium was assessed according to a previously established grading system, utilizing slit-lamp examination and slit-lamp photography[21]. The grading system involved recording the extension, vascularity, and thickness of the pterygia, which were then translated into severity scores. Subsequently, participants were categorized into mild (3–5), moderate (6–8), and severe (9–11) pterygium subgroups based on their respective severity scores.

Patients Specimen Collection Specimens for patients were collected through surgical extraction of pterygium and normal conjunctival tissues from the superior area of the same patient's eye. The dimensions of both pterygium and normal conjunctival tissues were approximately 2 mm×2 mm. The freshly obtained tissue was meticulously washed with normal saline, transferred to an RNASE-free EP tube, rapidly frozen in a liquid nitrogen box for transportation, and then preserved at -80°C in a refrigerator for subsequent quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis.

Primary Human Pterygium Fibroblasts and Conjunctival Fibroblasts Culture Patients diagnosed with severe pterygium were specifically chosen for the extraction of fresh subepithelial tissues from the pterygium's body. These tissues were then utilized for the cultivation of primary human pterygium fibroblasts (HPFs). Human conjunctival fibroblast cells (HCFs) were cultured from normal subconjunctival connective tissues in the superior area. The cultivation processes for both HPFs and HCFs followed a previously described method[21]. The collected tissues underwent thorough washing with 0.9% normal saline and were then cut into small pieces measuring 1–2 mm^3. These tissue specimens were placed in a culture flask with high-glucose DMEM medium containing 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The flask was incubated at 37°C in 5% CO_2, and the medium was replenished every three days. Upon reaching approximately 90% confluence, the cells were trypsinized with 0.25% trypsin-ethylenediaminetetraacetic acid and transferred to a new culture flask at a ratio of 1:2 or 1:3. Cells from the third to fifth generations were utilized in the experiments, and all procedures were conducted in triplicate.

Immunofluorescence Staining HPFs, cultured in twelve-well plates, were transfected with either siRNA negative control (si-NC) or si-hsa_circ_0007482 for 48h, followed by fixation and permeabilization. Subsequent to blocking with 1% bovine serum albumin, HPFs or HCFs were incubated overnight at 4°C with rabbit anti-vimentin (Cell Signaling Technology, Inc., USA), rabbit anti-cytokeratin (ProteinTech, USA), and mouse anti-Ki67 (Cell Signaling Technology, Inc., USA). Following this, goat anti-rabbit IgG H&L (Abcam, USA) or CoraLite594-conjugated goat anti-mouse IgG(H+L) (ProteinTech) was used to incubate HPFs and HCFs for 1h. The cell nuclei were

Table 1 Forward and reverse primers used in real-time quantitative PCR

Gene	Forward primer	Reverse primer
hsa_circ_0007482	5′-ACCACCAAATTCCTCGACCG-3′	5′-GCGCAAAAGCCTGTTGTCTT-3′
Ki67	5′-CCTCAGCACCTGCTTGTTTG-3′	5′-AGCCGTACAGGCTCATCAAT-3′
PCNA	5′-TCTGAGGGCTTCGACACCTA-3′	5′-CGCGTTATCTTCGGCCCTTA-3′
Cyclin D1	5′-GATGCCAACCTCCTCAACGA-3′	5′-ACTTCTGTTCCTCGCAGACC-3′
BAX	5′-CATGGGCTGGACATTGGACT-3′	5′-AAAGTAGGAGAGGAGGCCGT-3′
BCL-2	5′-CTTTGAGTTCGGTGGGGTCA-3′	5′-GAAATCAAACAGAGGCCGCA-3′
Caspase-3	5′-AAATACCAGTGGAGGCCGAC-3′	5′-TTCTGTTGCCACCTTTCGGT-3′
GAPDH	5′-GTCAAGGCTGAGAACGGGAA-3′	5′-AAATGAGCCCCAGCCTTCTC-3′

PCR: Polymerase chain reaction; PCNA: Proliferating cell nuclear antigen; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

stained using NucBlue™ Fixed Cell Stain ReadyProbes™ reagent (DAPI; Invitrogen, USA). Ultimately, a fluorescence microscope was employed to capture images of the cells.

Real-time Quantitative Polymerase Chain Reaction Total RNA was extracted from tissues or treated cells using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, China) and subsequently reverse-transcribed into cDNA. The resulting cDNA was diluted 10-fold in ddH_2O and subjected to amplification using the SYBR Green expression master mix in a Bio-Rad CFX96 quantitative PCR system. Primer sequences for all mRNAs are provided in Table 1 and were synthesized by Tsingke Biotechnology Co. Ltd., China Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene. The $2^{-\Delta\Delta CT}$ method was employed to calculate the relative gene expression levels.

Cell Transfection Three siRNAs (siRNA-1, siRNA-2, siRNA-3) targeting hsa_circ_0007482 and a negative control sequence (si-NC) were designed and synthesized by Ruibo Biotechnology (Guangzhou, China). According to the RiboFECT CP Transfection Kit protocol, four siRNA (100 mmol/L) were transfected into HPFs when the cell confluence reached 60%-70% for a 48-hour incubation period. Subsequently, transfected HPFs were harvested for further experimental procedures.

Cell Proliferation Assay To assess the impact of hsa_circ_0007482 on HPF proliferation, the cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was employed. HPFs were seeded into 96-well plates and incubated overnight. Following the protocol of riboFECT CP Transfection Kit, HPFs were transfected with si-NC or siRNA-1 targeting hsa_circ_0007482 for 24, 48, and 72h. HPFs were incubated for 2h at 37°C after adding 10 μL CCK-8 solution per well. The absorbance was measured at 450 nm, and the cell proliferation curve was plotted.

Flow Cytometry The apoptosis rates in HPFs with or without hsa_circ_0007482 knockdown were tested using flow cytometry with Annexin V-FITC/PI reagent (BioLegend, USA). After transfection with si-NC or siRNA-1 targeting hsa_circ_0007482 for 48h, HPFs were collected *via* centrifugation. Subsequently, the HPFs were treated with the Annexin V-FITC/PI reagent according to the manufacturer's protocol. Apoptosis was analyzed by flow cytometry (Agilent NovoCyte, USA).

Statistical Analysis All data were obtained from at least three experiments and presented as mean±standard deviation (SD). Statistical analyses were performed using the GraphPad Prism 9.4 software (GraphPad Software Inc., San Diego, CA, USA). Normal distribution was tested using the Shapiro-Wilk test, and the Student's *t*-test or a nonparametric test was used to analyze the two groups. A one-way analysis of variance was used to analyze more than two groups. Correlations assessed using the Spearman's rank correlation test. Statistical significance was considered at $P<0.05$.

RESULTS

Hsa_circ_0007482 was Highly Expressed in Pterygium Tissues and Correlates with Severity Grades The demographic characteristics of the 33 enrolled patients with pterygia are summarized in Table 2. The study included 17 male and 16 female participants, with 19 having monocular pterygium and 14 having binocular pterygia. The age of the participants ranged from 46 to 83y, with a median age of 63.24±8.18y. Based on the severity of pterygium, 13 patients (39.4%) were categorized as having moderate cases, while 20 patients (60.6%) were classified as severe. Notably, the study exclusively included cases where pterygium excision, coupled with conjunctival autograft surgery, was conducted when the pterygium had invaded the corneal limbus by a minimum of 3 mm. Consequently, individuals with mild pterygium were excluded from the study.

To assess the relative expression levels of hsa_circ_0007482 in both pterygium tissue and adjacent normal conjunctival tissue, RT-qPCR was employed. The findings revealed a significantly elevated relative expression level of hsa_circ_0007482 in the 33 pterygium tissues compared to the control group with normal conjunctival tissue (Figure 1A, 1B). Furthermore, the expression level of hsa_circ_0007482 demonstrated a positive correlation with the severity of pterygium (Figure 1C). While the expression level of hsa_circ_0007482 did not exhibit a significant correlation with the degree of pterygium extension

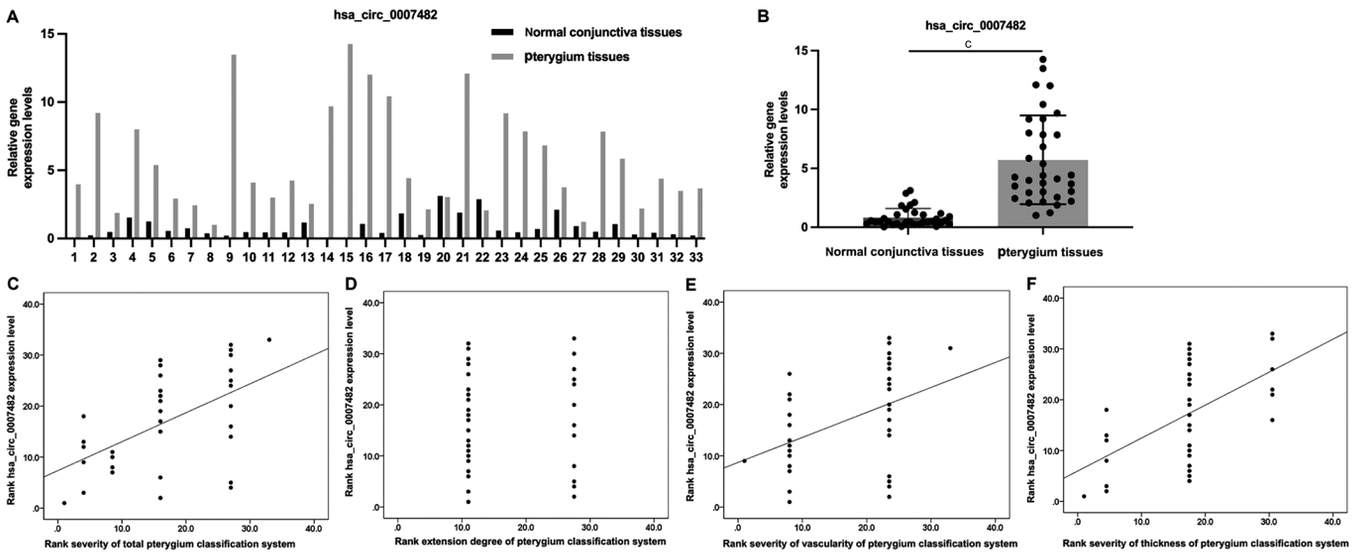

Figure 1 Impact of hsa_circ_0007482 expression on severity, extension, vascular density, and thickness of pterygium tissues A: The expression of hsa_circ_0007482 in human pterygium and contralateral normal conjunctiva samples from 33 patients assessed by RT-qPCR; B: Pterygium tissues exhibited significantly higher expression of hsa_circ_0007482 compared to the control conjunctiva; C: The expression level of hsa_circ_0007482 was positively correlated with the severity of pterygium (P<0.01); D: No correlation found between the expression level of hsa_circ_0007482 and the degree of pterygium extension (P=0.884); E: The expression level of hsa_circ_0007482 was positively correlated with the vascular density of pterygium (P<0.05); F: The expression level of hsa_circ_0007482 was positively correlated with the thickness of pterygium (P<0.01). All values are presented as mean±SD of thirty-three independent experiments. ^{c}P<0.001. RT-qPCR: Real-time quantitative polymerase chain reaction; SD: Standard deviation.

Table 2 Clinical characteristics and grading of pterygium classification in patients

Parameters	*n*
Number of patients	33
Male	17
Female	16
Monocular	19
Biocular	14
Grading of classification	
Mild	0
Moderate	13
Severe	20
Extension	
Grade 1	0
Grade 2	0
Grade 3	21
Grade 4	12
Vascularity	
Grade 1	1
Grade 2	16
Grade 3	16
Thickness	
Grade 1	1
Grade 2	9
Grade 3	17
Grade 4	6

(Figure 1D), it did show a positive correlation with both the vascular density and thickness of the pterygium (Figure 1E, 1F). These findings suggest a potential association between the expression of hsa_circ_0007482 and specific severity characteristics of pterygium.

Successful Separation and Identification of Primary HPFs and HCFs A simple flowchart of the isolation and culturing of primary HPFs and HCFs is shown in Figure 2A. HPFs, obtained from pterygium tissues, exhibited an elongated and fusiform phenotype after five days of culture, as observed under an inverted microscope (Figure 2A). Immunofluorescent staining of primary HPFs and HCFs revealed positive vimentin expression and negative cytokeratin staining (Figure 2B). These immunofluorescence results for HPFs and HCFs were consistent with the characteristic features of fibroblasts. In summary, we successfully isolated and identified primary HPFs, which were subsequently utilized to explore the functional aspects of hsa_circ_0007482 in HPFs.

Screening of the Most Effective siRNA Sequences for hsa_circ_0007482 Knockdown in HPFs After transfection with different siRNA sequences targeting hsa_circ_0007482 or the siRNA negative control sequence (si-NC), the mRNA expression levels of hsa_circ_0007482 were assessed by RT-qPCR in HPFs transfected for 48h to select the siRNA sequence that most effectively knocked down hsa_circ_0007482. The results revealed a significant downregulation of hsa_circ_0007482 mRNA expression with all three pairs of siRNA sequences targeting hsa_circ_0007482 (Figure 3A). Notably, the most substantial reduction in hsa_circ_0007482 expression occurred when HPFs were transfected with siRNA-1. The

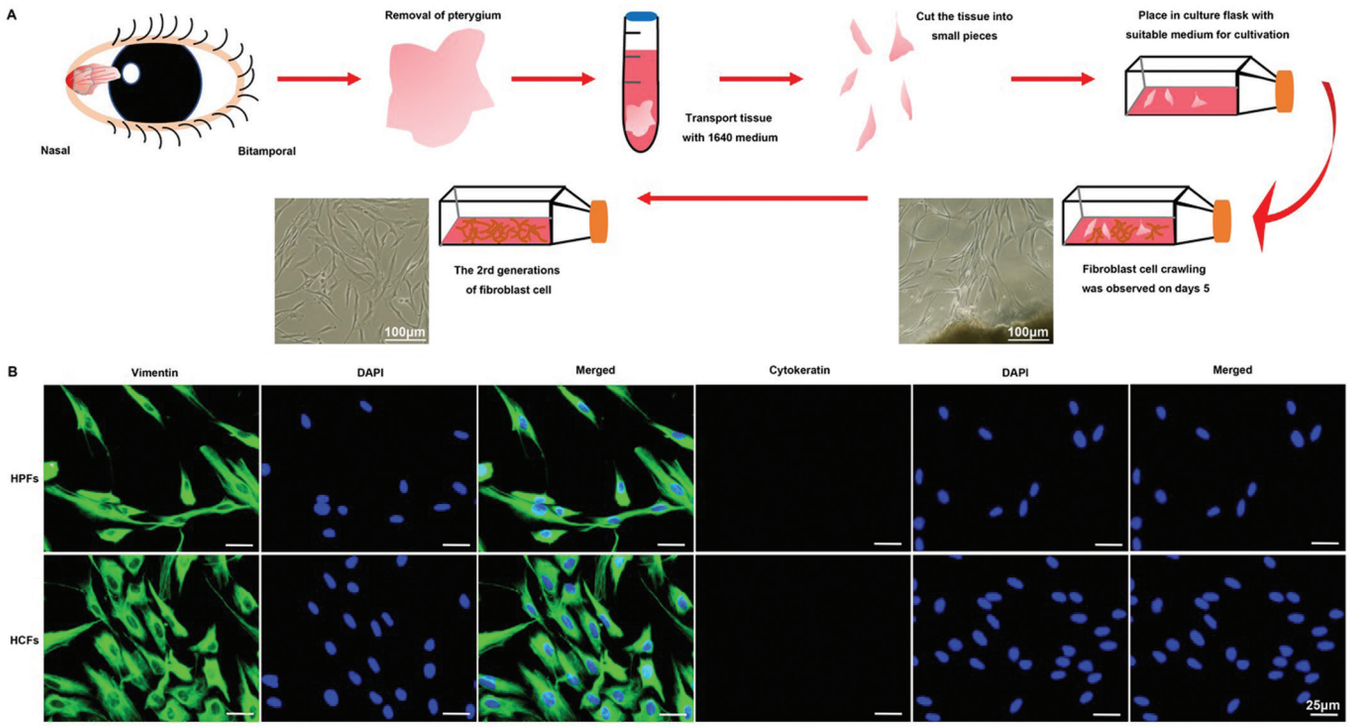

Figure 2 Successful isolation and identification of primary HPFs and HCFs A: Simple flow chart illustrating the primary cell culture process, accompanied by representative phase-contrast images of HPFs. Scale bar: 100 μm. B: Immunofluorescence depicting vimentin (green) and cytokeratin expression in both HPFs and HCFs. DAPI (blue) was used to stain the nucleus. Scale bar: 25 μm. HPFs: Human pterygium fibroblasts; HCFs: Human conjunctival fibroblasts; DAPI: 4′, 6-diamidino-2-phenylindole.

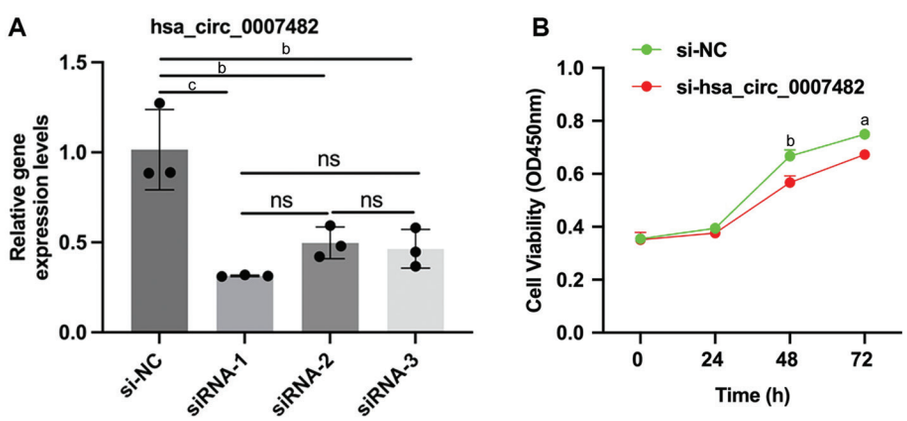

Figure 3 Confirmation of the most effective siRNA sequences and optimal transfection duration for hsa_circ_0007482 knockdown in HPFs A: The mRNA expression levels of the hsa_circ_0007482 in HPFs transfected with three siRNAs (siRNA-1, siRNA-2, siRNA-3) and a negative control sequence (si-NC) to knockdown the expression of hsa_circ_0007482 for 48h; B: After transfection with si-NC or si-hsa_circ_0007482 for 24, 48, and 72h, the absorbance of HPFs was detected by CCK-8 at 450 nm. All values are presented as mean±SD of three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. HPFs: Human pterygium fibroblasts; NC: Negative control; SD: Standard deviation.

expression levels of hsa_circ_0007482 did not show significant differences between HPFs transfected with siRNA-2 and siRNA-3. Based on these findings, siRNA-1 emerged as the most effective sequence for downregulating hsa_circ_0007482 and was subsequently employed in all subsequent experiments.

Silencing of hsa_circ_0007482 Inhibited Proliferation and Attenuated Vimentin Expression in HPFs After transfection of HPFs with si-NC or si-hsa_circ_0007482 for 24, 48, or 72h, significant differences in HPF proliferation inhibition were evident, as detected by CCK-8 assays at 48 and 72h. Notably, the most pronounced inhibitory effect on HPF proliferation occurred 48h post-transfection (Figure 3B). Therefore, we deemed 48h as the optimal duration for HPF transfection and employed it in all subsequent experiments.

To assess the impact of hsa_circ_0007482 knockdown on HPF proliferation, we measured the expression of proliferation-related marker genes, including Ki67, PCNA, and Cyclin D1. Following transfection with si-NC or si-hsa_circ_0007482, the mRNA levels of Ki67, PCNA, and Cyclin D1 in the si-hsa_circ_0007482 group were significantly lower than those

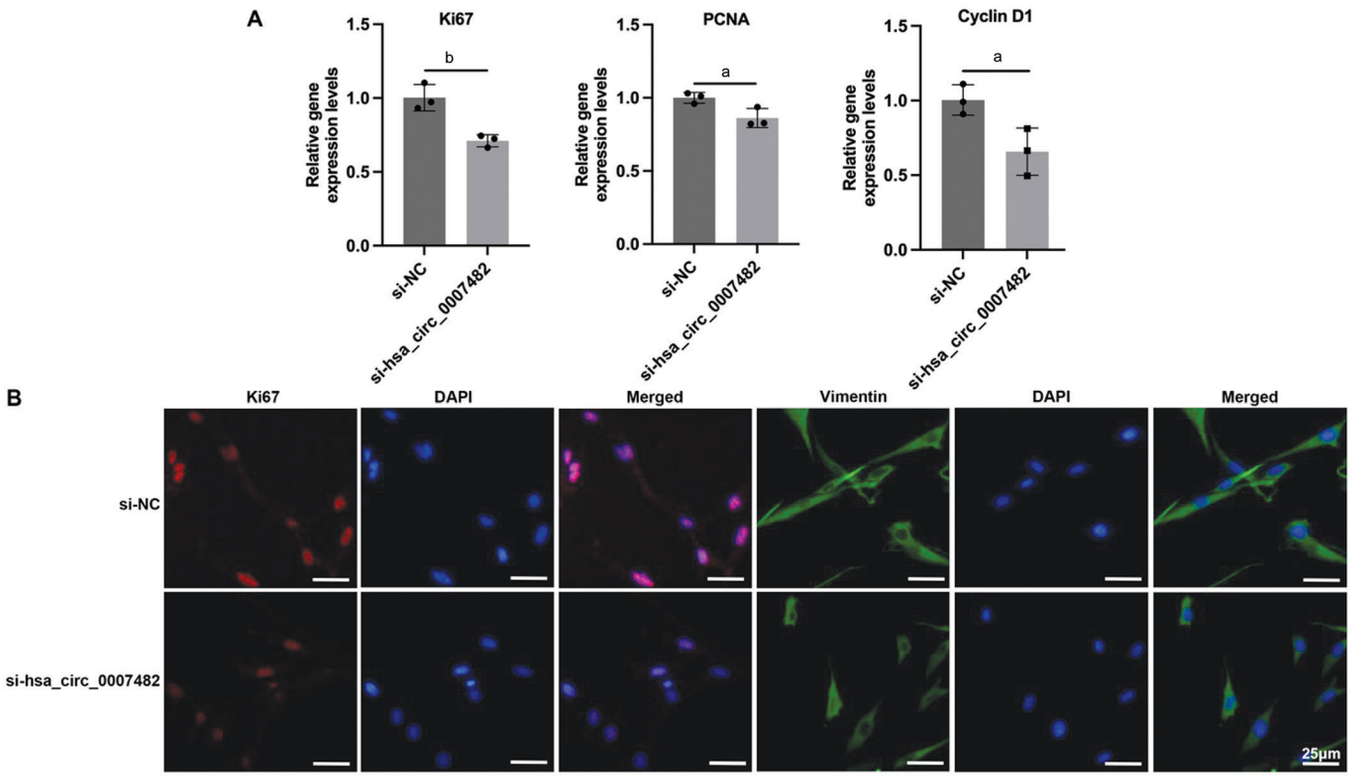

Figure 4 Knockdown of hsa_circ_0007482 suppresses the proliferation of HPFs A: The mRNA expression levels of Ki67, PCNA, and Cyclin D1 were detected in HPFs transfected with si-NC or si-hsa_circ_0007482; B: Effect of hsa_circ_0007482 on the expression of Ki67 (red) and vimentin (green) were detected using immunofluorescence staining. DAPI (blue) was used to stain the nucleus. Scale bar: 25 μm. All values are expressed as mean±SD of three independent experiments. [a]*P*<0.05, [b]*P*<0.01. HPFs: Human pterygial fibroblasts; PCNA: Proliferating cell nuclear antigen; NC: Negative control; DAPI: 4′,6-diamidino-2-phenylindole; SD: Standard deviation.

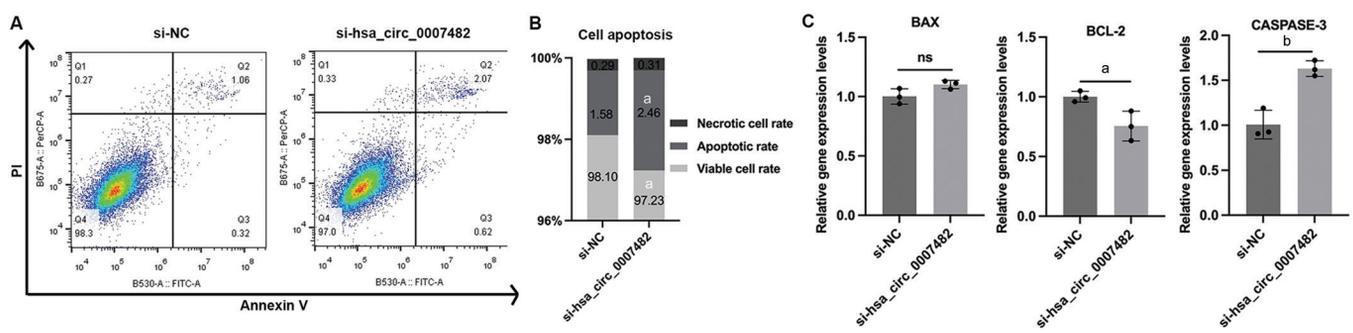

Figure 5 Knockdown of hsa_circ_0007482 induces apoptosis in HPFs A: HPFs were transfected with si-NC and si-hsa_circ_0007482 before staining with annexin V-FITC and PI. The effect of hsa_circ_0007482 on HPFs apoptosis was detected using flow cytometry. Q1: Necrotic, Q2: Late apoptotic cells, Q3: Early apoptotic cells, Q4: Viable cells. B: Effect of si-NC or si-hsa_circ_0007482 on the necrotic-cell, apoptosis, and viable-cell rates of HPFs; C: The mRNA expression levels of BAX, BCL-2, and Caspase-3 were detected in HPFs transfected with si-NC or si-hsa_circ_0007482. All values are expressed as mean±SD of three independent experiments. [a]*P*<0.05, [b]*P*<0.01. HPFs: Human pterygium fibroblasts; NC: Negative control; SD: Standard deviation.

in the si-NC group. Immunofluorescence staining further demonstrated that hsa_circ_0007482 knockdown markedly attenuated Ki67 expression in HPFs compared to the si-NC group (Figure 4A). We also investigated the impact of hsa_circ_0007482 on the expression of the mesenchymal marker vimentin in HPFs through immunofluorescence staining. In comparison to the si-NC group, hsa_circ_0007482 knockdown significantly reduced vimentin expression in HPFs (Figure 4B).

Silencing of hsa_circ_0007482 Promotes Apoptosis in HPFs The impact of hsa_circ_0007482 on apoptosis in HPFs was assessed through flow cytometry (Figure 5A). Hsa_circ_0007482 knockdown influenced necrotic cells (Q1), late apoptotic cells (Q2), early apoptotic cells (Q3), and viable cells (Q4). Following hsa_circ_0007482 knockdown, the apoptosis rate of HPFs significantly higher that of cells in the si-NC group (Figure 5B). Moreover, the viable cell rate in HPFs with hsa_circ_0007482 knockdown was notably lower

than in the si-NC group. However, necrotic cell rates showed no significant difference between the si-NC and si-hsa_circ_0007482 groups.

To further understand the effects of hsa_circ_0007482 knockdown on HPF apoptosis, we measured the expression of apoptosis-related marker genes, including BAX, BCL-2, and CASPASE-3. Post-transfection with si-NC or si-hsa_circ_0007482, the mRNA expression levels of BCL-2 significantly decreased, while those of Caspase-3 significantly increased in HPFs compared to the si-NC group (Figure 5C). Although there was no significant difference in BAX expression levels between HPFs transfected with si-NC or si-hsa_circ_0007482, the expression level of BAX was upregulated compared to that in the si-NC group (Figure 5C). These results align with the conclusions drawn from flow cytometry. In summary, silencing of hsa_circ_0007482 influenced the biological function of HPFs by inducing apoptosis.

DISCUSSION

Given the commonality and high recurrence rate of pterygium, addressing its devastating effects on quality of life necessitates innovative therapeutic strategies[22]. Recent studies have emphasized the crucial role of circRNAs in the diagnosing and prognosing various eye diseases[13]. CircRNAs play a regulatory role in cell proliferation, apoptosis, and migration in eye diseases *via* multiple factors. Previous studies have found that overexpression of circHIPK3 promotes the proliferation and migration of human retinal vascular endothelial cells[23], while knockdown of circHIPK3 suppresses EMT, proliferation and accelerates apoptosis of human lens epithelial cells[24]. Additionally, a prior study suggested that circRNAs may play a vital role in pterygia by targeting miRNAs to regulate EMT-related genes[25]. However, limited research has explored the relationship between circRNAs and pterygium development, and little is known about the expression and function of hsa_circ_0007482 in HPFs. In this study, we determined the correlation between the severity, vascular density, and thickness of pterygium and the expression levels of hsa_circ_0007482. Furthermore, we found that the knockdown of hsa_circ_0007482 affected the proliferation and apoptosis of HPFs.

We previously conducted a transcriptome analysis on paired normal conjunctiva and pterygium tissues, revealing an increased expression of hsa_circ_0007482 in pterygium tissues. This finding suggested the involvement of hsa_circ_0007482 in pterygium development[19]. Additionally, hsa_circ_0007482 has been reported to be associated with keloid development[20]. However, the specific biological function of hsa_circ_0007482 in pterygia remains unexplored. Therefore, this study aimed to assess the effects of hsa_circ_0007482 in HPFs and its correlation with the severity, vascular density, and thickness of pterygium. In our study, a positive correlation was observed between the severity, vascular density, and thickness of pterygium and the expression levels of hsa_circ_0007482. Moreover, the knockdown of hsa_circ_0007482 was found to regulate the proliferation and apoptosis of HPFs. These findings indicate a role for hsa_circ_0007482 in pterygium development, aligning with our previous study results. Despite providing valuable insights into the role of hsa_circ_0007482 in pterygium tissues and its impact on HPF functionality, it is essential to acknowledge a limitation in our study. Specifically, we did not explore the exact molecular mechanisms of hsa_circ_0007482 on pterygium development. Future investigations will aim to delve deeper into the specific regulatory role of hsa_circ_0007482 in HPFs and its overall impact on pterygium development. This focused approach will contribute to a more comprehensive understanding of the molecular mechanisms underlying the involvement of hsa_circ_0007482 in pterygium pathogenesis.

Cell proliferation serves as the foundation for the development, reproduction, and inheritance of organisms, playing a crucial role in their life activities[26]. It is also believed to be a key factor in the formation and growth process of pterygium[27]. In our study, we observed a significant decrease in the cell proliferation rate in HPFs 48h after transfection with hsa_circ_0007482 siRNA. Examining certain proliferation-related marker genes, the expression levels of Ki67, PCNA, and Cyclin D1 were notably reduced in HPFs with hsa_circ_0007482 knockdown. Additionally, the knockdown of hsa_circ_0007482 led to a reduction in vimentin expression. Recent studies have indicated that circ_0043144 knockdown represses retinal pigment epithelial cell proliferation and migration[17]. In addition, circ_0085020 silencing significantly inhibited HPFs proliferation and migration[18], and hsa_circ_0002406 was also found to promote the EMT process in pterygium[25]. These findings collectively highlight the significant regulatory role of circRNAs in cell proliferation and EMT, aligning with the results of our study.

Apoptosis, a physiological process essential for maintaining normal development in multicellular organisms by regulating survival, inflammation and proliferation of apoptotic cells, becomes disrupted in various diseases, including cancer, when abnormalities occur[28]. The regulation of cell apoptosis involves three crucial proteins: the pro-apoptotic BAX, the anti-apoptotic BCL-2, and the executioner Caspase-3. Our results indicate that the knockdown of hsa_circ_0007482 promoted cell apoptosis in HPFs, consistent with our previous study that revealed the association of dysregulated circRNAs with the apoptotic process[19]. Furthermore, the expression level of Caspase-3 was significantly upregulated, whereas that

of BCL-2 was significantly downregulated in HPFs with hsa_circ_0007482 knockdown. Previous reports have highlighted the impact of circ_0085020 silencing on apoptosis in HPFs and epithelial cells, influencing pterygium development[18]. Once again, our results underscore the involvement of circRNAs in pterygium development by influencing the apoptosis of HPFs. However, we acknowledge that our study did not delve into the specific mechanisms through which hsa_circ_0007482 knockdown modulates HPFs apoptosis. Future investigations will aim to address this aspect for a more comprehensive understanding.

In conclusion, our study revealed a significant upregulation of hsa_circ_0007482 in pterygium tissues, with its expression level positively correlating with pterygium severity, vascular density, and thickness. The silencing of hsa_circ_0007482 was found to inhibit HPF proliferation and promote HPF apoptosis. Our investigation not only shed light on some biological functions of hsa_circ_0007482 in HPFs but also laid the groundwork for further explorations into its role. Consequently, hsa_circ_0007482 emerges as a promising biomarker and a potential non-surgical treatment target for managing pterygium.

ACKNOWLEDGEMENTS

Foundation: Supported by Guangdong Basic and Applied Basic Research Foundation (No.2021A1515111012).

Conflicts of Interest: Zhang LY, None; **Liu X,** None; **Zheng S,** None; **Xi WQ,** None; **Wu XP,** None; **Nie DY,** None; **Hu HL,** None; **Wang JT,** None; **Liu XH,** None; **Zhang J,** None.

REFERENCES

1 Song P, Chang X, Wang M, An L. Variations of pterygium prevalence by age, gender and geographic characteristics in China: a systematic review and meta-analysis. *PLoS One* 2017;12(3):e0174587.

2 Rezvan F, Khabazkhoob M, Hooshmand E, Yekta A, Saatchi M, Hashemi H. Prevalence and risk factors of pterygium: a systematic review and meta-analysis. *Surv Ophthalmol* 2018;63(5):719-735.

3 Fernandes AG, Salomão SR, Ferraz NN, Mitsuhiro MH, Furtado JM, Muñoz S, Cypel MC, Cunha CC, Vasconcelos GC, Sacai PY, Morales PH, Cohen MJ, Cohen JM, Watanabe SS, Campos M, Belfort Junior R, Berezovsky A. Pterygium in adults from the Brazilian Amazon Region: prevalence, visual status and refractive errors. *Br J Ophthalmol* 2020;104(6):757-763.

4 Minami K, Tokunaga T, Okamoto K, Miyata K, Oshika T. Influence of pterygium size on corneal higher-order aberration evaluated using anterior-segment optical coherence tomography. *BMC Ophthalmol* 2018;18(1):166.

5 Fonseca EC, Rocha EM, Arruda GV. Comparison among adjuvant treatments for primary pterygium: a network meta-analysis. *Br J Ophthalmol* 2018;102(6):748-756.

6 Alsarhani W, Alshahrani S, Showail M, Alhabdan N, Alsumari O, Almalki A, Alsarhani A, Alluhaidan A, Alqahtani B. Characteristics and recurrence of pterygium in Saudi Arabia: a single center study with a long follow-up. *BMC Ophthalmol* 2021;21(1):207.

7 Zheng K, Cai J, Jhanji V, Chen H. Comparison of pterygium recurrence rates after limbal conjunctival autograft transplantation and other techniques: meta-analysis. *Cornea* 2012;31(12):1422-1427.

8 Wanzeler ACV, Barbosa IAF, Duarte B, Borges D, Barbosa EB, Kamiji D, Huarachi DRG, Melo MB, Alves M. Mechanisms and biomarker candidates in pterygium development. *Arq Bras Oftalmol* 2019;82(6):528-536.

9 Zhong X, Tang J, Li H, Shi X, Wu Y, Xia D, Zhang H, Ye J, Wu H. MiR-3175 promotes epithelial-mesenchymal transition by targeting Smad7 in human conjunctiva and pterygium. *FEBS Lett* 2020;594(7):1207-1217.

10 Masitas C, Peng Z, Wang M, Konai MM, Avila-Cobian LF, Lemieux L, Hovanesian J, Grady JE, Mobashery S, Chang M. Matrix metalloproteinase-14 as an instigator of fibrosis in human pterygium and its pharmacological intervention. *ACS Pharmacol Transl Sci* 2022;5(8):555-561.

11 Pisignano G, Michael DC, Visal TH, Pirlog R, Ladomery M, Calin GA. Going circular: history, present, and future of circRNAs in cancer. *Oncogene* 2023;42(38):2783-2800.

12 Yu X, Tong H, Chen J, Tang C, Wang S, Si Y, Wang S, Tang Z. CircRNA MBOAT2 promotes intrahepatic cholangiocarcinoma progression and lipid metabolism reprogramming by stabilizing PTBP1 to facilitate FASN mRNA cytoplasmic export. *Cell Death Dis* 2023;14(1):20.

13 Zhang C, Hu J, Yu Y. CircRNA is a rising star in researches of ocular diseases. *Front Cell Dev Biol* 2020;8:850.

14 Conn SJ, Pillman KA, Toubia J, Conn VM, Salmanidis M, Phillips CA, Roslan S, Schreiber AW, Gregory PA, Goodall GJ. The RNA binding protein quaking regulates formation of circRNAs. *Cell* 2015;160(6):1125-1134.

15 Beilerli A, Gareev I, Beylerli O, Yang G, Pavlov V, Aliev G, Ahmad A. Circular RNAs as biomarkers and therapeutic targets in cancer. *Semin Cancer Biol* 2022;83:242-252.

16 Kour B, Gupta S, Singh R, Sophiarani Y, Paul P. Interplay between circular RNA, microRNA, and human diseases. *Mol Genet Genomics* 2022;297(2):277-286.

17 Yao J, Hu LL, Li XM, Shan K, Zhou RM, Ge HM, Yao MD, Jiang Q, Zhao C, Yan B. Comprehensive circular RNA profiling of proliferative vitreoretinopathy and its clinical significance. *Biomed Pharmacother* 2019;111:548-554.

18 Li XM, Ge HM, Yao J, Zhou YF, Yao MD, Liu C, Hu HT, Zhu YX, Shan K, Yan B, Jiang Q. Genome-wide identification of circular RNAs as a novel class of putative biomarkers for an ocular surface disease. *Cell Physiol Biochem* 2018;47(4):1630-1642.

19 Liu X, Zhang J, Nie D, Zeng K, Hu H, Tie J, Sun L, Peng L, Liu X, Wang J. Comparative transcriptomic analysis to identify the important coding and non-coding RNAs involved in the pathogenesis of pterygium. *Front Genet* 2021;12:646550.

20 Shi J, Yao S, Chen P, Yang Y, Qian M, Han Y, Wang N, Zhao Y, He Y, Lyu L, Lu D. The integrative regulatory network of circRNA and microRNA in keloid scarring. *Mol Biol Rep* 2020;47(1):201-209.

21 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(2):133-138.

22 Baheran SS, Alany RG, Schwikkard S, Muen W, Salman LN, Freestone N, Al-Kinani AA. Pharmacological treatment strategies of pterygium: Drugs, biologics, and novel natural products. *Drug Discov Today* 2023;28(1):103416.

23 Shan K, Liu C, Liu BH, Chen X, Dong R, Liu X, Zhang YY, Liu B, Zhang SJ, Wang JJ, Zhang SH, Wu JH, Zhao C, Yan B. Circular noncoding RNA HIPK3 mediates retinal vascular dysfunction in diabetes mellitus. *Circulation* 2017;136(17):1629-1642.

24 Liu X, Liu B, Zhou M, Fan F, Yu M, Gao C, Lu Y, Luo Y. Circular RNA HIPK3 regulates human lens epithelial cells proliferation and apoptosis by targeting the miR-193a/CRYAA axis. *Biochem Biophys Res Commun* 2018;503(4):2277-2285.

25 Yu J, Luo J, Li P, Chen X, Zhang G, Guan H. Identification of the circRNA-miRNA-mRNA Regulatory Network in Pterygium-Associated Conjunctival Epithelium. *Biomed Res Int* 2022;2022:2673890.

26 Wang W, Zou H, Chen N, Tian Y, Hao H, Pang Y, Zhao X, Zhu H, Yu D, Du W. Knockdown of CLAUDIN-6 inhibited apoptosis and induced proliferation of bovine cumulus cells. *Int J Mol Sci* 2022;23(21):13222.

27 Kim KW, Park SH, Wee SW, Kim JC. Overexpression of angiogenin in pterygium body fibroblasts and its association with proliferative potency. *Invest Ophthalmol Vis Sci* 2013;54(9):6355-6362.

28 Riley JS, Bock FJ. Voices from beyond the grave: The impact of apoptosis on the microenvironment. *Biochim Biophys Acta Mol Cell Res* 2022;1869(11):119341.