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Inhibition of heat shock protein 47 suppressed collagen production in Tenon's capsule fibroblasts

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Abstract

Introduction. Glaucoma is the leading cause of irreversible blindness worldwide, and conjunctival bleb scarring remains the most frequent reason for the failure of glaucoma filtration surgery. Excessive proliferation of fibroblasts from Tenon's capsule and excessive deposition of collagen contribute to the scarification of the conjunctival bleb. Heat shock protein 47 (HSP47) is assumed to act as a collagen-specific molecular chaperone, and thereby involved in the pathogenesis of fibrotic diseases. Therefore, we investigated the effect of HSP47 knockout against collagen type I (COLI) production in rat Tenon's fibroblasts.

Material and methods. Newborn rat Tenon's fibroblasts were cultured and verified by anti-vimentin antibody. Transfection efficiency of small interference RNA targeted against HSP47 was confirmed by quantitative real-time polymerase chain reaction (RT-qPCR) at 48 h after siRNA transfection and by western blot at 72 h after transfection. The mRNA and protein expression of HSP 47 and COLI were detected by RT-qPCR and western blot. The proliferation of cells was measured by cell counting kit-8 assay.

Results. HSP47 siRNA down-regulated the mRNA and protein levels of HSP47 in rat Tenon's fibroblasts, and suppressed the mRNA and protein expression of COLI. Moreover, HSP47 siRNA had no significant effect on proliferation of rat Tenon's fibroblasts.

Conclusions. HSP47 siRNA inhibits the production of COLI in rat Tenon's fibroblasts, and may be the potential therapeutic method in bleb scarring after glaucoma filtration surgery. *(Folia Histochemica et Cytobiologica 2023, Vol. 61, No. 3, 153–159)*

Keywords: heat shock protein 47; collagen; Tenon's capsule; fibroblast; glaucoma; scar

Introduction

Glaucoma is the most frequent cause of irreversible blindness worldwide characterized by elevation of intraocular pressure (IOP) and visual field defects. Glaucoma filtration surgery is suggested as one of the most effective and economical surgeries to reduce IOP for glaucoma patients who fail medical treatment [1, 2]. Nevertheless, conjunctival bleb scarification often leads to the failure of this surgical procedure. Excessive proliferation of fibroblasts from Tenon's capsule and excessive accumulation and deposition of extracellular matrix components represented by collagen contribute to the scarring of the conjunctival bleb and uncontrolled IOP [3]. Although antimetabolites such as 5-fluorouracil and mitomycin C are clinically effective in preventing bleb failure, the accompanied complications such as bleb leaks, corneal toxicity as well as endophthalmitis limit its extensive use [4,5]. Therefore, alternative agents are needed for anti-scarring and to improve the success rate of glaucoma filtration surgery.

Various researchers have demonstrated that heat shock protein 47 (HSP47), a collagen-specific molecular chaperone, plays a profound role in the pathogenesis of numerous fibrotic diseases [6–10]. Furthermore, we reported previously that increased expression of HSP47 is in close proximity to the growing accumulation of collagen type I (COLI) in a rat conjunctival bleb model after filtration surgery

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[11], and HSP47 is upregulated in the aqueous humor of patients with acute primary angle closure [12], suggesting that HSP47 may have an important effect on scarring after glaucoma filtration surgery. Recent evidence showed that HSP47 may be a therapeutic target for anti-fibrotic treatment and against breast cancer metastasis [7, 13, 14]. Moreover, recent clinical trials revealed that it also exhibits therapeutic effects in patients with advanced hepatic fibrosis [15]. However, whether inhibiting HSP47 could suppress conjunctival bleb scarring remains unknown. Hence, we designed this study to investigate the effect of HSP47 knockout against conjunctival bleb scarification in rat Tenon's fibroblast (RTF).

Material and methods

Isolation and culture of rat Tenon's fibroblast (RTF). This research was approved by the Ethics Committee of Xi'an Jiaotong University (No. 2015-107). Newborn Sprague–Dawley rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal care and experimental procedures were conducted in accordance with the ARVO Statement for the Use of Animals.

To isolate RTF, we accompanied the set-up protocols with minor adjustments [16, 17]. Briefly, the Tenon's tissue removed from the newborn rat eye was cut into small, round pinhead size pieces of approximately 1 × 1 mm, treated with high glucose Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, New York, NY, USA) and 1% penicillin–streptomycin (Gibco) in an incubator at 37°C with 5% carbon dioxide. The medium was changed every 2 days. When the RTF cells reached about 70% to 80% confluence, they were passaged with a 1:3 split using 0.25% trypsin and 1 mM EDTA (Gibco). RTF at passages 3 to 6 were used for this study.

Immunohistochemical staining for vimentin. The purity of the cultured RTF cells was verified by anti-vimentin antibody (Bioss, China). Fibroblasts at passage 3 were used for immunostaining. Briefly, cells were fixed with 4% paraformaldehyde for 2 h at room temperature, then permeated with 0.4% Triton X-100 for 15 min, and blocked by 10% bovine serum albumin for 1 h at 37°C. After that, the cells were incubated with vimentin rabbit anti-rat antibody (1:100, bs-0756R, Bioss, China) at 4°C overnight, washed in phosphate-buffered saline (PBS), and then were incubated with goat secondary antibody to rabbit (Abcam, Cambridge, UK) for 1 h at 37°C. Immunoreactivity was detected *via* a diaminobenzidine method and counterstained with hematoxylin. The incubation of slides without primary antibody was used as a negative control.

Transfection with small interference (si)RNA targeted against HSP47. Proliferating RTF cells were incubated with HSP47 siRNA (General Biosystems, Chuzhou, China) in complete DMEM containing lipofectamine 3000 (Invitrogen) following the manufacturer's protocols. Negative control siRNA was used as siRNA control. The transfection efficiency was confirmed by real-time quantitative PCR (RT-qPCR) at 48 h after siRNA transfection and by western blot at 72 h after transfection. The siRNA sequences are listed in Table 1.

Table 1. Sequences of heat shock protein 47 siRNA

Primer Name	Sequence (5′ to 3′)
siHSP47-1	GCGCAAUGUGACCUGGAAATT
siHSP47-2	GCAACUAAAGACCUGGAUGTT
siHSP47-3	CCAUGACCUGCAGAAACAUTT
siNCC[1]	UUCUCCGAACGUGUCACGUTT

[1] negative control

Cell counting kit-8 assay. The RTF cell viability was detected through cell counting kit-8 (CCK8) assay (Dojindo, Japan). The samples of RTF were seeded into 96-well plates. CCK8 was then added to each well at 0, 24, 48, 72, and 96 h after transfection. Absorbance was measured at 450 nm when incubated in the dark at 37°C for 1 h.

Real-time quantitative PCR (RT-qPCR). RNA extraction from the RTF was conducted using an RNA-Isolation Kit (Takara, Kusatsu, Japan). A Prime Script RT Reagent Kit (Takara) was utilized for reverse transcription, followed by amplification with an ExTaq Kit (Takara). Cycling conditions were initial denaturation at 95°C for 2 min, followed by 36 cycles consisting of denaturation at 95°C for 10 s, annealing at 55°C for 30 s and elongation at 60°C for 30 s. Primer sequences are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a control housekeeping gene. The results were normalized to GAPDH expression. The quantity of mRNA was calculated by normalizing the cycle threshold (Ct) of HSP47 and collagen type I to the Ct of the housekeeping gene Gapdh of the same RNA sample, according to the following formula: the average GAPDH Ct was subtracted from the average HSP47 and collagen I Ct. This result represents the ΔCt, which is specific and can be compared with the ΔCt of a calibration sample (negative control). The subtraction of control ΔCt from the ΔCt of the RTF was referred to as ΔΔCt. The relative expression of HSP47 and collagen I (in comparison to the control) in the RTF was determined by $2^{-\Delta\Delta Ct}$.

Western blot analysis. Total protein was extracted from the RTF cells by a lysis buffer and a bicinchoninic acid (BCA) protein assay (Applygen, Beijing, China) was used for total protein concentration quantification. Subsequently, the total protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), and blocked using 5% non-fat milk in Tris-buffered saline with Tween-20 for 1 h at room temperature. The membrane was incubated at 4°C overnight with the primary antibodies: monoclonal mouse anti-HSP47 (1:1000; Enzo, ADI-SPA-470, New York, NY, USA), polyclonal rabbit anti-collagen 1 (1:5000; Abcam, ab34710), and polyclonal rabbit anti-GAPDH

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Table 2. Sequences of PCR primers

Primer name	Reverse sequence (5′ to 3′)	Forward sequence (5′ to 3′)
HSP47	GCAGCTTCTC CTTCTCGTCGTC	GATGGTAGACAACCGTGGCTTC
Collagen 1	CGGGCAGGGTTCTTTCTA	TGACCAGCCTCGCTCACA
GAPDH	GCCTTGGCAGCACCAGTGGATGC	GTCAGCAATGCATCCTGCACCACC

GAPDH — Glyceraldehyde-3-phosphate dehydrogenase

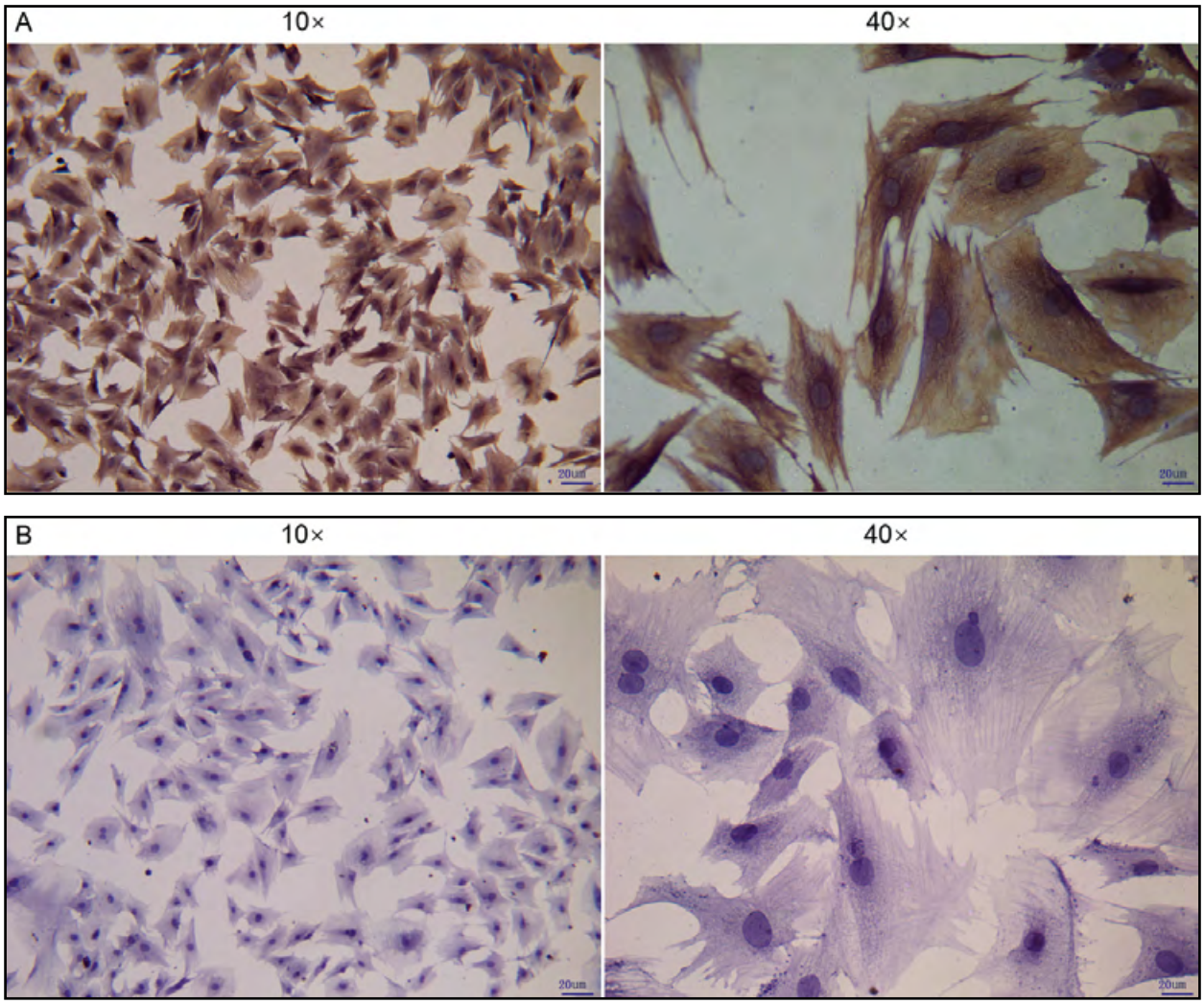

Figure 1. Immunostaining of cultured fibroblasts cells isolated from rat Tenon's capsule. **A.** The cytoplasm of rat Tenon's fibroblasts (RTFs) stains positively to vimentin. **B.** Negative control to vimentin Scale bar: 20 μm.

(1:2000; GoodHere, AB-P-R001, Hangzhou, China). GAPDH was used as the internal control and the protein bands were visualized using chemiluminescence. The relative intensities were calculated as the densitometric proportion between the protein band and GAPDH using Image J.

Statistical analysis. GraphPad Prism software version 9 (Graph Pad Software, San Diego, CA, USA) was used to conduct Student's *t*-test and one-way analysis of variance (ANOVA) between control and experimental groups. All data are expressed as the means ± standard deviation (SD) of at least three independent trials. The $P < 0.05$ was considered statistically significant.

Results

Immunostaining of cultured RTFs

To identify RTF, immunocytochemical staining of vimentin which is a unique cell marker of RTF was utilized [18]. All the fibroblasts derived from Tenon's capsule expressed vimentin in the cytoplasm (Fig. 1).

Inhibition of HSP47 by siRNA in RTFs

The transfection efficiency of siHSP47-1, siHSP47-2, and siHSP47-3 in RTF cells was determined *via*

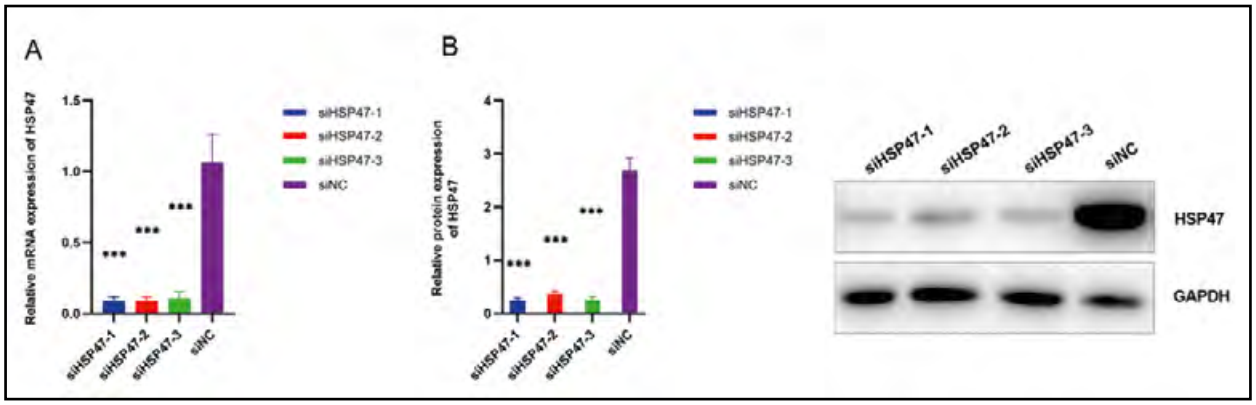

Figure 2. siRNA inhibited HSP47 expression. **A.** As in Fig. 1. RTFs were treated with siHSP47-1, or siHSP47-2, or siHSP47-3, or negative control (siNC) for 48 h, and the levels of HSP47 mRNA were analyzed by RT-qPCR. **B.** RTFs were treated with the studied siRNAs or siNC for 72 h, and the expression of HSP47 protein was detected by western blot. $^{***}P < 0.001$ *vs*. siNC group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control housekeeping gene and internal control protein.

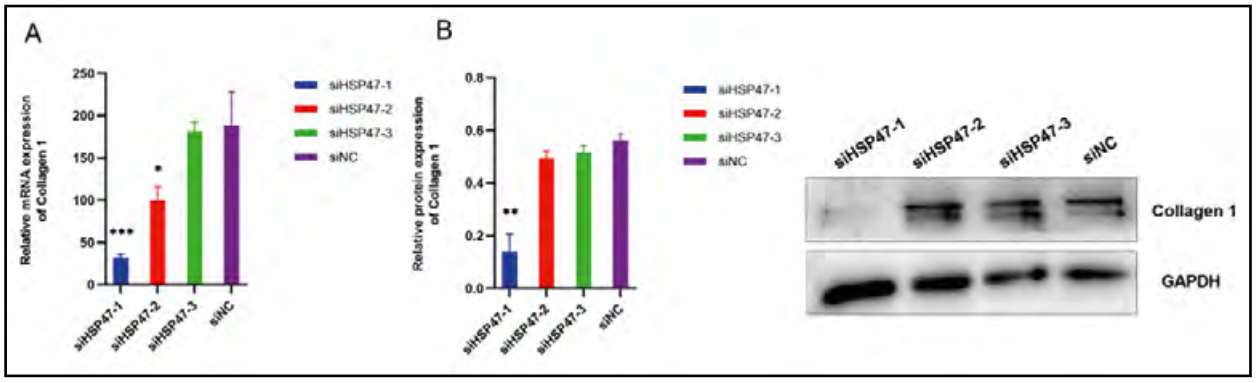

Figure 3. HSP47 siRNA downregulated collagen I expression. **A.** RTFs were treated with siHSP47-1, or siHSP47-2, or siHSP47-3, or negative control (siNC) for 48 h, and the expression of collagen I mRNA was analyzed by RT-qPCR. **B.** RTF cells were cultured with different siRNA or siNC for 72 h, and the levels of collagen I were determined by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* siNC group.

RT-qPCR at 48 h after siRNA transfection and by western blot at 72 h after transfection. The mRNA and protein levels of HSP47 were significantly down--regulated in RTFs by all used siRNAs (Fig. 2).

Downregulation of COLI by HSP47 siRNA in RTF

Collagen is recognized to play an essential role in conjunctival bleb scarring postoperatively via excessive deposition within the filtration region. To determine HSP47's potential effect on bleb scarification, we explored the expression of COLI in RTFs at the mRNA and protein levels. The RT-qPCR data showed that siHSP47-1 and siHSP47-2 downregulated the mRNA levels of COLI, although to a different degree (Fig. 3a), while only siHSP47-1 significantly suppressed the expression of COLI protein as shown by the Western blot analysis (Fig. 3b).

Effects of HSP47 siRNA on RTFs' viability

The results shown in Fig. 4 revealed that HSP47 silencing had no effect on the proliferation of RTFs.

Discussion

In the present study, we found that HSP47 knockout suppressed the protein and mRNA expression of COLI in RTFs. To the best of our knowledge, this study provides the first evidence that HSP47 siRNA reduces the levels of collagen I in RTF cells. These *in vitro* results indicate the possibility of using HSP47 siRNA for the therapy of conjunctival bleb scarring after glaucoma filtration surgery.

HSP47 is a molecular chaperone and is involved in the processing, assembly, folding, and secretion of collagens [6, 7]. Various studies have demonstrated that HSP47 plays a crucial role in regulating collagen in numerous fibrotic diseases. What is more, in our previous study, we found that increased expression

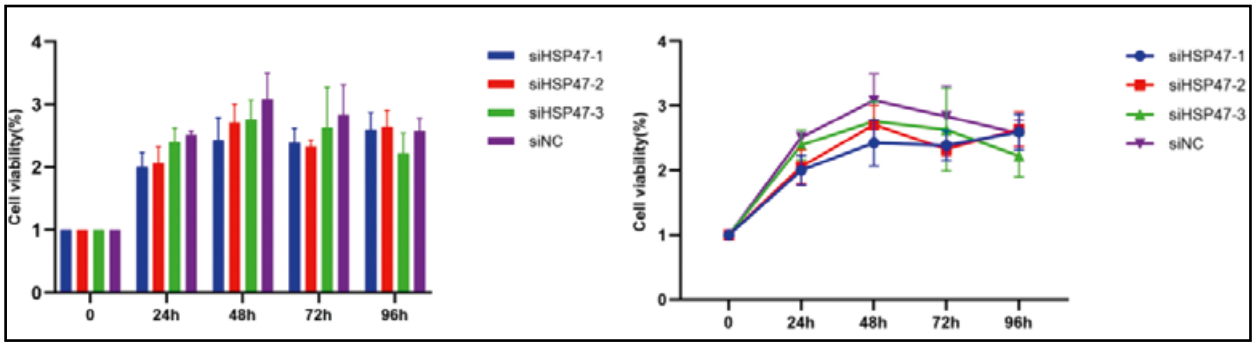

Figure 4. The effects of HSP47 siRNAs on RTFs proliferation. RTFs were treated with siHSP47-1, siHSP47-2, siHSP47-3 or negative control (siNC) for 96 h, and cell viability was detected by CCK8 assay. Compared to the siNC, the cell proliferation slightly decreased, but there were no statistical differences.

of HSP47 correlates with the growing deposition of COLI in a rat conjunctival bleb model [11], suggesting the importance of HSP47 in the pathologic tissue remodeling progression of conjunctival bleb scarification. Thus, we hypothesize that HSP47 may be a potential target to prevent conjunctival bleb scarring postoperatively.

Collagen accumulation in the extracellular matrix is a hallmark of fibrogenesis. Previous studies have shown that COLI is one of the core factors in the process of conjunctival bleb scarification postoperatively which is actually an abnormal wound healing procedure in response to tissue injury [3, 11, 19–22]. It is well known that fibroblasts from Tenon's capsule have a key effect on the pathology of conjunctival bleb scarification [3, 20, 22].

Small interfering RNAs (siRNAs) can effectively inhibit the expression of a specific gene. Preclinical studies revealed that down-regulation of HSP47 through siRNAs leads to decreased collagen deposition [23–26]. The safety, tolerability, and pharmacokinetics of lipid nanoparticle encapsulating HSP47 siRNA have been evaluated in clinical trials [15]. Therefore, we conducted the *in vitro* study aimed to determine the role of HSP47 siRNA in the regulation of COLI expression in RTFs, which could provide valuable information about its potential application against bleb scarring after glaucoma filtration surgery.

We found that the down-regulation of HSP47 by siRNAs resulted in a decreased expression of COLI in RTFs, which is in accord with the results of earlier studies. Guo *et al.* [10] showed that down-regulating HSP47 significantly reduced the mRNA and protein expression of COLI in guinea pig scleral fibroblasts. Tang *et al.* [27] demonstrated that microRNA-29 significantly inhibited HSP47 and COLI expression in human fetal scleral fibroblasts. Our data suggest that HSP47 siRNA has potential application in postoperative anti-scarring therapy. In contrast to the suppressive effects of three siRNAs on the HSP47 mRNA and protein expression, only one of the tested siRNA decreased COLI protein levels. Thus, it is plausible to draw a conclusion that collagen 1 down-regulation may not entirely depend on HSP47 silencing only. It is obvious that HSP47 is only one of many factors affecting COLI protein expression.

The limitations of our study should be mentioned. Various profibrotic factors have the potential to mediate fibrogenesis postoperatively, and further studies are required to investigate the interactions among HSP47 and other agents including fibrogenic cytokines such as transforming growth factor-beta1 [8], connective tissue growth factor [20], and to explore the exact mechanisms of HSP47 siRNA down-regulation of COLI after glaucoma filtration surgery.

In conclusion, we found that one type of the designed HSP47 siRNAs down-regulates COLI expression in rat Tenon's fibroblast RTF cells. Therefore, our data may provide information on the potential therapeutic targets of HSP47 in bleb scarring after glaucoma filtration surgery. Further basic and clinical studies should be crucial to explore precise mechanisms of HSP47 siRNA to suppress bleb scarification.

Article information

Data availability statement

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Ethics statement

This research was approved by the Ethics Committee of Xi'an Jiaotong University (No. 2015-107).

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Author contributions

Weiwei Wang designed the study, and conducted the statistical analysis. Haiyan Li carried out the experiment. Weiwei Wang drafted and revised the manuscript. All the authors have read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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