Original Article
Role of heat shock protein 47 in epithelial-mesenchymal transition of retinal pigment epithelial cells stimulated by TGF-β2

Dong-Chang Zhang*, Li-Li Yuan*, Zhe Zhang, Ya-Nan Lu

*Shanxi Eye Hospital, Taiyuan 030002, Shanxi, China. *Co-first authors.*

Abstract: Epithelial-mesenchymal transition (EMT) plays critical in proliferative retinopathy (PVR) characterized by the formation of fibrotic contractile membranes, which composed by extracellular matrix (ECM) and several types of cells especially RPE cells on the epi- and sub-retinal surface. In EMT, epithelial cells lose the epithelial phenotype and acquire the mesenchymal like phenotype, the trans-differentiation is characterized by increased cell invasion, migration, and excess production of ECM. Heat shock protein (HSP) 47 is a collagen specific chaperone, it is likely that increased expression of HSP47 contributes to the EMT and the accumulation of procollagen and, then cause an excessive accumulation of ECM in some fibrotic diseases, but the role of HSP47 in PVR and the underlying mechanism has not been clearly delineated. Our study aims to investigate the role of HSP47 in EMT of RPE cells. ARPE-19 cells were used in the experiments. After 4 h of serum starvation, the cells were divided into four groups: untreated, treated with 5 ng/ml TGF-β2 for 36 h, treated with 5 ng/ml TGF-β2 for 12 h then transfected with HSP47 siRNA or control siRNA for 24 h respectively. The cytomorphological change was observed under a microscope and the cell migration was studied by trans-well assay. The expression of HSP47 and ECM proteins (αSMA, FN and Col-I) were tested by quantitative real time PCR and Western blot analysis. All experiments were repeated three times. The ARPE-19 cells became long, looked like shuttle-shaped, and the number of migrated cells increased by 2.28 folds when stimulated by 5 ng/ml TGF-β2 for 36 h (P<0.05). The expression of HSP47 and ECM proteins (αSMA, FN and Col-I) were up-regulated by 2.56±0.17 folds, 3.01±0.31 folds, 2.42±0.21 folds and 2.74±0.16 folds respectively when stimulated by TGF-β2 for 36 h. Knocking down HSP47 with siRNA transfection could inhibit the changes in cell morphology, cell migration and the expressions of HSP47 and ECM proteins significantly. This study demonstrated that HSP47 modulates TGF-β2-induced EMT, migration and ECM proteins accumulation of ARPE-19 cells specifically, the chaperoning effect of which may consequentially contribute to PVR.

Keywords: EMT, HSP47, RPE cells, ECM

Introduction

Proliferative vitreoretinopathy (PVR) is a major cause of failure after retinal detachment surgeries. Fibrotic lesions on retinal surface reduce the flexibility of retina, result in retinal detachment, and lead to difficulty in retinal reattachment and severe vision loss [1]. PVR is characterized by the formation of contractile membranes composed of extracellular matrix (ECM) and different types of cells especially retinal pigment epithelial (RPE) cells [2, 3].

Previously, there were no effective approaches for preventing the onset of PVR, until mounting evidence indicated an important role for the epithelial-mesenchymal transition (EMT). Ex-

cessive wound healing and stimulation of inflammatory cytokines result in EMT. Once activated, trans-differentiated RPE cells could migrate into the vitreous cavity, produce ECM proteins (collagen type I and fibronectin) [4], and transform into fibroblast-like cells, which results in the formation of contractile membranes. Therefore, it may be of great value for PVR patients to prevent the EMT of RPE cells. Heat shock proteins are expressed under the influence of various stresses, and they are deemed as important modulators of many pathological processes. HSP47 is a 47-KD collagen-specific molecular chaperone, which belongs to the serpin superfamily [5]. HSP47 is a basic glycoprotein binds to collagen type I in collagen-producing cells and it has a crucial

Table 1. Primer sequence for real time RT-PCR

Gene Name	Type	Sequence
HSP47	Forward	5'-TCCAGCCTCATCATCCTCA-3'
	Reverse	5'-GCCATCTGTGTCCAACTCA-3'
αSMA	Forward	5'-GTGTTATGTAGCTCTGGACTTTGAAAA-3'
	Reverse	5'-GGCAGCGGAAACGTTCATT-3'
FN	Forward	5'-TGGAGGAAGCCGAGGTTT-3'
	Reverse	5'-CAGCGGTTTGCGATGGTA-3'
Col-I	Forward	5'-GGCGACAGAGGCATAAAGGG-3'
	Reverse	5'-GAGGTGGCTGGGGCAGGAAG-3'

role in the synthesis of ECM proteins [6]. It has been found overexpressed in many fibrotic diseases, including renal fibrosis [7], scar hypertrophy [8], hepatic fibrosis [9], and idiopathic pulmonary fibrosis [10]. Previously, we found HSP47 was highly expressed in PVR preretinal membranes and vitreous specimens taken from PVR patients (our unpublished data). So, it is logical to infer HSP47 is associated with the pathogenesis of PVR, and conclusions from several recent studies have supported this contention. However, how does HSP47 regulate EMT of RPE cells and the mechanism is not clear. In this study, we investigated the putative role of HSP47 in EMT of ARPE-19 cells at cytomorphological and molecular levels by using siRNA technique then discussed the feasibility of HSP47 siRNA as a specific therapeutic method for PVR.

Materials and methods

Cell culture and stimulated by TGF-β2

ARPE-19 cell line was obtained from the American Type Culture Collection (ATCC, USA) and cultured in DMEM-F12 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% penicillin-streptomycin in an atmosphere of 5% CO_2 at 37°C. Cells between the third and fifth passages were used in this study, and cultures were allowed to reach 70~80% confluence. After 4h of serum starvation, ARPE-19 cells were incubated with 5 ng/ml recombinant human TGF-β2 (Abcam, Britain) for 12 h. All experiments were performed at least three times with independent cell cultures.

SiRNA and cell transfection

Small interfering RNA (siRNA) sequence (TGG-CTTCATGGTGACTCGGTC) targeting HSP47 was

purchased from FuNeng Gene Biotechnology, Inc. (Guang Zhou, China). ARPE-19 cells were transfected with HSP47 siRNA according to the manufacturer's instructions and correlative studies [11, 12]. The scrambled siRNA which might cause degradation of any known cellular mRNA instead of HSP47 were used as the negative control.

The ARPE-19 cells were cultured in 6-well plates at the cell density of 2.5 × 105 cells per well in DMEM. The 50 nM HSP47 siRNA duplex was mixed with 2 μg/ml Lipofectamine TM 2000 (Life Tech, USA) to form the transfection complexes. The mixture was then added into the cells for 24 h in a CO_2 incubator at 37°C. As negative control, the scrambled siRNA was used instead of the HSP47 siRNA.

Morphology assay

In morphology assay, ARPE-19 cells were divided into four groups: untreated, treated with 5 ng/ml TGF-β2 only, 5 ng/ml TGF-β2 added HSP47 siRNA or control siRNA for 24 h respectively. The morphological changes of the cells in each group were observed with microscope.

Transwell assay

Migration of the ARPE-19 cells was measured by transwell assay. 12 well ThinCertTM cell culture inserts in the wells of a 12-well plate. 500 μl serum-free culture medium and 200 μl cell suspension of each group were added to each cell culture insert (1 × 105 cells/well), incubated for 24 h at 37°C, 5% CO_2. Then, the migrated cells were fixed with 4% paraformaldehyde, stained with crystal violet (1%), excess dye were removed, then observed under a microscope.

Expression analysis in mRNA level

Quantitative real time PCR was used to evaluate mRNA expression of HSP47 and ECM proteins (αSMA, FN and Col-I) in ARPE-19 cells stimulated by TGF-β2. Total RNA was extracted from the cells by using Trizol reagent (Takara, Shiga, Japan). 500 ng RNA was reverse transcribed into cDNA with PrimeScript RT Master Mix (Takara). All the qRT-PCR samples were performed using SYBR Green PCR Master Mix (Takara) on an Applied Biosystems Step One Plus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). The condi-
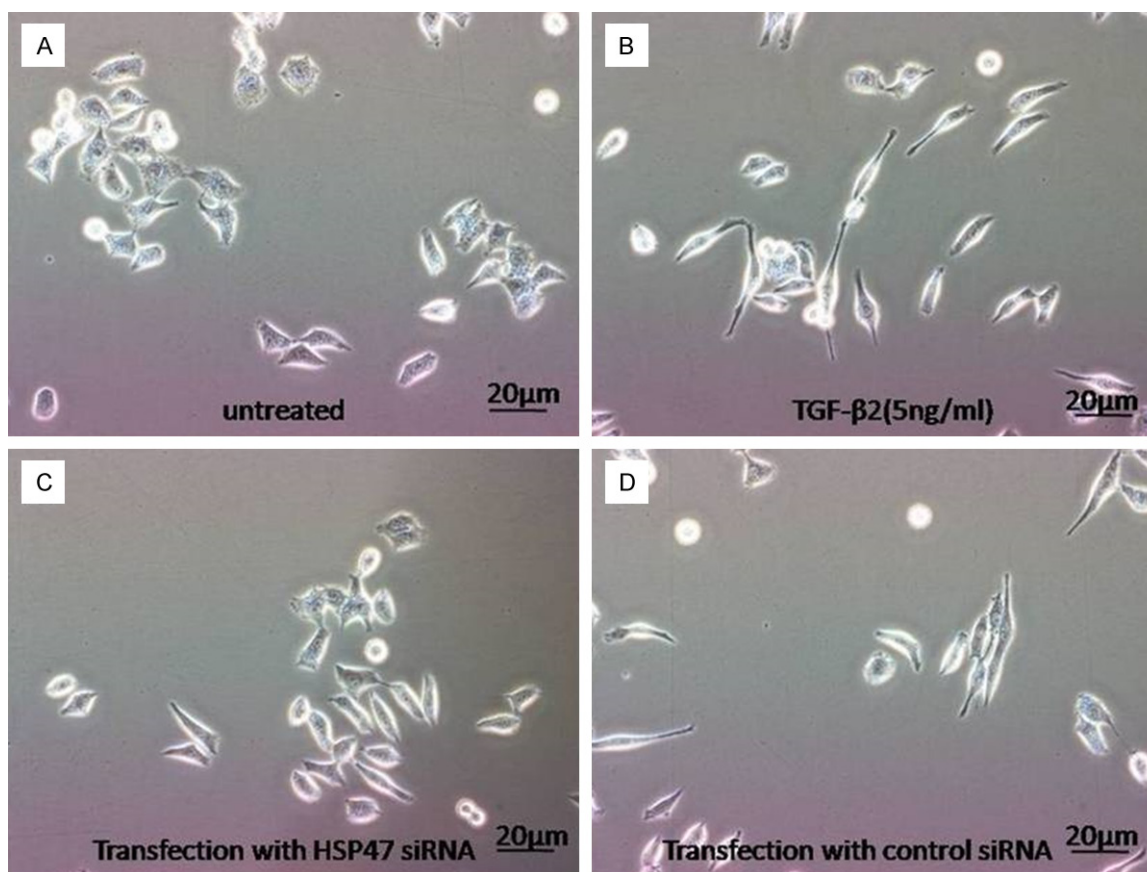
Figure 1. A: Cultured ARPE-19 cells presented similar round or polygonal; B: ARPE-19 cells changed into long shuttle shaped appearance stimulated by TGF-β2 (5 ng/ml); C: Transfection with HSP47 siRNA inhibited the change in cell morphology significantly; D: Transfection with control siRNA did not exert a significant influence in cell morphology.

tions were as following: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 64°C for 34 s, and a melt curve step. The target gene expression levels for each experiment were normalized to GAPDH. The relative gene expression levels were detected and calculated by using the ΔΔCt comparative method. The RT-PCR primer sequences are listed in **Table 1**.

Expression analysis in protein level

Proteins were extracted from ARPE-19 cells and homogenized in lysis buffer containing a cocktail of protease inhibitors. Protein concentrations were measured by the BCA protein assay (Millipore, USA). Blots were probed overnight at 4°C with the following primary antibodies: anti-HSP47 (1:500 dilution), anti-αSMA (1:400 dilution), anti-FN (1:400 dilution), anti-collagen type I (1:1,000), and anti-GAPDH (1:1,000), after being transfered onto the nitrocellulose membranes. Following washing, the HRP-conjugated secondary antibody was added to the membranes for 2 h at room temperature

and then washed with TBST solution. The reaction signal was detected by enhanced chemiluminescence with a chemiluminescence analyzer (Kodak Medical X-Ray Processor, Rochester, NY). Rabbit anti-goat IgG in PBS containing 1% FCS served as a negative control. GAPDH were used as internal gel loading controls.

Statistical analysis

Analysis was done with standard statistical software (SPSS for Windows, version 17.0). The Data from transwell assay, RT-PCR and western blot were expressed as means ± SD, One-way analysis of variance was used to determine significant difference in a multiple comparison. Statistical significance was set at *P*<0.05.

Results

HSP47 siRNA affects cell morphology in EMT of ARPE-19 cells

To examine the effect of HSP47 siRNA on the EMT of ARPE-19 cells, cytomorphology was
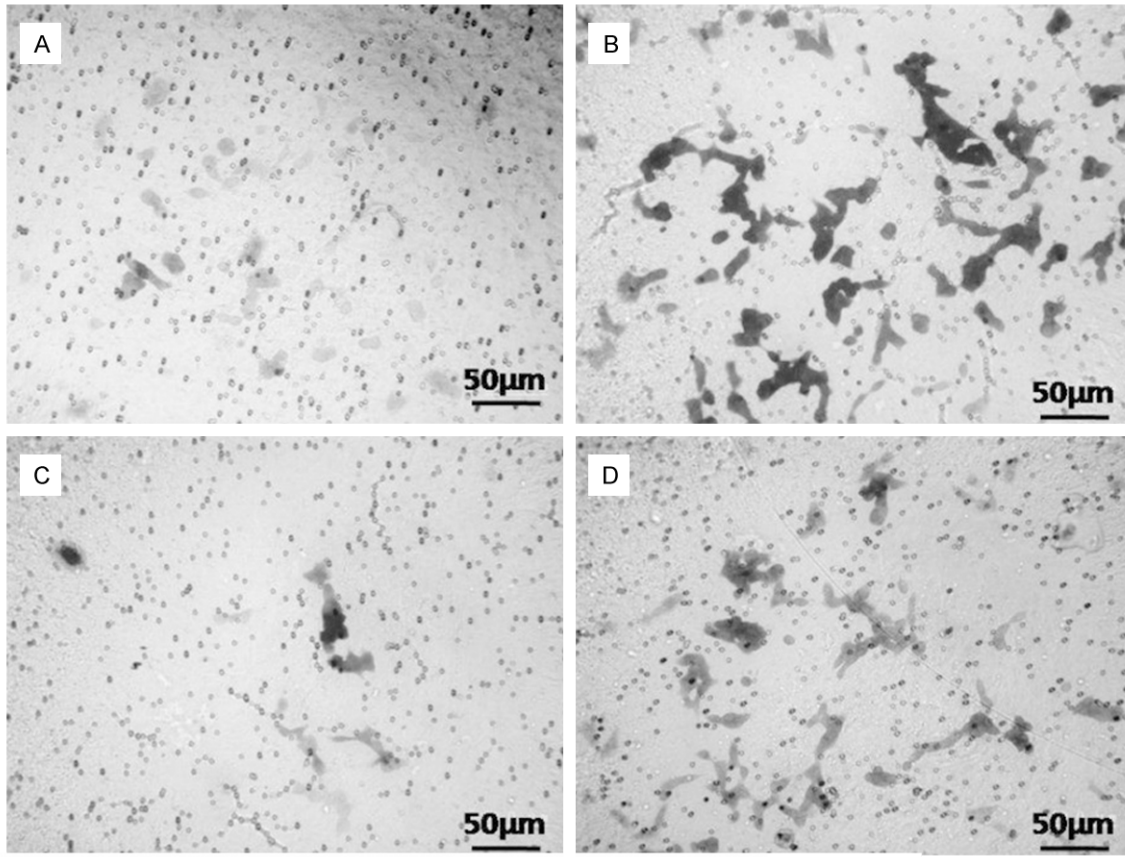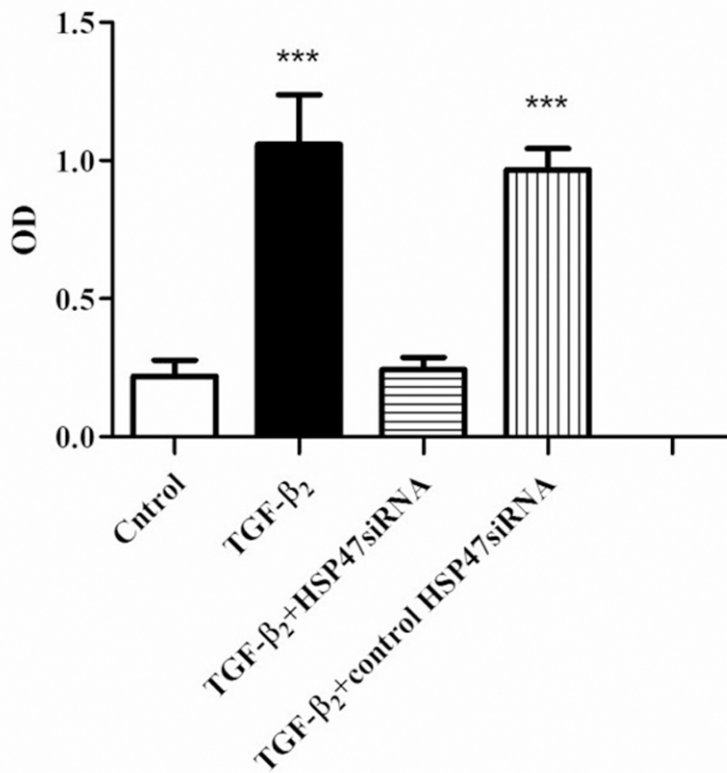
Figure 2. Attenuation of EMT markers by knocking down HSP47 gene. A: Less transmembrane ARPE-19 cells were observed by transwell assay with TGF-β2 (0 ng/ml); B: significantly increased transmembrane cells stimulated by TGF-β2 (5 ng/ml); C: Transfection with HSP47 siRNA inhibited the cells transmembrane mobility; D: Transfection with control siRNA did not exert a significant influence on the transmembrane cells. E: Results are expressed as OD value of ARPE-49 cells transmembrane mobility. Error bars showed mean ± SD. Statistical significances when compared to control samples are denoted as follow: *p<0.05; **p<0.01; ***p<0.001.
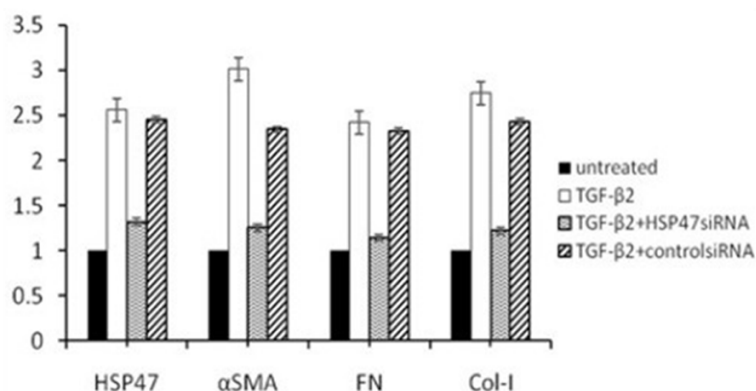
Figure 3. Treatment with recombinant TGF-β2 (5 ng/ml) induced 2.56±0.17 -fold, 3.01±0.31 fold, 2.42±0.21 fold, and 2.74±0.16 fold increase in HSP47, αSMA, FN, Col-I mRNA expression in 24h respectively; transfection with HSP47 siRNA resulted in a significant reduction in the TGF-β2-induced mRNA expression of the four proteins in ARPE-19 cells to 1.32±0.09 fold, 1.25±0.12 fold, 1.15±0.21 fold, and 1.22±0.11 fold as analyzed by RT-PCR.
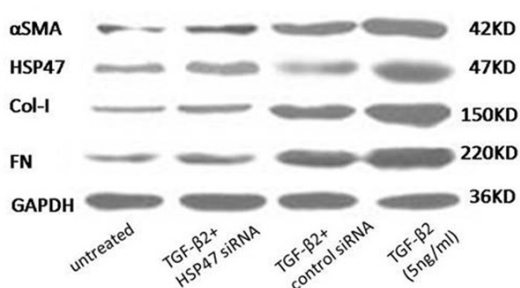

Figure 4. By western blot, significantly reduced protein expressions of αSMA, HSP47, Col-I, and FN were observed in HSP47 siRNA group (P<0.05 vs. the control group); Transfection with control siRNA did not show discernable effect on the expressions of the four proteins (P>0.05 vs. the control group).

investigated firstly. As shown in **Figure 1**, induction of ARPE-19 cells with 5 ng/ml TGF-β2 resulted in a significant change in cell morphology, which was transition from an epithelial phenotype to a mesenchymal phenotype. Transfection of HSP47 siRNA could markedly inhibit the cell morphology changing, while the negative control did not exert a significant influence.

Effect of HSP47 siRNA on migration of ARPE-19 cells

From the transwell chamber assay, stimulated by TGF-β2 (5 ng/ml), the number of transmembrane ARPE-19 cells increased significantly, which meant the cell migration ability got

enhanced (P<0.05). Transfection of HSP47 siRNA could inhibit cell migration significantly, while the control siRNA did not show such an effect (**Figure 2**, *P*>0.05).

Attenuation of EMT markers expression by transfection of ARPE-19 cells with HSP47 siRNA

Treatment with recombinant TGF-β2 (5 ng/ml) induced 2.56±0.17 fold, 3.01±0.31 fold, 2.42±0.21 fold, and 2.74±0.16 fold increased in HSP47, αSMA, FN and Col-I mRNA expression in 24 h respectively (**Figure 3**, P<0.05 vs. the control group respectively). To investigate whether the increased expressions of these EMT markers (αSMA, FN, Col-I) were related to TGF-β2-induced HSP47 protein synthesis specifically, the ARPE-19 cells were transfected with HSP47 siRNA before the stimulation with TGF-β2. HSP47 siRNA resulted in a significant reduction in the TGF-β2-induced mRNA expression of the four proteins in the ARPE-19 cells to 1.32±0.09 fold, 1.25±0.12 fold, 1.15±0.21 fold and 1.22±0.11 fold orderly as analyzed by RT-PCR (**Figure 3**, P<0.05 vs. the control group respectively). Transfection with control siRNA did not show significant reduction.

A similar trend appeared in further western blot assay, in which significantly reduced protein expressions of the above EMT markers were observed in HSP47 siRNA group (**Figure 4**, P<0.05 vs. the control group). Transfection with control siRNA did not show discernable effect on the expressions of these ECM proteins (P>0.05 vs. the control group). These results can support the hypothesis that HSP47 modulates TGF-β2-induced EMT and ECM protein accumulation of ARPE-19 cells specifically.

Discussion

In this study, we investigated the role of HSP47 in regulating the EMT and ECM proteins synthesis by TGF-β2 using a human RPE cell line, ARPE-19. TGF-β2, as a stimulator, can promote the fibrosis process of several cell types includ-

ing ARPE-19, but the mechanism remains unknown. The results demonstrate that HSP47 associated with fibrogenesis by regulating morphological changing, migration and collagen synthesis. This is the first study to confirm that knocking down HSP47 can attenuate EMT process and ECM proteins accumulation in ARPE-19 cells stimulated by TGF-β2.

Increasing evidence indicates that EMT is a crucial pathophysiologic procedure in the development and progression of PVR, in which RPE cells are the major contributors [13]. Various inflammatory cytokines are involved in this process, in which TGF-β2 has been reported to play an important role [14]. However, TGF-β2 may not be an ideal therapeutic target because it has some important biologic functions else including tissue regeneration [15] and immunosuppressive [16]. HSP47, as a specific collagenic chaperone and a potent regulator of EMT, may provide a sharper and more pertinent target based on the present study.

Our previous study (unpublished data) showed significantly upregulation of TGF-β2, FN and Col-I in specimen of preretinal proliferative membrane taken from PVR patients, which was accompanied by over expression of HSP47. According to several studies that HSP47 played a regulatory role in EMT process in various cells [17-19] and tissue fibrotic disorders [20, 21], we thought that HSP47 played a role in PVR through regulating EMT process of ARPE-19 cells.

The present study demonstrated that the ARPE-19 cells stimulated by TGF-β2 (5 ng/ml) were changed into an elongated shape and got a mesenchymal-like phenotype. Furthermore, we observed that more active cells migrated through the polycarbonate membrane by Transwell Chamber assay. The acquired results showed that the ARPE-19 cells had possessed a stronger ability of migration. More importantly, our study indicates that HSP47 siRNA transfection can inhibit migration ability of the ARPE-19 cells, and the enhancement of migration is considered to be a prerequisite for EMT in RPE cells [22, 23].

In the present study, we also found that transfection of siRNA targeting HSP47 could reduce ECM protein accumulation, such as αSMA, FN and Col-I in ARPE-19 cells after being stimulat-

ed by TGF-β2. As the three proteins are deemed to be the main markers of mesenchymal cells [24], the results suggests that HSP47 plays an important role in EMT of ARPE-19 cells stimulated by TGF-β2 and transfection of HSP47 siRNA can prevent the development of PVR.

Promisingly, the fibrotic lesions can be significantly reduced by knocking down HSP47 in the specific organs of animals, according to some reports of experimental organic fibrotic disorders models [25-28]. As a downstream protein of TGF-β2, HSP47 is more specific to the synthesis of collagen proteins, and HSP47 may become a perspective target for regulating EMT in RPE cells and fibrotic process in PVR. Further studies *in vivo* are expected to set up an animal model of PVR and observe the inhibition of PVR with HSP47 siRNA transfection into vitreous of the animals.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Dongchang Zhang, Shanxi Eye Hospital, 92. N. Fudong Road, Taiyuan 030002, Shanxi Province, China. Tel: +861500-3431945; Fax: +86 35 1413 1791; E-mail: zhangdongchanghome@163.com

References

[1] Morescalchi F, Costagliola C. Proliferative vitreoretinopathy after eye injuries: an overexpression of growth factors and cytokines leading to a retinal keloid. Mediators Inflamm 2013; 2013: 269787.

[2] Pastor JC, de la Rua ER, Martin F. Proliferative vitreoretinopathy: risk factors and pathobiology. Prog Retin Eye Res 2002; 21: 127-144.

[3] Kroll P, Rodrigues EB, Hoerle S. Pathogenesis and classification of proliferative diabetic vitreoretinopathy. Ophthalmologica 2007; 221: 78-94.

[4] Cui JZ, Ma P, Samad A. Stage specificity of novel growth factor expression during development of proliferative vitreoretinopathy. Eye (Lond) 2007; 21: 200-208.

[5] Whisstock J, Skinner R, Lesk AM. An atlas of serpin conformations. Trends Biochem Sci 1998; 23: 63-7.

[6] Satoh M, Hirayoshi K, Yokota S, Hosokawa N, Nagata K. Intracellular interaction of collagen specific stress protein HSP47 with newly synthesized pro-collagen. J Cell Biol 1996; 133: 469-483.

[7] Xiao HB, Liu RH, Ling GH. HSP47 regulates ECM accumulation in renal proximal tubular cells induced by TGF-β1 through ERK1/2 and JNK MAPK pathways. Am J Physiol Renal Physiol 2012; 303: 757-765.

[8] Taguchi T, Razzaque MS. The collagen-specific molecular chaperone HSP47: is there a role in fibrosis? Trends Mol Med 2007; 13: 45-53.

[9] Li L, Wu T, Huang J. Expression of heat shock protein 47, transforming growth factor-beta 1, and connective tissue growth factor in liver tissue of patients with Schistosoma japonicum-induced hepatic fibrosis. Parasitology 2014; 11: 1-11.

[10] Kakugawa T, Yokota S, Ishimatsu Y. Serum heat shock protein 47 levels are elevated in acute exacerbation of idiopathic pulmonary fibrosis. Cell Stress Chaperones 2013; 18: 581-90.

[11] Lin G, Yang L. Delivery of siRNA by MRI-visible nanovehicles to overcome drug resistance in MCF-7/ADR human breast cancercells. Biomaterials 2014; 35: 9495-507.

[12] Tang F, Li W, Chen Y, Wang D, Han J, Liu D. Downregulation of hnRNP K by RNAi inhibits growth of human lung carcinoma cells. Oncol Lett 2014; 7: 1073-1077.

[13] Pastor JC, de la Rúa ER, Martin F. Proliferative vitreo-retinopathy: risk factors and pathobiology. Prog Retin Eye Res 2002; 21: 127-144.

[14] Cui Jz, Chiu A, Maberley D, Ma P, Samad A, Matsubara JA. Stage specificity of novel growth factor expression during development of proliferative vitreoretinopathy. Eye 2007; 21: 200-208,

[15] Kita T, Hata Y, Arita R, Kawahara S, Miura M, Nakao S, Mochizuki Y, Enaida H, Goto Y, Shimokawa H, Hafezi-Moghadam A, Ishibashi T. Role of TGF-beta in proliferative vitreoretinal diseases and ROCK as a therapeutic target. Proc Natl Acad Sci U S A 2008; 105: 17504-17509.

[16] Walsh MF, Ampasala DR, Hatfield J. Transforming growth factor-beta stimulates intestinal epithelial focal adhesion kinase synthesis via Smad- and p38-dependent mechanisms, Am J Pathol 2008; 173: 385-399.

[17] Rubtsov YP, Rudensky AY. TGF beta signalling in control of T-cell-mediated self-reactivity. Nat Rev Immunol 2007; 7: 443-453.

[18] Nakayama S, Mukae H, Sakamoto N, Kakugawa T, Yoshioka S, Soda H, Oku H, Urata Y, Kondo T, Kubota H, Nagata K, Kohno S. Pirfenidone inhibits the expression of HSP47 in TGF-β1-stimulated human lung fibroblasts. Life Sci 2008; 82: 210-217.

[19] Xiao HB, Liu RH, Ling GH, Xiao L, Xia YC, Liu FY, Li J, Liu YH, Chen QK, Lv JL, Zhan M, Yang SK, Kanwar YS, Sun L. HSP47 regulates ECM accumulation in renal proximal tubular cells induced byTGF-β1 through ERK1/2 and JNK-MAPK pathways. Am J Physiol Renal Physiol 2012; 303: F757-65.

[20] Hong S, Park K, Kim JH, Han SH, Lee JB, Seong GJ. Role of Heat Shock Protein 47 in Transdifferentiation of Human Tenon's Fibroblasts to Myofibroblasts. BMC Ophthalmol 2012; 12: 49.

[21] Kitamura H, Yamamoto S, Nakase H. Role of heat shock protein 47 in intestinal fibrosis of experimental colitis. Biochem Biophy Res Commun 2011; 404: 599-604.

[22] Li L, Wu T, Huang J, Ma K, Xu L, Wang H, Fan X, Tao R, Ai G, Ning Q. Expression of heat shock protein 47, transforming growth factor-beta 1, and connective tissue growth factor in liver tissue of patients with Schistosoma japonicum-induced hepatic fibrosis. Parasitology 2014; 11: 1-11.

[23] Su CC, Chan CM, Chen HM, Wu CC, Hsiao CY, Lee PL, Lin VC, Hung CF. Lutein inhibits the migration of retinal pigment epithelial cells via cytosolic and mitochondrial Akt pathways (lutein inhibits RPE cells migration). Int J Mol Sci 2014; 15: 13755-67.

[24] Zhao HM, Sheng MJ, Yu J. Expression of IGFBP-6 in a proliferative vitreoretinopathy rat model and its effects on retinal pigment epithelial cell proliferation and migration. Int J Ophthalmol 2014; 7: 27-33.

[25] Ma B, Kang Q, Qin L. TGF-β2 induces transdifferentiation and fibrosis in human lens epithelial cells via regulating gremlin and CTGF. Biochem Biophys Res Commun 2014; 447: 689-95.

[26] Leung VY, Aladin DM, Lv F. Mesenchymal stem cells reduce intervertebral disc fibrosis and facilitate repair. Stem Cells 2014; 32: 2164-77.

[27] Wang Z, Li L. Adenovirus-mediated RNA interference against collagen-specific molecular chaperone 47-KDa heat shock protein suppresses scar formation on mouse wounds. Cell Biol Int 2008; 32: 484-493.

[28] Xia Z, Abe K, Furusu A. Suppression of renal tubulointerstitial fibrosis by small interfering RNA targeting heat shock protein 47. Am J Nephrol 2008; 28: 34-46.