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Hsp70 protects human trabecular meshwork cells injury induced by UVB through Smad pathway

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Aim: Glaucoma is a universal eye disease which is commonly associated with increased intraocular pressure caused by impaired aqueous humor (AH) drainage. This study aimed to investigate the effects of Hsp70 on trabecular meshwork (TM) injury induced by UVB. *Methods:* Real-time quantitative PCR (qRT-PCR) was used to examine the mRNA levels of Hsp70. siRNA was used to downregulate Hsp70 expression in the TM cells to inspect changes in cell proliferation and apoptosis. Cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8) assay and the number of apoptotic cells was assessed using annexin V-FITC/PI apoptosis detection kit. The Smad signaling pathway was investigated using western blotting analyses. *Results:* The overexpression of Hsp70 promoted cell proliferation and suppressed apoptosis. What's more, the overexpression of Hsp70 suppressed the expression of Smad-2, Smad-3 and Smad-7. *Conclusion:* Hsp70 might improve cell viability and inhibit TM apoptosis by inhibition of the Smad pathway. Hsp70 is a potential therapeutic target for the treatment of glaucoma.

1. Introduction

UVB (280-320 nm) plays an important role in photo-damage including clinical sunburns, loss of skin tone, hyperpigmentation, plaque-like thickening, erythema, deep furrowing, and fine wrinkle formation, via the induction of apoptosis, inflammatory responses, and DNA damage. Various stressors, such as UVB, induce heat shock proteins (HSPs) and the induction provides cellular resistance to such stressors.

Heat shock proteins (HSPs) are molecular chaperones which can protect cells from extreme pathological, physiological and environmental injuries (Kiang and Tsokos 1998). Hsp70 not only survey the folding status of proteins as part of the quality control function, which is very important under stress conditions, but are also involved in the regulation of fundamental cellular processes, such as cell cycle regulation, signal transduction, apoptosis, and innate immunity (Mayer 2010). Some studies have discovered that HSPs play a crucial role in the survival defense of cardiac myocytes (Yenari et al. 1999), internal epithelial cells (Tsuruma et al. 1996), lung fibroblasts, hepatocytes, fibroblasts (Gutsmann-Conrad et al. 1998) and skin melanocytes.

Glaucoma is a universal eye disease which is commonly associated with increased intraocular pressure caused by impaired aqueous humor (AH) drainage (Tamm and Fuchshofer 2007). Some studies have discovered that the increased resistance to AH outflow through the trabecular pathway including the trabecular meshwork (TM), juxta canalicular connective tissue and Schlemm's canal (Tektas and Lutjen-Drecoll 2009), is the primary cause for increased intraocular pressure in glaucoma patients (Keller et al. 2009). It has been reported that the biomechanical changes of the trabecular meshwork tissue such as tissue contraction and stiffness will lead to increased resistance to AH outflow and elevated intraocular pressure (Russell and Johnson 2012). Hsp70 has been reported to play an important role in corneal wound recovery (McKee et al. 2011; Mushtaq et al. 2011). Up to now, it has not been reported the effect of Hsp70 on the apoptosis of trabecular meshwork cells. In the present study, we investigated the protection effect of Hsp70 on human trabecular meshwork cells injury induced by UVB.

2. Investigations and results

2.1. Production levels of Hsp70 in trabecular meshwork cells exposed to UVB

To examine the influence of UVB radiation on the expression of Hsp70, we analyzed the content of Hsp70 in trabecular meshwork cells by western blot at different time intervals, namely, 0, 6, 12, 24 h, along with UVB (40 mJ/cm^2). We can see from Fig. 1 that the expression of Hsp70 reached the peak after 6 h with treatment of UVB, and decreased with the increase of time.

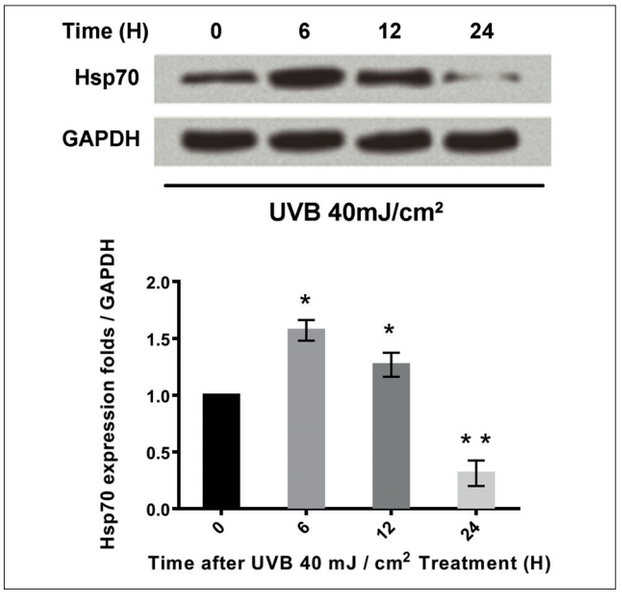

Fig. 1: The protein levels of Hsp70 after UVB radiation. Cells were exposed to UVB light (40 mJ/cm^2) and collected at various time intervals. The expression of Hsp70 protein was analyzed by Western blot. Results are presented as the mean ± SD of three independent experiments. *P values < 0.05; **P values < 0.01.

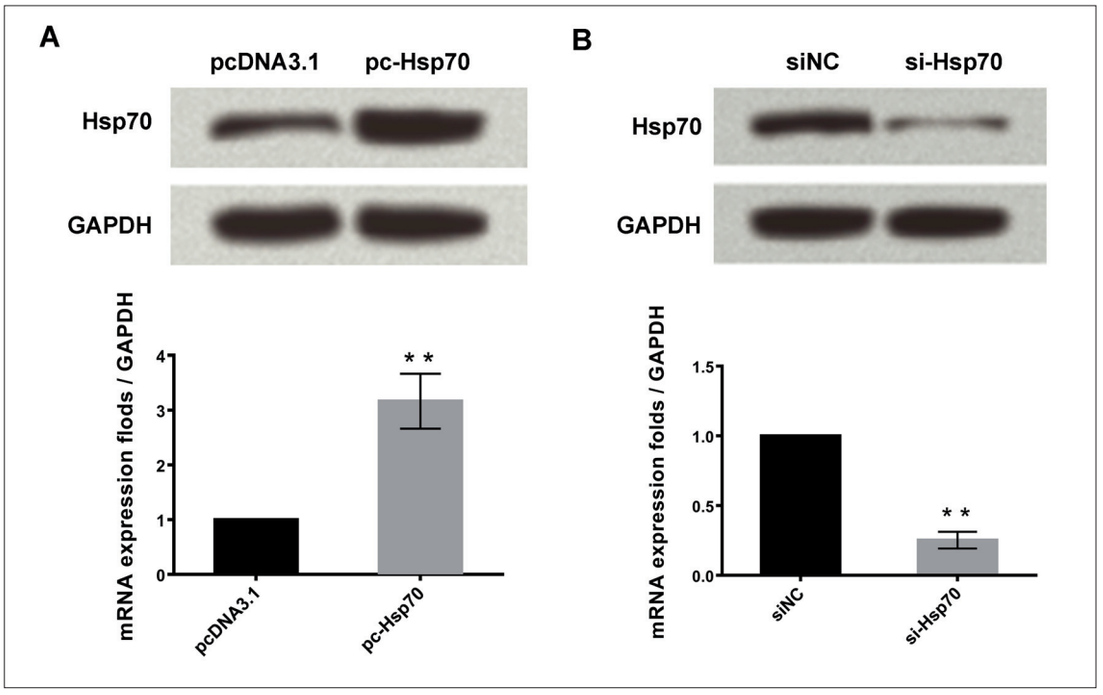

Fig. 2: The effect of overexpression and knockout of Hsp70 on TM cells. Results are presented as the mean ± SD of three independent experiments. *P values < 0.01.

2.2. Upregulation and downregulation of Hsp70 in trabecular meshwork cells

To investigate the function of Hsp70, we transfected cells with Hsp70 specific siRNA. Overexpression of Hsp70 upregulated Hsp70 protein expression in these cells (Fig. 2A), while the knockout of Hsp70 downregulated Hsp70 protein expression respectively compared to control cells (Fig. 2B). The changes of Hsp70 mRNA expression were similar to protein expression (Figs. 2A and 2B).

2.3. Hsp70 gene could enhance cell proliferation of trabecular meshwork cells

To test the protective effect of Hsp70 on trabecular meshwork cells injury induced by UVB, we detected cell viability with MTT assay. As shown in Fig. 3, exposure of cells to UVB light decreased cell viability; while this effect was inhibited by the overexpression of Hsp70 and accelerated by the knockout of Hsp70.

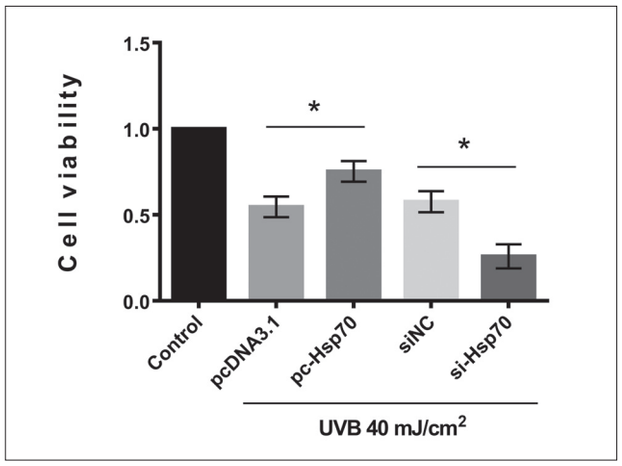

Fig. 3: Cell viability of TM cells with different treatment of Hsp70. Results are presented as the mean ± SD of three independent experiments. *P values < 0.05.

2.4. Overexpression of Hsp70 inhibited apoptosis and downregulation of Hsp70 promoted apoptosis

To determine whether Hsp70 has association with apoptosis induced by UVB, we used Annexin V-FITC/PI apoptosis detection kit to identify and quantify the apoptotic cells. As shown in Fig. 4, UVB irradiation increased the number of apoptotic cells, while the overexpression of Hsp70 resulted in a significant decline in apoptotic cells, which indicated that the overexpression of Hsp70 inhibited apoptosis. However, the downregulation of Hsp70 obviously increased apoptotic cells, which indicated that the knockout of Hsp70 induced apoptosis in trabecular meshwork cells.

2.5. Overexpression of Hsp70 suppressed the Smad pathway, while downregulation of Hsp70 activated the Smad pathway

To explore the protection mechanism of Hsp70 on human trabecular meshwork cells injury induced by UVB, we analyzed the expression of Smad proteins by western blotting. The protein levels of Smad were obviously increased in UVB-treated groups, while the overexpression of Hsp70 suppressed the expression of Smad-2, Smad-3 and Smad-7, which indicated that the overexpression of Hsp70 suppressed Smad pathway. On the contrary, the downregulation of Hsp70 enhanced the expression of Smad-2, Smad-3 and Smad-7 to a certain extent, indicating that the down-regulation of Hsp70 activated Smad pathway (Fig. 5).

3. Discussion

It is generally known that UV radiation contributes to several pathological conditions including epidermal photo-aging, photo-carcinogenesis, free radical reactions, immunosuppression, inflammation, DNA damage and apoptosis (Cui et al. 2004; Taylor et al. 1990). Large amounts of studies have found that UVB can induce apoptosis in keratinocytes (Yoshihisa et al. 2012). In the present study, we investigated the protection effect of Hsp70 on human trabecular meshwork cells injury induced by UVB.

Hsp70 family proteins were widely studied due to its anti-apoptotic, anti-inflammatory, molecular chaperone, and cytoprotective properties. The induction of HSP70 has been reported for various

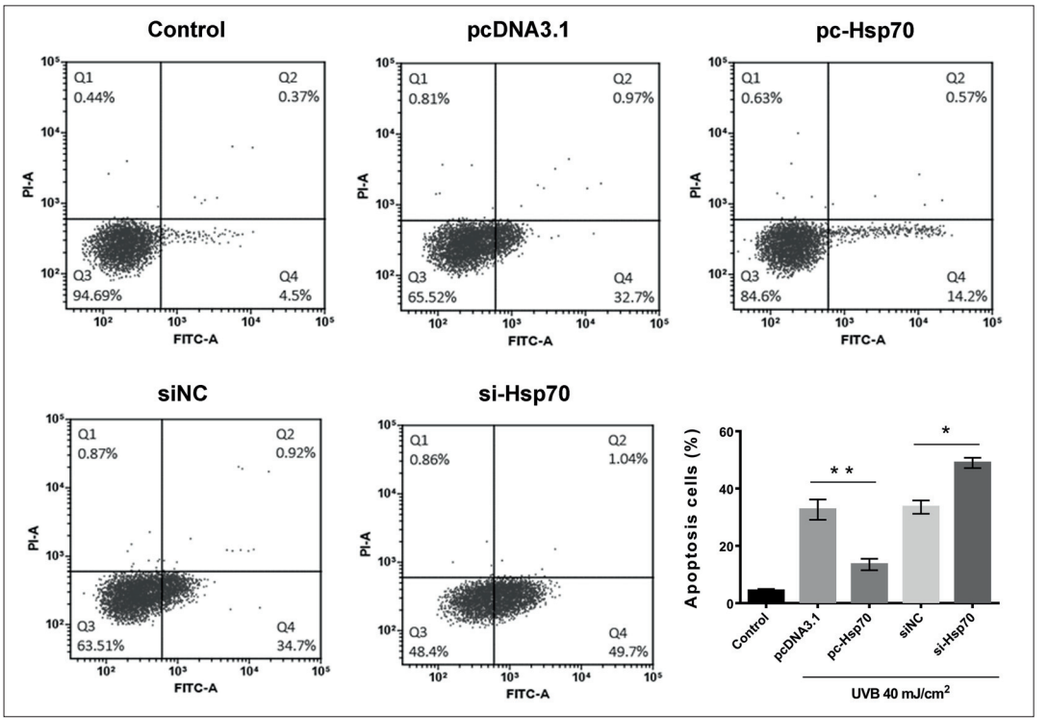

Fig. 4: The percentage of apoptotic cells was reduced after transfection with pc- Hsp70, and was increased after transfection with si-Hsp70. Results are presented as the mean ± SD of three independent experiments. *P values <0.05. **P values <0.01.

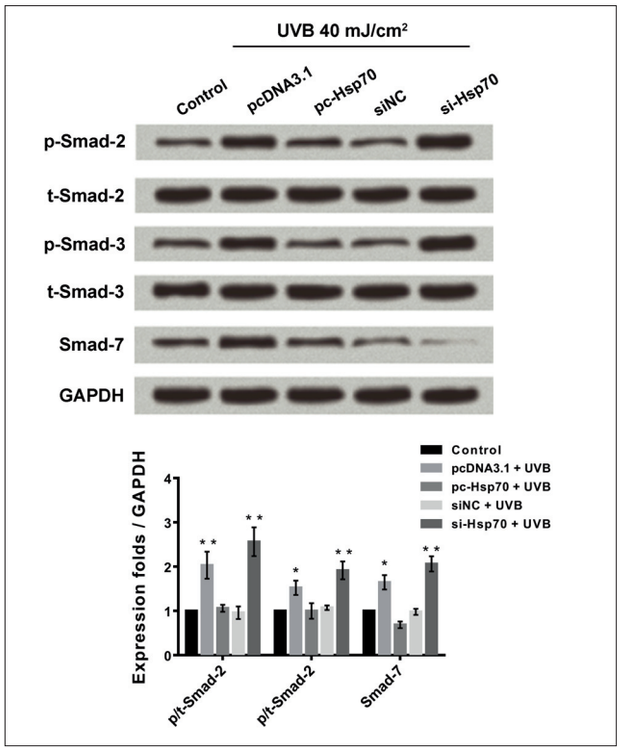

Fig. 5: Detection and quantitative analysis of Smad pathways by western blotting. GAPDH was used as the loading control. Results are presented as the mean ± SD of three independent experiments. *P values <0.05. **P values <0.01.

diseases that caused by exposure to arsenic, infrared laser radiation, heavy metals and heat shock (Roh et al. 2008; Souil et al. 2001; Wang et al. 2011). However, research related to the effects of UV radiation on the expression of these proteins is scarce (Merwald et al. 2006). Some researchers discovered that the expression of Hsp70 in keratinocytes protects effect against UVB light (Kwon et al. 2002; Trautinger 2001). Our study showed that the expression of Hsp70 reached the peak after 6 h with the treatment of UVB, and decreased with the increase of time. To understand the function of Hsp70 in trabecular meshwork (TM) cells injury induced by UVB, Hsp70-specific siRNA was used to downregulate Hsp70 expression in TM injury cells and cell proliferation was analysed with the CCK-8 assay. The results showed that the exposure of cells to UVB light decreased cell viability, while this effect was inhibited by the overexpression of Hsp70 and accelerated by the knockout of Hsp70. We hypothesized that the inhibition of apoptosis might be a pathway which can explain the cell proliferation results in TM cells. Our study showed that the upregulation of Hsp70 resulted in a significant decline in apoptotic cells, indicating that the Hsp70 gene had anti-apoptotic functions. The results were consistent with previous studies (Raj et al. 2006), which suggested that Hsp70 suppressed various steps in the molecular pathways governing apoptosis.

Activation of the Smad pathway has been described in many diseases, including renal tumor progression (Park et al. 2013), hypertensive-induced renal damage, and diabetic nephropathy (Chen et al. 2011). To further understand the mechanism responsible for the induction of cell proliferation, we analyzed the expression of Smad-2, Smad-3 and Smad-7 by qRT-PCR and western blotting. The results showed that the overexpression of Hsp70 activated the Smad pathway characterized by increased phosphorylation of Smad-2, Smad-3, a critical downstream mediator of fibrosis (Ruiz-Ortega et al. 2007) and Smad-7 proteins. From the above results, we found that Hsp70 inhibited apoptosis and promoted cell proliferation by suppressing the Smad pathway.

In the present study, we explored the protective effects of Hsp70 on trabecular meshwork (TM) cells injury induced by UVB. The expression of Hsp70 was found to be obviously repressed in TM injury cells induced by UVB. Hsp70 inhibited apoptosis and promoted cell proliferation by suppressing Smad pathway, which indicated that Hsp70 protected TM injury induced by UVB though the Smad pathway. Our results laid a foundation for further study of Hsp70 proteins, as well as provided a theoretical basis for the clinical treatment of glaucoma.

4. Experimental

4.1. Trabecular meshwork (TM) cell culture and UVB radiation

The primary human TM cell line was obtained from ScienCell Research Labs (Catalog#6590; Carlsbad, CA, USA). Primary human TM cells were grown in Fibroblast Medium (Catalog No. 2301; ScienCell Research Labs) and were used at the third to sixth passage. For maintenance, the cells were incubated at 37 °C in the environment with 5% CO_2. TM cells were exposed to UVB irradiation with a double bank of UVB lamps (peak emis-sion at 312 nm, VL-215LM lamp, Vilber Lourmat). The UV dose was measured with a UVX Radiometer (UVP, Inc., Upland, CA). TM cells were irradiated at a dose of 40 mJ/cm^2 in phosphate buffer saline (PBS).

4.2. Plasmids and siRNA transfection

An Hsp70 expression vector (pc-Hsp70) was constructed by sub-cloning the full-length wild-type Hsp70 coding sequence into pcDNA3.1 (+), and confirmed by sequencing. The empty construct pcDNA3.1 was transfected as a control. The target sequence for Hsp70 specific siRNA was (5' CACGGCAACCUGGAGAUCA'3, and 5' UGAUCUCCACC UUGCCGUG'3), and control siRNA (no silencing) was synthesized by GenePharma Co (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol. Stable Hsp70 transfection was generated under G418 (Gibco, Paisley, UK) selection as described.

4.3. Cell proliferation assay

TM cells were seeded in 96-well plate with 5000 cells/well. Cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8, DOJINDO Molecular Technologies, Gaithersburg, MD) assay. After stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for one hour at 37 °C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

4.4. Apoptosis assay

Apoptosis analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells (100,000 cells/well) were seeded in a six well-plate. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

4.5. Quantitative real-time RT-PCR analysis

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega). Reverse transcription was performed by using the Multiscribe RT kit (Applied Biosystems) and random hexamers or oligo(dT). The reverse transcription conditions were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C. The sequences of the primers were as follows, Hsp70 forward primer: 5' GCCGGATCCATATGGCCAAAG CC'3, reverse primer: 5' CCCTC-GAGCTAATCTACCTCCT'3. GAPDH forward primer: 5'GCACCGTCAAGGCT-GAGAAC'3, reverse primer: 5'TGGTGAAGACGCCAGTGGA3'.

4.6. Western Blot analysis

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Rabbit-anti-human Hsp70 antibody for chromatin immunoprecipitation was purchased from Abcam (Shanghai, China). GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.7. Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean±SD. Statistical analyses were performed using Graphpad statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Conflict of Interest: Authors declare that there is no conflict of interests.

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