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Effect of HSF4b on age related cataract may through its novel downstream target Hif1α

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ABSTRACT

Our previous study identified five new heat shock factor 4 (HSF4) mutations in 150 age-related cataract (ARC) patients which indicated that HSF4 mutations may be associated with this disease. Hypoxia-inducible factor (Hif1α) is an important downstream target of HSF4b. It has been found that Hif1α play also important roles in cataract development. To identify if HSF4b play it role in cataract development through HIF1α, we transfected SRA01/04 lens epithelial cells with small hairpin RNA of HSF4b and measured expressions Hif1α after transfection. Then, we perform chromatin immunoprecipitation quantitative PCR to see the relationship between HSF4b and HIF1α. We found that HSF4 downregulation led to decrease of HIF1α mRNA expression. Furthermore, we demonstrated by ChIP followed by quantitative PCR (ChIP-qPCR) that these HIF-1α is bound by HSF4b near promoters, not gene bodies.

1. Introduction

Heat shock transcription factor 4 (HSF4) plays a crucial role in maintaining lens transparency [1–6]. In our previous studies we identified five new HSF4 mutations in 150 age-related cataract (ARC) patients, suggesting that HSF4 mutations may play a role in opacifications of human lenses [7]. HSF4 is a member of the HSF family that mediates an inducible transcription response. It is derived by alternative RNA splicing events into HSF4a and HSF4b isoforms, but only the HSF4b isoform acts as a transcriptional activator [8], with predicted amino acid sequence in humans [9]. In this study, we investigated the effect of HSF4b on ARC.

To characterize the role of HSF4b in lens physiology, it is necessary to identify its downstream targets. Candidate targets of HSF4b include γs-crystallin, SKAP2, Crygf, Fgf7, Hspb2, and Bfsp2 [6,10,11]. It has been reported that Hif1α is a novel downstream target of HSF4 in human breast cancer cells [12]. Recently, the role of Hif1α in proliferation of older lenses has received considerable attention [13–16]. In aged lenses, it represses proliferation of epithelial cells [13]. We hypothesize that HSF4b plays a role in regulation of Hif1α expression in lens epithelial cells. To test this hypothesis, we suppressed HSF4b expression using shRNA and measured gene expression of the hypoxia-inducible factor Hif1α in lens epithelial cells, to determine if HSF4b can affect expression of this gene. We also performed chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) to identify whether Hif1α is one of the downstream target genes of HSF4b.

2. Materials and methods

2.1. Detection of HSF4b and HIF-1a mRNA expression by real-time PCR

This study was approved by the Ethics Committee of Shanghai Eye and ENT Hospital. The human lens epithelial cells (HELCs) from lens anterior subcapsule of age-related cataract groups were gathered from cataract surgery. As most age-related cataract of class I and II are not needed surgery, here we gathered only class III, IV, and V groups according to LOCSIII. The HELCs of control group were gathered from eye bank, which were divided into young normal lens group and old normal group according to age of the donor. Total RNA from HLECs was prepared using Trizol Reagent (Invitrogen). The cDNA synthesis was performed according to the RNA PCR kit protocol (Takara, Dalian, China). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used as a normalizing control. The designed paired primers were as follows: HSF4b,

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5′-GCGTGATTGGGCG ACTTCT-3′ (forward), 5′-GATGAGTGGGAGAC TTGGGTT-3′ (reverse); HIF-1a, 5′-CCATTAGAAAG CAGTTCCGC-3′ (forward), 5′-TGGGTAGGAGATGGAGATGC-3′ (reverse); GAPDH, 5′-GGAGTCCACTGGCGTCTTC-3′ (forward), 5′-GCTGATGATCTTGA GGCTGTTG-3′ (reverse). The PCR reaction was performed in a volume of 20 μl with SYBR green mix (Takara) using an MXP3000 instrument (Stratagene Laboratories, La Jolla, CA, USA). PCR results were analyzed using Opticon Monitor Analysis 2.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each sample was analyzed at least three times.

2.2. Detection of HSF4b and HIF-1a protein level expression by Western blot

To detect levels of HSF4b in HLECs, Sample of lens epithelia were rapidly dissected in medium kept on ice, then lysed directly in 2× electrophoresis sample buffer. After electrophoresis in 4–12% gradient gels, the proteins were transferred to nitrocellulose membranes for 4 h and probed with a goat polyclonal antibody to HSF4b (Santa Cruz Biotechnology) diluted at 1:1000. Detection used horseradish peroxidase (HRP)-labeled anti-goat secondary antibody (Santa Cruz Biotechnology) diluted with 0.1% Tween-20 (TBS-T) (The 4th Reagent Factory of Shanghai, Shanghai, China) to 1:1000. Chemiluminescence (Hyperfilm ECL; GE Healthcare, Buckinghamshire, UK) of band intensities was recorded and quantified with a gel documentation system (EDAS 290; Eastman Kodak, Rochester, NY, USA). The relative levels of Hif1α lens epithelial cells from different classifications of ARCs and control groups were determined by western blotting with a rabbit polyclonal antibody to Hif1α (Santa Cruz Biotechnology) diluted at 1:500. Detection was with horseradish peroxidase (HRP)-labeled anti-goat and anti-rabbit secondary antibody, respectively (Santa Cruz Biotechnology) diluted with 0.1% Tween-20 (TBS-T), followed by chemiluminescence. The results of two experiments, with duplicate blots for each experiment, were normalized, averaged, and plotted as the mean ± SEM.

2.3. HSF4b small hairpin RNA preparation and plasmid construction

One pair of shRNA were designed according to the *HIF4b* sequence in GenBank (No. NM001040667), mainly following Tuschl's rules [17]. Its hairpin sequence was 5′-CCGGCCGGGTCA TTGGCAAGCTGATCTCGAGATCAGCTTGCCAATGAC CCGGTTTTT-3′, its mature sense was 5′-CCGGGTCATTGGCAAGCTGAT-3′, and its mature antisense was 5′-ATCAGCTTGCCAATGACCCGG-3′. The sequence was transcribed with DNA polymerase III U6 promoter in plasmid pGCsi purchased from GeneChem Co. Ltd. (Shanghai, China). The pGCsi plasmid contained the GFP (green fluorescent protein) gene.

2.4. Cell culture and transfection with the small hairpin RNA expression vector

The HLEC line, SRA01/04, was cultured in DMEM medium supplemented with 10% fetal bovine serum, 2.05 mmol/l L-glutamine, 100 U/ml of penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. For transfection, the cells were seeded in 6-well plates at 1×10^6 cells/well and allowed to grow overnight to 90–95% confluency. They were transfected with a mixture of 2 μg plasmid DNA and 4 μl Lipofectamine™ 2000 (Invitrogen) in 2 ml serum-free medium. At 6 h after transfection, the medium was replaced by normal medium containing 10% fetal bovine serum and antibiotics, for up to 48 h. At 24, 48, and 72 h after transfection, the samples were digested with trypsin and centrifuged at 20,000g for 10 min. After removing the supernatant, 200 μl of buffer was added and the cells were analyzed by flow cytometry. Transfected cells appeared green when viewed in a fluorescent microscope [18].

2.5. Detection of HIF-1a mRNA expression by real-time PCR after transfection with HSF4b shRNA expression vector

Total RNA from the HELCs transfected with HSF4b shRNA expression vector was prepared using Trizol Reagent (Invitrogen, Carlsbad, CA). HIF-1a mRNA expression was detected the same way as above.

2.6. Detection of HSF4b mediated HIF-1a expression by chromatin immunoprecipitation (Chip)

SRA01/04 cells were treated with 10% formaldehyde for 10 min at 37 °C. Then the cells were washed twice with ice-cold PBS and suspended in the lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.5, w/protease inhibitors). After 10 min incubation on ice, cells were centrifuged to pellet the nuclei. Nuclei were then suspended in the nuclei lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, w/protease inhibitors), incubated on ice for 10 min and sonicated for 1.5 min (30 s each time, then cooled on ice and repeated twice). The sheared chromatin was then immunoprecipitated by using anti-HSF4b antibody or IgG control. After extensive washing and then elution, the crosslink was reverted by heat treatment (65 °C overnight and protease K digestion). The captured genomic DNA fragments were then purified by using Qiagen PCR purification kit. Identification of the captured HIF-1a promoter fragments was performed by PCR analysis by using the promoter primers. The sequences of primers covering region −915 to−658, are 5′-ACTCTTTGCCACGGAGCACA (forward) and 5′-GCTTGCAAAGTTGCC AAAGG (reverse); the sequences of primers covering region −1455 to −995, are 5′-TTGAGCCCA ACAAAGTAG CATT (forward), 5′-CTTCTCTTCAGGCA TTTCCCA (reverse). Thirty cycles of PCR were performed and the amplified products were analyzed on a 2% agarose gel [12].

2.7. Statistical analysis

All experiments were performed in triplicate. One-way ANOVA was used to determine statistical significance of the data, using SPSS software (SPSS for Windows, version 11.0, SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. HSF4b mRNA expression in HLECs of ARCs

Real-time PCR showed that HSF4b mRNA expression in HLECs of ARCs was greatly reduced compared with HLECs of young HLECs (Fig. 1A). Relative quantities (HSF4b/GAPDH) in HLECs of different classifications of ARCs were as follows: young normal lens, 8.06E−05 ± 1.67E−05; old normal lens, 4.36E−05 ± 6.23E−06; ARC (class III), 2.92E−05 ± 2.24E−06; ARC (class IV), 2.10E−05 ± 2.476E−06, ARC (class V), 1.70E−05 ± 5.14E−06 (Fig. 1A). The relative gene expressions of HSF4b in HLECs of the three ARC groups were all significantly lower than the old normal lens group and young normal lens group ($p < 0.01$). Regarding gene expression of HSF4b, there was a significant difference between normal senile and young lens epithelial cells ($p < 0.01$). HSF4b gene expression in HLECs of ARC (class V) was slightly reduced compared with HLECs of ARC (class IV) and ARC (class III) ($p < 0.05$), and there was no significant difference between ARC (class IV) and ARC (class III) ($p > 0.05$, Fig. 1A).

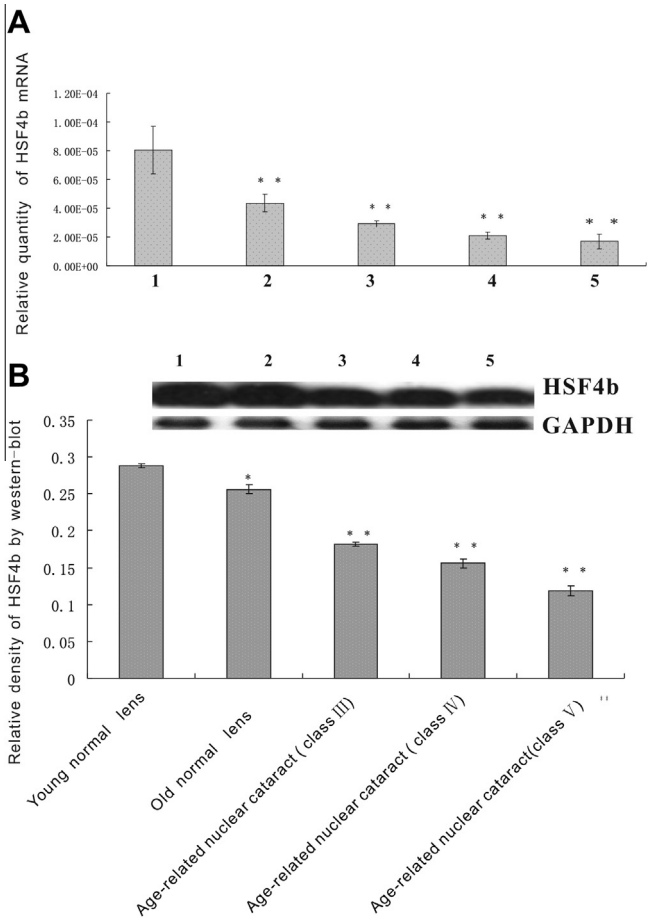

Fig. 1. Expression of HSF4b in HLECs. (A) HSF4b mRNA expressions in HLECs of the three ARC groups were all significantly lower than old normal lens group and young normal group ($p < 0.01$), and HSF4b mRNA expression in HLECs of old normal lens group was significantly lower than young normal group ($p < 0.01$). HSF4b mRNA expression in HLECs of ARC (class V) was slightly reduced compared with HLECs of ARC (class IV) and ARC (class III) ($p < 0.05$). There was no significant difference between ARC (class IV) and ARC (class III) ($p > 0.05$). (B) HSF4b protein expression in HLECs of the ARC group was greatly reduced compared with the HLECs of the young group ($p < 0.01$), and HSF4b protein expression in HLECs of old normal lens group was slightly reduced compared with the HLECs of young group ($p < 0.05$). HSF4b protein expression in HLECs of ARC (class V) was slightly reduced compared with ARC (class IV) and ARC (class III) ($p < 0.05$), and there was no significant difference between ARC (class IV) and ARC (class III) ($p > 0.05$) (*p<0.05, **p<0.01).

3.2. Protein expression of HSF4b in HLECs of ARCs

Western blotting confirmed that HSF4b protein expression in HLECs of the ARC group was reduced compared with the HLECs of young HLECs (Fig. 1B). The ratios of optical density (OD) (HSF4b/GAPDH) in HLECs of different classifications of ARCs were as follows: young normal lens, 0.29 ± 0.003; old normal lens, 0.25 ± 0.006; ARC (class III), 0.18 ± 0.002; ARC (class IV), 0.16 ± 0.005; ARC (class V), 0.12 ± 0.007 (Fig. 1B). OD values of HSF4b in HLECs of the three ARC groups were all significantly lower than the old normal lens group and young normal lens group ($p < 0.01$). There was a significant difference between normal senile and young lens epithelial cells in protein expression of HSF4b as measured by western blotting ($p < 0.05$, Fig. 1B). OD values in HLECs of ARC (class V) was slightly reduced compared with ARC (class IV) and ARC (class III) ($p < 0.05$), and there was no significant difference between ARC (class IV) and ARC (class III) ($p > 0.05$, Fig. 1B).

3.3. HIF-1α mRNA expression in HLECs of ARCs

Real time PCR showed that the HIF-1α mRNA expressions of in HLECs of the ARC groups were greatly reduced compared with the HLECs of young HLECs (Fig. 2A). Relative quantity (HIF-1α/GAPDH) in HLECs were as following: young normal lens 6.056E−05 ± 6.70E−06, old normal lens 4.96E−05 ± 5.23E−06, ARC (class III) 3.32E−05 ± 3.24E−06, ARC (class IV) 3.10E−05 ± 2.17E−06, ARC (class V) 2.70E−05 ± 1.14E−06, respectively (Fig. 2A). The relative quantitative gene expression of HIF-1α in HLECs of the three ARC groups were all significantly lower than old normal lens group and young normal group ($p < 0.01$). There was significant difference between normal senile and young lens epithelial cells in relative quantitative gene expression of HIF-1α ($p < 0.01$). HIF-1α mRNA expression in ARC (class V) was slightly lower than ARC (class III) ($p < 0.05$). There were no significant difference between the ARC (class III) and ARC (class IV), and there were no significant difference between the ARC (class IV) and ARC (class V) ($p > 0.05$, Fig. 2A).

3.4. HIF-1α protein expression in HLECs of ARCs

Western blot confirmed that the HIF-1α protein expressions in HLECs of the ARC groups were greatly reduced compared with the HLECs of young HLECs (Fig. 2B). Ratios of optical density (O.D) (HIF-1α/GAPDH) in HLECs of different classification of ARC were as following: young normal lens 0.19 ± 0.009, old normal lens 0.17 ± 0.007, ARC (class III) 0.09 ± 0.003, ARC (class IV) 0.08 ± 0.004, ARC (class V) 0.07 ± 0.003, respectively (Fig. 2B). Optical density (O.D) value of HIF-1α in HLECs of the three ARC groups

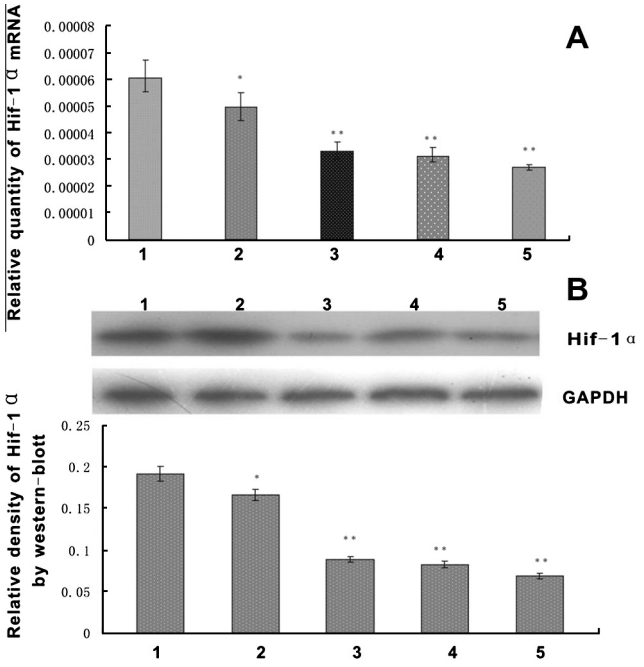

Fig. 2. Expression of HIF-1α in HLECs. (A) Hif1α mRNA expression in HLECs of the three ARC groups were all lower than the old normal lens group and young normal lens group ($p < 0.01$). HIF-1α mRNA expression in ARC (class V) was slightly lower than ARC (class III) ($p < 0.05$). There were no significant difference between the ARC (class III) and ARC (class IV), and there were no significant difference between the ARC (class IV) and ARC (class V) ($p > 0.05$). (B) Hif1α protein expression in HLECs of the three ARC groups were all lower than the old normal lens group and young normal lens group ($p < 0.01$). HIF-1α protein expression of HIF-1α in HLECs of normal senile group was slightly lower than young lens epithelial cells ($p < 0.05$). There were no significant difference among the three ARC groups in protein expression of HIF-1α ($p > 0.05$ for all comparisons) (*p<0.05, **p<0.01).

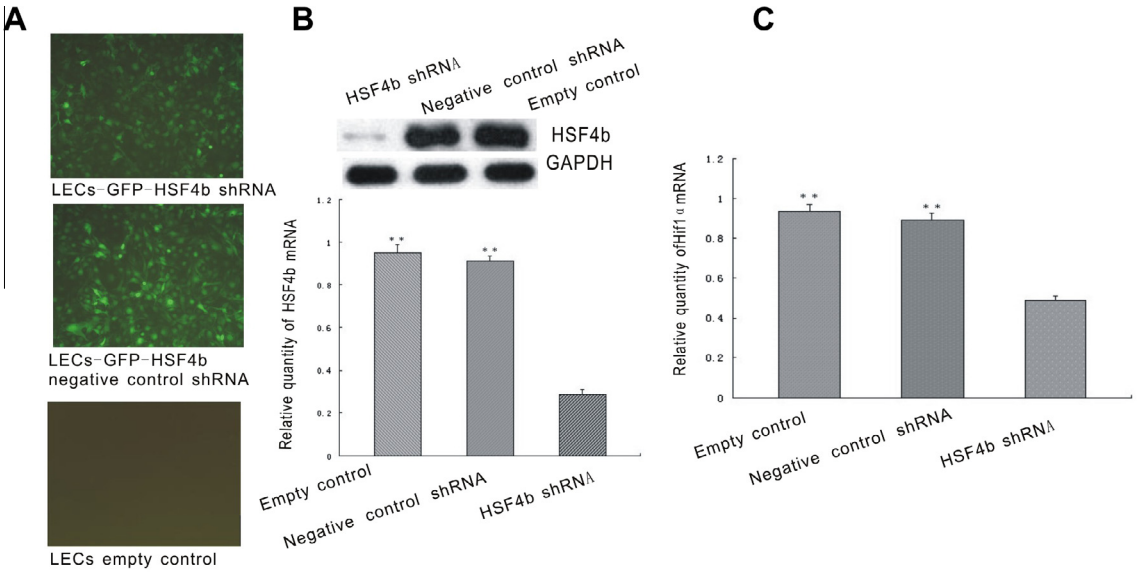

Fig. 3. HSF4b siRNA suppresses expression of HSFb and downstream HIF-1α. (A) Transfection rate at 48 h after transfection was 69.6 ± 2.3%. (B) At 48 h after transfection, HSF4b mRNA and protein expression in the transfected group was reduced compared with the negative control shRNA group and empty control group ($p < 0.01$). (C) Downregulation of HSF4b reduced Hif1α mRNA expression ($p < 0.01$) ($^{**}p<0.01$).

were all significantly lower than old normal lens group and young normal group($p < 0.01$). Optical density (O.D) value of HIF-1α in HLECs of normal senile group was slightly lower than young lens epithelial cells ($p < 0.05$, Fig. 2B). There were no significant difference among the three ARC groups in protein expression of HIF-1α ($p > 0.05$ for all comparisons).

3.5. HSF4b deficiency leads to repression of Hif1a transcription

To investigate the effect of HSF4b on Hif1α, we knocked down HSF4b expression in human HLECs with shRNA, and then examined Hif1α expression by PCR. The knockdown efficiency is shown in Fig. 3A and B. Transfection rates at 24 h, 48 h, and 72 h after transfection were 57.4 ± 4.7%, 69.6 ± 2.3%, and 85.3 ± 5.2%, respectively. Fluorescent microscopy showed numerous green cells in the HSF4b shRNA group and negative control shRNA group, there were almost no green cells in the empty control group (Fig. 3A). There were almost no green cells in the empty control group (Fig. 3A). HSF4b mRNA and protein expression in the transfected groups were greatly reduced compared with the negative control shRNA group and empty control group (Fig. 3B). In addition, downregulation of HSF4b greatly reduced Hif1α expression (Fig. 3C). Together, these results indicated that HSF4b deficiency leads to repression of Hif1α transcription.

3.6. HSF4b regulates Hif1α through binding to the promoter region of Hif1α

We further investigated whether anti-HSF4b can bind to the promoter region of Hif1α to find out the relationship between HSF4b and Hif1α gene. We demonstrated by ChIP followed by quantitative PCR (ChIP-qPCR) that HSF4b-regulated Hif1α is bound by HSF4b near promoters (Fig. 4), but not gene bodies.

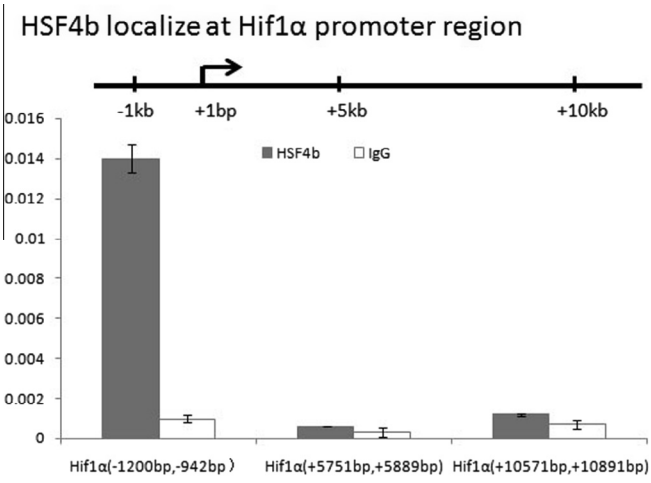

Fig. 4. HSF4b regulates Hif1α through binding near the promoter region of Hif1α. HSF4b but not IgG control was bound near the transcription start point upstream of Hif1α.

4. Discussion

We have previously identified five new HSF4 mutations in 150 ARC patients, indicating that HSF4 mutations may associate with a small fraction of ARC patients. It has been reported that deletion of HSF4 leads to cataract development in mice [6]. However, two missense mutations that have been associated with ARC as described in our previous study [7] did not or only slightly altered HSF4 activity, implying that other factors may affect the functions of these proteins [4].

The present study found that HSF4b was mainly expressed in the nucleus of HLECs, and that the expression of HSF4b was less in HLECs of ARCs than HLECs of young lenses. In addition, expression of HSF4b gradually decreased with seriousness of the ARC, and HSF4 downregulation led to apoptosis of HLECs. From these results we suggest that the amount of HSF4b in the lens may play a more important role in ARCs.

Because of this possible involvement, it is important to characterize the function of HSF4b. As a transcriptional factor, it is imperative to identify its downstream targets. Hif1α is a downstream target of HSF4 in human breast cancer cells [12], and Hif1α has been reported to repress the proliferation of lens epithelial cells in old lenses [13]. In addition, most cells respond to hypoxia by stabilizing the hypoxia-dependent transcriptional factor HIF1α, which is essential for hypoxia-induced inhibition of cell proliferation [13].

Regarding the two isoforms of HSF4, HSF4a is highly expressed in breast cancer cells, but HSF4b is expressed more in the lens, which indicates that HSF4a and HSF4b have at least slightly different functions [1]. Our finding that reduction of HSF4b expression in lens epithelial cells using shRNA technology indicated that HSF4 downregulation decreases the production of Hif1α mRNA and protein, implying that HSF4 functions as an activator of Hif1α expression. We further performed ChIP and found that medications specific for HSF4b were bound to an area of the Hif1α gene promoter, suggesting that the Hif1α gene expression may be regulated by HSF4b in lens epithelial cells.

It has been reported that HSF4 downregulation increases the production of Hif1α mRNA and protein, implying that HSF4 functions as a repressor of HIF1α expression [12]. However, in our study, HSF4 downregulation decreased the production of Hif1α mRNA and protein, implying that HSF4 functions as an activator of Hif1α expression. Together, these studies suggest that HSF4 effects on Hif1α expression are different in different tissues, with associated different functions. A possible reason could be that individual HSFs are known to function in concert with, or in opposition to, other members of the HSF family [12], and HSF4 actions on Hif1α expression are more complex than simple transcriptional repression or promotion.

We further measured the expression of Hif1α in lens epithelial cells and found that its expression was less in HLECs of ARC than HLECs of young lenses. In addition, expression of Hif1α was reduced gradually with seriousness of the ARC. This trend of Hif1α change was synchronized with HSF4b, which suggests that expression of Hif1α is promoted rather than suppressed by HSF4b in lens epithelial cells. Presently, there is no report of Hif1α expression in lens epithelial cells of ARCs.

We hypothesize that HSF4b plays a role in regulation of Hif1α expression in lens epithelial cells. To test this hypothesis, we suppressed HSF4b expression using shRNA and measured gene expression of the hypoxia-inducible factor Hif1α in lens epithelial cells, to determine if HSF4b can affect expression of this gene. We also performed chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) to identify whether Hif1α is one of the downstream target genes of HSF4b.

We therefore suggest that HSF4b can affect Hif1α expression by binding to HIF-1α gene near promoters. We propose that in the senile lens, expression of HSF4b decreases, leading to reduction of Hif1α expression and affecting proliferation and differentiation of HLECs [13], eventually leading to nuclear and cortical cataracts. Further studies should therefore emphasize in ARCs whether the effect of HSF4b is through traditional or nontraditional signaling pathways of Hif1α, and should further emphasize characterization of the effects of the HSF family on Hif1α expression in ARCs.

Acknowledgments

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