• Basic Research •

Bioinformatics analysis of microarray data to explore the key genes involved in *HSF4* mutation-induced cataract

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Abstract

• AIM: To reveal the mechanisms of *heat-shock transcription factor* 4 (*HSF4*) mutation-induced cataract.

• METHODS: GSE22362, including 3 *HSF4*-null lens and 3 wild-type lens, was obtained from Gene Expression Omnibus database. After data preprocessing, the differentially expressed genes (DEGs) were identified using the limma package. Based on Database for Annotation, Visualization and Integrated Discovery (DAVID) tool, functional and pathway enrichment analyses were performed for the DEGs. Followed by protein-protein interaction (PPI) network was constructed using STRING database and Cytoscape software. Furthermore, the validated microRNA (miRNA)-DEG pairs were obtained from miRWalk2.0 database, and then miRNA-DEG regulatory network was visualized by Cytoscape software.

• RESULTS: A total of 176 DEGs were identified in *HSF4*-null lens compared with wild-type lens. In the PPI network, FBJ osteosarcoma oncogene (FOS), early growth response 1 (EGR1) and heme oxygenase (decycling) 1 (HMOX1) had higher degrees and could interact with each other. Besides, *mmu-miR-15a-5p* and *mmu-miR-26a-5p* were among the top 10 miRNAs in the miRNA-DEG regulatory network. Additionally, *mmu-miR-26a-5p* could target *EGR1* in the regulatory network.

• CONCLUSION: *FOS, EGR1, HMOX1, mmu-miR-26a-5p* and *mmu-miR-15a-5p* might function in the pathogenesis of *HSF4* mutation-induced cataract.

• KEYWORDS: cataract; *heat-shock transcription factor 4*; differentially expressed genes; protein-protein interaction network; regulatory network

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INTRODUCTION

As a clouding of the lens in the eye, cataract may result in blurry vision, faded colors, trouble with bright lights, halos around light, and trouble seeing at night[1]. Cataract-caused poor vision may further lead to an increased risk of falling into depression[2]. The risk factors for cataract include smoking tobacco, diabetes, alcohol, and prolonged exposure to sunlight[3]. Globally, cataract accounts for one-third of visual impairment and half of blindness[4-5]. Cataract results in blindness of about 1 to 4 per 100 000 children and 10 to 40 per 100 000 children in the developed countries and the developing countries, respectively[6]. In worldwide, cataract can induce disability in 53.8 million people, and 52.2 million of them are living in poor countries[7]. Thus, it's important to explore the mechanisms of cataract and develop novel therapies.

Previous study reports that *heat-shock transcription factor 4* (*HSF4*) is critical for lens development and its disruption can cause cataract through reducing the expression of lens beaded filament, down-regulating γ-crystallin, and decreasing the post-translational modification of αA-crystallin[8-9]. *HSF4* pathogenic mutations result in nuclear cataracts *via* abrogating the induction of expression and DNase activity of *DLAD* (DNase 2β)[10]. Mou *et al*[11] deem that vimentin is targeted by *HSF4* in lens and plays a role in the aberrant lens development and cataractogenesis induced by *HSF4* mutation. Based on a p53-dependent manner, *HSF4* mutations may induce cataract through affecting the switch between the proliferation of lens epithelial cell (LEC) and the differentiation of secondary fiber cell[12]. In 2010, He *et al*[13] deposited GSE22362 and analyzed the differentially expressed genes (DEGs) in *HSF4* homozygous lens, finding many genes co-regulated by *HSF4* (especially the down-regulated DNase Iiβ, an enzyme for the denucleation of lens fiber cells). However, the action mechanisms of *HSF4* mutations in lens development and cataract formation remaining largely unknown.

Using the microarray data deposited by He *et al*[13], we further analyzed the DEGs between *HSF4*-null lens and wild-type

lens. Subsequently, the functions of the DEGs were predicted using enrichment analysis. Furthermore, the key genes co-regulated by *HSF4* in lens were deeply investigated by protein-protein interaction (PPI) network and microRNA (miRNA)-DEG regulatory network analyses. This study might contribute to finding the mechanisms regarding how *HSF4* mutations induce cataract formation.

SUBJECTS AND METHODS

Microarray Data The dataset of GSE22362 was downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database, which was sequenced on the platform of GPL8321 [Mouse430A_2] Affymetrix Mouse Genome 430A 2.0 Array. The dataset included 3 *HSF4*-null lens and 3 wild-type lens. Lens of wild-type and day E15.5 transgenic embryos were isolated and then kept in RNA Later (Ambion, Woodlands, TX, USA). He *et al*[13] deposited GSE22362, and their study was performed according to the approved protocol of the Association for Research in Vision and Ophthalmology and the Albert Einstein College of Medicine Animal Institute Committee.

Data Preprocessing and Differential Expression Analysis The raw data were preprocessed by the Robust MultiArray Averaging (RMA) method[14] in R package oligo (version 1.38.0, http://bioconductor.org/packages/release/bioc/html/oligo.html), including background correction, normalization and expression calculation. Probes were annotated based on platform annotation files, and the probes without corresponding gene symbols were removed. For the probes mapped to one and the same gene symbol, their average value was obtained as the gene expression value. Using the limma (Linear Models for Microarray Data) package[15] (version3.10.3, http://www.bioconductor.org/packages/2.9/bioc/html/limma.html) in R language, the DEGs between *HSF4*-null and wild-type lens were identified. Genes with |logFC (fold-change)| >1 and *P*-value <0.05 were identified as DEGs.

Functional and Pathway Enrichment Analysis Gene Ontology (GO) is a database developed for describing biological process, molecular function and subcellular location of gene products[16]. Kyoto Encyclopedia of Genes and Genomes (KEGG) database, which is composed by genes and their corresponding functions, can be used for predicting potential functions of gene lists[17]. Using database for annotation, visualization and integrated discovery (DAVID) tool (version 6.8, https://david-d.ncifcrf.gov/)[18], the up-regulated and down-regulated genes were conducted with GO functional and KEGG pathway enrichment analyses, respectively. The terms with *P*-value <0.05 and count (the number of enriched genes) ≥2 were selected as significant terms.

Construction of Protein-protein Interaction Network The PPI pairs among the DEGs were predicted by the Search Tool for the Retrieval of Interacting Genes (STRING, version 10, http://www.string-db.org/)[19] database, with PPI score (medium confidence) was set as 0.4. Then, PPI network was constructed using Cytoscape software (version 3.2.0, http://www.cytoscape.org)[20]. Subsequently, degree centrality of the nodes in the PPI network were calculated by the CytoNCA plug-in[21] in Cytoscape software, with parameter was set as without weight. The nodes with higher degrees were identified as the hub proteins[22].

MicroRNA-gene Regulatory Network Analysis Using miRWalk2.0 database (validated gene-miRNA interaction information retrieval system, http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/)[23], the validated miRNA-DEG pairs were obtained. Afterwards, miRNA-DEG regulatory network was visualized by Cytoscape software[20].

RESULTS

Differential Expression Analysis The gene expression distribution before and after normalization are shown in Figure 1. The median values were almost at the same level, indicating that the effect of data preprocessing was good. With |logFC| >1 and *P*-value <0.05 as the thresholds, a total of 176 DEGs (51 up-regulated and 125 down-regulated genes) were identified in *HSF4*-null lens compared with wild-type lens. The number of down-regulated genes exceeded that of up-regulated genes. The heatmap of the DEGs is shown in Figure 2.

Functional and Pathway Enrichment Analysis The top 10 GO terms enriched for the up-regulated genes were listed in Table 1, including cytoskeleton organization (*P*=2.06E-03), cellular response to fibroblast growth factor stimulus (*P*=2.41E-03) and cellular response to tumor necrosis factor (*P*=2.42E-03). Besides, 4 pathways were enriched for the up-regulated genes, including malaria (*P*=2.39E-04), rheumatoid arthritis (*P*=1.87E-02), Chagas disease (American trypanosomiasis) (*P*=2.86E-02) and tumor necrosis factor (TNF) signaling pathway (*P*=3.18E-02) (Table 1).

Meanwhile, enrichment analysis was also performed for the down-regulated genes. For the down-regulated genes, 10 GO terms including cellular response to cisplatin (*P*=3.18E-04), negative regulation of inclusion body assembly (*P*=6.62E-04) and lipid storage (*P*=1.03E-02) were enriched (Table 2). Moreover, protein processing in endoplasmic reticulum (*P*=2.00E-02) was the only pathway enriched for the down-regulated genes (Table 2).

Protein-protein Interaction Network Analysis The PPI network constructed for the DEGs included 88 nodes and 121 interactions (Figure 3).

According to the degrees of nodes, the top 20 nodes with higher degrees were identified, including FBJ osteosarcoma oncogene (FOS, degree=16), early growth response 1 (EGR1, degree=10) and heme oxygenase (decycling) 1 (HMOX1, degree=9) (Table 3).

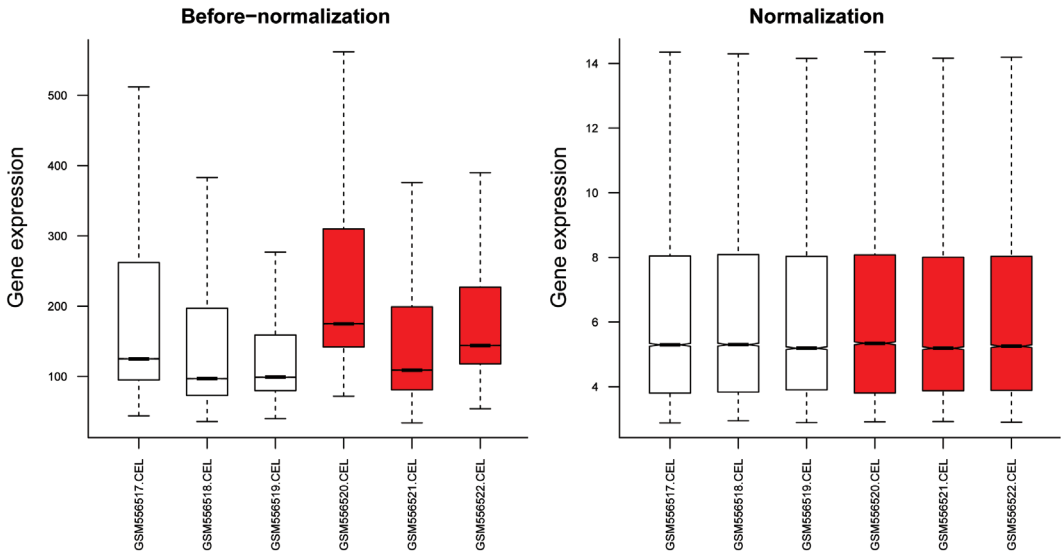

Figure 1 The gene expression distribution before and after normalization Red and white boxes represent *HSF4*-null and wild-type lens, respectively.

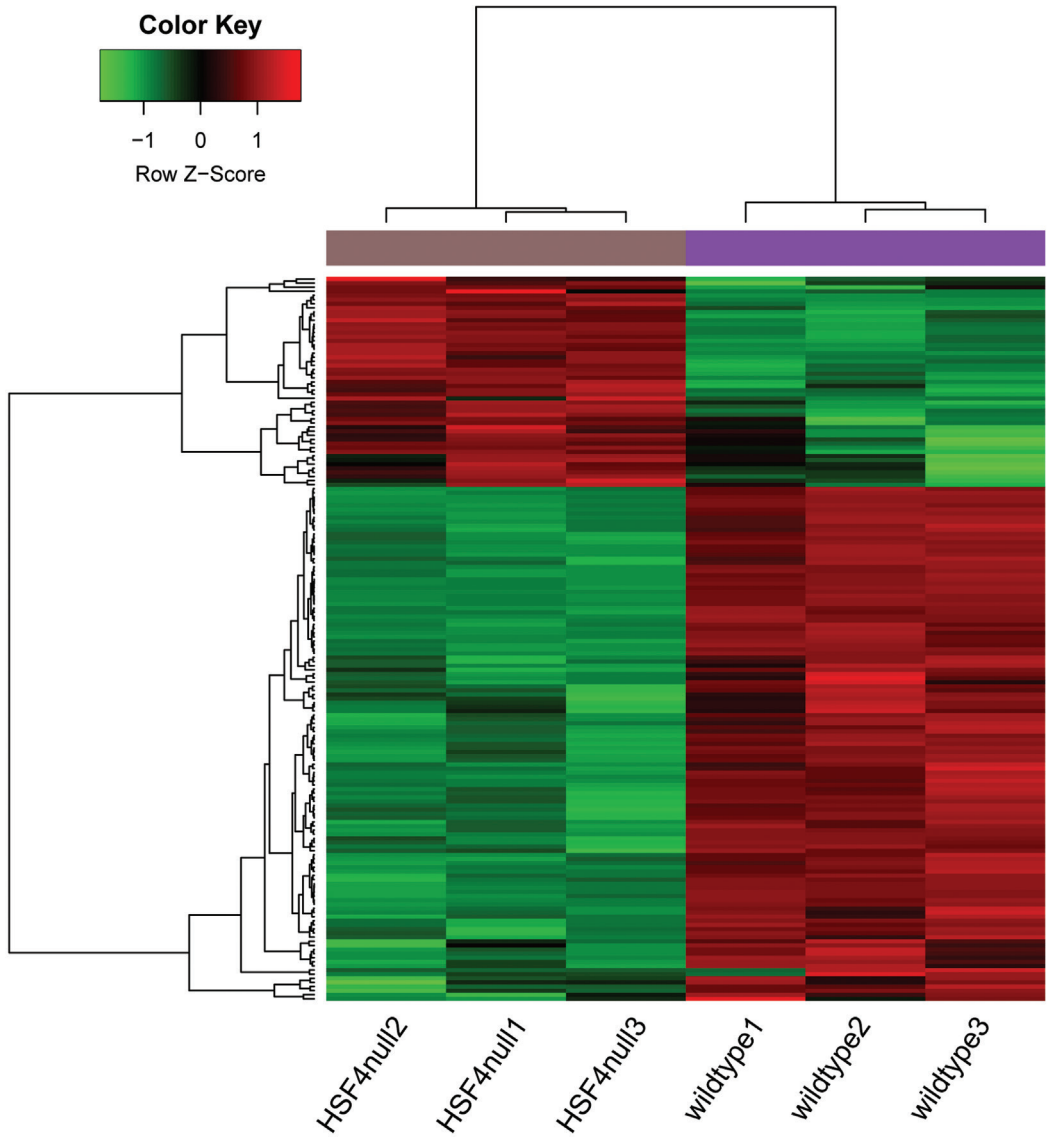

Figure 2 The heatmap of the DEGs Color changes from green to red indicates that expression values range from low to high.

Especially, FOS, EGR1 and HMOX1 had interactions with each other in the PPI network. Afterwards, pathway enrichment analysis was performed for the top 20 nodes, the enriched pathways mainly included TNF signaling pathway

Table 1 The terms enriched for the up-regulated genes

ID	Description	Count	*P*	Genes
GO				
0007010	Cytoskeleton organization	4	2.06E-03	*CCL12, CCL2, TAGLN, MICAL2*
0044344	Cellular response to fibroblast growth factor stimulus	3	2.41E-03	*HYAL1, CCL2, COL1A1*
0071356	Cellular response to tumor necrosis factor	4	2.42E-03	*CCL12, HYAL1, CCL2, COL1A1*
0006935	Chemotaxis	4	2.95E-03	*CCL12, CCL2, CMTM3, CYR61*
0030199	Collagen fibril organization	3	4.05E-03	*SFRP2, COL1A1, GREM1*
0051591	Response to cAMP	3	6.84E-03	*FOS, PYGM, COL1A1*
2000502	Negative regulation of natural killer cell chemotaxis	2	7.28E-03	*CCL12, CCL2*
0001666	Response to hypoxia	4	1.13E-02	*EGR1, CCL2, PYGM, BNIP3*
0006915	Apoptotic process	6	1.21E-02	*DNASE1, SGK1, CHAC1, SFRP2, BNIP3, GREM1*
0048023	Positive regulation of melanin biosynthetic process	2	1.21E-02	*TYRP1, PMEL*
KEGG				
mmu05144	Malaria	4	2.39E-04	*HBA-A1, CCL12, CCL2, HBB-B1*
mmu05323	Rheumatoid arthritis	3	1.87E-02	*CCL12, FOS, CCL2*
mmu05142	Chagas disease (American trypanosomiasis)	3	2.86E-02	*CCL12, FOS, CCL2*
mmu04668	TNF signaling pathway	3	3.18E-02	*CCL12, FOS, CCL2*

ID: Identification; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TNF: tumor necrosis factor.

Table 2 The terms enriched for the down-regulated genes

ID	Description	Count	*P*	Genes
GO				
0072719	Cellular response to cisplatin	3	3.18E-04	*TIMELESS, HMOX1, SLC31A1*
0090084	Negative regulation of inclusion body assembly	3	6.62E-04	*DNAJB2, DNAJB1, DNAJA4*
0019915	Lipid storage	3	1.03E-02	*CRY2, DGAT2, BSCL2*
0043029	T cell homeostasis	3	1.34E-02	*SPNS2, RAG1, FAS*
0002246	Wound healing involved in inflammatory response	2	1.70E-02	*CD44, HMOX1*
0045766	Positive regulation of angiogenesis	4	3.19E-02	*HMOX1, HSPB1, CELA1, ANGPT2*
0042752	Regulation of circadian rhythm	3	3.54E-02	*CRY2, TIMELESS, MAPK10*
0006457	Protein folding	4	3.67E-02	*HSPA4L, DNAJB1, DNAJB4, DNAJA4*
0008630	Intrinsic apoptotic signaling pathway in response to DNA damage	3	3.80E-02	*PGAP2, HMOX1, EPHA2*
0014066	Regulation of phosphatidylinositol 3-kinase signaling	2	4.47E-02	*1190002N15RIK, CEACAM1*
KEGG				
mmu04141	Protein processing in endoplasmic reticulum	5	2.00E-02	*MAP3K5, HSPA4L, DNAJB2, DNAJB1, MAPK10*

ID: Identification; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genome.

(*P*=1.37E-06), Chagas disease (American trypanosomiasis) (*P*=3.64E-05) and influenza A (*P*=2.62E-04) (Table 4).

MicroRNA-gene Regulatory Network Analysis The constructed miRNA-DEG regulatory network had 243 nodes (including 18 up-regulated genes, 44 down-regulated genes and 181 miRNAs) (Figure 4). Among the nodes involved in the regulatory network, the top 10 genes and miRNAs (including *mmu-miR-15a-5p* and *mmu-miR-26a-5p*) with higher degrees were listed in Table 5. In addition, *mmu-miR-26a-5p* could target *EGR1* in the regulatory network.

DISCUSSION

This study was aimed to reveal the genes and miRNAs involved in *HSF4* mutation-induced cataract, which provided targets for the further experimental researches and the clinical therapy of this disease. In this study, a total of 176 DEGs were identified in *HSF4*-null lens, including 51 up-regulated and 125 down-regulated genes. FOS, EGR1 and HMOX1 had relatively higher degrees in the PPI network. Besides, the pathways of TNF signaling pathway, Chagas disease (American trypanosomiasis) and influenza A were enriched for the top 20 nodes in the PPI network. Moreover, *mmu-miR-15a-5p* and *mmu-miR-26a-5p* also were among the top 10 miRNAs in the miRNA-DEG regulatory network.

FOS protein was temporarily expressed in equatorial LECs following anterior capsule rubbing, indicating that equatorial LECs are transcriptionally activated after the anterior lens surface experience minor mechanical stimuli[24]. *FOS*, *UBC* (E2 ubiquitin-conjugating protein UBC), *EGR1* and *PTGS2* (prostaglandin-endoperoxide synthase 2) expression levels are mediated by *HSF4* mutations in cataract and can serve as

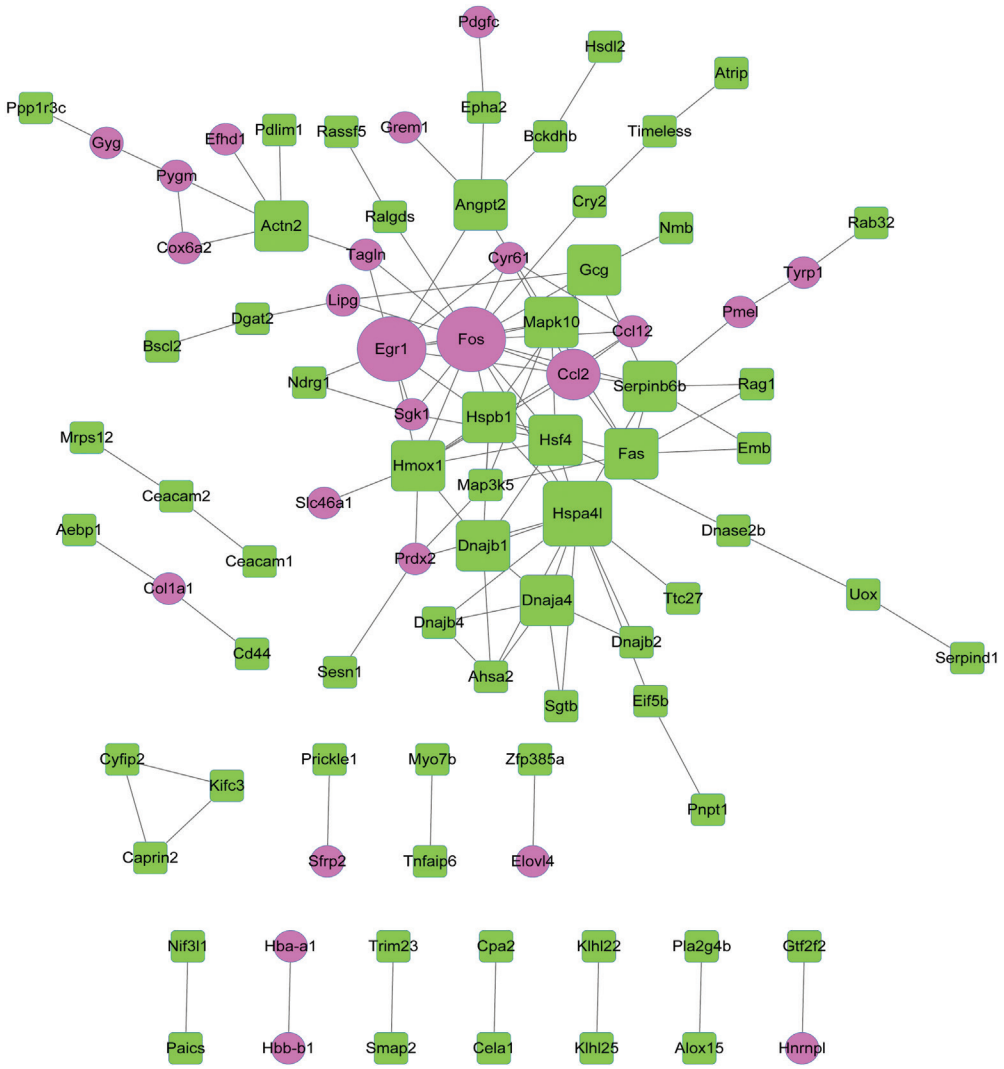

Figure 3 The PPI network constructed for the DEGs Red circles and green squares separately represent up-regulated and down-regulated genes. A larger size indicates a higher degree of the node.

Table 3 The top 20 nodes with higher degrees in the PPI network

Gene	Description	Degree
FOS	Up	16
HSPA4l	Down	13
EGR1	Up	10
HMOX1	Down	9
HSPB1	Down	9
HSF4	Down	8
SERPINB6B	Down	8
CCL2	Up	7
FAS	Down	7
MAPK10	Down	6
DNAJB1	Down	6
DNAJA4	Down	6
GCG	Down	5
ACTN2	Down	5
ANGPT2	Down	5
MAP3K5	Down	4
CYR61	Up	4
CCL12	Up	4
SGK1	Up	4
AHSA2	Down	4

therapeutic targets for the disease[25]. The expression of *FOS* and *JUN* (jun proto-oncogene) are dysregulated in the terminal differentiation process of lens fiber cells[26]. *FOS* can interact with *JUN* to constitute the *AP-1* (activating protein 1) transcription factor, and *AP-1* may act in mediating the behavior of lens cells during lens wound repair[27]. These declared that *FOS* might function in the pathogenesis of *HSF4* mutation-induced cataract.

EGR1 may function in the embryonic fibrotic phenotype in the β1MLR10 (β1 F/F and homozygous for MLR10-Cre) lens, and may also be involved in the pathogenesis of posterior capsular opacification (PCO) and other lens diseases[28]. The mRNA levels of *EGR1* in a mammalian retina exhibiting a biphasic response to adversing ocular growth stimuli, indicating that retinal *EGR1* may serve as a signal for directing ocular growth in various species[29]. Nakajima *et al*[30] demonstrate that increased activity of *EGR1* may play a role in selenite-induced LEC death. Ma *et al*[31] find that *HMOX1* protects LECs from oxidant stress induced by hydrogen peroxide (H_2O_2) through decreasing the generation of reactive oxygen species (ROS)

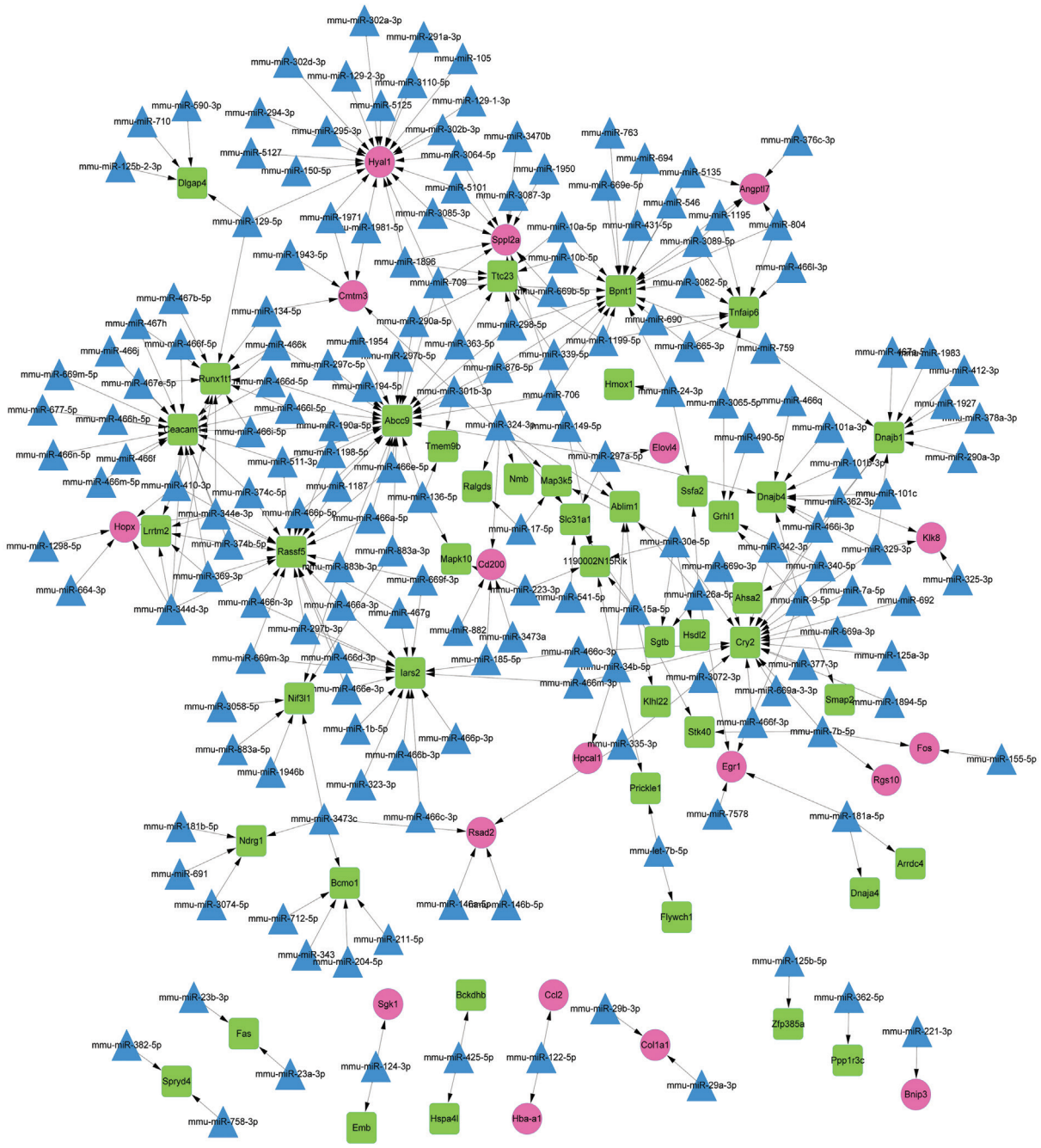

Figure 4 The microRNA-DEG regulatory network Red circles, green squares and blue triangles represent up-regulated genes, down-regulated genes and miRNAs, respectively.

Table 4 The pathways enriched for the top 20 nodes in the PPI network

ID	Description	Count	*P*	Genes
mmu04668	TNF signaling pathway	6	1.37E-06	*CCL12, FOS, MAP3K5, CCL2, FAS, MAPK10*
mmu05142	Chagas disease (American trypanosomiasis)	5	3.64E-05	*CCL12, FOS, CCL2, FAS, MAPK10*
mmu05164	Influenza A	5	2.62E-04	*CCL12, CCL2, FAS, DNAJB1, MAPK10*
mmu05168	Herpes simplex infection	5	5.53E-04	*CCL12, FOS, CCL2, FAS, MAPK10*
mmu04010	MAPK signaling pathway	5	1.16E-03	*FOS, MAP3K5, HSPB1, FAS, MAPK10*
mmu04141	Protein processing in endoplasmic reticulum	4	3.80E-03	*MAP3K5, HSPA4L, DNAJB1, MAPK10*
mmu04621	NOD-like receptor signaling pathway	3	5.11E-03	*CCL12, CCL2, MAPK10*
mmu05323	Rheumatoid arthritis	3	1.07E-02	*CCL12, FOS, CCL2*
mmu05146	Amoebiasis	3	2.10E-02	*SERPINB6B, HSPB1, ACTN2*
mmu05161	Hepatitis B	3	3.17E-02	*FOS, FAS, MAPK10*
mmu04932	Non-alcoholic fatty liver disease	3	3.63E-02	*MAP3K5, FAS, MAPK10*

ID: Identification.

Table 5 The top 10 genes and microRNAs in the regulatory network

Gene	Description	Degree	miRNA	Degree
ABCC9	Down	26	*mmu-miR-324-3p*	6
HYAL1	Up	21	*mmu-miR-344d-3p*	5
BPNT1	Down	21	*mmu-miR-410-3p*	5
CEACAM1	Down	21	*mmu-miR-344e-3p*	5
CRY2	Down	20	*mmu-miR-30e-5p*	5
RASSF5	Down	20	*mmu-miR-15a-5p*	5
IARS2	Down	15	*mmu-miR-26a-5p*	4
RUNXLT1	Down	12	*mmu-miR-9-5p*	4
SPPL2A	Up	11	*mmu-miR-297a-5p*	4
DNAJB1	Down	10	*mmu-miR-7b-5p*	4

and enhancing the activity of antioxidant enzyme, and therefore restraining caspase family-dependent apoptosis. Therefore, *EGR1* and *HMOX1* might play roles in formation of *HSF4* mutation-induced cataract. FOS, EGR1 and HMOX1 could interactions with each other in the PPI network, indicating that *FOS*, *EGR1* and *HMOX1* might also act in *HSF4* mutation-induced cataract through interacting with other genes.

Hsa-miR-15a-5p, *hsa-miR-15a-3p*, and *hsa-miR-16-1-5p* are overexpressed in the LECs of age-related cataract, and may promote the progression of age-related cataract through inhibiting the expression levels of the anti-apoptotic genes *myeloid cell leukemia sequence 1* (*MCL1*) and *B-cell CLL/lymphoma 2* (*BCL2*)[32]. Previous studies report that *miR-26b* plays an important role in growth and proliferation of LECs in cataract rat[33-34]. Downregulation of *miR-26b* can postpone the progression of oxidative cataract *via* mediating cell proliferation, and suppressing LEC apoptosis, nuclear factor kappa B (NF-κB) expression and inflammatory factors[35]. *mmu-miR-26a-5p* could target *EGR1* in the miRNA-DEG regulatory network, suggesting that *mmu-miR-26a-5p* targeting *EGR1* and *mmu-miR-15a-5p* might also be involved in the procession of *HSF4* mutation-induced cataract.

In conclusion, a total of 176 DEGs were identified in *HSF4*-null lens through a series of bioinformatics analyses. Besides, *FOS*, *EGR1*, *HMOX1*, *mmu-miR-26a-5p* and *mmu-miR-15a-5p* might play roles in the pathogenesis of *HSF4* mutation-induced cataract. However, these results were obtained from bioinformatics analyses and in-depth experimental researches should be done in the future.

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