Identification of long non-coding RNA and mRNA expression in βB2-crystallin knockout mice

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Abstract. βB2-crystallin (CRYBB2) is expressed at an increased level in the postnatal lens cortex and is associated with cataracts. Improved understanding of the underlying biology of cataracts is likely to be critical for the development of early detection strategies and new therapeutics. The present study aimed to identify long non-coding RNAs (lncRNAs) and mRNAs associated with CRYBB2 knockdown (KO)-induced cataracts. RNAs from 3 non-treated mice and 3 CRYBB2 KO mice were analyzed using the Affymetrix GeneChip Mouse Gene 2.0 ST array. A total of 149 lncRNAs and 803 mRNAs were identified to have upregulated expression, including Snora73b, Klk1b22 and Rnu3a, while the expression levels of 180 lncRNAs and 732 mRNAs were downregulated in CRYBB2 KO mice, including Snord82, Snhg9 and Foxn3. This lncRNA and mRNA expression profile of mice with CRYBB2 KO provides a basis for studying the genetic mechanisms of cataract progression.

Introduction

Congenital cataracts area common cause of blindness, with the incidence estimated to be 1-6/10,000 infants in most populations (1,2). There are 13,000-200,000 patients with bilateral congenital cataract who go blind each year worldwide, with an increase of 2,000-40,000 per year (3). The primary clinical manifestation of the disease is the occurrence of lens opacity in the first year (4). Although surgical techniques and visual prognosis have improved, congenital cataracts remain the leading cause of visual disability in children worldwide (5). Previous studies have revealed that almost one-third of congenital cataracts are caused by genetic mutations (6), and 13 genes have previously been confirmed to be associated with congenital cataracts (7). These include crystallin genes [αA-crystallin (CRYAA), αB-crystallin, βA1-crystallin (CRYBA1), βB1-crystallin, βB2-crystallin (CRYBB2), γC-crystallin and γD-crystallin (CRYGD)], membrane transport protein genes [major intrinsic protein (MIP), gap junction protein (GJ)A3 and GJA8], a cytoskeletal protein gene [beaded filament structural protein 2 (BFSP2)], and transcription factor genes (paired-like homeodomain 3 and heat shock transcription factor 4).

Evidence indicates that gene expression in the lens epithelium is significantly altered during cataract formation. Sheets *et al* (8) reported the downregulation of CRYAA and CRYBA1/CRYBA3 and the upregulation of the receptor tyrosine kinase adhesion-related kinase (ARK) in the Emory mouse, a well-characterized model of age-dependent cataracts. Furthermore, metallothionein-IIA, osteonectin and ARK are upregulated in cataractous lenses relative to transparent lenses (9-11). Ruotolo *et al* (12) identified extensive downregulation of genes, including GCS1, GRB7, FST and POLR2E, in the lens associated with the development of age-related cataracts in humans. Although these changes in gene expression are informative, further gene identifications are required to elucidate the molecular mechanism of cataract formation. Crystallins, CRYBB2 in particular, are considered to act primarily as structural proteins of the lens (13). Previously, it was demonstrated that the relative amounts of CRYBB2 protein expression in the lens change markedly, increasing from 12 to 24% (14), suggesting that CRYBB2 serves a contributive function in lens development. Moreover, targeted knockout (KO) of CRYBB2 in mice has been demonstrated to induce age-related (15) and congenital cataracts (16); however, its functional significance is not yet known.

Long non-coding RNAs (lncRNAs) are defined as non-coding RNA molecules >200 nucleotides in length with limited protein coding potential (17,18). Previous studies have indicated that lncRNAs are deregulated in numerous diseases and associated with a wide range of biological processes, such as proliferation, apoptosis and cell migration (19,20). Recently, some lncRNAs have been identified to serve critical functions in eye development and diseases. Shen *et al* (21) reported that 38 lncRNAs were differentially expressed between transparent and cataractous lenses, among which one of the most abundant lncRNAs, myocardial infarction associated transcript, was specifically upregulated in the plasma fraction of whole blood and the aqueous humor of cataract patients. However, the function of lncRNAs in human lenses remains unknown.

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In the present study, differences in lncRNA and mRNA expression between the lenses of untreated mice and CRYBB2 KO-induced cataract mice models were evaluated. A total of149 lncRNAs and 803 mRNAs were identified whose expression was upregulated, while the expression levels of a further 180 lncRNAs and 732 mRNAs were downregulated in CRYBB2 KO mice lenses. These findings suggest a potential function for these lncRNAs and mRNAs in cataract formation.

Materials and methods

Animals. A total of 3 male wild type (WT) and 3 male CRYBB2 KO BALB/c mice (age, 12 weeks old; weight, 25 g) were provided by in Genious Targeting Laboratory, Inc. (Ronkonkoma, NY, USA) (22). Mice with targeted disruption of the CRYBB2 gene were generated at the company by inserting a neo expression cassette to replace the first and second exons, preventing the production of a functional transcript from this locus. Mice were maintained in an animal facility at 25˚C, with a relative humidity of 60-70%, under a 12-h light/dark cycle with free access to food and water at the Laboratory Animal Center of the Changhai Hospital, Second Military Medical University (Shanghai, China). All procedures were carried out in accordance with the Chinese legislation on the Use and Care of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (23) and were approved by the Institutional Animal Care and Use Committee of Changhai Hospital, Second Military Medical University (Shanghai, China).

RNA extraction. Following the sacrifice of the mice, the lenses were collected and RNA was isolated from the lenses of mice using the Chomczynski method (24) and was further purified using an RNeasy MinElute Clean-up kit (Qiagen GmbH, Hilden, Germany). The RNA concentration was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The A260/A280 ratio was 1.8-2.0 and the quality of the RNA was verified by agarose gel electrophoresis.

Microarray processing. LncRNA and mRNA expression profiling was performed using the Affymetrix GeneChip Mouse Gene 2.0 ST array (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Intensities of target hybridization to respective probe features were detected by laser scanning of the array. First, quantile normalization of the microarray data of the 3 untreated and 3 CRYBB2 KO mice was performed. The data was then log2-scale transformed. Hierarchical clustering of the lncRNA and mRNA profiles was performed using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) (25). The normalized expression values of the lncRNAs and mRNAs were centered on the median before unsupervised hierarchical clustering was performed. Clustering was performed with complete linkage and centered Pearson correlation. To estimate the accuracy of the measurements, the coefficient of variance for each measured parameter was determined.

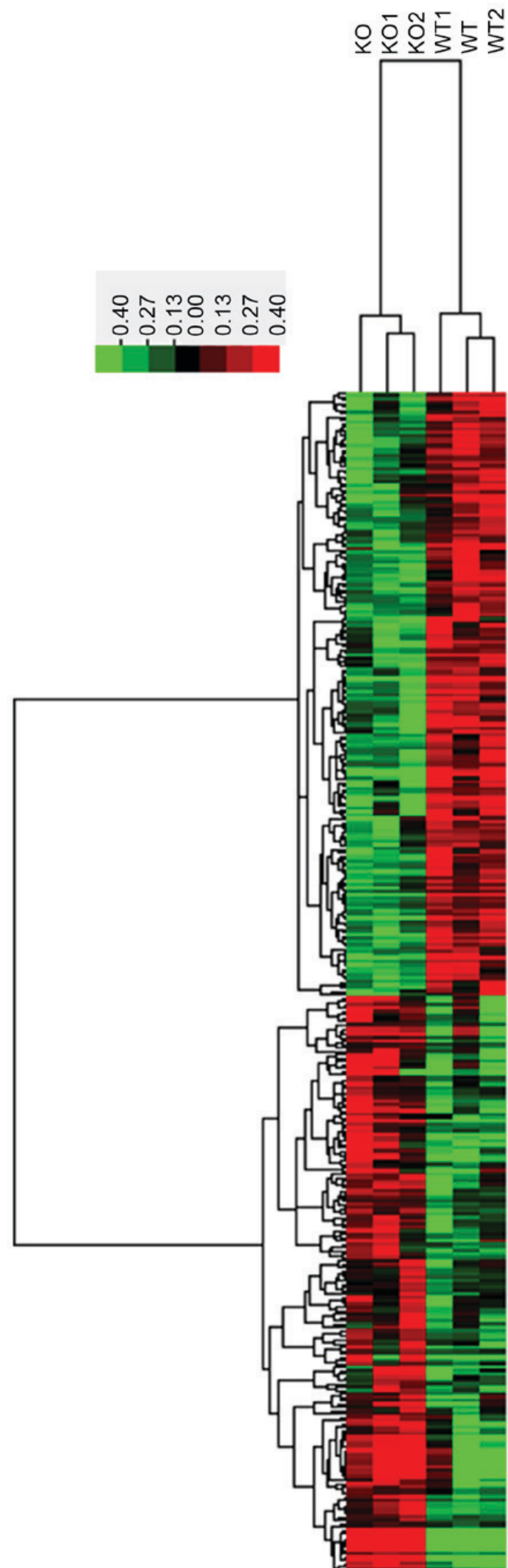

Figure 1. Differential expression of lncRNAs in WT and βB2-crystallin KO cataractous lens samples. Heatmaps were generated from hierarchical cluster analysis to indicate differential expression. The color scale illustrates the relative expression level of lncRNAs across different samples. Red denotes upregulation; green denotes downregulation. KO, knockout; WT, wild type; lncRNA, long non-coding RNA.

Table I. Top 20 upregulated long non-coding RNAs in βB2-crystallin KO mice.

Probe set ID	P-value	Fold-change (KO/WT)	MGI gene symbol	Gene description	GenBank accession no.
17296979	$2.00x10^{-7}$	5.84	Gm10409	Predicted gene 10409	NR_033121
17303368	$1.00x10^{-6}$	5.21	Gm3002	α-takusan pseudogene	NR_033388
17296602	$2.60x10^{-6}$	3.75	Gm3020	Predicted gene 3020	NR_033117
17430831	$3.57x10^{-5}$	3.55	Snora73b	Small nucleolar RNA, H/ACA box 73b	NR_028513
17303147	$1.70x10^{-6}$	3.52	Gm3591	Predicted gene 3591	XR_141206
17434158	$6.77x10^{-3}$	3.51	-	-	ENSMUST00000169242
17337152	$4.01x10^{-3}$	3.38	-	-	ENSMUST00000174425
17424407	$1.21x10^{-2}$	2.98	4933409K07Rik	RIKEN cDNA 4933409K07 gene	NR_033123
17413061	$3.08x10^{-3}$	2.87	-	-	ENSMUST00000169242
17480922	$3.86x10^{-4}$	2.68	Mir139	MicroRNA139NR_029791	
17302054	$3.04x10^{-2}$	2.49	Snora31	Small nucleolar RNA, H/ACA box 31	NR_028481
17232731	$3.02x10^{-2}$	2.4	Rnu3a	U3A small nuclear RNA	NR_002842
17412952	$9.63x10^{-3}$	2.32	Gm3893	Predicted gene 3893	NR_033506
17342996	$2.00x10^{-3}$	2.21	Gm16197	Predicted gene 16197	NR_036469
17421488	$1.19x10^{-2}$	2.06	-	-	ENSMUST00000172415
17348121	$3.41x10^{-2}$	2.04	4833419F23Rik	RIKEN cDNA4833419F23 gene	NR_040328
17347279	$3.47x10^{-2}$	2.01	-	-	ENSMUST00000157334
17430833	$1.42x10^{-3}$	1.98	Snora73a	Small nucleolar RNA, H/ACA box 73a	NR_028512
17221923	$2.92x10^{-2}$	1.91	-	-	ENSMUST00000083191
17523680	$4.81x10^{-4}$	1.89	Mir101c	MicroRNA101cNR_039546	

KO, knockout; WT, wild type; MGI, mouse genome informatics database.

Statistical analysis. Statistical analyses were performed with the use of GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The Significance Analysis of Microarray method was used to identify significant gene expression changes between CRYBB2 KO mice and controls (26). P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of lncRNA expression patterns in CRYBB2 KO mice. The lncRNA expression profiles of lens tissues were compared using unsupervised hierarchical clustering in 3 untreated and 3CRYBB2 KO mice. As demonstrated in Fig. 1, in total, 329 lncRNAs with a coefficient of variance >0.10 were selected for clustering analysis. Hierarchical clustering of these 329 lncRNAs based on centered Pearson correlation indicated notable differential lncRNA expression in CRYBB2 KO and untreated mice (Fig. 1). Among these lncRNAs, 17 exhibited at least a two-fold change in the CRYBB2 KO mice compared with the untreated mice (all upregulated in CRYBB2 KO mice). A total of 149 out of 329 lncRNAs were upregulated in CRYBB2 KO mice compared with the untreated mice (Table I presents the top 20 most upregulated lncRNAs), whereas 180 out of 329 lncRNAs were downregulated in CRYBB2 KO mice compared with the untreated mice (Table II presents the top 20 most downregulated lncRNAs).

Analysis of mRNA expression patterns in CRYBB2 KO mice. The mRNA expression profiles of lens tissues were compared using unsupervised hierarchical clustering in the 3 untreated and 3 CRYBB2 KO mice. In total, 1,535 mRNAs with a coefficient of variance >0.10 were selected for clustering analysis. Hierarchical clustering of these 1,535 mRNAs based on centered Pearson correlation indicated notable differential mRNA expression between CRYBB2 KO and untreated mice (Fig. 2). Among these mRNAs, 52 exhibited at least a two-fold change in the CRYBB2 KO mice compared with the untreated mice (all upregulated in CRYBB2 KO mice). A total of 803 out of 1,535 mRNAs were upregulated in CRYBB2 KO mice compared with the untreated mice (Table III presents the top 20 most upregulated mRNAs), whereas 732 out of 1,535 mRNAs were downregulated (Table IV presents the top 20 most downregulated mRNAs).

Discussion

In the present study, the lncRNA and mRNA profiles of untreated and CRYBB2 KO cataractous lenses were evaluated. A total of 149 lncRNAs and 803 mRNAs were identified to be upregulated, while 180 lncRNAs and 732 mRNAs were identified to be downregulated in CRYBB2 KO mice lenses, implying a potential role of these lncRNAs and mRNAs in cataract formation.

In previous research, an increasing number of lncRNAs have been identified and associations between lncRNAs and

Table II. Top 20 downregulated long non-coding RNAs in βB2-crystallin KO mice.

Probe set ID	P-value	Fold-change (KO/WT)	MGI gene symbol	Gene description	GenBank accession no.
17251898	4.62×10^{-2}	0.86	Mir324	MicroRNA 324	NR_029758
17547715	4.84×10^{-2}	0.86	-	-	ENSMUST00000117972
17468138	4.35×10^{-2}	0.85	-	-	ENSMUST00000145420
17329209	4.58×10^{-2}	0.85	A830060N17	Uncharacterized LOC328646	NR_046162
17365718	3.37×10^{-2}	0.84	-	-	ENSMUST00000162724
17403967	3.43×10^{-2}	0.84	-	-	ENSMUST00000158662
17362668	3.62×10^{-2}	0.84	-	-	ENSMUST00000169060
17315735	3.71×10^{-2}	0.84	-	-	ENSMUST00000160698
17527984	4.10×10^{-2}	0.84	A730043L09	Uncharacterized protein A730043L09	NR_040769
17278612	4.40×10^{-2}	0.84	Mir342	MicroRNA342	NR_029771
17448958	2.17×10^{-2}	0.83	-	-	ENSMUST00000158856
17269866	3.17×10^{-2}	0.83	-	-	ENSMUST00000102272
17225173	3.59×10^{-2}	0.83	Snord82	Small nucleolar RNA, C/D box 82	NR_002851
17230480	3.98×10^{-2}	0.83	-	-	ENSMUST00000128545
17532275	4.53×10^{-2}	0.83	-	-	ENSMUST00000082463
17345664	4.84×10^{-2}	0.83	-	-	ENSMUST00000122623
17280661	2.01×10^{-2}	0.82	F730043M19Rik	RIKEN cDNA F730043M19 gene	NR_015602
17395003	2.17×10^{-2}	0.82	-	-	ENSMUST00000133525
17342024	2.90×10^{-2}	0.82	Snhg9	Small nucleolar RNA host gene (non-protein coding) 9	NR_027900
17232800	3.09×10^{-2}	0.82	-	-	ENSMUST00000104610

KO, knockout; WT, wild type; MGI, mouse genome informatics database.

Table III. Top 20 upregulated mRNAs in βB2-crystallin KO mice.

Probe set ID	P-value	Fold-change (KO/WT)	MGI gene symbol	Gene description	GenBank accession no.
17477347	3.60×10^{-2}	7.22	Klk1b22	Kallikrein 1-related peptidase b22	NM_010114
17303018	1.00×10^{-7}	7.14	Gm3500	Predicted gene 3500	NM_001256886
17296943	2.52×10^{-4}	5.78	-	-	ENSMUST00000163719
17303117	1.00×10^{-7}	5.58	Gm3696	Predicted gene 3696	ENSMUST00000167923
17296896	4.00×10^{-7}	5.14	Gm5796	Predicted gene 5796	NM_001029930
17548311	1.22×10^{-5}	4.56	Gm3579	Predicted gene 3579	AY140896
17373996	1.35×10^{-4}	4.53	BC048594	cDNA sequence BC048594	BC048594
17331088	4.22×10^{-5}	4.48	Gm19797	Predicted gene 19797	XM_003085996
17303024	3.66×10^{-4}	4.13	Gm10021	Predicted gene 10021	AK084071
17413352	5.14×10^{-3}	3.46	Car9	Carbonic anhydrase 9	ENSMUST00000030183
17296849	9.00×10^{-6}	3.4	Gm2897	Predicted gene 2897	NM_001177715
17335467	1.21×10^{-2}	3.22	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669
17412962	7.94×10^{-3}	3.2	Gm3893	Predicted gene 3893	BC059060
17496857	2.59×10^{-2}	3.16	Cox6a2	Cytochrome c oxidase, subunit VI a, polypeptide 2	NM_009943
17466743	9.47×10^{-4}	3.13	Npvf	Neuropeptide VF precursor	ENSMUST00000031853
17331078	3.77×10^{-4}	3.06	Tmem45a	Transmembrane protein 45a	NM_019631
17296836	2.00×10^{-6}	3.01	Gm5458	Predicted gene 5458	NM_001024706
17280292	5.05×10^{-4}	2.93	-	-	ENSMUST00000169148
17296595	2.52×10^{-5}	2.9	D830030K20Rik	RIKEN cDNA D830030K20 gene	ENSMUST00000169218
17303315	5.57×10^{-5}	2.78	Gm5797	Predicted gene 5797	ENSMUST00000100886

KO, knockout; WT, wild type; MGI, mouse genome informatics database.

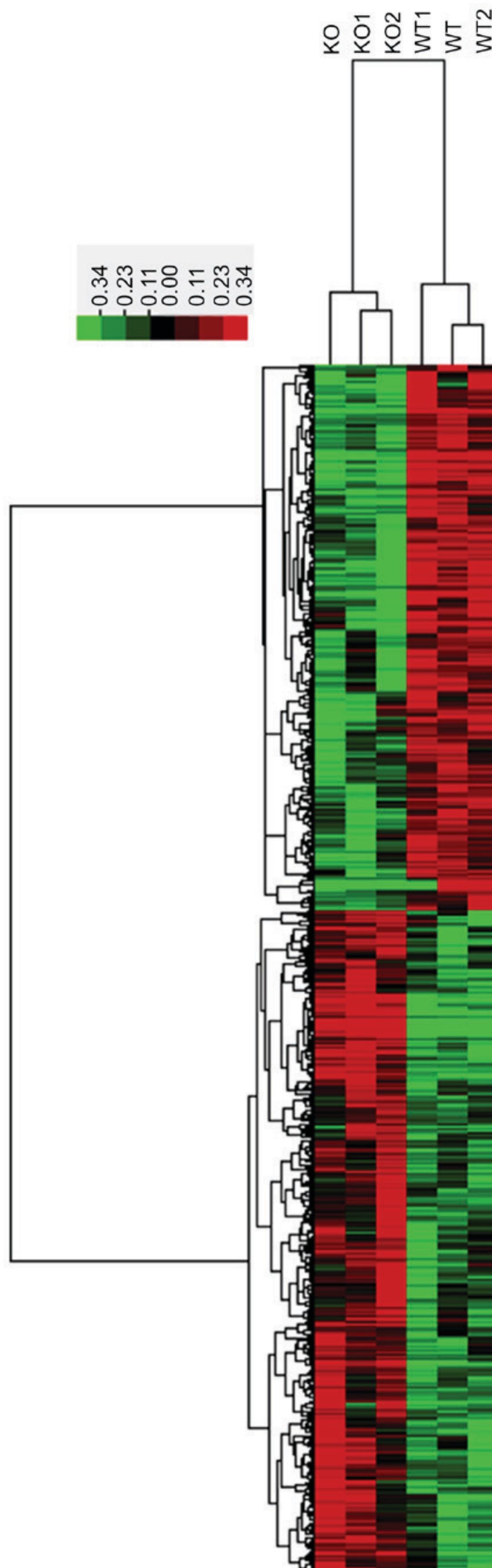

Figure 2. Differential expression of mRNAs in WT and βB2-crystallin KO cataractous lens samples. Heatmaps were generated from hierarchical cluster analysis to indicate differential expression. The color scale illustrates the relative expression level of mRNAs across different samples. Red denotes upregulation; green denotes downregulation. KO, knockout; WT, wild type.

numerous diseases, including cardiovascular and neurodegeneration diseases, have been reported (27). The roles of lncRNAs in cancer development are being studied (28,29). However, the function of lncRNAs in disease, particularly in cataracts, has not yet been reported. To the best of our knowledge, the current study presents the first report on differential lncRNA expression in a cohort of mice with or without CRYBB2 KO. Through an analysis of lenses, it was identified that 329 lncRNAs were differentially expressed in CRYBB2 KO and untreated mice, suggesting that lncRNAs may serve critical functions in cataract formation. Among the top 20 most upregulated lncRNAs, five were predicted genes and a further six were unnamed. Among the top 20 most downregulated lncRNAs, 13 lncRNAs were unnamed and the others were known (identified with Mouse Genome Informatics gene symbols). These results indicated that these lncRNAs were linked with CRYBB2-associated cataract formation. Notably, the expression changes of lncRNAs in the upregulated group (maximum change, 5.84-fold) were higher compared with those in the downregulated group (maximum change, 0.86-fold), suggesting a higher susceptibility of lncRNAs to be upregulated rather than downregulated in cataracts.

Differential mRNA expression was also examined in the cohort of mice with or without CRYBB2 KO. Through an analysis of lenses, it was identified that 1,535 mRNAs were differentially expressed between CRYBB2 KO and untreated mice. Among the top 20 most upregulated mRNAs, 10 mRNAs were predicted genes and two mRNAs were unnamed. These results indicated that these mRNAs may serve critical functions in cataract formation. Notably, the expression changes of mRNAs in the upregulated group (maximum change, 7.22-fold) were higher compared with those in the downregulated group (maximum change, 0.86-fold), suggesting a higher susceptibility of mRNAs to be upregulated rather than downregulated in cataracts.

A previous limited microarray survey with a panel of cell cycle-regulated genes illustrated that irradiation with protons altered the gene expression pattern of human lens epithelial cells (30), such as cyclin-dependent kinase inhibitor 1 (CDKN1A), which codes for a protein that is involved in several pathways functionally associated with linear energy transfer-responsive radiation damage. Cytochrome C oxidase 6A2 (COX6A2) was identified to be upregulated during cataract development in mice with a mutation in MIP, a functional water channel that serves a key role in establishing lens fiber cell architecture and is associated with inherited and age-related forms of cataracts (31). Consistent with these results, the present study also identified upregulated expression of CDKN1A and COX6A2 in a CRYBB2 KO-induced cataract mice model. Furthermore, Fas-mediated apoptosis in human lens epithelial cells of cataracts is associated with diabetic retinopathy (32), suggesting a role for Fas in cataract formation, which is contrary to the finding of the present study that Fas was downregulated in a CRYBB2 KO-induced cataracts mouse model (data not shown). BFSP2, a gene for a lens-specific beaded filament structural protein, was down-regulated in CRYBB2 KO-induced cataract mice (33,34), which is in agreement with the findings of the present study: BFSP2 expression is restricted to the lens fiber cells, and a deletion mutation of BFSP2 is associated with

Table IV. Top 20 downregulated mRNAs in βB2-crystallin KO mice.

Probe set ID	P-value	Fold-change (KO/WT)	MGI gene symbol	Gene description	GenBank accession no.
17256388	3.86×10^{-2}	0.86	Ttc25	Tetratricopeptide repeat domain 25	NM_028918
17283203	4.91×10^{-2}	0.86	Foxn3	Forkhead box N3	ENSMUST00000046859
17357502	4.94×10^{-2}	0.86	Cpsf7	Cleavage and polyadenylation specific factor 7	NM_172302
17440775	4.31×10^{-2}	0.86	Dao	D-amino acid oxidase	ENSMUST00000112292
17473796	4.34×10^{-2}	0.86	Rps5	Ribosomal protein S5	NM_009095
17528663	4.79×10^{-2}	0.86	Polr2m	Polymerase (RNA) II (DNA directed) polypeptide M	NM_178602
17231003	4.52×10^{-2}	0.85	Mfsd7b	Major facilitator superfamily domain containing 7B	NM_001081259
17219005	4.68×10^{-2}	0.85	Creg1	Cellular repressor of E1A-stimulated genes 1	NM_011804
17225580	4.98×10^{-2}	0.85	Olfr1415	Olfactory receptor 1415	NM_001011525
17256716	4.83×10^{-2}	0.85	Rundc1	RUN domain containing 1	NM_172566
17291143	3.31×10^{-2}	0.85	-	-	AK029074
17304186	4.98×10^{-2}	0.85	Plac9	Placenta specific 9	NM_207229
17358640	4.57×10^{-2}	0.85	Mbl2	Mannose-binding lectin (protein C) 2	ENSMUST00000025797
17368499	4.89×10^{-2}	0.85	Dbh	Dopamine beta hydroxylase	ENSMUST00000000910
17425160	3.58×10^{-2}	0.85	Erp44	Endoplasmic reticulum protein 44	NM_029572
17451345	4.98×10^{-2}	0.85	2900026A02Rik	RIKEN cDNA 2900026A02 gene	NM_172884
17501800	3.53×10^{-2}	0.85	Hapln4	Hyaluronan and proteoglycan link protein 4	NM_177900
17499224	3.82×10^{-2}	0.85	F10	Coagulation factor X	NM_001242368
17504309	3.96×10^{-2}	0.85	Ccdc113	Coiled-coil domain containing 113	NM_172914
17502071	4.01×10^{-2}	0.85	-	-	ENSMUST00000050921

KO, knockout; WT, wild type; MGI, mouse genome informatics database.

cataracts. CRYGD mutation has previously been observed to cause autosomal dominant congenital cerulean cataracts, suggesting an inhibitory role of CRYGD in cataract formation (35). This is consistent with the current findings that CRYGD is downregulated in a CRYBB2 KO-induced cataracts mouse model (data not shown).

The present study has some limitations, including the relatively small number of mice in each cohort, and the fact that only RNA samples from the lens were utilized for hybridizations. Furthermore, the differentially expressed lncRNAs and mRNAs require further clarification in future investigations.

In conclusion, knowledge of the changes in lncRNA and mRNA expression associated with cataracts may contribute to a better understanding of the opacification process. The findings of the present study demonstrate that there are notable lncRNA and mRNA differences between mice with or without CRYBB2 KO induction. The data indicate that the response of the lens to the development of CRYBB2 KO-related cataract is characterized by an extensive upregulation of numerous mRNAs and lncRNAs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YJ conceived of and designed the experiments. KX, H-XR and W-JL performed the experiments and analyzed the data. W-JL and KX obtained the reagents, materials and analysis tools. YJ and W-JL wrote the study. All authors read and approved the final study.

Ethics approval and consent to participate

All procedures were carried out in accordance with the Chinese legislation on the Use and Care of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (23) and were approved by the Institutional Animal Care and Use Committee of Changhai Hospital, Second Military Medical University (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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