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RNA-seq reveals transcriptome changes of the embryonic lens cells in Prox1 tissue specific knockout mice

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Abstract. – **OBJECTIVE:** Prox1 is expressed in both lens epithelial cells and fiber cells and is essential for lens fiber cell elongation. This study aimed to explore the molecular mechanisms of how Prox1 mutations influence lens fiber cells development.

MATERIALS AND METHODS: Comparative transcriptomes analysis of Prox1 conditional knockout (cKO) lens and wild-type (WT) lens were performed using the data GSE69940 downloaded from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were determined by the R package "edgeR" of Trinity software. GO (Gene Ontology) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes databases) enrichment analysis were performed using the cluster Profiler R package. Then, the protein-protein interaction (PPI) network was predicted using Cytoscape, and the Module analysis of the PPI network was analyzed through the Cytoscape MCODE plugin. Moreover, MotifDb package in R was used to predict the transcription factors binding to Prox1 promoter regions.

RESULTS: In total, 2263 differentially expressed genes were identified between the two groups. GO and KEGG analysis showed that the down-regulated genes were enriched in camera-type eye term, nucleosome assembly, lens fiber cell differentiation, and cell modified and amino acid metabolism. The KEGG pathway of up-regulated genes was associated with lens development, including Hedgehog signaling pathway and MAPK signaling pathway. GO terms of up-regulated DEGs were mainly relevant to bone morphological development, muscle development, and sensory organ morphological development. Next, the PPI network of DEGs was constructed, and 4 modules were analyzed. Moreover, 30 transcription factors were predicted, which are likely to be downstream targets of Prox1 with potential roles in lens development in mice.

CONCLUSIONS: This study provides insights into the unique transcriptome profile of lens cells in Prox1 conditional knockout mice, which is a valuable resource for further study of mouse lens genomics.

Key Words:
Prox1, RNA-seq, Differentially expressed gene (DEGs), Protein-protein interaction (PPI), Motif.

Introduction

The lens, a sensory organ that transmits and focuses light onto the retina, derive from the head surface of the embryo ectoderm in vertebrates[1-4]. The anterior cells of the lens vehicle (LV) maintain the epithelial morphology; the posterior cells extend anteriorly and terminally and differentiated into lens fibers cells[5]. The *cMaf*, *Sox1,* and *Prox1* mutant mice fail to form lens fibers, and these transcription factors are required during lens fiber cell differentiation[6-8]. The sequence-specific DNA-binding transcription factor (TF), *Prox1,* was known to be required for lens fiber differentiation and morphogenesis[8,9]. *Prox1* homeobox gene was first detected in the lens placode[9]. It has been verified that *Prox1* cKO mice showed normal development until the posterior LV cells fail to elongate into lens fibers and reduced expression of lens fiber cell markers, such as *beta-gamma-cry*, *MIP*, and *Fgfr3*. *Prox1* participates in forming a positive-feedback loop controlling gene expression and lens fiber cells differentiation[10]. RNA-sequencing (RNA-seq) is a fast, highthroughput method for detecting mRNA expression in cells or tissues. Recent studies[11,12] have shown that bioinformatics data mining and gene network analysis play a major role in predicting and studying the mechanism of genes in develop-

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ment biology. Our study analyzed the RNA-seq data from GEO database to identify the DEGs between *Prox1* cKO and WT lens[10], and we used a cutoff threshold of log2 fold change >1 to identify the differentially expressed genes (DEGs). Possible biological functions were predicted by enrichment analysis; proteinprotein interaction (PPI) networks were visualized, and module analysis was conducted for screening key genes. Furthermore, we predicted a few TFs binding sites at promotor regions of *Prox1*. Computational bioinformatics analysis of gene expression was used to explore the pathogenesis of *Prox1* cKO and explore potential target for gene therapy

Materials and Methods

RNA-Seq Data Set Preparation for Analysis

The gene expression profiles with accession number GSE69940 was downloaded from GEO database (http://www.ncbi.nlm.nih.-gov/geo/). The GSE69940 dataset contains transcriptome data from Prox1cKO lens tissue samples and WT lens tissue samples (30 lenses/sample, three biological replicates/genotype; doi:10.1242/dev.127860).

Quality Control and Read Mapping to the Reference Genome

For quality control (QC) and read cleaning, we use the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and PRINSEQ (lite version 0.20.4). The Q20, Q30, and GC contents of the clean data were calculated. For mapping, paired-end clean reads were aligned to the reference genome GRCm38 (Mus musculus) using the Hisat software.

Gene Expression

Differentially expressed genes (DEGs) were determined by the R package "edgeR" of Trinity software with a cutoff threshold of log2 fold change >1 and FDR (false discovery rate) < 0.05[13]. Known genes from GENCODE database were annotated mapping to GRCm38[14].The counts of the read numbers mapped to each gene, and the FPKM of each gene were calculated. StringTie tool was used to quantify all the transcripts provided in an annotation file[15].

DEGs Ontology and Functional Analysis

GO (Gene Ontology) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes databases) enrichment analysis were performed using cluster Profiler R package (v3.0.4). GO terms with corrected p-values < 0.05 were considered significantly enriched by DEGs. Pathway enrichment analysis and pathway-pathway network generation of KEGG Reactome and WIKI pathway databases were performed by cluePedia cytoscape Plugin (https://dl.acm.org/citation.cfm?id=2479276)

Identification of DEGs Regulatory Proteins and Their Interaction Network

STRING database (http://www.string-db.org/) is a known useful tool to predict protein-protein interactions network (PPI network) with cutoff criterion of combined score >0.4[16,17]. Module analysis of the PPI network was analyzed through Cytoscape MCODE plugin (http://www.cytoscape.org/)[18].

Analysis of Transcriptional Regulation Networks

We selected TRANSFAC database, one of the most commonly used platform for the analysis of gene regulatory networks and for the description and prediction of transcription factors[19]. Using the MotifDb package in R (fully accessible and operational on the website http://datalab.njit.edu/bioinfo/) to predict the transcription factors binding to *Prox1* promoter regions. We used a position specific weight matrix (PWM) score >75% as the threshold to predict as reliable transcriptional binding sites.

Results

High Throughput RNA Sequencing (RNA-Seq) Data Set Preparation for Analysis

We first processed quality control and found a very high percentage of clean reads (more than 98%). Then, we mapped the clean reads to the murine reference genome GRcm38. We found that in each sample, more than 70% of reads could be mapped to the reference genome. The clean reads percentage, and the mapping percentage suggested good sequence quality (data not shown).

RNA-Seq Data Analysis to Find DEGs

The read count data obtained from the RNA-seq experiment were used to analyze differentially expressed genes (DEGs). We obtained 2263 DEGs between *Prox1* cKO and *Prox1* WT group (1260 up-regulated genes and 1003 down-regulated

Table I. The top 10 up-regulated and down-regulated genes with higher connectivity degrees.

Up-regulated	Name	Degree	Down-regulated	Name	Degree
1	Egfr	117	1	Lrguk	58
2	Alb	115	2	Prkca	45
3	Acta2	97	3	Myo5a	44
4	Actg2	89	4	Arvcf	42
5	Smarca2	83	5	Gcg	41
6	Mapk4	60	6	Kat2b	40
7	Met	60	7	Stat1	40
8	Igf1	59	8	Aldh1a7	39
9	Cd44	56	9	Mapk10	38
10	Col1a1	54	10	Pde6b	38

genes). Volcano plots and heat map of the DEGs are presented in supplementary data **(Supplementary Figure S1)**. The 40 most up-regulation and down-regulation DEGs are shown in supplementary data **(Supplementary Tables S1, S2)**.

GO and KEGG Enrichment

GO and KEGG enrichment analysis was used to identify the DEGs and determine their potential functions of metabolic pathways. The GO enrichment analysis of DEGs was divided into three categories: biological process (BP), cellular component (CC), and molecular function (MF). GO and KEGG analysis of down-regulated DEGs are mainly associated with many functions (Figure 1A). DEGs down-regulated were enriched in lens development in camera-type eye term (*Fgfr3/Cryba2/Mip/Tmod1/Lim2/Gje1/Tdrd7/Cryge/Crygd/Cryaa/Crygs/Cryga/Bfsp2/Crygb/Bfsp1/Cdkn1c/Cryab*), nucleosome assembly, lens fiber cell differentiation (*Fgfr3/Tmod1/Tdrd7/Crygd/Cryaa/Bfsp2/Crygb/Bfsp1/Cdkn1c*) and cell modified, and amino acid metabolism. KEGG pathway of up-regulated gene and down-regulated gene are shown in supplementary data **(Supplementary Tables S1, S2, S3).** The KEGG pathway of up-regulated gene associated with lens development, including the Hedgehog signaling pathway and MAPK signaling pathway. Comparing to the gene list of *Prox1* cKO and WT group, GO terms of up-regulated DEGs mainly relevant to bone morphological development, muscle development, and sensory organ morphological development were identified (Figure 1B). The top 5 most enriched GO terms and KEGG are shown (Figure 1C).

PPI Network Construction

According to the PPI network predicted by STRING, 1194 DEGs formed 5988 interaction proteins pairs. The PPI network of DEGs was constructed (**Supplementary Figure S3**). The green spots represent the down-regulated genes, and the red spots represent the up-regulated genes. Several nodes showed higher connectivity degrees in the PPI network, and the top 10 up-regulated and down-regulated genes are exhibited in Table I.

Screening of Modules

Molecular complex detection (MCODE) was performed utilizing MCODE plugin of cytoscape software to screen modules of the large PPI network, with the cutoff criterion of degrees≥10. We analyzed the functions of top 4 MCODE score modules (**Supplementary Figure S2**). *Col1a1, Smarca2*, *Cxcl12*, and *Wnt3a* were selected as hub genes. Remarkably, there were higher degrees in *Egfr* (degree=117) and *Alb* (degree=115) of up-regulated genes, and in *Lrguk* (degree=58) and *Prkca* (degree=45) of down-regulated genes in the PPI network. Module one contained 23 genes and 253 interaction proteins pairs (Figure 2A). Biological functional enrichment analysis showed that genes in this module were markedly enriched in extracellular matrix organization, protein heterotrimerization, collagen fibril organization, extracellular structure organization and cellular response to amino acid stimulus. GO analysis results showed that *Col5a1/Col8a2/Col8a1/Col11a1/Col2a1/Col5a2* were markedly enriched in eye morphogenesis and eye development. Module two contained 19 genes and 161 interaction proteins pairs (Figure 2B), mainly related to nucleosome assembly or disassembly, chromatin assembly, nucleosome organization, chromatin assembly, and protein-DNA complex subunit organization. Module three contained 24 genes and 138 interaction proteins pairs (Figure 2C), mainly related

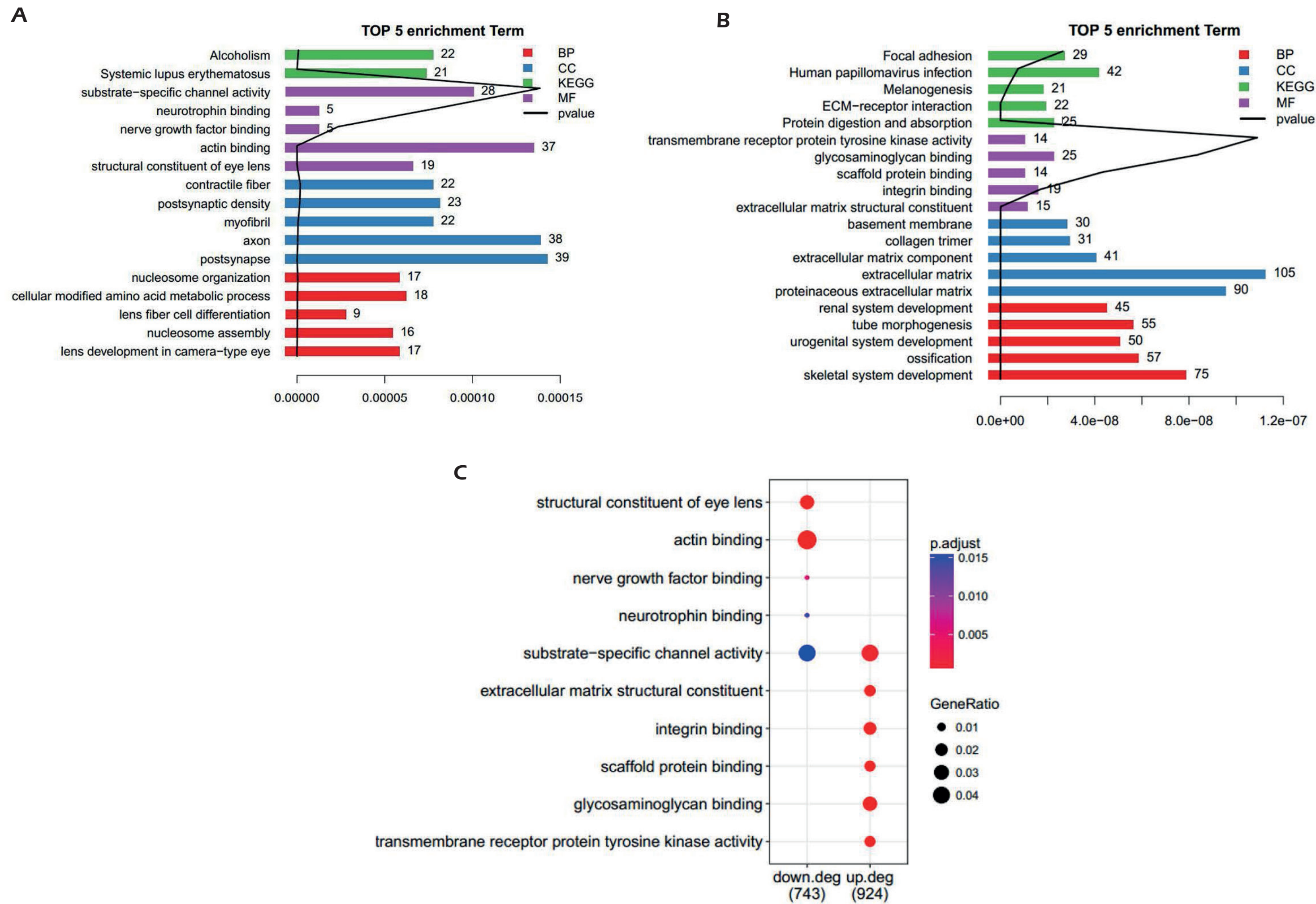

Figure 1. Gene ontology analysis summary. ***A,*** The top5 most enriched GO terms of down-regulation DEGs. ***B,*** The top5 most enriched GO terms of up-regulation DEGs. ***C,*** Compare of the top 5 most enriched Go terms between up-regulation DEGs and down-regulation DEGs.

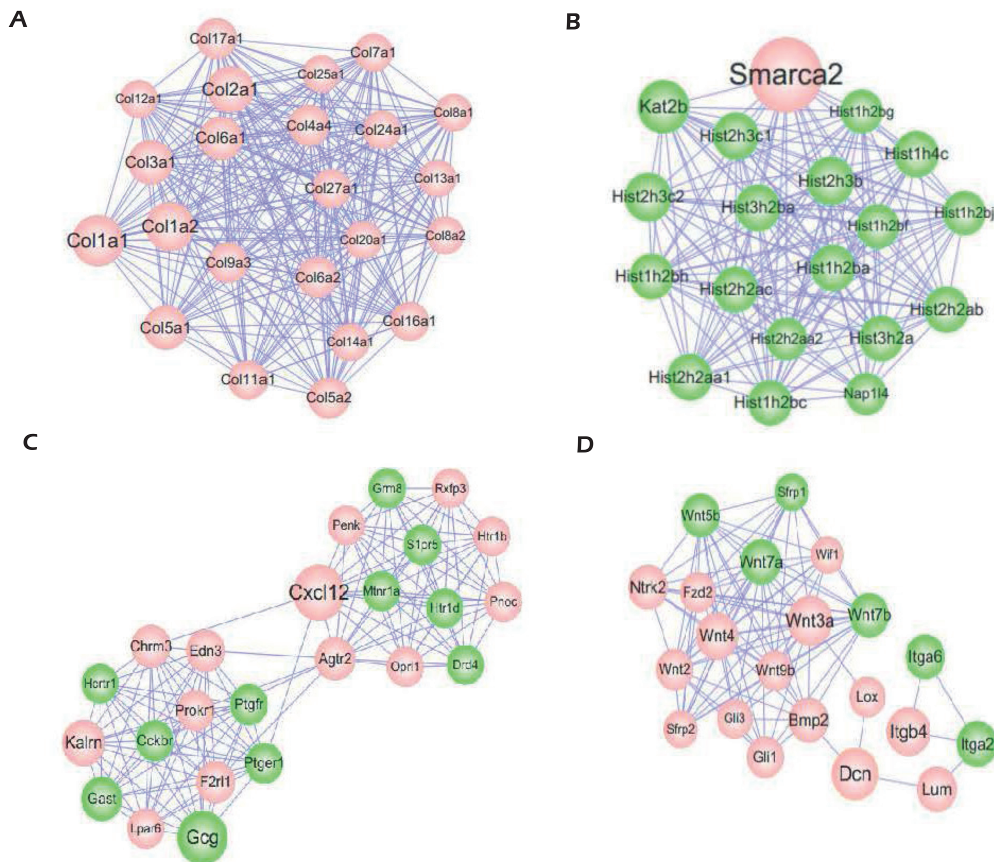

Figure 2. The top 4 significant modules selected from the PPI network by utilizing MCODE in Cytoscape. Lines represent an interacting relationship between nodes. **A,** Col1a1. **B,** Smarca2. **C,** Cxcl12. **D,** Wnt3a.

to adenylate cyclase-modulating G-protein coupled receptor signaling pathway and regulation of cyclic nucleotide biosynthetic process. Module four contained 21 genes and 93 interaction proteins pairs (Figure 2D), mainly related to the Wnt signaling pathway participating in cell signaling transduction and regulation of organ morphogenesis. Top 10 GO BP enrichment of each modules are shown in supplementary data **(Supplementary Figure S2)**.

Transcription Factors Prediction of DEGs

We searched the transcription factors (TFs) of the *Prox1* gene from the TRANSFAC. We found 60 murine transcription factors related to lens development (14 down-regulated and 46 up-regulated, shown in supplementary data **(Supplementary Table S1)**. "Binding motif" is a transcription factor binding preferences to a specific part of DNA sequences. Sequence logo of *Prox1* motif is shown in Figure 3A. Using the MotifDb package in R, we predict 30 transcription factors which may be regulated by *Prox1* (Figure 3B). 30 TFs were predicted to be regulated by *Prox1.* Among these genes, *Arntl2 Foxn1, Hsf4, Mafg, Me1, Nobox, Stat1*, and *Stat5a* were down-regulated in *Prox1* cKO lens, and *Ahr*, *Atf3*, *Ebf2*, *Emx2*, *Etv4*, *Foxc2*, *Foxg1*, *Gli3*, *Hic1*, *Hlx*, *Irf6*, *Irx3*, *Irx5*, *Klf5*, *Pax7*, *Pitx2*, *Pou3f3*, *Prrx1*, *Ptx3*, *Relb*, *Sox3*, and *Sox9* were up-regulated in *Prox1* cKO lens.

Discussion

Bioinformatics analysis of GSE69940 was carried out; we identified a total of 2263 DEGs in *Prox1* cKO lens compared with wild-type lens, including 1260 upregulated and 1003 downregulated genes.We aim to identify the influence of lens development caused by *Prox1* specific targeting genes, so we use a different strategy and get more DEGs than the original article. Upon analysis by MCODE in Cytoscape, top 4 modules were selected from the PPI network, and *Col1a1*,

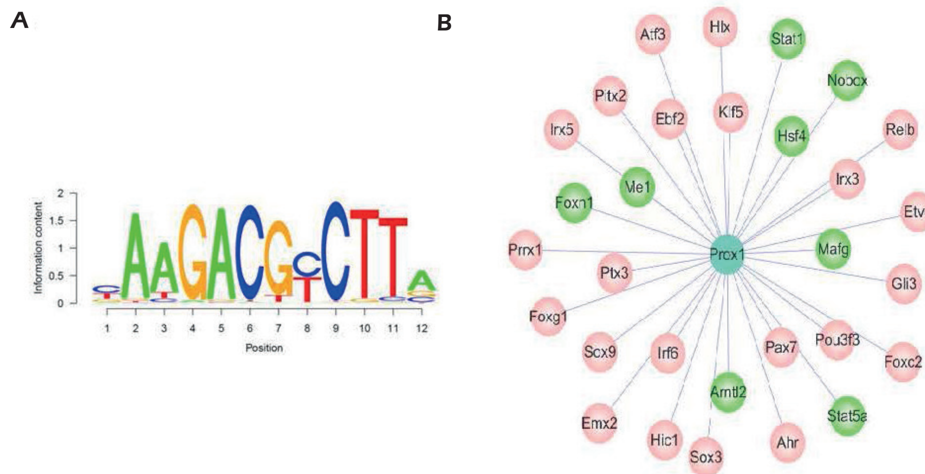

Figure 3. Transcription factors prediction of DEGs. **A,** Sequence logo of the PWM created by ChIPMunk39 using 17,781 binding site regions predicted for PU.1/Spi-1 using ChIP sequencing (ChIP-seq) data40. **B,** 30 transcription factors may be regulated by Prox1. Red stands for up-regulated TFs, and green stands for down-regulated Tfs.

Smarca2, *Cxcl12*, and *Wnt3a* genes were identified as hub genes. *Col1a1* gene encoded a protein forming heterotrimeric type I procollagen that functions as proteolytic processing during fibril formation. Dominant inherited *COL1A1* mutations caused connective tissue disorders, related phenotypes of varying severity, including early-onset glaucoma[20]. Epithelial-mesenchymal transition (EMT) of lens epithelial cells (LECs) is a key pathologic mechanism of cataract. Increased expression of *Col1a1*, a *TGF-β*-responsive gene, showed inducing EMT in the lens cells of transgenic mice and along with the activation of signaling pathways related to EMT[21]. For example, *TGF-β* pathway activation is a distinctive mark of some forms of human cataracts which displays EMT[22]. Moreover, *TGF-β2* induces the development of cataract *in vitro* in rat lens[23]. Other types collagen fibers encoded genes (*Col5a1/Col8a2/Col8a1/Col11a1/Col2a1/Col5a2*) are enriched in eye morphogenesis and eye development. These results revealed the fact that these types of collagens, may have an essential role in lens development and structural component of the connective tissue. *Smarca2* (SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 2) with *Brg1* are subunits of SWI/SNF complex that play vital roles in regulating genomic architecture during developmental processes. The function of DNA methylation may regulate the recruitment of histone modification enzymes or transcription factor binding and contribute to silencing gene expression[24]. Multiple binding motifs of *Smarca2* displayed altered by DNA methylation are notably critical for regulating neural specification and neuronal development[25]. Understanding the role of DNA methylation in lens development and disease shall shed new lights on a new therapeutic approach to clinical treatment of cataract[26]. *Cxcl12* signaling mediated by the receptors *Cxcr4* and/or *Cxcr7* plays a crucial role during embryonic development[26]. *Cxcl12* mutant mice are embryonic lethal between day (E)15 and birth with defects in neurogenesis and cardiovascular development[27]. *Cxcl12* is initially expressed in the periocular mesenchyme at E11.5 in the mouse eye, then it located in the cells adjacent to the iris stroma, retinal pigment epithelium, and ocular blood vessels. As *Cxcl12* is involved in the migration of neural crest cells, its expression by periocular neural crest cells indicates that it may play a similar role during lens and cornea development[27]. Further studies are necessary to elucidate the roles of *Cxcl12* during lens development. *Wnt3a* Wnt signaling is activated during lens fiber cells differentiation through β-catenin-independent signaling pathways or β-catenin-dependent direct signaling[28,29]. *Wnt3a* induces lens differentiation by regulating the accumulation of lens proteins through β-catenin-independent Wnt signaling pathway related to *FGF* and *BMP*[30]. The complex cytoskeletal reorganization (a key feature of EMT and fiber elongation in the lens) maybe regulated by non-canonical Wnt signaling pathway[31]. But the mechanism of how Wnt stimulation of EMT

in the normal lens is still not yet clear. *Prox1* cKO down-regulated DEGs were also enriched in nucleosome assembly and cell modified and amino acid metabolism. *Prox1* cKO up-regulated DEGs were also enriched in bone morphological development, muscle development, and sensory organ morphological development. The most enriched GO category of down DEGs included genes involved camera-type eye term (*Fgfr3/Cryba2/Mip/Tmod1/Lim2/Gje1/Tdrd7/Cryge/Crygd/Cryaa/Crygs/Cryga/Bfsp2/Crygb/Bfsp1/Cdkn1c/Cryab*), lens fiber cell differentiation (*Fgfr3/Tmod1/Tdrd7/Crygd/Cryaa/Bfsp2/Crygb/Bfsp1/Cdkn1c*). In our present study, KEGG pathways, including Hedgehog signaling pathway and MAPK signaling pathway are associated with lens development. Though we didn't perform confirmation of these potential pathways in the present study, they are significant for understanding the abnormal lens morphogenesis in *Prox1* cKO mice. Each transcription factor (TF) has a DNA binding domain (DBD) that prefers to bind to a specific DNA sequence, which is a motif. Searching for the location of the motif on the genome to identify the nearby genes which might be regulated by the TF[32]. Thirty of these transcription factors are potentially regulated by *Prox1*. *Hsf4*, *Sox3*, *Mafg*, *Pitx2*, and *Pitx3* genes were expressed in the lens but were not reported to regulate Prox1[33-37]. *FoxC2* expression was induced by *Prox1* in retinal pigment epithelial cell[38]. The other 24 genes were not reported to expressed in the lens and didn't regulate *Prox1* in other tissue. Although the PWM-based search is sensitive, its biggest drawback is that the false positive rate is too high, and there are many combinations in the predicted results that do not really have a biological function. Furthermore, we cannot rule out the possibility of the contamination of retina or other ocular tissue, since some of these genes are expressed in either retina, optic nerve, or muller cell.

Conclusions

We investigated the underlying mechanisms of *Prox1* gene using bioinformatics and prediction tools. A total of 2263 DEGs were identified, and 4 hub genes including *Col1a1*, *Smarca2*, *Cxcl12*, and *Wnt3a* genes may be involved in the abnormal lens development. Meanwhile, we found several motifs that may be involved in regulating *Prox1* gene expression. All these findings shed light on the role of *Prox1* in morphogenesis of lens development and provide a potential novel strategy for gene therapy. Nonetheless, the bioinformatics results obtained in this study require further experimental confirmation. Screening of DEGs in larger cohorts of patients with congenital lens disease would open new avenues to establish a detailed phenotype/genotype correlation.

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Conflict of interest

The authors declare no conflicts of interest.

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