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RNA-sequencing-based transcriptomic profiles of embryonic lens development for cataract gene discovery

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Abstract

Isolated or syndromic congenital cataract are heterogeneous developmental defects, making the identification of their associated genes challenging. In the past, mouse lens expression microarrays have been successfully applied in bioinformatics tools (*e.g.* iSyTE) to facilitate human cataract-associated gene discovery. To develop a new resource for geneticists, we report high-throughput RNA-sequencing (RNA-seq) profiles of mouse lens at key embryonic stages (E)10.5 (lens pit), E12.5 (primary fiber cell differentiation), E14.5 and E16.5 (secondary fiber cell differentiation). These stages capture important events as the lens develops from an invaginating placode into a transparent tissue. Previously, *in silico* whole-embryo body (WB)-subtraction-based “lens-enriched” expression has been effective in prioritizing cataract-linked genes. To apply an analogous approach, we generated new mouse WB RNA-seq datasets and show that *in silico* WB-subtraction of lens RNA-seq datasets successfully identifies key genes based on lens-enriched expression. At 2 counts-per-million expression, 1.5 log₂ fold-enrichment ($p < 0.05$) cut-off, E10.5 lens exhibits 1401 enriched-genes (17% lens-expressed genes), E12.5 lens exhibits 1937 enriched-genes (22% lens-expressed genes), E14.5 lens exhibits 2514 enriched-genes (31% lens-expressed genes), and E16.5 lens exhibits 2745 enriched-genes (34% lens-expressed genes). Biological pathway analysis identified genes associated with lens development, transcription regulation and signaling pathways, among other functional groups. Furthermore, these new RNA-seq data confirmed high expression of established cataract-linked genes and identified new potential regulators in the lens. Finally, we developed new lens stage-specific UCSC Genome Browser annotation-tracks and made these publicly accessible through iSyTE (<https://research.bioinformatics.udel.edu/iSyTE/>) for user-friendly visualization of lens gene expression/enrichment to prioritize genes from high-throughput data from cataract cases.

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Keywords

Cataract; Lens; RNA-sequencing; Transcriptome; Gene discovery; Development

Introduction

Tissue or cell-specific gene expression information has been important for prioritization of candidate genes associated with developmental defects of the eye (Blackshaw et al. 2001; Lachke et al. 2012b; Kakrana et al. 2018). Thus far, high-throughput transcript profiling analysis of lens development has largely been performed using microarray analysis (Chauhan et al. 2002b, a; Hawse et al. 2003, 2004, 2005; Ivanov et al. 2005; Xiao et al. 2006; Greiling et al. 2009; Lachke et al. 2011, 2012b; Sousounis and Tsonis 2012; Wolf et al. 2013b, a; Agrawal et al. 2015; De Maria and Bassnett 2015; Anand et al. 2015; Kakrana et al. 2018; Siddam et al. 2018). However, microarray-based expression profiling, while effective, is limited by the number/type of probes on an array, which in turn is limited by the existing knowledge of the transcriptome. Furthermore, probe-hybridization kinetics plays major role in gene expression quantification unlike the direct detection of transcripts using RNA-sequencing. Thus, microarrays do not necessarily inform on the full repertoire of the transcriptome in a given cell or tissue type (Anand and Lachke 2017).

To address these shortcomings, recent studies have begun to apply high-throughput RNA-sequencing (RNA-seq) to investigate the mouse lens transcriptome. However, these efforts have largely focused on mid to late embryonic and early postnatal stages (Hoang et al. 2014; Manthey et al. 2014a, b; Khan et al. 2015, 2016, 2018; Audette et al. 2016; Wang et al. 2017; Cavalheiro et al. 2017; Budak et al. 2018; Zhao et al. 2018). Further, the principle challenge in leveraging high-throughput expression profiles for disease-associated gene discovery lies in the application of effective downstream analysis for the identification of high-priority candidate genes that potentially function in the morphogenesis, homeostasis or pathology of the specific tissue of interest. Recently, we demonstrated that for mouse lens microarray datasets, an approach termed “*in silico* WB-subtraction”, involving comparative analysis to a reference mouse whole-embryo body (WB) tissue dataset, allows the identification of genes with lens-enriched expression (Lachke et al. 2012b; Kakrana et al. 2018). Lens-enriched expression is an indicator of the significance of genes to lens biology and is the basis of the web-tool iSyTE (integrated Systems Tool for Eye gene discovery), which has led to the identification of several new genes as well as the characterization of multiple regulatory pathways associated with cataract and other eye-defects (Lachke et al. 2011; Kasaikina et al. 2011; Lachke et al. 2012a, b; Aldahmesh et al. 2012, 2013; Wolf et al. 2013b; Manthey et al. 2014a; Patel et al. 2014; Agrawal et al. 2015; Dash et al. 2015; Chograni et al. 2015; Greenlees et al. 2015; Evers et al. 2015; Audette et al. 2016; Zhang et al. 2016; Rothe et al. 2017; Patel et al. 2017; Cavalheiro et al. 2017; Kakrana et al. 2018; David et al. 2018; Siddam et al. 2018; Krall et al. 2018).

To develop a matching resource for ocular geneticists, we report generation and integrated analyses of new RNA-seq datasets for mouse early embryonic lens at lens pit stage (embryonic day (E) 10.5), at primary fiber cell differentiation stage (E12.5) and at secondary

fiber differentiation stages (E14.5 and E16.5). Further, we generated new mouse WB RNA-seq datasets and tested the hypothesis that “*in silico* WB-subtraction” can be extended to process lens RNA-seq data and prioritize candidates important to lens biology and cataract. Indeed, *in silico* WB-subtracted datasets for all four lens stages effectively identified all known genes linked to lens development and cataract. When tested for gene ontology (GO) clustering using DAVID analysis, in contrast to un-subtracted lens expression profiles, *in silico* WB-subtracted lens expression profiles were highly enriched in GO categories for “lens development in camera-type eye” and “structural constituent of eye lens”, among others, indicating the utility of this approach. Furthermore, these analyses led to the identification of several new high-priority candidate genes for the lens. Finally, to enable effective downstream analyses, we developed new lens stage-specific custom UCSC Genome Browser annotation-tracks that are publicly accessible through iSyTE (<https://research.bioinformatics.udel.edu/iSyTE/>), which allow ready visualization and deeper investigation of genes with respect to their expression and enrichment in the lens.

Materials and Methods

Tissue collection and RNA isolation

Mice were bred and maintained at the University of Delaware Center for Animal research and the animal protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC). Experiments were performed according to the guidelines established by the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research. Mouse embryos were staged by designating the day of the detection of the vaginal plug as embryonic day (E) 0.5. Mouse C57BL/6NTac strain (Taconic Biosciences) lenses were micro-dissected at stages E10.5, E12.5, E14.5 and E16.5. Total RNA was isolated using Qiagen RNeasy kit from three biological replicates for each stage. Each biological replicate consisted of multiple lenses (E10.5, $n=50$ lenses/replicate; E12.5, $n=29$ lenses/replicate; E14.5, $n=18$ lenses/replicate; E16.5, $n=17$ lenses/replicate). Mouse whole embryos at E10.5, E11.5 and E12.5 were dissected and total RNA was isolated from each stage separately in three biological replicates. An equimolar mixture of total RNA from each stage was considered as individual “whole-embryo body (WB)” sample and three independent WB replicates were generated. RNA quality was analyzed using fragment analyzer (Advanced Analytical Technologies, AATI FEMTO Pulse) and samples with RNA quality number (RQN) greater than 8.5 were used for library preparation and RNA-sequencing.

Mouse lens transcriptome assembly and analysis

RNA isolated from three biological replicates for each of the E10.5, E12.5, E14.5 and E16.5 mouse lens stages was used to generate strand-specific, paired-end 100 bp-libraries. All libraries were sequenced on an Illumina HiSeq 2500 instrument at the University of Kansas Medical Center Genome Sequencing Facility following standard protocols. Sequencing quality specifically the per-base sequence quality, sequence length distribution, overrepresented adapter/ primer sequences were evaluated using FastQC tool (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Adapters were trimmed using the Trimmomatic tool following recommended cutoffs (Bolger et al. 2014). Reads were aligned

against *Mus musculus* reference genome (mm10) using Tophat v2.0.9 (Trapnell et al. 2009) and gene-, cds-, and transcripts-level expression were computed through Cufflinks (Trapnell et al. 2012) using the mouse genome annotations (GRCm38.p6 assembly) as reference. The transcript expression raw counts generated using “classic-FPKM” method implemented in Cufflinks were imported in edgeR package (Robinson et al. 2010) for R statistical environment. Lowly expressed reads (< 1 count per million in less than 2 samples) were filtered out, library size was reset and normalization was performed using default Trimmed Mean of M-values (TMM) method implemented in edgeR package, dispersion estimates were computed using the recommended Cox-Reid profile-adjusted likelihood (CR) method in edgeR. All new RNA-seq data is submitted to NCBI Gene Expression Omnibus (GEO) Database under series GSE119596.

***In silico* WB-subtraction for RNA-seq datasets**

The *in silico* WB-subtraction approach, previously described for estimating lens-enrichment of genes from microarray datasets (Lachke et al. 2012b; Kakrana et al. 2018), was applied to the lens RNA-seq datasets. The newly generated RNA-seq WB data was used as reference and the preferential accumulation of transcripts was computed as $\log_2$ Fold-Change (FC) using the “quasi-likelihood” (QF-) test included in edgeR package for WB vs. lens stages E10.5, E12.5, E14.5 and E16.5 contrasts. Genes displaying statistically significant preferential accumulation between lens vs. WB (1.5-fold, expression cut-off 2.0 counts, $p < 0.05$) were identified using *in-house* Python scripts and considered as “lens-enriched”. Top 500 lens-enriched genes from each stage were used as an input for gene ontology (GO) based clustering and comparative analysis, performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource tool (<https://david.ncifcrf.gov/>) (Huang et al. 2009).

Lens-enriched gene cluster analysis

Lens-enriched genes ($n=638$, normalized expression 2 counts-per-million (CPM), lens enrichment 1.5 $\log_2$ -fold change *i.e.* lens stage vs. WB) identified from all four stages (E10.5, E12.5, E14.5, E16.5). These were used for enrichment level based clustering as previously described (Kakrana et al. 2018); Self-Organizing Tree Algorithm (SOTA) method was used from the “clValid” package available in the Bioconductor (<https://www.bioconductor.org>). This analyses identified gene clusters exhibiting distinct lens enrichment patterns across E10.5 to E16.4 developmental window.

Web-access for lens development RNA-seq data through iSyTE 2.0

Publicly available web-based custom annotation tracks were developed to access gene expression and enrichment scores for E10.5, E12.5, E14.5, and E16.5 lens stages using the University of California at Santa Cruz (UCSC) Genome Browser. Lens expression and enrichment scores were converted into BED (Browser Extensible Data) format and displayed as annotation track in the UCSC genome browser (Mouse GRCm38/mm10 assembly). The custom tracks for Human GRCh38/hg38 assembly were also developed to the corresponding genes in the mouse. The links to access these tracks is made available through iSyTE 2.0 under the tab “Mouse lens RNA-seq” at <https://research.bioinformatics.udel.edu/iSyTE/>.

Results and Discussion

Generation of mouse embryonic lens RNA-seq datasets and transcriptome assembly

We performed RNA-seq on micro-dissected wild-type mouse lenses at embryonic stages E10.5, E12.5, E14.5 and E16.5 as they inform on key stages of lens development, namely, lens pit (E10.5), primary fiber cell differentiation (E12.5), through secondary fiber cell differentiation (E14.5, E16.5). To perform *in silico* WB-subtraction (see section below), we also generated RNA-seq data from mouse WB as described in details in the methods section. We designed an appropriate experimental and integrative computational pipeline for these analyses (Fig. 1). Strand-specific, paired-end 100 bp RNA-seq libraries were prepared and sequenced from mouse lenses at the above stages and analyzed using the bioinformatics pipeline described in Fig. 1. A total of 276.6 million reads, on average 18.4 million reads per replicate of lens and WB, were generated and aligned using splice junction mapper, Tophat (Trapnell et al. 2009) (Table 1). On average, 97.7% of the reads were aligned to the *Mus musculus* reference genome (mm10) (Table 1). Using criteria of p -value 0.05 (edgeR) (Robinson et al. 2010) and normalized expression counts ≥ 2 CPM in at least one stage (two replicates) of the lens, we identify a total of 9,733 transcripts (mapped at the gene level) from 8,480 loci (Table 2, Table S1). Interestingly, the number of distinct transcripts (mapped at the gene level) expressed by E10.5 and E12.5 lenses was higher than the number of distinct transcripts (mapped at the gene level) expressed by E14.5 and E16.5 lens (Table 2). As an important quality control step, principle component analysis (PCA) was performed on expression levels of all the datasets (lens and WB), which showed that individual mouse lens RNA-seq datasets clustered according to their specific stage (Fig. 2A). These data also show that the mouse lens replicates of a specific stage are distinct from those at other stages (Fig. 2A). Further, these data indicate that all the lens datasets, regardless of their stage, are distinct from the WB datasets.

In silico WB-subtraction can be effectively applied for RNA-seq data

The principal challenge in high-throughput expression profiling studies is the careful implementation of downstream analytical strategies to effectively prioritize or identify genes that likely function in the tissue of interest. Successful strategies can identify genes linked to cell or tissue morphogenesis/homeostasis and prioritize promising candidates relevant to their associated developmental defects/disease. Recently, we demonstrated that for tissue-specific microarray datasets, an approach termed “*in silico* WB-subtraction”, involving comparative analysis of the tissue in question to a “reference” WB dataset, provides estimates of tissue-specific enrichment that can be applied for prioritization of candidate genes. For the lens, we showed that tissue-enrichment is an excellent predictor of significance to lens biology, which has led to the identification and/or characterization of several new cataract-associated genes (Lachke et al. 2012b; Anand and Lachke 2017; Kakrana et al. 2018). Here, we tested the hypothesis that *in silico* WB-subtraction can be extended to process lens RNA-seq data and prioritize candidates important to lens biology and cataract. RNA-seq datasets on mouse E10.5, E12.5, E14.5 and E16.5 lens tissue was compared to the newly generated WB reference RNA-seq dataset as described in detail in the methods section. Previously, we showed that WB datasets from different developmental stages are similarly effective in identifying lens-enriched genes. Therefore, to make the data

interpretation consistent with the previous analysis, as well as, across the four lens developmental stages in this study, we used a single WB reference RNA-seq dataset comprising of the embryonic stages E10.5, E11.5 and E12.5. To test the effectiveness of this strategy to identify lens-relevant genes, we performed gene ontology (GO) based clustering via DAVID using the top 500 lens-enriched gene profiles from E10.5, E12.5, E14.5 and E16.5 stages generated in this study. *In silico* WB-subtracted lens expression profiles were enriched in GO categories for “Eye lens protein”, “Structural constituent of eye lens” “Lens development in camera-type eye”, “lens fiber differentiation”, “Eye-development”, and “Embryonic eye-morphogenesis”, indicating the utility of the present approach in identifying the genes relevant to lens biology and development (Fig. 2B). In sharp contrast to lens-enriched genes, the non-subtracted lens expression profiles from all four stages were rich in GO categories for the general functioning of the cell, namely, “Acetylation”, “Structural constituent of ribosome”, “Translation” and “Oxidative phosphorylation”. These data demonstrate that *in silico* WB subtraction analysis can be successfully applied to lens RNA-seq data to prioritize new candidate genes important for lens biology and cataract.

Lens-enrichment identifies known genes linked to lens development and cataract

We evaluated the effectiveness of the *in silico* WB-subtraction approach by analyzing lens-enrichment of genes with known function in lens development, homeostasis and cataract (Cvekl and Zhang 2017). A vast majority of genes (58/61 genes; 95%) associated with cataract or lens defects were found to be highly enriched in the lens at one or more stages (Fig. 3). Further, all of these genes ($n=61$) were significantly expressed in the lens datasets (Fig. 3). These include established lens-associated genes that encode crystallins (*Cryaa*, *Cryab*, *Cryba1*, *Cryba2*, *Cryba4*, *Crybb1*, *Crybb2*, *Crybb3*, *Cryga*, *Crygb*, *Crygc*, *Crygd*, *Crygs*), transcription factors (*Foxe3*, *Hsf4*, *Mab21l1*, *Maf*, *Mafg*, *Meis1*, *Meis2*, *Pax6*, *Pitx3*, *Prox1*, *Six3*, *Sox1*, *Sox2*, *Sox11*, *Tfap2a*, *Zeb2*), structural proteins (*Bfsp1*, *Bfsp2*), gap-junction proteins (*Gja3*, *Gja8*), RNA-binding proteins (*Caprin2*, *Celf1*, *Tdrd7*), enzymes (*Adam9*, *Adamts18*, *Dnase2b*, *Pxdn*), and proteins with other function (Fig. 3). We next examined the lens-enrichment of genes as the lens develops from E10.5 through E16.5 stages. Using stringent criteria of lens-enrichment cutoff 1.5-fold change (lens vs. WB), lens- expression cutoff 2 CPM expression ($p<0.05$), we identified 1401 lens-enriched genes at E10.5 (Table S2), 1937 lens-enriched genes at E12.5 (Table S3), 2514 lens-enriched genes at E14.5 (Table S4), and 2745 lens-enriched genes at E16.5 (Table S5) (Fig. 4). This analysis showed that while the total number of lens expressed genes showed a reduction between E10.5 and E16.5, the number and percent of lens-enriched genes progressively increased with development, with E10.5 having 17% lens-enriched genes and E16.5 having 34% lens-enriched genes (Fig. 4). Finally, the present RNA-seq analysis is more effective in identifying the human cataract-linked genes *FYCO* and *PXDN* (Fig. 3), compared to our previous microarray analysis on embryonic lens tissue (Lachke et al. 2012b).

Dynamics of “lens-enriched” gene expression in embryonic development

To gain biological insights from the cohort of lens-enriched genes identified by *in silico* WB-subtraction, we examined these candidates from all the embryonic stages by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource. Expectedly, this analysis revealed enrichment of gene groups with known function

in the lens, such as “Lens development in camera-type eye” and “Structural constituent of eye lens” in all the stages (Fig. 5). Further, this analysis also revealed gene groups with functional significance in the lens as it transits from early to late developmental stages. For example, “Homeodomain” and “Notch signaling pathway” were enriched at E10.5 (Fig. 5A, Table S6), as would be expected based on earlier studies (Ashery-Padan et al. 2000; Liu et al. 2006; Rowan et al. 2008; Saravanamuthu et al. 2012). Further, gene groups for “Transcription regulation”, “Cell-cell adherens junction”, “Phosphatidylinositol signaling”, “Basic-leucine zipper domain” and “Pleckstrin homology-like domain” were identified in all four stages, indicating the general importance of these factors in lens development. For example, “Cell-cell adherens junctions” at E10.5 included genes such as *Cdh1*, *Epha2*, and *Ezr* that are known to be important in the lens. Interestingly, “Transcription regulation” at E10.5 includes 175 genes involved in the process of transcription, which in addition to the known key lens regulators (e.g. *Foxe3*, *Pax6*, *Pitx3*, *Prox1*, *Six3*, *Tfap2a*), also identifies new transcription factor genes to be expressed in the lens. At E10.5, these are *Crebzf* (CREB/ATF bZIP transcription factor), *Gabpa* (GA repeat binding protein, alpha), *Sox7* (SRY (sex determining region Y)-box 7), *Atf1*, *Atf2* (activating transcription factors), *Alx3* (aristaless-like homeobox 3), *Sall4* (sal-like 4) and *Tcf3* (transcription factor 3) (Table S6). Interestingly, “Pleckstrin homology (PH)-like domain” was identified in 50 genes expressed at E10.5. PH domains are present in a variety of signaling proteins and also function as “lipid-binding” domains. These genes encoded Rho GTPase activating proteins (*Arhgap21*, *Arhgap24*, *Arhgap27*), Rho guanine nucleotide exchange factors (*Arhgef5*, *Arhgef6*, *Arhgef16*, *Arhgef19*, *Arhgef28*) and Pleckstrin homology domain containing proteins (*Plekha1*, *Plekha5*, *Plekha1*, *Phlda1*, *Phlda2*) (Table S6). In addition to the above pathways, KEGG analysis of the E10.5 lens-enriched genes identified several signaling pathways such as “cGMP-PKG signaling pathway”, “G-protein coupled receptor signaling”, “Jak-STAT signaling”, “MAPK signaling” and “TNF signaling pathway”, among others, which suggests their importance to early lens developmental events (Fig. 5).

Starting from stage E12.5, gene groups for “Cell differentiation”, “Actin binding”, “Gap junction” and “Spectrin/alpha-actinin” were identified among lens-enriched genes, which correlate with fiber cell differentiation (Fig. 5) (Bassnett et al. 2011). The “Actin binding” category included myosin encoding genes *Myo6*, *Myo7a*, *Myo7b* as well as other understudied genes in the lens such as *Ablim1*, *Ablim2*, *Mylk* and *Sptbn1*. At stages E12.5, E14.5, and E16.5, gene groups for “Fibronectin, type III”, “Glutathione metabolism”, “PDZ domain”, “Spectrin/alpha-actinin” and “ErbB signaling pathway” are enriched (Fig. 5, Tables S7, S8, S9). This highlights interesting new candidates in these stages, for example, the “ErbB signaling pathway” comprises of receptor tyrosine kinases (RTKs) that are involved in the regulation of diverse cellular aspects including survival, proliferation and differentiation. Interestingly, only at stages E14.5 and E16.5, genes for the categories “Intracellular ribonucleoprotein complex”, “Catenin complex”, “PPAR signaling pathway”, “EF-hand like domain” and “Src homology-3 domain” were identified, indicating the importance of these genes in these later stages.

Additionally, genes displaying highly dynamic expression patterns that correlate with the specific lens developmental stage(s) were identified as well. For example, at early lens stages E10.5 and E12.5, the gene group belonging to “Epidermal growth factor-like domain”

was enriched in the lens, which was not represented in later stages. Interestingly, only at stage E12.5, the gene categories “Semaphorin-plexin signaling pathway” and “Calcium signaling pathway” were enriched (Table S7). The latter pathway identified 18 candidates that include genes for the calcium voltage-gated channel subunit alpha proteins (*Cacna1d*, *Cacna1a*), calcium/calmodulin dependent protein kinase II alpha protein (*Camk2a*) and phospholipases (*Plcd3*, *Plcb1*, *Plcb4*) that require calcium as a cofactor. Further, at stages E12.5 and E14.5, “Biosynthesis of unsaturated fatty acids” was enriched (Tables S7, S8), while only stage E16.5 showed enrichment of the gene groups for “Basement membrane” and “Phospholipid biosynthetic process”, which correlates with lens differentiation (Table S9). Moreover, gene groups for “autophagosome assembly” and “mitophagy” were identified only at E16.5; genes identified in these autophagy/mitophagy-related GO clusters, for example *Atg4a*, *Atg7*, *Pink1*, have been previously reported in the lens (Brennan et al. 2012) and were also identified by a recent RNA-seq based study of isolated lens fiber cells (Zhao et al. 2018). In addition to validating these known autophagy-related genes, our lens RNA-seq data identifies high expression in the lens of new candidate genes such as *Atg4c*, *Atg9a*, *Ctsd*, *Pacs2* and *Wipi1* (Table S9). Finally, in agreement with previous reports (Zhao et al. 2018), we find that the autophagy/mitophagy-related genes *Bnip3*, *Gabap1l*, *Mtor*, *Pink1*, *Plekhm1*, and *Rblcc1* show an overall increase in expression with progression of lens development (Table S1, Table S5).

In addition to the dynamics of these lens-enriched genes, the present lens RNA-seq data also validates the “lens signature genes” previously identified by microarray analysis (Kakrana et al. 2018). Indeed, the lens signature genes *Aldoc*, *Dhx32*, *Fabp5*, *Gprc5b*, *Grifin*, *Gstm1*, *Hmgcn3*, *Mboat1*, *Mocs2*, *Npl*, *Ogn*, *Pgam2*, *Tmem40* and *Zbtb8b* are found to be highly expressed in the lens by the present analysis as well (Table S1).

Lens-enriched genes exhibit distinct clusters based on developmental expression pattern

We next sought to identify specific gene clusters that share dynamics of expression patterns as the lens develops from E10.5 through E16.5. To achieve this goal, we first identified the top lens-enriched genes ($n=638$) in all four stages of lens development based on the filtering criteria of expression score >2 CPM and lens enrichment score >1.5 fold change ($p<0.05$). Next, we applied Self Organizing Tree Algorithm (SOTA) method to identify clusters ($n=7$) with distinct expression patterns across the four stages. These clusters revealed remarkably distinct expression patterns (Fig. 6, Table S10) and included: 66 genes in Cluster 1, 26 genes in Cluster 2, 11 genes in Cluster 3, 207 genes in Cluster 4, 223 genes in Cluster 5, 56 genes in Cluster 6 and 49 genes in Cluster 7. Cluster 1 (for lens pit formation) showed high expression of genes at E10.5 and comparatively low expression at other stages (E12.5, E14.5 and E16.5). Cluster 2 (for primary fiber cell differentiation) genes showed high expression at E12.5 and low at other stages. Cluster 3 (from lens pit formation to fiber cell differentiation) genes had high expression at E10.5, E12.5 and E14.5 and low expression at E16.5. Similarly, Cluster 4 (for primary and secondary fiber cell differentiation) genes had high expression at E12.5, E14.5 and E16.5 and low expression at E10.5. Cluster 5 (for secondary fiber cell differentiation and elongation) genes had high expression at E14.5 and E16.5 and low expression at E10.5 and E12.5. Cluster 6 and Cluster 7 had varied expression at all stages, and therefore did not classify as distinct compared to Clusters 1–5 (Fig. S1).

Analysis of genes in these Clusters using the AnimalTFDB 2.0 database (Zhang et al. 2015) identified several new genes related to transcription regulation in distinct clusters, which may potentially function in embryonic lens development. For example, in Cluster 1, *Pax6* was identified among the other new TF genes *L3mbtl3*, *Myef2* and *Zfp507*. In Cluster 2, five new TF genes *Arnt2*, *Mef2a*, *Platr25*, *Tgif1* and *Zfp369* were identified, and in Cluster 3, *Zbtb33* was identified as a new TF candidate gene for further investigation. Cluster 4 contained known lens TFs genes such as *Foxe3*, *Hmx1*, *Maf*, *Mitf*, *Pitx3*, *Prox1* and *Zeb2* and predicted new TF genes such as *Aff4*, *Dnajc1*, *Etv1*, *Kmt2c*, *Mbd2*, *Mta3*, *Pbrm1*, *Satb1*, *Sox13*, *Srebf1*, *Zbtb8b*, *Zfp385a*, *Zfp445*, *Zfp672*, *Zfp84*, *Zfp874a*, *Zfp932* and *Zfp933*. Cluster 5 identified twelve new TFs, namely, *Bhlhe41*, *Dmtf1*, *Gabpa*, *Plag1*, *Rcor3*, *Ttf1*, *Zbtb20*, *Zbtb26*, *Zbtb6*, *Zfp280d*, *Zfp420* and *Zfp62*. In addition to these TFs, SOTA clustering approach revealed co-factors potentially important in transcription regulation in the lens. For example, in Cluster 1, *Ccnk*, *Mtf2*, *Mrip1*, *Ogt*, *Rbm15*, *Taf9* and *Tb11x* were identified. Cluster 3 identified *Ino80b* and *Rnf2*, Cluster 4 identified *Anp32a*, *Ezr*, *Kdm5a*, *Lanc12*, *Malt1*, *Med10*, *Med12l*, *Sirt7* and *Trim*, and Cluster 5 identified *Ash2l*, *Brms1l*, *Clu*, *Ddx5*, *Hspa8*, *Jade1* and *Mlk2*. Several other interesting candidate genes were highlighted in stage-specific clusters. For example, Sfpq (splicing factor proline and glutamine rich) and a known syndromic microphthalmia gene *Stra6* (stimulated by retinoic acid 6) were identified in cluster 1). Thus, SOTA clustering analysis of genes based on lens enrichment profiles led to the identification of several new potential regulators in lens development.

Web-based public resource to prioritize lens-enriched candidate genes from RNA-seq data

Next, we sought to make this rich knowledge of RNA-seq-based lens gene expression widely applicable and easily accessible to the research community for prioritization of gene candidates that are relevant to lens biology and cataract through our iSyTE 2.0 tool. To achieve this goal we developed a new interactive web page that allows direct public access to both, the RNA-seq-based lens expression as well as lens enrichment data, in a user-friendly manner. The interactive web page named as “RNA-seq Lens-enrichment” is accessible under the under “Lens Gene Expression” section of iSyTE 2.0 web resource at <https://research.bioinformatics.udel.edu/iSyTE/ppi/expression.php>. The “RNA-seq Lens enrichment” page provides direct access to our eight newly developed custom iSyTE tracks for lens gene expression and enrichment at E10.5, E12.5, E14.5 and E16.5 loaded to the University of California at Santa Cruz (UCSC) Genome Browser (Fig. 7). Four new custom iSyTE tracks provide color-codes for lens enrichment based on *in silico* WB-subtracted gene expression profiles at E10.5, E12.5, E14.5 and E16.5. Further, four separate custom tracks provide color-codes for lens gene expression (without *in silico* WB-subtraction). Thus, lens enrichment as well as lens expression for individual genes can now be viewed in the mouse genome GRCm38/mm10 assembly in the context of the other rich genome-level information available. Functionally, the user can browse a specific genomic interval, such as a mapped interval, and use the color-codes for lens expression or enrichment to effectively visualize and prioritize the best candidate genes in the region of interest for further investigation (Fig. 7). Alternately, a user can search for specific genes, such as those from exome-seq data, to estimate their lens expression and enrichment. Concurrent visualization of these tracks for four embryonic stages allows analysis of the dynamic expression of genes as lens

development progresses. As an example to illustrate the utility of these RNA-seq-based custom tracks, the human cataract-linked gene *BFSP2* shows high lens-enrichment at early embryonic stages (e.g. E10.5) prior to its progressive upregulated expression in the lens at later stages of development (Fig. 7).

Conclusion

The present study reports the development and utility of high-throughput RNA-seq-based developmental transcriptomic profiles for mouse lens at the embryonic stages E10.5, E12.5, E14.5 and E16.5. These data demonstrate that *in silico* WB-subtraction analysis can be successfully applied to lens RNA-seq data to prioritize genes important for lens biology and cataract. The rich early lens developmental expression information generated in this study is made publicly accessible as a new web resource through iSyTE 2.0 in form of user-friendly custom-tracks on the UCSC Genome Browser. The inclusion of several key embryonic stages facilitates effective visualization of the developmental dynamics of gene expression and enrichment in the lens. Further, visualization of this data in the UCSC Genome Browser allows comparative analysis with other publicly available genome-level resources such as evolutionary conservation, SNP positions, and epigenetic information. Thus, this new lens RNA-seq data and its accessibility through iSyTE 2.0 serves as a new integrative resource for prioritization of lens defects and/or cataract-linked candidate genes identified by other high-throughput analyses such as exome-seq and GWAS. Importantly, by generating a new RNA-seq based WB dataset and showing that RNA-seq datasets can be effectively WB-subtracted, we have developed a new resource that can be used by the research community to apply *in silico* subtraction to a wide range of cells and tissues for prioritization of genes with high tissue-enriched expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. RNA-seq experimental design for mouse embryonic lens transcriptome analysis.
A flowchart of experimental design and integrative computational pipeline to determine the mouse lens developmental transcriptome at embryonic stages E10.5, E12.5, E14.5 and E16.5.

Fig. 2. Quality analysis of RNA-seq data and efficacy of *in silico* WB-subtraction.

(A) Principle component analysis (PCA) of normalized expression counts from RNA-seq datasets for the lens and WB. The plots demonstrate that data generated from individual replicates for specific lens stages (E10.5 (black), E12.5 (red), E14.5 (green) and E16.5 (blue)) clustered in a stage-specific manner and were distinct from the WB datasets that clustered together (turquoise). (B) The *in silico* WB-subtraction approach effectively identifies genes with lens-enriched expression that are linked to lens biology. The top 500 genes identified by *in silico* WB-subtraction and the top 500 genes identified based on just high expression (but which were not subjected to *in silico* WB-subtraction) from E10.5, E12.5, E14.5 and E16.5 mouse lens were examined for significant enrichment of gene ontology (GO) terms. Heat map intensity reflects significant *p*-values for GO term enrichment with high significance represented by red and low significance represented by white. This shows that while non *in silico*-subtracted genes are enriched for GO terms for

housekeeping function and are not specific to the lens, *in silico*-subtracted genes are significantly enriched for lens-related GO terms.

Fig. 3. In silico WB-subtraction and expression analysis effectively identify genes linked to cataract and lens development.

Genes known for lens development and cataract formation were examined for lens expression and lens-enrichment in the RNA-seq data. Genes are grouped based on gene ontology (GO) terms and presented as a heatmap. Lens expression scores (expression counts) are presented as 'red' for high expression to 'white' for low expression. Lens enriched-expression scores (fold-change) are presented as 'red' for high enrichment to 'green' for low enrichment.

Fig. 4. Lens-enriched gene expression dynamics in mouse embryonic development. Lens-enriched genes at different embryonic stages were identified by *in silico* WB-subtraction approach using criteria of: expression counts ≥ 2.0 , 1.5 fold-change in the lens compared to WB, $p < 0.05$. (A) At E10.5, 17% ($n=1401$) genes showed lens enrichment. (B) At E12.5, 22% ($n=1937$) genes showed lens enrichment. (C) At E14.5, 31% ($n=2514$) genes showed lens enrichment. (D) At E16.5, 34% ($n=2745$) genes showed lens enrichment.

Fig. 5. Identification of Gene Ontology (GO), KEGG pathway and Interpro terms for lens-enriched genes.

Enrichment of gene groups related to lens function from GO analysis of lens-enriched genes identified at mouse embryonic stages (**A**) E10.5, (**B**) E12.5, (**C**) E14.5 and (**D**) E16.5. The genes were subjected to GO annotation clustering method implemented in DAVID bioinformatics tool to identify various GO terms, KEGG pathway and InterPro entries.

Fig. 6. Developmental expression clusters reveal dynamics of lens-enriched genes.

Self-organizing tree algorithm (SOTA)-based clustering of 638 common lens-enriched genes at stages E10.5, E12.5, E14.5 and E16.5 were used to identify stage-specific clusters. **(A)** Heatmap of cluster (1–5) genes showing dynamic expression (normalized expression generated by SOTA) in the lens at stages E10.5, E12.5, E14.5 and E16.5. Transcription factors identified in each cluster are listed. **(B)** Clusters of genes with distinct expression patterns in the developing lens are shown as Cluster 1 (green), Cluster 2 (blue), Cluster 3 (purple), Cluster 4 (brown) and Cluster 5 (red). x-axis represents normalized expression

generated by SOTA and y-axis represents mouse lens embryonic stage. The red line represents the data trend average for the genes in each cluster.

Fig. 7. RNA-seq data based custom tracks for UCSC browser to visualize lens expression and enrichment.

To access the RNA-seq-based lens expression and lens enrichment data, we developed UCSC custom tracks on the human genome GRCh38/hg38 and the mouse genome GRCm38/mm10 assembly and have made these publicly accessible through iSyTE 2.0 at <https://research.bioinformatics.udel.edu/iSyTE/>. Under the “RNAseq lens-enriched” tab, a link is given that allows access to eight UCSC custom-tracks. Four tracks provide visual representation of lens gene expression and four tracks provide visual representation of lens gene enrichment data for mouse embryonic stages E10.5, E12.5, E14.5 and E16.5. A color key is provided on iSyTE 2.0 to estimate lens enrichment (numbers indicate fold-change gene expression in the lens compared to WB) and expression (numbers indicate counts for expression). As an example, the human cataract-linked gene *BFSP2* can be analyzed for expression and enrichment in the lens by following steps 1 through 3.

Table 1.

Summary of high-confidence embryonic lens and WB transcriptome assembly

Lens stage	Total Reads	Mapped reads (left)	Mapped reads (right)	Matched reads (pairs)	Matched reads (%)
E10.5 rep1	18871557	18576519	18658583	18511891	98.1
E10.5 rep2	15595659	15372775	15445696	15317953	98.2
E10.5 rep3	18822247	18520863	18607527	18452371	98.0
E12.5 rep1	12982302	12787835	12852258	12732162	98.1
E12.5 rep2	15428028	15218508	15289138	15156543	98.2
E12.5 rep3	17764700	17545231	17624089	17475411	98.4
E14.5 rep1	13588817	13348064	13423278	13290339	97.8
E14.5 rep2	18388032	17996804	18103009	17915106	97.4
E14.5 rep3	18836743	18451243	18559893	18373856	97.5
E16.5 rep1	18196526	17771816	17875908	17689187	97.2
E16.5 rep2	18877953	18449119	18550864	18358392	97.2
E16.5 rep3	19476511	18980010	19093548	18887815	97.0
WB rep1	24311823	23887833	23852761	23648047	97.3
WB rep2	23208285	22864444	22874050	22690142	97.8
WB rep3	22345072	21984202	21989833	21810851	97.6

Table 2.

Summary of high-confidence embryonic lens transcriptome assembly

Lens Stage	Total expressed transcripts
E10.5	8476
E12.5	8721
E14.5	8022
E16.5	8078