Identification of novel transcripts and peptides in developing murine lens

Shahid Y. Khan1, Muhammad Ali1, Firoz Kabir1, Ruiqiang Chen2, Chan Hyun Na2, Mei-Chong W. Lee3, Nader Pourmand3, Sean F. Hackett1 & S. Amer Riazuddin1

We previously investigated the transcriptome and proteome profiles of the murine ocular lens at six developmental time points including two embryonic (E15 and E18) and four postnatal time points (P0, P3, P6, and P9). Here, we extend our analyses to identify novel transcripts and peptides in developing mouse lens. We identified a total of 9,707 novel transcripts and 325 novel fusion genes in developing mouse lens. Additionally, we identified 13,281 novel alternative splicing (AS) events in mouse lens including 6,990 exon skipping (ES), 2,447 alternative 3′ splice site (A3SS), 1,900 alternative 5′ splice site (A5SS), 1,771 mutually exclusive exons (MXE), and 173 intron retention (IR). Finally, we integrated our OMIC (Transcriptome and Proteome) datasets identifying 20 novel peptides in mouse lens. All 20 peptides were validated through matching MS/MS spectra of synthetic peptides. To the best of our knowledge, this is the first report integrating OMIC datasets to identify novel peptides in developing murine lens.

Next-generation RNA sequencing (RNA-Seq) has significantly enhanced our ability to decipher whole transcriptomes through the gene expression quantification, identification of novel transcripts, detection of fusion genes, and isoform diversity1–7. The mouse genome encodes 53,715 genes, including 21,981 protein-coding genes (GENCODE Ver. M17). However, the total number of transcripts encoded by these genes is believed to be much higher suggesting multiple layers of complexity at the transcriptome level8,9.

Fusion genes describe a phenomenon of hybrid RNA resulting from read-through transcripts, composed of two different genes formed during chromosomal re-arrangements10,11. Fusion genes could be a product of cis-splicing as well as trans-splicing12–15. Alternative mRNA splicing, a phenomenon more prevalent in higher eukaryotes, provides additional diversity in gene expression8,16, and according to some estimates >95% of human multi-exonic mRNAs undergo mRNA splicing17,18.

The ocular tissue especially the retina has been characterized extensively using multiple next-generation based transcriptome studies that revealed highly diverse annotated and novel transcriptome and novel isoforms8,9,20. In contrast, the characterization of the lens expression profile has received less attention and fewer next-generation RNA sequencing-based studies have been completed. Recently, Srivastava and colleagues identified novel transcripts and splicing alterations in developing murine lens21.

We previously reported the mouse lens coding and non-coding transcriptome at six developmental time points including two embryonic (E15 and E18) and four postnatal stages (P0, P3, P6, and P9)22,23. More recently, we reported a comprehensive proteome of the mouse lens at the same six developmental time points24. Here, we extend our analyses to identify novel transcripts and peptides in developing mouse lens.

Results

Here, we extend our analysis of the RNA-Seq data using multiple bioinformatics tools to identify novel transcripts, fusion genes, and alternative splicing (AS) in developing mouse lens (Fig. 1). Additionally, we integrate our OMIC (Transcriptome and Proteome) datasets to identify novel peptides in mouse lens and subsequently validated them through matching MS/MS spectra of synthetic peptides (Fig. 1).
First, the raw reads were mapped to the *Mus musculus* genome resulting in >93% alignment to the genome.

Next, the aligned reads were examined for PCR duplicates identifying ~19% of total reads as PCR duplicates that were removed. The remaining mapped reads were processed using StringTie to convert the RNA-Seq alignments into potential transcripts and the expression of each transcript was measured and normalized using transcripts per million (TPM) algorithm. Our analysis identified both annotated and novel transcripts in mouse lens transcriptome. We divided the novel transcripts further into two categories: first, transcripts that map entirely to the unannotated regions of the mouse genome and second, transcripts that partially align to both annotated and unannotated regions of the mouse genome.

We identified a total of 21,265 annotated transcripts expressed in at least one of the six developmental time points (Table 1 & Supplementary Table 1). Of these, we identified 9,707 novel transcripts present in at least one of the six developmental stages mapping entirely to unannotated regions of the mouse genome (Table 1 & Supplementary Table 2). Additionally, we identified 14,113 transcripts aligned to both the annotated and unannotated regions of the mouse genome (Table 1 & Supplementary Table 3) termed hereafter as semi-novel transcripts.

We further investigated our mRNA sequencing data to identify fusion genes expressed in the mouse lens transcriptome. The analysis identified 325 novel fusion genes including 195, 161, 275, 218, 280, and 223 fusion genes in the mouse lens at E15, E18, P0, P3, P6, and P9, respectively (Table 1 & Supplementary Table 4). Gene ontologies (GO) based functional and mammalian phenotype enrichment analysis of novel fusion genes revealed...
(q-value ≤ 0.01) unique molecular function, biological process, cellular component and mammalian phenotypes (Supplementary Tables 5–6).

Next, we examined our RNA-Seq dataset using the rMATS pipeline (≤ 0.01 FDR) to identify the novel AS events across the six developmental time points in mouse lens. In total, we identified five AS events including exon skipping (ES), alternative 3′ splice site (A3SS), alternative 5′ splice site (A5SS), mutually exclusive exons (MXE), and intron retention (IR) in developing mouse lens (Table 2). The analysis identified 6,990 novel ES splicing events (≤ 0.01 FDR) across the six developmental time points in mouse lens (Supplementary Table 7). Of these 2,023 events present in at least one developmental time point, and 4,967 ES events in ≥2-time points (Supplementary Table 7).

We identified 2,447 novel A3SS splicing events (≤ 0.01 FDR) including 809 events detected in a single developmental time point and 1,638 events in ≥2-time points (Supplementary Table 8). Likewise, we identified 1,900 novel A5SS splicing events including 719 events detected in a single developmental time point and 1,181 events in ≥2-time points (Supplementary Table 9). Furthermore, we identified 1,771 novel MXE splicing events (≤ 0.01 FDR) including 387 events detected in a single developmental time point and 1,384 events in ≥2-time points (Supplementary Table 10). Lastly, our analysis identified 173 IR splicing events (≤ 0.01 FDR) in mouse lens (Supplementary Table 11).

Our RNA-Seq datasets are critical in identifying novel transcripts; however, the biological significance of these events is incomplete without knowing the corresponding changes at the protein level. We recently investigated the proteome profile of developing mouse lens through mass spectrometry-based protein sequencing25. We integrated our OMIC datasets to identify novel peptides in mouse lens. As mentioned above, we identified a total of 9,707 novel transcripts that were translated into three open reading frames (ORFs) to identify all theoretical peptides translated by the novel transcripts. This theoretical peptide dataset was interrogated against the mouse lens proteome to identify peptides originating from a sequence of the novel transcripts (9,707 novel transcripts identified in the mouse lens transcriptome). The analysis identified 55 peptides in the mouse lens proteome based on TMT spectra. All of the 55 candidate peptides were screened against the mouse non-redundant (nr) protein database (NCBI) and peptides with ≥2 amino acids mismatches and an XCorr score ≥ 2.5 were considered novel. This criterion identified a total of 20 novel peptides that were retained for further analysis.

All 20 novel peptides along with three control peptides were synthesized commercially and the respective spectra of these synthetic peptides were generated using the Orbitrap Fusion Lumos Tribrid Mass Spectrometer. The MS/MS fragmentation patterns of synthetic peptides were manually compared with MS/MS spectra generated from the proteomic analysis of mouse lens. The control peptides revealed similar spectra consistent with the MS/MS fragmentation patterns originating from mouse lens proteome (Supplementary Figs 1–3). The MS/MS fragmentation patterns of all 20 synthetic peptides (representing 20 novel peptides) exhibited spectrum consistent with the MS/MS fragmentation patterns originating from mouse lens proteome dataset (Table 3, Fig. 2, Supplementary Figs 4–20, and Supplementary Table 12).

Discussion

We previously investigated mouse lens transcriptome at two embryonic (E15 and E18) and four postnatal (P0, P3, P6, and P9) time points using next-generation RNA sequencing, which identified a total of 14,465 genes along with 12 different classes of non-coding RNAs (ncRNAs) in mouse lens22,23. More recently, we completed a comprehensive proteome of mouse lens at the same six developmental time points identifying 5,404 proteins24. We integrated our OMIC datasets to identify novel transcripts (9,707 novel transcripts identified in six developmental time points (Table 1, and Supplementary Table 2). Of these, ~25% are multi-exonic and ~40% are expressed in all six developmental time points (Supplementary Table 2). In addition to novel transcripts, we identified a total of 14,113 semi-novel transcripts in lens transcriptome (Table 1, and Supplementary Table 3). In contrast to the novel transcripts where a majority (~75%) of the transcript are single exon, most of the semi-novel transcripts (~85%) revealed a multi-exonic structure i.e. ≥2.0 exons (Supplementary Tables 2 and 3).

We identified 325 novel fusion genes in developing mouse lens (Table 1). Among these, we identified multiple fusion transcripts for CryoA, CryoB, CryA1, CryA2, CryA4, CryB1, CryB2, CryB3, CryYA, CryYC, CryYD, CryYE, and CryYS (Supplementary Table 4). Additionally, we identified fusion transcripts for Bfsp1, Bfsp2, Tdrd7, Mip, Lim2, Pax6, and Dnase2 in mouse lens (Supplementary Table 4). Multiple studies have reported fusion genes in normal human and mouse tissues and more importantly, in tumorigenesis25–28. We did not find any reports in literature, and therefore this would be the first report describing fusion genes in the ocular lens.

Multiple transcriptome-based studies have revealed extensive AS in normal human and mouse ocular tissues29–33. We identified 13,281 novel AS events in developing mouse lens with a predominant contribution from ES, A3SS, A5SS, and MXE while a small number of IR splicing events identified in developing mouse lens (Table 2). The ES events contribute to the diversity of the transcriptome and consistent with this notion, we identified a total of 6,990 ES events in developing mouse lens. Interestingly, most of the ES were identified at embryonic time points (Table 2).

Recently, Srivastava and colleagues reported the identification of 1,241 AS events in developing mouse lens21, examining RNA-Seq datasets recently published by our group22. We identified a total of 13,281 AS events in developing mouse lens analyzing the same RNA-Seq datasets. The difference in the numbers of AS events published by Srivastava and colleagues and identified in the current study may be attributed to different cut-off values. Srivastava and colleagues used PSI (Percent Spliced Index) cut-off value for significant (<1% FDR) AS events21, whereas we used a cut-off value of ≤0.01 FDR for the identification of AS events in developing mouse lens.
| Developmental Stage | Types of AS Events | Total AS Events | Significant Novel AS Events* |
|---------------------|--------------------|----------------|-------------------------------|
| E15 vs. E18         | ES                 | 22160          | 416                           |
|                     | MXE                | 3069           | 93                            |
|                     | A5SS               | 9753           | 162                           |
|                     | A3SS               | 13755          | 306                           |
|                     | IR                 | 575            | 23                            |
| E15 vs. P0          | ES                 | 26560          | 521                           |
|                     | MXE                | 4379           | 124                           |
|                     | A5SS               | 10743          | 97                            |
|                     | A3SS               | 14818          | 93                            |
|                     | IR                 | 646            | 11                            |
| E15 vs. P3          | ES                 | 23261          | 336                           |
|                     | MXE                | 3881           | 107                           |
|                     | A5SS               | 10247          | 106                           |
|                     | A3SS               | 14215          | 107                           |
|                     | IR                 | 605            | 8                             |
| E15 vs. P6          | ES                 | 22898          | 459                           |
|                     | MXE                | 3827           | 129                           |
|                     | A5SS               | 10119          | 110                           |
|                     | A3SS               | 14093          | 119                           |
|                     | IR                 | 617            | 8                             |
| E15 vs. P9          | ES                 | 21982          | 444                           |
|                     | MXE                | 3872           | 121                           |
|                     | A5SS               | 9859           | 97                            |
|                     | A3SS               | 13623          | 94                            |
|                     | IR                 | 559            | 13                            |
| E18 vs. P0          | ES                 | 23579          | 1107                          |
|                     | MXE                | 3470           | 194                           |
|                     | A5SS               | 9295           | 259                           |
|                     | A3SS               | 12849          | 309                           |
|                     | IR                 | 557            | 26                            |
| E18 vs. P3          | ES                 | 20212          | 810                           |
|                     | MXE                | 3321           | 219                           |
|                     | A5SS               | 8888           | 233                           |
|                     | A3SS               | 12271          | 287                           |
|                     | IR                 | 515            | 19                            |
| E18 vs. P6          | ES                 | 19789          | 931                           |
|                     | MXE                | 3109           | 198                           |
|                     | A5SS               | 80692          | 238                           |
|                     | A3SS               | 12123          | 300                           |
|                     | IR                 | 503            | 21                            |
| E18 vs. P9          | ES                 | 18776          | 921                           |
|                     | MXE                | 2938           | 188                           |
|                     | A5SS               | 8384           | 241                           |
|                     | A3SS               | 11645          | 290                           |
|                     | IR                 | 452            | 15                            |
| P0 vs. P3           | ES                 | 24298          | 281                           |
|                     | MXE                | 4296           | 131                           |
|                     | A5SS               | 9669           | 82                            |
|                     | A3SS               | 13227          | 108                           |
|                     | IR                 | 586            | 5                             |
| P0 vs. P6           | ES                 | 23843          | 194                           |
|                     | MXE                | 4205           | 33                            |
|                     | A5SS               | 9581           | 46                            |
|                     | A3SS               | 13119          | 88                            |
|                     | IR                 | 583            | 6                             |

Continued
Finally, we adopted a proteogenomics approach to identify expression of novel transcripts in mouse lens proteome and subsequently validated these novel peptides through MS/MS spectra of corresponding synthetic peptides. In silico analyses identified a total of 55 novel peptides in mouse lens proteome. Of these, only 20 peptides passed a more stringent criterion i.e. ≥2 amino acids mismatches and an XCorr score ≥2.5. These 20 novel peptides were validated through MS/MS spectra of corresponding synthetic peptides. We are currently investigating the identity of the proteins harboring these novel peptides and examining their biological significance in lens morphogenesis.

In conclusion, we represent a comprehensive developing mouse lens profile through the identification of novel transcripts, novel fusion genes, and novel AS events. Additionally, we integrate our OMIC datasets to identify novel peptides in developing mouse lens.

### Materials and Methods

#### mRNA sequencing data.

The Illumina paired-end mRNA sequencing data (GEO series accession number GSE69221) was used for the downstream bioinformatics analysis. The paired-end raw reads were processed to remove the adapter sequences using SeqPrep (https://github.com/jstjohn/SeqPrep). The quality of the pre-processed reads was evaluated with FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc) and low-quality reads were removed prior to the downstream analysis.

#### Mapping and transcript annotation of mRNA sequencing data.

HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts), a spliced alignment tool (Ver. 2.1.0-beta) was used to map pre-processed reads to the mouse genome (NCBI37/mm9) with default parameter settings. The BAM output files were generated for each sample and PCR duplicates were marked and removed from BAM files using Picard software (Ver. 2.8.3; https://github.com/broadinstitute/picard). Subsequently, StringTie algorithm (Ver. 1.3.3b) was used with default parameter settings to assemble RNA-Seq alignments into annotated and novel transcripts and estimate their respective expression level. The expression of these transcripts was normalized using transcripts per million (TPM) algorithm and the number of known, and novel transcripts were estimated from the output GTF file generated by StringTie with expression threshold (≥1.0 TPM).

#### Identification of AS events.

AS events were identified through rMATS software (Ver. 3.2.5). The mRNA-Seq alignment files (bam files) generated by the HISAT2 was used as an input for the rMATS analysis. The Mus musculus RefSeq gene annotations (GRcm38/mm10) was used as a reference with default parameter settings. Finally, the rMATS was used to calculate p-value and false discovery rate (FDR) for AS events among different developmental time points.

#### Identification of fusion genes.

JAFFA (Ver. 1.08), a multi-step pipeline was used in a hybrid mode to detect fusion genes in mRNA sequencing data. The Mus musculus reference genome (GRcm38/mm10),

| Developmental Stage | Types of AS Events | Total AS Events | Significant Novel AS Events* |
|---------------------|--------------------|-----------------|------------------------------|
| P0 vs. P9           | ES                 | 22985           | 214                          |
|                     | MXE                | 4137            | 49                           |
|                     | A5SS               | 9293            | 67                           |
|                     | A3SS               | 12681           | 114                          |
|                     | IR                 | 533             | 9                            |
| P3 vs. P6           | ES                 | 20632           | 149                          |
|                     | MXE                | 4121            | 108                          |
|                     | A5SS               | 9142            | 62                           |
|                     | A3SS               | 12526           | 89                           |
|                     | IR                 | 542             | 3                            |
| P3 vs. P9           | ES                 | 19721           | 143                          |
|                     | MXE                | 3816            | 60                           |
|                     | A5SS               | 8832            | 54                           |
|                     | A3SS               | 12003           | 78                           |
|                     | IR                 | 499             | 4                            |
| P6 vs. P9           | ES                 | 19208           | 64                           |
|                     | MXE                | 4083            | 17                           |
|                     | A5SS               | 8672            | 46                           |
|                     | A3SS               | 11934           | 65                           |
|                     | IR                 | 492             | 2                            |

Table 2. Alternative splicing events identified in developing mouse lens. Note: AS: alternative splicing; ES: exon skipping; MXE: mutually exclusive exon; A5SS: alternative 5′ splice site; A3SS: alternative 3′ splice site; and IR: intron retention. *Significant events based on false discovery rate (FDR) <0.01. E15 and E18 are embryonic days 15, and 18, and P0, P3, P6, and P9 are postnatal days 0, 3, 6, and 9, respectively.
transcripts annotations and sequences from mouse GENCODE Ver. M15 (GRCm38) were used as a reference for fusion gene identification using default JAFFA parameter settings.

Gene ontologies functional enrichment analysis. A functional annotation analysis of mouse lens genes was investigated using Visual Annotation Display (VLAD; Ver. 1.6.0), a web-based tool from the Mouse Genome Informatics (MGI)40. The VLAD tool performs the statistical analysis to test the enrichment of gene ontology (GO) terms based on their annotations to gene function and mammalian phenotype 40. A complete set of mouse genes was used as a reference annotation dataset and ontological terms annotated with the evidence code ND (no biological data) were excluded from the enrichment analysis. The statistically significant enriched terms were sorted based on their corrected \( p \)-value \( \leq 0.01 \) calculated using multiple testing and positive false discovery rate for each term.

Proteogenomic analysis of novel transcripts in lens proteome. The mRNA sequencing data was used to extract Fasta sequences of novel transcripts using bedtools getfasta tool (Ver. 2.25.0; http://bedtools.readthedocs.io/en/latest/content/tools/getfasta.html) with the transcript coordinates output by StringTie. The novel transcripts were translated into three open reading frames (ORFs) using in-house generated Python script to generate a dataset of all potential proteins resulting from the translation of the novel transcripts. The theoretical protein dataset was screened to remove shorter proteins (<6 amino acids long) and the resulting dataset was used as a reference database.

The MS/MS spectra from mouse lens proteome were interrogated against the reference database (generated above, using the Python script) using the SEQUEST search algorithm through the Proteome Discoverer Suite.

### Table 3. Novel peptides identified in mouse lens through integration of OMIC (Transcriptome and Proteome) datasets22,24. All 20 peptides were validated through matching MS/MS spectra of synthetic peptides. Note: PSMs: Peptide Spectrum Matches.
Figure 2. Validation through matching MS/MS spectra of synthetic peptides. (A) MS/MS spectra of a novel peptide (WLIEISK) shown with a similar fragmentation pattern observed from the corresponding synthetic peptide. (B) MS/MS spectra of a novel peptide (SMGEDTVPK) shown with a similar fragmentation pattern observed from the corresponding synthetic peptide. (C) MS/MS spectra of a novel peptide (LCGACGTASGTK) shown with a similar fragmentation pattern observed from the corresponding synthetic peptide. Note: the term “peptide identified from sample” refers to the MS/MS spectra identified in mouse lens proteome\textsuperscript{24}, and the synthetic peptide refers to MS/MS spectra of the peptide synthesized by JPT Peptide Technologies (Berlin, Germany).

| Stage | Replicates | Genes | Total Genes | Stage | TMT | Proteins | Total Proteins |
|-------|------------|-------|-------------|-------|-----|----------|----------------|
| E15   | E15A E15B  | 13,274| Set 1       | E15   |     | 4,630    |                |
|       |            |       |             | E18   |     |          |                |
| E18   | E18A E18B  | 13,900|             | P0    |     | 4,426    | 5,404          |
| P0    | P0A P0B    | 12,560|             | E15   |     |          |                |
|       |            |       |             | E18   |     |          |                |
| P3    | P3A P3B    | 12,940|             | P0    |     |          |                |
|       |            |       |             | P3    |     |          |                |
| P6    | P6A P6B    | 12,130|             | P6    |     |          |                |
| P9    | P9A P9B    | 12,229|             | P9    |     |          |                |

Table 4. Data retrieved from previously published OMIC (Transcriptome and Proteome) datasets\textsuperscript{22,24}. Note: E15 and E18 are embryonic days 15, and 18, and P0, P3, P6, and P9 are postnatal days 0, 3, 6, and 9, respectively.
Validation of novel peptides through LC-MS/MS analysis of synthetic peptides. All synthetic peptides were purchased from JPT Peptide Technologies (Berlin, Germany). The synthetic peptides were pooled and labeled with 1-plex TMT reagents according to the manufacturer's instructions (Thermo Fisher Scientific). The labeling reaction was performed for one hour at room temperature followed by quenching of the labeling reaction with 100 mM Tris-HCl (pH 8.0). The labeled peptides were desalted with C18 Sep-Pak (Waters Corporation, Milford, MA), dried and resuspended in 0.1% formic acid. Peptides were subjected to Orbitrap Fusion Lumos Trifid Mass Spectrometer coupled with the Easy-nLC 1200 nano-flow liquid chromatography system (Thermo Fisher Scientific) with similar parameters used for the mouse lens proteome profiling.

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**Author Contributions**
S.Y.K., C.H.N. and S.A.R.: conceived and designed the experiments; C.H.N. and N.P. and S.A.R.: contributed reagents, materials, and analytical tools; S.Y.K., M.A., F.K., R.C., C.H.N., S.F.H. and S.A.R.: performed experiments; S.Y.K., R.C., C.H.N., M.C.W.L. and S.A.R.: analyzed the data; S.Y.K., M.A., F.K., R.C., C.H.N., M.C.W.L., N.P., S.F.H. and S.A.R.: contributed to writing the manuscript.

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