The Long Noncoding RNA Landscape of the Mouse Eye

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Ongoing advances in sequencing technology as well as findings by the ENCODE project revealed that the vast majority of the mammalian genome is transcribed and results in the generation of a diverse large population of noncoding RNAs (ncRNAs), whereas only a small proportion of genomic DNA constitutes protein-coding genes.1-3 The realization of the importance of ncRNAs in contributing to the regulation of an ever growing number of wide ranging physiologic and pathophysiologic scenarios is prompting numerous studies delineating their significance as potential drug targets to treat disease.

MicroRNAs (miRNAs) are the most widely studied subclass of ncRNAs, being endogenously single-stranded RNAs of approximately 22 nucleotides that mediate posttranscriptional gene silencing by controlling mRNA translation into proteins. MiRNAs control entire physiologic processes critical for maintenance of tissue identity and function by either suppressing the translation or promoting degradation of mRNA transcripts.4 Our previous studies identified a number of miRNAs having essential roles in the regulation of normal eye development whose altered expression levels underlie some of the symptoms characteristic of uveal melanoma and dysfunctional corneal epithelial wound healing.5-9

In contrast with miRNAs, the largest but poorly characterized subclass of long ncRNAs (lncRNAs) whose lengths range from 200 nucleotides to multiple kilobases are emerging as key regulators in diverse biologic pathways through targeting appropriate genomic regions in local cis or distal trans sites.10 Several functional lncRNAs in mammalian eyes have been suggested in different murine eye disease models to underlie some of the symptoms. Various broad analyses of results obtained with custom lncRNA microarrays of murine eye disease models including age-related cataract, ocular neovascularization, and early diabetic retinopathy identified tens to hundreds of aberrantly expressed lncRNAs suggesting that they may contribute to ocular pathologic processes.11-13 Another recent study identified a repertoire of lncRNAs in the murine lens at two embryonic and four postnatal developmental stages.14 A lncRNA MIAT (myocardial infarction-associated transcript) is upregulated in the diabetic retina, functioning as a competing endogenous RNA (ceRNA). It may sequester miR-150-5p, thereby relieving its repressive effect on VEGF expression.15,16 Subsequently, mouse lncRNA-MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) was reported to interact with CREB to regulate cell proliferation via maintaining CREB phosphorylation status during the development of retinal neurodegeneration.17 In another study of proliferative retinopathy (PVR), MALAT1 was upregulated in the cellular and plasma fractions of peripheral blood in PVR patients.18 Although such studies highlight the relevance of

AUTHOR CONTRIBUTIONS

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ABBREVIATIONS

MALAT1: metastasis-associated lung adenocarcinoma transcript 1
miR: microRNA
P0: postnatal day 0
PVR: proliferative retinopathy
RNA: ribonucleic acid
时间和生物的科学研究的发展。

MicroRNA (miRNA) 是最广泛研究的ncRNA子类，是长度约为22个核苷酸的单链RNA，能通过调节mRNA翻译来抑制基因表达。miRNA控制了广泛的生理过程，包括维持组织身份和功能。我们的前期研究识别了一些在正常眼发育过程中起关键作用的miRNA，其表达水平的变化导致了一些症状。

长非编码RNA（lncRNA）是最长度的但特征最低的ncRNA子类，长度从200个核苷酸到多个kilobase不等。它们在多种生物路径中起作用，通过靶向特定的基因区域。我们的前期研究识别了一些在正常眼发育过程中起关键作用的lncRNA，其表达水平的变化导致了一些症状。

协同作用的靶基因。这可能导致眼病的路径。

此外，miRNA在糖尿病视网膜病变中的作用被研究，miRNA-MALAT1（metastasis-associated lung adenocarcinoma transcript 1）被发现上调，可能通过竞争性内源性RNA（ceRNA）与CREB结合，调节细胞增殖。

总的来说，这些研究强调了ncRNA在眼病发展中可能的作用，特别是长非编码RNA。然而，这些研究还需要进一步的验证和理解，以确定它们在眼病治疗中的潜在应用。
lncRNAs in regulating basic biologic and pathologic processes, a comprehensive analysis of their expression profiles and function under physiologic conditions in the whole mammalian eye is still lacking. In order to define how lncRNAs function in different ocular developmental and disease states, it is indispensable to systematically characterize stage-dependent and tissue-specific lncRNA expression patterns under these conditions.

In the present study, we performed comprehensive microarray analysis combined with advanced computational approaches to gain insight into the individual stage-dependent and tissue-specific lncRNA expression profiles of six different ocular tissue subsets (i.e., retina, RPE, lens, cornea, choroid, and sclera) from newborn and normal 8-week-old mice. Building on this foundation, we performed bioinformatic analysis to integrate expression profiles and genomic locations of distinctive lncRNAs and protein-coding genes. Such insight made it possible for us to suggest their possible functional roles in the developing ocular tissues. We also provide the first comprehensive landscapes of stage-dependent and tissue-specific lncRNA expression in mouse eyes. This resource will most assuredly provide a solid foundation to better understand the role of lncRNAs in physiological and pathologic processes in mammalian eyes.

**Materials and Methods**

**Isolation of Ocular Tissues and RNA Extraction**

Ocular tissues (i.e., retina, RPE, lens, cornea, choroid, and sclera) from newborn (P0) and normal 8-week-old (adult) C57BL/6 mice were immediately and carefully dissected and pooled in RNAlater solution (detailed information is shown in Supplementary Table S1). It is very important to separate specific tissues free from contamination by other tissues, especially for RPE, choroid, and retina. Our procedure was carried out essentially as previously described19-20 and further optimized to ensure successful extraction of high-quality RNA. The main procedure for separating these tissues from adult mice is described below: Eyeballs were removed and dissected immediately in a cold Dulbecco’s (D)-PBS droplet in a petri dish kept on ice. The retinas along with partially dissociated RPE cell sheets were mechanically separated from choroid and sclera, and then cultured in a 0.25% trypsin solution, RPE cells were immediately scraped off from the retina in 1 to 2 minutes. The retina was taken out and the remaining RPE cell suspension was centrifuged for 2 minutes at 300 g, the suspension including a few photoreceptors was discarded and cell pellets were collected for RNA extraction. The choroid layer was peeled gently away from the sclera after carefully scraping off the RPE cells from the choroid. Other than that, our main procedure for separating these tissues from newborn mice is described below. We first mechanically separated retina from choroid. Both retinal and choroidal layers included partially dissociated RPE cell sheets, which were then scraped off from the retina or choroid, respectively, and these RPE cell pellets as well as the retina and choroid were collected separately for RNA extraction. Total RNA from these dissected tissues was isolated with the miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was quantified using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was assessed with a bioanalyzer system (Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA, USA). Quantitative RT-qPCR analysis was used to assess possible cross contamination of these tissues by evaluating the expression levels of recoverin, RPE65, and CD31, which are retina, RPE, and choroidal endothelial biomarkers, respectively.

All animal treatments were performed in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approval of the Wenzhou Medical University Animal Care and Use Committee.

**Microarray Experiment and Data Processing**

We used a mouse transcriptome assay (MTA 1.0; Affymetrix, Santa Clara, CA, USA) for digital lncRNA profiling using 500 ng total RNA extracted from each of the aforementioned murine ocular tissues. This microarray provides a whole-transcriptome expression data set, which contains approximately 600 million probes targeted against more than 23,000 protein-coding genes and more than 55,000 noncoding genes. RNA amplification, labeling, and array hybridization were performed according to the manufacturer’s instructions. The hybridization signaling was detected using a commercial scanner (Affymetrix GeneChip Scanner 3000; Affymetrix), and probe cell intensity data were summarized with the default settings of commercial software (Command Console Software; Affymetrix). After performing a quality assessment procedure, raw microarray data were then normalized by the expression console. Only probes perfectly matched to one gene were retained, while probes targeting several transcripts were discarded. To filter for expressed genes, a threshold cutoff-point of 5.0 was selected, and values for each gene in all biologic samples below this cutoff-point were considered to be indicative of relevant protein-coding or ncRNA expression. Accordingly, only those gene expressions satisfying this criterion were taken as candidates for the following bioinformatics analysis.

**Bioinformatic Analysis for Identifying lncRNA Signatures**

To pinpoint any age-dependent lncRNA signatures in each ocular tissue subset in newborn and 8-week-old mice, either protein-coding genes or lncRNA encoding genes, whose expression level differences in a specific ocular tissue had changed by ≥2-fold, were defined as being significant.21,22 To determine whether lncRNAs signatures possess tissue-enriched expression in a specific tissue, only genes whose expression levels differed by ≥2-fold from those in all other subsets in tissues obtained from the same age group were considered. This selection was done by first identifying the distinctive lncRNAs in the above data sets that had aggregated. Subsequently, K-means clustering identified candidate gene signatures specific for each of these two age groups. Accordingly, Venn analysis was employed to characterize the distinctive postnatal lncRNA signatures for the six different ocular tissues at two different ages.

**Functional Analyses of lncRNA Signatures**

To predict lncRNA functions in regulating the expression of protein-coding genes, their genomic relationships were determined. Accordingly, cis analysis was performed on the known differentially expressed lncRNAs. We used the algorithm searches for identifying target genes acting in the cis mode. With the help of gene annotations at UCSC (http://genome.ucsc.edu/, in the public domain), lncRNAs and potential target genes were paired and visualized using the UCSC genome browser. The genes transcribed within a 10-kb window upstream or downstream of IncRNAs were considered as cis target genes.23-25 Then Gene Ontology (GO) enrichment analyses were performed for biologic process terms associated with protein-coding genes that were proximal to lncRNA
signatures at the genomic level. For each lncRNA signature, the proximal protein-coding gene was selected regardless of the sense of transcription. We downloaded the GO annotations from the NCBI (http://www.ncbi.nlm.nih.gov/, in the public domain), UniProt (http://www.uniprot.org/, in the public domain), and GO (http://www.geneontology.org/, in the public domain) sites. Fisher’s exact test was applied to identify the significant GO categories and a false discovery rate (FDR) was used to correct the \( P \) values. The threshold of significance was defined based on its \( P \) value.

Quantitative RT-PCR

To validate the microarray results, quantitative RT-PCR analysis of selected lncRNA genes was performed using in total 12 different ocular tissue samples that were prepared following the aforementioned procedures and were different than those used in the microarray experiments. For reverse transcription, first-strand cDNA was synthesized using a reagent kit (Prime-Script RT; TaKaRa, Dalian, China) following the suggested instructions provided by the manufacturer. For confirmation of the signatures of differentially expressed lncRNAs in each ocular tissue from the mice of two different ages, diluted cDNA was then used as an input for quantitative RT-PCR performed with a SYBR Green PCR master mix (Life Technologies, Washington, DC, USA) on a commercial PCR system (7500 Fast Real-Time PCR; Applied Biosystems, Foster City, CA, USA). The specificity of each amplified product was evaluated by measuring their melting curves at the end of each amplification reaction. Gapdh or \( \beta \)-actin expression levels were used as a reference for lncRNA expression normalization, and the \( qRT-PCR \) results were analyzed by the \( 2^{-\Delta\Delta CT} \) method and expressed relative to threshold cycle (CT) values and then converted to log₂-fold changes. Each experiment was carried out at least in quadruplicate. The primers used in quantitative PCR are listed in Supplementary Table S2.

RESULTS

Overview of lncRNA Expression Profiles in Murine Eye Tissues

To assess lncRNA expression patterns in the murine eye, we first isolated RNA from six ocular tissue subsets (i.e., cornea, lens, retina, RPE, choroid, and sclera) from both newborn and 8-week-old adult mice (Fig. 1A). We then analyzed protein-coding gene and ncRNA expression profiles using a mouse transcriptome microarray. Our workflow for postnatal and tissue-specific lncRNA screening, validation, and further bioinformatic analyses is shown in Figure 1A. Summary statistics of the distribution of normalized microarray data in each sample is displayed as a box plot (Supplementary Fig. S1). Only genes satisfying the criterion (see Materials and Methods) were taken as candidates for the following bioinformatic analyses. As a result, a total of 47,332 protein-coding and
noncoding gene transcripts were identified (log2 signal value >5) in newborn and adult mouse ocular tissues. LncRNAs comprise 19,313 of these transcripts that are annotated in public data banks. On the basis of their genomic location relative to protein-coding genes, we classified the detected lncRNAs into the following five categories: sense, antisense, bidirectional, intronic, and intergenic lncRNAs (lincRNAs), and their numbers are shown in Figure 1B, respectively. Intronic lncRNAs and lincRNAs comprised a major proportion of total lncRNAs. The top 50 of the most highly expressed lncRNAs in each ocular tissue subset are shown in Supplementary Table S3. The most highly expressed lncRNAs overlapped with one another in the six tissue subsets. Some of the overlapping lncRNAs shared by all or most of these tissue types may reflect fundamental underlying similarities amongst the different tissue subsets during the period between birth and postnatal 8 weeks of age. In all tissues examined—especially cornea, lens, and RPE—there was apparent overlapping of highly expressed lncRNAs, which may reflect a commonality in maintaining epithelial integrity and regulating epithelial function.

Identification of Age-Dependent IncRNA Signatures in Murine Ocular Tissues

To determine if there are differences in IncRNA expression patterns between mice at birth and postnatal 8 weeks of age, dynamically regulated IncRNA profiles and genomic locations were obtained from these two different stages. This was done by setting the expression level threshold at a fold-change of ≥2.0 and the background threshold at 5 to optimize detecting bona fide active gene readout. The IncRNA expression patterns varied significantly between two samples of the same tissue type in the two different age groups. During the transition from birth to 8 weeks of age, more than 1000 of the known lncRNAs underwent ≥2-fold changes in their expression levels among each of the six different tissue subsets. For example, when comparing the expression patterns between the two age groups, 1870 lncRNAs in the 8-week-old mouse retina exhibited significantly altered expression levels from those in the P0 group. They included 992 upregulations and 878 downregulations (for other tissues, see Supplementary Fig. S2). Accordingly, more lncRNA genes were differentially expressed than protein-coding genes among the six ocular tissue subsets, as shown in Figure 2A. When we categorized transcripts on the basis of their age dependent-specific expression patterns, as observed in various samples, we found that the dynamically expressed lncRNAs generally displayed lower expression levels than protein-coding genes in most ocular tissues (Fig. 2B). To confirm our signature data, we assessed expression of the signature lncRNAs among the analyzed tissues by quantitative RT-PCR analysis. At least six of the distinctive lncRNAs in each interactome, were randomly selected for quantitative RTPCR analysis. There was a high level of consistency between the two procedures used in identifying lncRNAs contributing to development (Fig. 2C).

Delineation of Tissue-Specific IncRNA Signatures in Murine Ocular Tissues

Appropriate eye development depends on precise coordination and integration of morphogenetic and cell differentiation events. As lncRNAs are generally more species and tissue specific than protein-coding genes, we next assessed the ocular tissue-subset specificity of lncRNAs. We therefore sought protein-coding and lncRNA genes whose expression levels in a given subset differed by ≥2-fold from those in all other tissues obtained from the same age mice. These genes were classified according to their expression profiles by K-means clustering. This procedure resolved 910 IncRNAs plus 627 protein-coding genes from P0 groups, and 686 lncRNAs plus 418 protein-coding genes from the postnatal 8-week-old groups (Fig. 3, Supplementary Fig. S3). Furthermore, this analysis revealed distinct tissue-specific IncRNA clusters which are consistent with previous observations that IncRNA expression profiles display greater tissue specificity than mRNAs. As observed by comparing RNA expression patterns between P0 and 8 weeks of age, there are tissue-specific signature IncRNAs whose expression levels were also generally lower than those of the protein-coding genes (Supplementary Fig. S4). Similar patterns of expression variation among other tissues have been described in numerous studies. Furthermore, we found that most of these tissue-specific IncRNAs were expressed at much higher levels than in other tissues (Fig. 3; Supplementary Table S4). The numbers of shared and distinct lncRNAs or protein-coding genes among the same tissues are displayed in Venn diagrams (Fig. 4), which show that protein-coding genes constitute a larger proportion than the IncRNAs in the overlapping transcripts in each tissue. This difference might suggest that the expression profiles of tissue-specific mRNAs are less variable than those of lncRNAs between P0 and postnatal 8 weeks of age. Thus, based on the results of transcriptome microarray analyses of different ocular tissue subsets, we are able to provide a comprehensive landscape of IncRNA expression in murine eye tissues, which also suggests that IncRNA expression patterns are relevant markers of ocular tissue identity.

Bioinformatic Functional Analyses of the IncRNA cis-Associated Genes

Since lncRNAs can regulate the expression of neighboring genes, we then sought to identify potential protein-coding genes proximal to the lncRNAs characteristic of specific ocular tissues. To make this assessment, we performed cis analysis on all of the significantly altered known IncRNAs by mapping those less than 10 kb away from protein-coding genes in the mouse genome, and then applied GO analysis to these targets. Six IncRNA signatures present in P0 and 8-week-old postnatal mice in each of the six tissue subsets were first subjected to cis analysis, followed by GO enrichment analysis. Cluster analysis revealed subgroups of IncRNAs enriched in each of the above tissues. Such enrichment may be indicative of synteny between their constituents needed for cell fate commitment and stabilization in these six tissue subsets during their maturation into adulthood.

We then found that protein-coding genes adjacent to the genomic loci of the featured IncRNA showed enrichment in several GO biologic process terms, which were highly correlated with their own tissue-specific functional maturation between P0 and 8 weeks of age in the six tissue subsets (Fig. 5; Supplementary Fig. S5, left). This agreement suggests a possible role for featured IncRNAs in the six tissue subsets. We list here the 15 most enriched GO biological process terms in maturing retina which could indicate a close relationship of prominently expressed IncRNAs with genes encoding mRNAs that mediate control of neural development (Fig. 5, left). We also applied a similar analytical technique to identify tissue-specific IncRNA signatures. Our results indicate that enriched GO biological process terms are highly associated with tissue specificity at both the P0 and postnatal 8 weeks of age (Fig. 5; Supplementary Fig. S5, middle and right, respectively). For example, the 15 most enriched terms in the retina are largely involved in controlling elements such as cone cell development, axonogenesis, and neuronal...
development, suggesting that these lncRNAs could possibly contribute to determining retinal cell specificity and visual function (Fig. 5). Despite limited characterization of the functional roles of the numerous lncRNAs in the nervous system, their narrower expression pattern diversity and close proximity to genomic sites controlling neuronal responses suggests that they may be involved in regulating neuronal development. Regarding GO enrichments of other tissue-specific lncRNA signatures shown in Supplementary Figure S5, these results are supportive of the reliability of our analysis. Taken together, identified GO enrichments for specific processes are in agreement with known functions of ocular tissues in which the lncRNAs are predominantly expressed. This correspondence suggests that lncRNA cluster expression data analysis provides insight into the possible specific functional processes regulated by lncRNA-mRNA networks within the eye.

LncRNAs can act as cis regulators of their neighboring genes and their genomic location identifies possible functional lncRNA-mRNA relationships.30 Here we chose 12 tissue-specific lncRNA-mRNA pairs to identify potential lncRNAs mediating cis-regulation. In these lncRNA-mRNA pairs, both the lncRNA and mRNA were significantly altered, and shared similar expression pattern in six tissue subsets (Fig. 6). The abundance of these lncRNA-mRNA pairs is suggestive of their possible tissue specificity. Such correlation of lncRNA expression patterns with their neighboring genes constitutes a common feature of lncRNAs.
DISCUSSION

Mammalian genomes encode more lncRNAs than any other type of ncRNAs, and the identification of functional lncRNAs involvement in biologic processes is progressively increasing. As there are a very limited number of studies describing their contribution to mammalian eye development and disease, we provide a comprehensive landscape of lncRNAs in six distinct murine ocular tissues at two different maturation stages. To the best of our knowledge, this is the first combined analyses of lncRNAs in the main ocular tissues between P0 and 8-week-old mice, which allows for a direct semiquantitative assessment of tissue-specific differential lncRNA expression. In addition, we investigated the homology between mouse and human lncRNA transcripts listed at NONCODE (http://www.noncode.com/download.php; in the public domain). Only 651 lncRNAs (out of the 19,315 lncRNAs) were found to be conserved with the human lncRNAs by setting the alignment E value of BLASTN <10^{-5}. The sequence conservation of lncRNA genes between human and mouse (i.e., ~3.4%) is much less than that of protein-coding genes. Its low value, in accord with numerous other reports, suggests that these unique ncRNAs have species-specific roles in controlling gene expression and therefore organismal complexity.
A total of 218 highly expressed lncRNAs (log2 signal value >9) were detected in each subset, whereas only 25 of them were evolutionarily conserved (~11.5%). Among these conserved lncRNAs, six of them were also among the top 30 commonly highest expressed lncRNAs in ocular tissues (~20%). This commonality warrants additional evaluation in future studies to establish whether these lncRNAs play important roles in contributing to mammalian eye development.

As lncRNA signatures are both tissue and species specific, gaining meaningful insight into their tissue-specific roles during maturation is dependent on assessing their expression profiles (as well as those of any highly specific transcripts) in a series of tissues during postnatal maturation rather than in only one tissue at one time. Our analysis demonstrates that lncRNA expression patterns exhibit distinct changes during maturation between P0 and 8 weeks of age as well as tissue specificity within the cornea, lens, retina, RPE, choroid and sclera, which is consistent with the idea that lncRNA signatures may be important in maintaining tissue identity.

Previous studies have identified several lncRNAs that are uniquely expressed in developing murine ocular tissues. For example, retinal noncoding RNA 4 (Rncr4) was reported to be specifically expressed in maturing mouse photoreceptors and acts as a stimulator in pri-miR-183/96/182 processing.34 Here we also found that expression of this retina-specific lncRNA was restricted to the 8-week-old adult mouse retina. Maternally expressed gene 3 (MEG3) encodes a lncRNA, which is preferentially expressed in both newborn and adult mouse retina as well as sclera, was suggested to be associated with diabetic retinopathy.35 Another well characterized lncRNA, Malat1, is a commonly highly expressed regulator in each ocular tissue.36 Its widespread expression suggests that it may be involved in regulating fundamental biologic processes such as cell proliferation in murine eyes. A previous study showed that Malat1 functioned as a ceRNA and was upregulated in diabetic retinas.17 Our microarray data clearly shows that the
expression level of this ceRNA is much higher in newborn mouse retina than in either adult mouse retina or other tissues. All of our results agree with previous studies obtained using in situ hybridization and confirm the reliability of our microarray data.

As some lncRNAs exhibited tissue-specific patterns of expression during postnatal maturation, they may have essential roles in regulating this process. Indeed, our study provides additional support of high tissue-specificity of lncRNAs, because both age-dependent and tissue-specific lncRNA expression was restricted to a given subset. Notably, we found that lncRNAs expression patterns were more specific for the maturing process and tissue identity better than protein-coding gene expression patterns. By examining patterns of lncRNA coexpression with mRNAs eliciting a known function, we found strong enrichment for ocular tissue-related functions among all identified lncRNAs. This was most apparent for the lncRNAs that are differentially expressed in the retina. Our findings clearly demonstrate that lncRNAs exhibit distinct tissue and cell-type specificity. This is consistent with the known regulatory function of lncRNAs in ocular development and functional maintenance.

Moreover, we selected 12 pairs of tissue-specific lncRNAs and their highly related neighboring protein-coding genes that may have important roles in establishing tissue specificity. For example, in comparison to other tissues, the antisense lncRNA NONMMUT011969 and its potential target gene Krt12 are very highly expressed in the cornea. This association indicates that this cornea-specific lncRNA may be involved in regulating corneal epithelium differentiation as well as Krt12 expression, which is a definitive corneal epithelial biomarker. Another such pair is the very highly expressed cornea-specific lncRNA NONMMUT026302 and its cis target Muc4. This association suggests that this lncRNA may be involved in the protection of ocular surface epithelia and tear stability since Muc4 has this function. According to lens-specific lncRNA and mRNA pairs, antisense lncRNA ENSMUST00000180407 and its neighboring gene major intrinsic protein of lens fiber (Mip), antisense lncRNA Prox1os and its neighboring gene Prox1, are notably accumulated in newborn and adult lens. Mip is also known as Aquaporin-0, a lens fiber cell membrane protein, which has a dual function as a water channel protein and provides adhesion for maintaining fiber organization. These functions are both critical for lens integrity and transparency maintenance. Prox1, a transcription factor as well as a lens fiber cell marker, plays a key role in controlling lens fiber differentiation and gene expression. Our results suggest that their partner lncRNA ENSMUST00000180407 and Prox1os may be critical in maintaining the normal structure and function of the lens fibers. With regards to the retina, two retina-specific intronic lncRNAs NONMMUT015187 and NONMMUT019464 are in close proximity to their respective neighboring genes Nrxn3 and Hcn1. Their gene products exert critical functions in mediating nervous system or retinal cone cell development. In addition, a choroid-specific lncRNA NONMMUT023354 and its neighboring gene Angpt1 may function in the control of angiogenesis or vasculogenesis in the choroid. Two sclera-specific NONMMUT058728 and NONMMUT009498 lncRNAs along with their respective target genes Mfap5 and Sgcd, are probably involved in scleral extracellular matrix organization.

To precisely identify functional lncRNA is a daunting task due to the intricate molecular mechanisms underlying lncRNA coupled with a lack of a universal experimental approach to characterize in vivo biological function of a specific lncRNA. Nevertheless, there are still some strategies that can be followed for this purpose. LncRNA function can be initially assessed through loss-of-function and gain-of-function studies, using cell-based assays. To further characterize the in vivo function of lncRNAs, there are several different genetic strategies that can be employed. A canonical loss-of-function approach involves genetically deleting the candidate lncRNA locus or inserting a certain DNA fragment to inactivate a candidate lncRNA. Another loss-of-function approach is to knockdown the expression of candidate lncRNA using a RNAi-based technique. This approach is indispensable under some circumstances when it is extremely difficult to disentangle possible overlapping molecular contributions by a candidate lncRNA from its genomic DNA locus. The in vivo gain-of-function approach to directly identify functional lncRNA can be achieved by using transgenic technology, as reported in the study of Tmevpg1 lncRNA in T cells. In considering the limitation of any particular method, it is wise to integrate multiple approaches to decipher the responses controlled by a specific lncRNA. This particular strategy was used to characterize HAUNT lncRNA involvement in embryonic stem cell differentiation. Hence, we will use such strategy in a future study to elucidate the biologic functions of our identified lncRNAs in eye development and physiology.
Over all, our study highlights the potential importance of lncRNAs in ocular postnatal maturation and provides a valuable resource for follow-up investigations aimed at understanding the importance of lncRNA expression pattern changes occurring under both physiologic and possibly pathophysiologic conditions in mouse eyes.

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