Evolutionarily conserved long intergenic non-coding RNAs in the eye

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The discovery that the mammalian transcriptome encodes thousands of long intergenic non-coding (linc) RNA transcripts, together with recent evidence that lincRNAs can regulate protein-coding genes, has added a new level of complexity to cellular transcriptional/translational regulation. Indeed several reports now link mutations in lincRNAs to heritable human disorders. Here, we identified a subset of lincRNAs in terminally differentiated adult human retinal neurons based on their sequence conservation across species. RNA sequencing of eye tissue from several mammalian species with varied rod/cone photoreceptor content identified 18 lincRNAs that were highly conserved across these species. Sixteen of the 18 were conserved in human retinal tissue with 14 of these also conserved in the macular region. A subset of lincRNAs exhibited restricted tissue expression profiles in mice, with preferential expression in the retina. Mouse models with different populations of retinal cells as well as in situ hybridization provided evidence that these lincRNAs localized to specific retinal compartments, most notably to the photoreceptor neuronal layer. Computational genomic loci and promoter region analyses provided a basis for regulated expression of these conserved lincRNAs in retinal post-mitotic neurons. This combined approach identified several lincRNAs that could be critical for retinal and visual maintenance in adults.

INTRODUCTION

Genetic complexity in the ocular transcriptome of the adult retina (1) allows the encoding of a diverse set of proteins that support vision (2). However, work over the last few decades has revealed that non-coding RNAs can have a profound effect on the cellular transcriptional and translational landscape. Small non-coding RNAs such as microRNAs, first discovered in 1993 (3), were later reported in 2000 to have a universal post-transcriptional role across species (4) through translational regulation (5). Advances in next-generation sequencing along with closer examination of the human (6,7) and mouse (8) genomes revealed that the mammalian genome encodes thousands of long non-coding RNAs (9), including over 8000 long intergenic non-coding RNAs (lincRNAs) in the human genome (10). Transcribed from genomic loci flanked by two protein coding genes, lincRNAs are over 200 nucleotides long, undergo typical mammalian RNA processing involving 5′ capping, poly-adenylation and splicing, but have no protein-coding capability (11–13). LincRNAs reportedly regulate transcription of protein-coding genes by guiding and tethering chromatin modifying complexes to specific genomic loci in a trans-regulatory manner (13–15), but the precise mechanisms are still largely unknown as lincRNAs have also been shown to work in a cis-regulatory manner (16). Moreover, lincRNAs show a broad sub-cellular compartmentalization (17,18) implying that their functional contribution may occur at both the transcriptional and post-transcriptional levels. With increasing association of long non-coding RNAs with human disease (19–21) and recent evidence of lincRNAs related to Mendelian disorders and neurodevelopmental disabilities (22), there is a pressing need to understand the cellular roles of these molecules.

An importance of lincRNAs is supported by their highly tissue-specific expression (11) and location close to protein-coding genes associated with development and transcriptional...
regulation (16,23). Model organisms in which to study lincRNAs (24,25) together with emerging technologies (26) and collaboration with computational methods (27,28) will aid in delineating the cell-specific roles of these transcripts that are not only conserved at the sequence level, but also at the secondary structure level (29). But with no high-throughput methodology yet to assess lincRNA function, there exists a need to first identify and then filter lincRNAs in a cell-specific context. Identifying lincRNAs that display evolutionary sequence conservation across species in a particular tissue provides a first step in this endeavor.

The presence of lincRNAs in the adult retina would suggest a role in mediating vision in humans, a process dependent on strict transcriptional control of genes specific to terminally differentiated photoreceptor neurons. LincRNAs have been shown to influence both circadian control in the closely related pineal gland (30) and early stages of retinal development (31–36). The possible physiological roles of lincRNAs in post-mitotic neurons of the adult retina have yet to be investigated. Thus we carried out RNA-Sequencing (RNA-Seq) of eye tissue from a diverse set of adult mammalian species to document the conservation profile of lincRNAs relative to those of human retinal and macular tissue.

RESULTS

RNA-Seq identifies eye lincRNAs that exhibit sequence conservation in mammals and those that exhibit conservation in the human retina and macular region

We carried out RNA-Seq with eye tissue from a diverse set of mammalian species with varied retinal photoreceptor cellular compositions and distributions. We first analyzed three biological replicates of whole eye tissue from mice with three distinct genetic backgrounds: A/J, BALB/c and C57BL/6J (B6) (37). An RNA-Seq experiment of a retinal sample from B6 mice (38) was also included to guide transcript localization. Expression values were calculated as fragments per kilobase per million reads (FPKM) (39). With an expression cut-off of 1 FPKM, 103, 83 and 109 lincRNAs were detected in A/J, BALB/c and B6 mouse eyes, respectively, of which 48 were consistently expressed (Fig. 1A). These 48 lincRNAs were then investigated for conservation at the sequence level in eye tissue from three rodents with a range of photoreceptor populations and behavior patterning, namely Rattus norvegicus (rat, nocturnal, ~1% cones), Arvicanthis niloticus (Nile rat, diurnal, ~33% cones) and Ictidomys tridecemlineatus (ground squirrel, diurnal, ~97% cones), as well as the higher order mammal, Macaca fasicularis (monkey, diurnal, 5% cones, macula present). Data were analyzed by quality trimming the reads from each species and mapping them to the 48 consistently expressed mouse lincRNA sequences.

Using this methodology, we found 18 mouse lincRNAs to be conserved in the eye across all species examined (Fig. 1A and B). Many of these lincRNAs exhibited extremely high sequence conservation and further examination revealed that 16 of the 18 lincRNAs were also conserved in human retinal tissue subjected to RNA-Seq analysis (Fig. 1C). The conservation profile of lincRNAs in the eye and retina of the species (Supplementary Material, Tables S1 and S2) indicated that most localized to the retina, a perception further supported by examining the expression profile in B6 mice (Table 1). Although high-order mammals like monkeys and humans have only about 5% cone photoreceptors in their retina (40), a central region of the retina termed the macula, most specifically the fovea, is rich in cone photoreceptors and responsible for visual acuity and high-resolution vision (41). By specifically sequencing the macular region from four monkey biological replicates, we found that 14 of the 18 conserved lincRNAs across species were present (Fig. 2 and Supplementary Material, Table S3). Next, we probed these 18 transcripts in greater detail to determine their tissue-specific expression profiles in B6 mice.

Tissue and eye compartment expression of conserved lincRNAs

Expression profiles of the 18 conserved lincRNAs were investigated by isolating total RNA from a range of tissues in B6 mice and subjecting them to real-time polymerase chain reaction (RT-PCR) analysis. Eye, brain, heart, liver and lung tissue were chosen for their cellular complexity and diversity of biological function. Eye compartments including the lens/cornea, retina and retinal pigmented epithelium (RPE)/choroid were also examined separately. Specific primers used to amplify the product sequence of lincRNAs were subjected to Sanger sequencing which revealed 100% sequence overlap with the annotated sequence in Ensembl. Semi-quantitative RT-PCR (sqRT-PCR), which allowed us to establish transcript presence and absence at optimized cycles, showed the tissue enrichment profile of these lincRNAs (Supplementary Material, Table S4). These results were further evaluated with quantitative RT-PCR (qRT-PCR) of 6 selected lincRNAs that displayed broad organ and eye tissue distribution in expression profiles (Fig. 3). The sqRT-PCR and qRT-PCR results were in good agreement, with subtle differences attributable to the use of different custom primer sets for the experiments. A subset of lincRNAs evidenced expression restricted to just a few organs and specific eye compartments. Targets such as EyeLinc1 and EyeLinc4 showed enrichment in just a few tissues, whereas others like EyeLinc17 were expressed in most tissues assayed. Although many lincRNAs showed preferential expression in the retinal compartment of the eye, we sought to determine whether they were localized to specific retinal cell types.

Expression of some conserved lincRNAs is localized to specific retinal layers

To localize lincRNAs in the retina, we first used mouse models with different rod/cone ratios, a methodology previously employed to localize microRNAs to cellular components of the retina (42). Whereas in B6 mice all cellular components of the retina were preserved, in Cone-DTA+/− mice (Cone-DTA) cone photoreceptors were absent but rod photoreceptors were preserved (43), in Nrl−/− mice rod photoreceptors were absent and only cone-like photoreceptors were
present (44), and in aged (7-month-old) P23H mice there were no photoreceptors detected but other layers of the neural retina were retained (45) (Fig. 4A). Total RNA extracted from eyes of these mice was then subjected to RT-PCR analysis for lincRNA expression levels. The sqRT-PCR (Supplementary Material, Table S5) results, further quantified by qRT-PCR of selected targets (Fig. 4B), suggested possible localization of the lincRNAs to specific retinal layers. Enriched expression of EyeLinc2 and EyeLinc7/8 in B6 and Cone-DTA mice together with greatly reduced expression in Nr1−/− mice and some residual expression in P23H mice suggested their possible localization to the photoreceptor layer, as this pattern resembled the profile of Abca4 expression, with a gene product that resides in photoreceptor discs (46). EyeLinc14 displayed higher expression in B6 and Cone-DTA mice compared with reduced expression in Nr1−/− and P23H mice.
Markedly reduced EyeLinc14 expression in the latter two mouse models suggests that this lincRNA primarily localizes to both rod photoreceptors and other retinal cellular compartments such as the RPE, in agreement with the results shown in Figure 3.

To further investigate the lincRNA localization inferred from these mouse models, we carried out in situ hybridization of EyeLinc2, EyeLinc7/8, EyeLinc14 and EyeLinc17 in the mouse retina by establishing a new methodology in frozen mouse retinal sections that allowed sensitive single-molecule RNA detection with almost no background. Mouse ubiquitin c (Ubc) was used as a positive control, whereas E. coli dihydrolipocolinate reductase (dapB) was used as a negative control for these hybridization assays. All lincRNAs were detected above the negative control background and signals in the photoreceptor regions were localized to the inner photoreceptor cell segments (Fig. 4C). EyeLinc14 was notably detected in both the photoreceptor inner segment and RPE layers, consistent with localization findings from previous PCR experiments shown in Figures 3 and 4B. Therefore, the localization of these lincRNAs to specific retinal compartments, especially their enrichment in the neuronal photoreceptor cell layer, suggests that lincRNAs could have an important role in supporting vision. Next, we carried out a computational analysis of the conserved lincRNA loci and analyzed their promoter regions for binding motifs of transcription factors.

**DISCUSSION**

The hypothesis that long non-coding RNAs in intergenic regions of the human genome possess functional roles (54) in normal homeostasis of post-mitotic retinal neurons and that their disregulation can lead to human diseases (55) necessitates a detailed understanding of these transcripts. The sheer number of the lincRNA population makes it difficult to elucidate their physiological contribution to a specific tissue, but a first step would be to identify those that are conserved across species in specific tissues. Using high-throughput RNA-Seq methodology, we identified 18 lincRNAs in the adult eye that showed sequence conservation across a diverse range of species, from nocturnal rod-dominant rodents such as mouse.
Figure 3. LincRNAs display spatially restricted expression in adult B6 mouse organs and eye compartments. Semi-quantitative RT-PCR of all 18 conserved lincRNAs revealed tissue-specific expression, such as for EyeLinc14, which not only revealed enriched expression in the eye relative to other organs but was also more preferentially expressed in the retina and RPE/choroid. Rpe65, a gene expressed only in the RPE of the eye, is shown as a positive control, whereas Actin was used for a loading control. The heatmap below shows qRT-PCR results for six selected lincRNAs and two positive controls (Opin1sw and Rpe65) in the different organs and eye compartments. EyeLinc7 and EyeLinc8, identical in sequence from a duplication in the X chromosome, are listed as EyeLinc7/8. Areas are shaded according to the relative levels of transcription normalized to the tissue compartment with the highest expression levels ranging from 0% (white, undetectable) to 100% (black, highest).

and rat, to cone-dominant diurnal rodents such as the Nile rat and ground squirrel, to the diurnal monkey which like humans, possesses a cone-rich macula and rod-rich peripheral retina. Moreover, 16 of these 18 lincRNAs were found conserved in the human retina with 14 of these 18 also conserved in the macular region of the retina. A subset of these lincRNAs exhibited expression restricted to certain tissues in the mouse. Mouse models with different retinal cell populations along with in situ hybridization were used to further localize these lincRNAs to specific cellular layers of the retina, most notably the neuronal photoreceptor layer. Complementation of the cellular work with computational analysis of transcription factor-binding site motifs of the 18 conserved lincRNAs was a critical step in revealing a contribution of these transcripts in retinal homeostasis. In the absence of proper retinal cell lines to accurately identify in vitro functionality of these transcripts, more detailed roles of individual lincRNAs should become evident from cellular phenotypes that result when the cohort of conserved lincRNA loci identified in this work are disrupted.

Evolutionary pressure drives rapid sequence alterations of lincRNAs even in closely related species (56) highlighting the functional importance of those transcripts that remain conserved across species (57). Identification of 18 conserved lincRNAs from the initial 3133 mouse lincRNAs agrees with past studies that only a small minority of lincRNAs in the mouse or human have transcribed orthogonal sequences in the other species (11). Identification of conserved lincRNAs that persisted despite evolutionary pressure across the diverse adult species studied (Figs 1 and 2) implies that these transcripts could potentially coordinate essential transcriptional/translational roles in adults when all retinal components are fully developed. These conserved lincRNAs exhibited a 100-fold expression range in the B6 mouse eye and retina (Table 1), but even those marginally expressed lincRNAs could fulfill vital roles such as basal regulation of protein-coding genes (55).

Closer examination of these 18 lincRNAs in B6 mice revealed that whereas some transcripts such as EyeLinc17 displayed ubiquitous expression in the several tissues assayed, others such as EyeLinc14 displayed tissue-restricted expression. EyeLinc14 not only displayed enriched expression in the eye relative to other organs assayed, but it also was preferentially located in the retina and RPE, two layers that intimately collaborate to drive vision. Expression in tissues outside the eye does not preclude those lincRNAs from having retina-specific roles. Indeed Tug1, a non-coding RNA shown to be important at early stages of photoreceptor development, displayed robust expression in tissues besides the eye (35). As to the retina, we first localized lincRNAs in mouse models with subtle differences in retinal architecture (Fig. 4A and B) that had been previously used to localize non-coding RNAs (42). In situ hybridization (Fig. 4C) was then used to localize certain lincRNAs to specific retinal compartments, most notably to the neuronal photoreceptor and RPE layers of the retina.

Complementation of the above cellular studies with computational analysis of the genomic loci and promoter regions of all conserved lincRNAs provided insights into their possible roles in retinal homeostasis (Fig. 5). EyeLinc2 exhibited an enrichment and localization pattern suggestive of photoreceptor expression (Fig. 4B and C) and lies adjacent in the mouse genome to Abca4 (Fig. 5A), an essential photoreceptor protein product for removal of toxic retinoid metabolites (58) that can cause severe retinopathies if mutated (59, 60). Promoter analysis of EyeLinc2 revealed that it possesses binding sites for HMG-I(Y), which is rarely expressed in terminally differentiated cells, but it is enriched in photoreceptor cells and thought to accommodate the daily induction of phototransduction and visual cycle genes such as Abca4 (48). Consistent with the diurnal nature of gene expression in the retina, HMG-box proteins evidence diurnal rhythms in photoreceptors (61), and EyeLinc2 also displayed temporal changes in expression levels with a 1.7-fold elevation in the afternoon when compared with the morning (Fig. 5A). Temporal cycling of lincRNAs in the eye adds another dimension to their possible regulatory function (30). Examination of other lincRNAs revealed promoter regions for genes such as Pax4 and CTCF. Unlike the master regulator homeobox-containing Pax6 involved in eye morphogenesis (62), both Pax4 (50) and CTCF (52) are developmentally segregated and display highest expression in photoreceptor cells of the adult retina. Pax4 is thought to regulate gene expression in the mature retina (50) and can activate expression of rod-derived cone viability factor (63), a novel trophic factor that can protect cone photoreceptors from degenerating (64) and thus serve as alternative therapy for patients with retinitis pigmentosa (65). Meanwhile, recent evidence revealed that CTCF regulates the ataxin-7 gene through interactions with a non-coding RNA (53). Dysfunction in ataxin-7 leads to spinocerebellar ataxia type 7 and has been shown to produce photoreceptor dysfunction (66, 67) and retinal degeneration (68). Moreover, overlapping disease pathways recently demonstrated for this neurodegenerative disorder and age-related
Macular degeneration (69) highlight that these lincRNAs can have homeostatic roles beyond the retina. Thus, our in silico analysis highlights potential involvements of these conserved lincRNAs, not only in the maintenance of adult retinal homeostasis, but also in interacting with key retinal transcription factors for potential therapy of human retinopathies.

The dynamic process of vision requires a high level of expression to maintain photoreceptor specific genes that carry out phototransduction and the visual cycle (2), a process potentially affected by lincRNAs (70). This study suggests how in adult terminally differentiated post-mitotic retinal photoreceptor cells, lincRNAs could play a critical role in physiology of these cells. As understanding of the transcriptional landscape in human cells improves (71), accurate assembly of lincRNAs in tissue-specific contexts through high-throughput sequencing approaches constitutes the first step to identifying those transcripts that are likely to be important and warrant more detailed investigation. With increasing evidence that long non-coding RNAs such as lincRNAs are associated with common diseases (72) and constitute potential drivers of cancerous states (73), it is imperative to investigate those that could influence such pathology. That these transcripts can be targeted in mouse models of human disease to correct pathological states (74) also provides hope that their improved understanding will shape future therapeutics.

**MATERIALS AND METHODS**

**Eye and retina tissue collection**

A/J, BALB/c, C57BL/6 (B6) and Cone-DTA strains of mice, all 4 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). P23H knock-in mice generated in our laboratory (45) were 7 months of age. Four-week-old Nrl- deficient mice with a B6 background were obtained from Dr Anand Swaroop (University of Michigan, Ann Arbor, MI, USA). Long-Evans rats (Rattus norvegicus), 4 weeks of age, were purchased from Harlan Laboratories (Madison, WI, USA). Nile rats (Arvicanthis niloticus), 6
weeks of age, were obtained from the laboratory of Dr Laura Smale (Michigan State University, Lansing, MI, USA). Wild caught adult ground squirrels (Ictidomys tridecemlineatus) were purchased from TLS Research (Bloomingdale, IL, USA). Mice, rats and ground squirrels were housed in the Case Western Reserve University (CWRU) animal facility where they were maintained on a standard chow diet in a 12 h light (≏10 lux)/12 h dark cycle. After euthanizing animals, eye and retina tissue were collected and placed in a solution of RNAlater (Qiagen, Valencia, CA, USA) for processing. Enucleated macaque (Macaca fascicularis) eyes in RNAlater from 4-year-old animals were obtained from Ricerca Biosciences (Painesville, OH, USA). Clinical evaluations of the human patient from whom retinal tissue was obtained were carried out at the Cleveland Clinic Cole Eye Institute (Cleveland, OH, USA). This research conformed to the tenets of the Declaration of Helsinki. The retina was carefully dissected out of an untreated eye from a patient requiring enucleation for a large ocular melanoma and immediately placed in RNALater; the retinal sample was obtained from a hemi-retina free of tumor. This eye had no abnormal neovascularization of the iris or retina and lacked signs of inflammation.

Library preparation and Illumina RNA-Seq runs

Eye and retina tissue libraries were prepared as previously described (37,38). Each mouse library was run on the Illumina Genome Analyzer IIX (Illumina, San Diego, CA, USA) in the CWRU Genomics core facility using 36–79 bp single-end read lengths. The processed and raw fastq files were previously deposited in GEO (accession numbers GSE38359 and GSE29752). Prepared libraries of Long-Evans rat eye and retinal tissues and human retinal tissue were sequenced by single-end sequencing, whereas prepared libraries of Nile rat, ground squirrel and macaque eye and retina tissue were sequenced by paired-end sequencing technology with the Illumina Genome Analyzer IIX or HiScan SQ.

Expression of lincRNAs in mouse eye and retina

Sequence information of 3133 identified lincRNAs in the mouse was extracted from Ensembl release 67. The Illumina reads from each mouse replicated tissue sample were processed separately. A quality trimming step was performed to remove bases from both ends with quality scores equivalent to a Phred quality score lower than 20. Only reads with 20 bases or longer after such trimming were discarded. Reads from technical replicate
lanes were combined before being mapped to the mouse lincRNA sequences with TopHat v2.0.0 (75) and the resulting alignment files were processed with custom Perl scripts. Alignments with large (>3 base) insertions or deletions or reads of low complexity (≥80% of the reads were di-nucleotide repeats or contained 8 or more consecutive A- or T-bases) were removed before the number of original reads and unique reads aligned to mouse lincRNA transcripts were counted. Portions of mouse lincRNA transcripts covered by reads were calculated and presented as bases and portions of the entire length of the transcript. If a mouse lincRNA transcript was aligned to five or fewer unique reads from a sample or the read coverage of the lincRNA transcript was <10%, then the mouse lincRNA transcript was not considered to be conserved in that species sample.

Promoter analysis
Each 5 kb promoter sequence of the 18 conserved lincRNA transcripts was searched with FIMO (76) for motifs in the JASPAR CORE 2009 database (http://jaspar.genereg.net/) and only those that returned statistically significant P-values with a position-specific scoring matrix for each of the motifs in the promoter sequences were considered. The 5 kb promoter sequences of all annotated genes in mouse genome release mm9 were also searched for motifs in the JASPAR CORE 2009 database. Motif enrichment in the promoter regions of the 18 conserved lincRNAs compared with genome-wide promoter regions was assessed with the Fisher’s exact test using a Bonferroni correction.

Immunohistochemistry
All procedures used were reported previously (77). Cross-sections of mouse eyecups were incubated with primary antibodies, namely anti-mouse RPE65, biotinylated peanut agglutinin (PNA) and wheat germ agglutinin (WGA). Signals were detected with Cy3- or Alexa488-conjugated secondary antibody. Nuclear staining was achieved with 4′,6-diamidino-2-phenylindole (DAPI). Sections were analyzed with a Leica TCS SP5 II confocal microscope (Leica, Wetzlar, Germany).

In situ hybridization
The QuantiGene ViewRNA (Affymetrix, Santa Clara, CA, USA) in situ hybridization protocol was optimized by Affymetrix for use with fresh frozen mouse eye tissue samples. The protease concentration was increased 4-fold from a 1:100 to a 1:25 dilution and the incubation time was set at 40 min from the standard protocol for optimized signal strength and tissue morphology preservation. Slides were incubated with either EyeLinc2 (VB1-13468), EyeLinc7/8 (VB1-13469), EyeLinc14 (VB1-13470) or EyeLinc17 (VB1-13471) Fast Red probe sets for detection with a Leica TCS SP5 II confocal microscope. Slides incubated with the mouse Ubc (VB1-10202) Fast Red probe set were used for a positive control, whereas slides incubated with the E. coli dapB (VF1-10272) Fast Red probe set, employed as a negative control, were used to establish a background signal for the assay. Rat kidney FFPE tissue slides as a positive (Rat Ubc probe set, VC1-10190) and a negative (E. coli dapB probe set, VF1-10272) control were also analyzed to demonstrate that the assay reagents and the assay protocol were properly followed.

Semi-quantitative real-time PCR
Total RNA from B6 mouse tissues, including the eye, brain, heart, liver, lung, cornea/lens, retina and RPE/choroid, was purified by using the RNaseasy Mini Kit with On-column DNase treatment (Qiagen). Each of the 18 lincRNAs identified to be conserved in all species was probed with the Qiagen One-step RT-PCR Kit. Twenty-five nanograms of total RNA was used in each 12.5 μl reaction as per the manufacturer’s instructions. Primers used to probe lincRNAs were custom designed to span introns whenever possible to rule out genomic DNA contamination. Actin primers were designed for loading controls and primers against Nrl (retina) and Rpe65 (RPE) were employed to confirm the fidelity of tissue dissections.

Quantitative real-time PCR
One microgram of isolated eye, brain, heart, liver and lung tissue RNA from two pooled B6 mice, 1 μg of cornea/lens, retina and RPE/choroid tissue RNA from two pooled B6 mice and 1 μg of eye tissue RNA from B6, Cone-DTA, Nrl+/−, and P23H mice were converted to cDNA with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was done with TaqMan chemistry and Assays on Demand probes (Applied Biosystems) for mouse Abca4 (Mm00492035_m1), Opn1sw (Mm00432058_m1), Rpe65 (Mm00504133_m1) and ABI custom designed primers for EyeLinc1 (AIX00TR), EyeLinc2 (AIW2N1), EyeLinc4 (AIS074V), EyeLinc7 (AIW14H), EyeLinc14 (AIT96A3) and EyeLinc17 (AIR99YN). One 18S rRNA (4319413E) probe set (Applied Biosystems) was used as the endogenous control. All real-time experiments were done in triplicate with the ABI Step-One Plus qRT-PCR machine (Applied Biosystems). Fold changes were calculated based on differences in threshold cycle (Ct) after normalization to 18S rRNA. Percent relative expression is presented as a percent of the maximal normalized expression observed in the different samples.

Statistical analysis
Experimental results were analyzed by an independent two-sample t-test. A P-value of 0.05 or less was considered statistically significant. Data presented graphically in figures are shown as means ± standard deviations.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.
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