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Integrative human-mouse gene expression and phylogenetics analysis to prioritize genes of evolutionary and biomedical importance in the retina and retinal pigment epithelium

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Abstract

Mammalian models, such as mice, are often used to study human retinal diseases, but, owing to the evolutionary time-scale separating the two species, some physiological functions involved in vision differ between the two species. Here, public RNA-seq data sets were used to interrogate genome-wide gene expression patterns in mouse and human retina and retinal pigment epithelium in order to identify genes of significance underlying visual signal processing in the two species. Individual genes with distinct and conserved expression patterns across the retinal tissues were identified both within and between species, followed by an assessment of biomedical roles in visual functions, and their extent of sequence conservation among mammals. There was evidence that the conservation of expression patterns is linked to evolutionary sequence conservation, retinal cell-type specificity and disease association, suggesting that these parameters should be considered together when investigating the genetic and evolutionary underpinnings of mammalian eye function and pathology. The extent of sequence and expression pattern conservation observed at individual genes and at pathway level could highlight the relative importance of signaling pathways that control retinal cell development, differentiation and survival across species. This information may be crucial in providing the basis for which genes to prioritize in cross-species treatment testing, including gene therapy for retinal diseases, as well as providing deeper insights on the evolution of retinal diseases susceptibilities in different species.

Keywords: gene expression; retina; retinal pigment epithelium; phylogenetics

Introduction

Impaired vision adversely affects human productivity and well-being worldwide. About 30% of the world's population, over 1 billion people, is estimated to have either poor vision or are completely blind (WHO 2022). The disease burden attributable to visual impairment is projected to increase by over 80% in the next 10 years, due to an ever-increasing aging population associated with global population growth (Prince et al. 2015). The leading contributor to blindness for people of all social-economic status, across all age groups, is a range of retinal diseases (Yorston 2003). The cure for retinal disease states has proved elusive because the associated molecular mechanisms remain poorly understood.

The mouse has been an invaluable species for studies of human retinal diseases (Flannery 1999, Baird et al. 2002, Veleri et al. 2015). However, in the development and expression of some retinal diseases, there are notable differences between human and mice models. For example, not all features of human age-related macular degenerations (AMDs) are accurately replicated in the mice model (Pennesi et al. 2012). Likewise, the

murine model for autosomal dominant retininis pigmentosa (adRP) partially mimics adRP in humans carrying the same mutation (Olsson et al. 1992).

The vertebrate retina consists of two layers: the inner neurosensory retina and the outer retinal pigment epithelium (RPE) both tissues spanning the subretinal space. The two retinal layers exhibit an intricate functional relationship, as exemplified by the classical visual cycle, where the RPE regenerates the 11-*cis* retinal which is required by photoreceptors during light signal processing (Wald 1935, Moiseyev et al. 2005). It is therefore not surprising that primary neural retina degeneration often causes RPE dysfunctions and *vice versa* (Roepman et al. 2000; Longbottom et al. 2009). Marked differences exist between mice and the human with respect to cellular and anatomical aspects of retina and RPE. The higher rod-cone ratio and the absence of the macula in the mice retina as well as the thinner Bruch's membrane in mice RPE are among some of the notable differences in the neurosensory retina and RPE features between mice and humans (Volland et al. 2015). Furthermore, rodents' visual ecology is markedly different to human: in contrast to rodent species which can be arboreal, fossorial or semiaquatic, and thus less dependent on light for their sensory functions, humans are highly visually oriented, trichromatic, with half of the human brain directly or indirectly devoted to processing visual information (Withers et al. 2016). To what extent these cellular, anatomical and ecological differences are reflected at molecular level between and within both the human and the mouse is not entirely understood.

Some of the known genetic variations causing vision-related phenotypes in different species are sequence changes in specific genes acting in specific physiological pathways. For instance, the best understood visual physiological process in the retina is the molecular basis of phototransduction (Jacobs et al. 1993, Nathans 1999, Osorio et al. 2004). Sequence changes in particular genes, such as the genes encoding photoreceptors, lead to variations in the spectral tuning of light wavelengths in vertebrates (Tada et al. 2009, Zhao et al. 2009). However, the role of gene expression in the cognitive distinctions between species is becoming more valuable and important. For example, a study of gene expression levels in human and non-human primate cerebral cortices, reveals certain genes involved in essential physiological activities of the brain that are expressed in one species but not in others. In particular, genes expressed in the cortex were found to be expressed at higher levels in humans compared to non-human primates; this is consistent with superior visual cognitive qualities in humans (Cáceres et al. 2003). Orthologous genes in different species which deviate in their pattern of expression between neural retina and RPE may contribute to species-specific physiological processes in the normal or pathological states of the retina. Similarly, genes that remain co-expressed in the retina during evolution could participate in common pathways for normal or diseased retinal physiology.

One way of investigating this "similar co-expression patterns-similar function", hypothesis is to characterize gene expression pattern in the neural retina and RPE of humans and mice, making use of public gene expression data generated by recent advances in high-throughput RNA sequencing ("RNA-seq"). Therefore, the aims of this study were to evaluate how these expression patterns reflect sequence conservation across evolution and to assess possible association between subretinal gene expression pattern and retinal cell-type specificity and/or retinal disease.

Materials and Methods

Public mouse and human neural retina and RPE RNA-seq aata

To obtain target data, a systematic search of RNA-seq experiments of neural retina and RPE from wild-type mice and humans from various public sequence repositories was performed. The following search strategy was used to obtain the target data: taxon, human (9606) or mouse (10090), database source, Short Read Archive (SRA) or Gene

Expression Omnibus (GEO) or European Nucleotide Archive (ENA); data generation, RNA-Seq; tissue or cell type, retina and RPE. All experiments used the standard Illumina poly-adenylated (polyA+) mRNA-seq protocol. This is based on oligo(dT)-coated beads that select the polyadenylated fraction of the transcriptome including coding mRNAs. The human and mice datasets were generated in Li et al (2014) and Zhang et al (2014), respectively (Table 1).

Table 1. RNA-sequencing Data Sets Selected for analysis

Organism	Source (SRA Id)	Tissue type	#Samples	Instrument	Library prep	Read size	Disease Status
Mus musculus	SRP050429	Retina	x3	Illumina HiSeq 2500	Paired	101	normal
Mus musculus	SRP050429	RPE	x3	Illumina HiSeq 2500	Paired	101	normal
Homo sapiens	SRP034875	Retina	x3	Illumina HiSeq 2000	Paired	101	normal
Homo sapiens	SRP034875	RPE	x3	Illumina HiSeq 2000	Paired	101	normal

RNA-seq data analysis

The Exon Quantification Pipeline (EQP) (https://github.com/Novartis/EQP-cluster) was used to map reads to the reference genomes and to quantify gene expression (Schuierer and Roma 2016). The alignment of the mouse and human reads to the respective reference genome sequences (GRCm38 and GRCh38) was performed using Bowtie version 2 (Langmead and Salzberg 2012). The quantification of gene expression was based on gene models from the Ensembl database. Gene counts were normalized by library size and represented as counts per million (cpm).

To increase the power of detection of differentially expressed genes, genes with a cpm value of less than 1 in more than 75% of the samples across all groups were filtered out. Differential expression (DE) analysis was performed using LIMMA voom (Ritchie et al. 2015). First voom normalization to log transformed cpm data for linear modeling was performed, as applied in the LIMMA package. Using RPE samples as a reference design, the difference between the retina and RPE for both mouse and human data sets was estimated followed by a linear modeling of the expression value for each gene. Empirical Bayes statistics to moderate the standard error of the estimated log-fold changes was also applied to generate more accurate inference and improved power (Smyth 2004).

Next, genes with absolute log fold change of 2 or greater were selected, meaning that they have higher expression in the RPE than in the neural retina (if the log fold change is positive), or have lower expression in the RPE than in the neural retina (if the log fold change is negative). An adjusted p-value of less than 0.05 after Benjamini-Hochberg correction for multiple hypothesis testing was selected as cut-off value for statistically significant difference (*i.e.* 5% false positives). Two data subsets per species (human and mouse) were created so that each contained all genes that show a significant higher expression in RPE compared to the neural retina, or each contained a significantly lower expression in RPE compared to the retina. Differentially expressed genes (DEGs) were visualized as volcano plots. These genes were then used for downstream analyses.

Definition of human-mouse orthologs

Homology groups of DEGs from humans and mice were obtained from the NCBI Homologene database (http://www.ncbi.nlm.nih.gov/homologene/). There are several homologous relationships between human and mice genes, as annotated by NCBI. To avoid ambiguity in assigning homologous relationships based on gene names, only mouse-human gene pairs with one-to-one relationships were considered based on blast searches.

Although targeting only genes with a one-to-one relationship greatly reduced the number of genes to compare both species against, it has the advantage of confidently yielding those genes with orthologous functional relationships between the two

species. This homology information proved important in the comparison of the gene expression profiles of the neural retina and RPE between mice and human.

Relationship between subretinal expression pattern and gene sequence evolution

To gain insight if natural selection has acted differently among the genes expressed in the retina and RPE, top upregulated DEGs were classified into four sub-classes of subretinal expression pattern between the two species: genes that exhibited consistent upregulation in the retina in both species, genes that exhibited species-specific upregulation pattern in the retina, genes that exhibited consistent upregulation in the RPE in both species, and genes that exhibited species-specific upregulation pattern in the RPE. Next, sequence evolution was defined by assembling a set of 28 species spanning mammalian evolution from rodents to primates, followed by applying tests of conservation constraints on each gene (Table 2). Briefly, for each gene in the subclass of upregulation pattern, multiple sequence alignments of coding sequences were constructed, gene trees were generated through maximum likelihood framework in RaxML (Stamatakis 2014), followed by estimation of overall evolutionary rates averaged across the entire gene length using the one-ratio model under *codeml* implementation in PAML (Álvarez-Carretero et al. 2023). Differences in evolutionary pressure across these genes were analyzed and plotted for visualization within and between subclasses.

Table 2. Common names, species name and rhodopsin sequence accession for the *mammalian* species considered in this study

Common name	Species name	NCBI Accession
Alpaca	*Vicugna pacos*	XM_006206787.2
Camel	*Camelus ferus*	XM_006180073.2
Sheep	*Ovis aries*	XM_004018534.5
Horse	*Equus caballus*	XM_023619934.1
Lemur	*Otolemur garnettii*	XM_003796229.3
Rhinoceros	*Ceratotherium simum simum*	XM_004442424.2
Sperm whale	*Physeter catodon*	XM_007126220.3
Dolpjin	*Tursiops trancutus*	NM_001280659.1
Minke whale	*Balaenoptera acutorostrata*	XM_007192608.2
Macaque	*Macaca mulatta*	XM_001094250.3
Lion	*Panthera leo*	XM_042927549.1
Pig	*Sus scrofa*	NM_214221.1
Dog	*Canis lupus familiaris*	NM_001008276.1
Hedgehog	*Echinops telfairi*	XM_004702378.2
Cow	*Bos taurus*	NM_001014890.2
Microbat	*Myotis lucifugus*	XM_006083811.3
Gorilla	*Gorilla gorilla*	XM_004036292.5
Human	*Homo sapiens*	NM_000539.3
Panda	*Ailuropoda melanoleuca*	XM_002921249.4
Elephant	*Loxodonta africana*	NM_001280858.1

Megabat	*Pteropus alecto*	XM_006917646.1
Galago	*Otolemur crassicaudatus*	AB112591.1
Mole rat	*Heterocephalus glaber*	XM_004870461.2
Rat	*Rattus norvegicus*	NM_033441.1
Pika	*Ochotona princeps*	XM_004581320.2
Guinea pig	*Cavia porcellus*	NM_001173085.1
Mouse	*Mus musculus*	NM_145383.2
Shrew	*Tupaia chinensis*	XM_006160664.1

Association of DEGs with retina cell type specificity and retinal disease

The neural retinas *vs.* RPE DEGs were compared to the already published neural retina and RPE gene signatures. The comparison used Refseq Gene identifiers to investigate the extent to which this analysis reproduced the retina/RPE signature genes published in Strunnikova et al (2010), Siegert et al (2012) and Bennis et al (2015). By identifying the differential expression status of these genes in mice and humans, an assessment of the relationship of gene cell-type specificity, disease association and subretinal expression pattern in the two species was determined.

Results

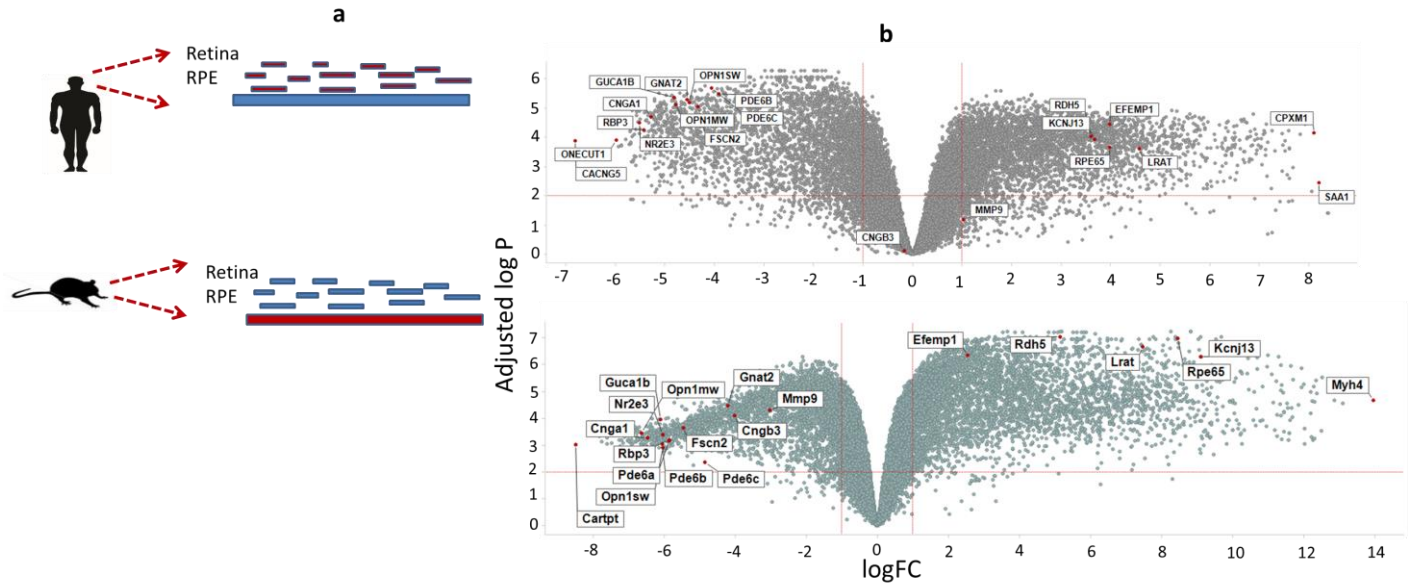
Gene expression pattern in healthy subretinal tissues of mice and humans

RNA-seq data generated from two pertinent studies and available in SRA was obtained and analyzed (Table 1). The mouse RNA-seq data set is part of the SRA series SRP050429 and consists of paired-end reads from three retina and three RPE wild-type samples; likewise, the human data set, accession number SRP034875, refers to three retina and three RPE RNA samples from healthy donors. A schematic overview of the gene expression analysis process is presented in Figure 1.

Applying EQP, 12–30 million sequencing reads (corresponding to ~65% of the average of 26 million total reads per sample) were aligned to gene exons and obtained gene expression counts. A total of 19367 transcripts had an expression value greater than 1 cpm in the human retina when compared to the 17087 transcripts expressed in mice retina. This suggests that over 80% of the transcriptome is expressed in the mammalian retina, which is consistent with the original studies (Li et al. 2014, Zhang et al. 2014). These expression profiles allowed for further examination of gene expression differences between retina and RPE tissues in mice and humans.

Subretinal differential expression pattern as a proxy for visual differences in mice and humans

The differential expression analysis identified more than 2000 genes that exhibited remarkable expression differences between the neural retina and RPE for each species (Figure 1b). More precisely, the numbers of genes that were downregulated in the neural retina, relative to RPE, were 2231 in human and 2647 in mice. In contrast, the number of genes that were found to be upregulated in the neural retina, relative to RPE, was 1558 in humans and 1447 in mice. Secondly, DEGs that exhibited species-specific pattern, or consistent pattern in both species, was used as a proxy for insights into the degree of conservation of retinal functions between the mice and humans. Overall, the results indicate that 75% of all DEGs have a species-specific subretinal expression pattern in mice and humans while 25% exhibited consistent gene expression pattern. The latter genes are likely to represent a conserved co-expression pattern of significance in the use of mice as models for the investigation of human diseases, and have therefore been given more detailed evolutionary scrutiny in this study



Figur

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iew of comparative differential expression analysis between the retina and RPE in mouse and human. (a) RNA-Seq studies on relevant tissues in both human and mouse were identified and raw sequence data were downloaded, converted and aligned on respective species reference genomes. (b) Differential expression analysis was performed which generated a pattern of up- and down-regulated genes in the retina relative to RPE of both species, as represented by their volcano plots.

To identify the most differentially expressed genes, when compared to the neural retina and RPE for both species, those genes that exceeded the 75th percentile (75th P) threshold were selected based on their fold change. Again, only genes with a one-to-one homologous relationship between mice and humans, categorized as species-specific and species-shared genes in terms of pattern of gene expression were considered. Then Fisher's exact test was applied to assess the significance of those genes with the strongest subretinal expression pattern that overlapped between mice and humans. The results show that the overlap in the genes with the strongest expression in the neural retina of mice and humans is significantly greater than that expected by chance (97 genes, p = 1.65e-08). However, the number of intersecting genes with strongest expression in the RPE between the two species was not found to be significant (72 genes, p = 0.6096). The latter finding may be a reflection of the physiological and functional differences between the RPE of mice and humans.

Expression pattern conservation and evolutionary constraint on subretinal visual genes in mammals

After seeing that mouse *vs*. human subretinal differences might be mirrored at a molecular level in terms of patterns of gene expression, the next step was to explore whether the DEGs pattern observed between mouse and human reflects selective constraints on visual genes. The robustness of this investigation required first the definition of mouse-human one-to-one orthologs. In this case, the proportions of genes with a one-to-one humans-mice homologous relationship and with significantly lower expression in RPE than in the neural retina were 32.5% of the total genes expressed in mice and 40.1% of the total genes expressed in humans. The respective proportions of the genes with significantly higher expression in RPE than in the neural retina were 51% in mice and 60.4% in humans. Secondly the specification of the evolutionary context of coding sequence of each DEG in the four subclasses of subretinal expression pattern followed after collating additional sequences from other mammalian species used in the evolutionary analysis (Figure 2a, Table 2, see Materials and Methods for details). It could be seen that that the DEGs sequence conservation, as indicated by the Ka/Ks (ω) *ratio*, almost follows a particular trend of expression pattern between species and between subretinal tissues. For example, the conservation rates of DEGs exhibiting consistent pattern of upregulation in the two species was remarkably higher for those DEGs upregulated in the retina compared to those highly expressed in the RPE (Figure 3b, P < 0.001, Wilcoxon test). This, again, could suggest the relative significance of the roles of the genes expressed in the retina and those expressed in the RPE in their visual and other functions. The degree of sequence conservation between genes with a shared pattern and those with a species-specific expression pattern can also be used to investigate the evolutionary importance of the subretinal tissue specific genes. Whilst upregulated DEGs in the retina did not show significant changes in terms of divergence rates between species-specific and species-shared categories, those upregulated in the RPE did (Figure 3b). Furthermore, describing the four subclasses of upregulation patterns in terms of co-expression networks could also offer another way of inferring the evolutionary significance of DEGs from a system's biology perspective. This was achieved by mapping the DEGs to the STRING database of protein-protein interaction networks, allowing for a sense of association of individual genes in terms of expression levels, evolutionary conservation and topological position within a network (Figure 1b, Figure 2c, see Discussion for details).

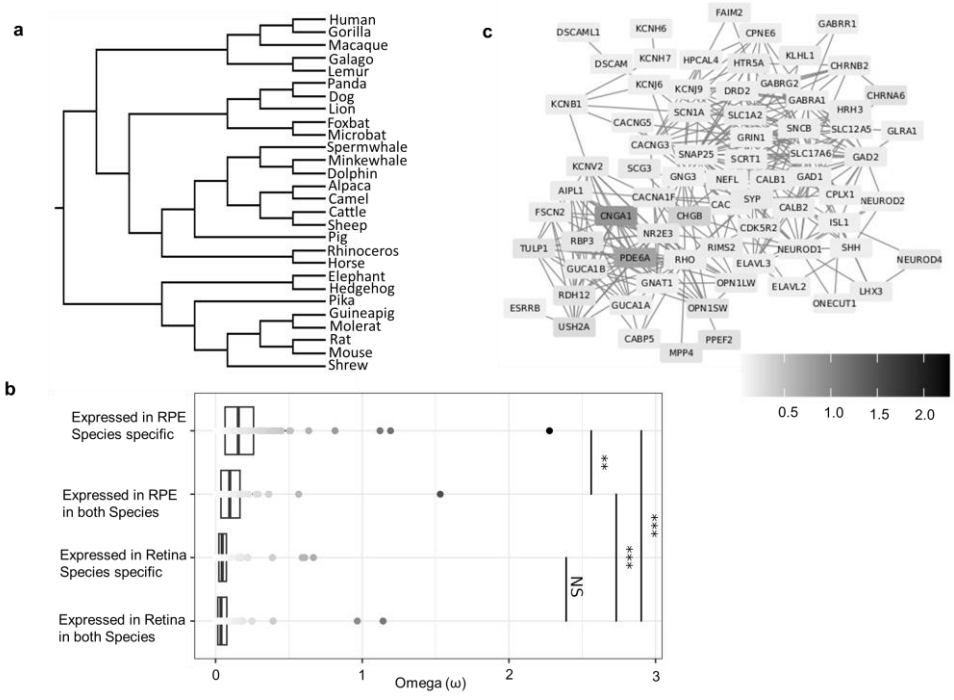

Figure 2: Evolutionary perspective of subretinal gene expression. (a) A maximum-likelihood generated cladogram based on mammalian rhodopsin (*RHO*) gene representing all of the mammalian species used in examining evolutionary rate analyses of subretinal genes expressed in mouse and human. (b) Boxplot of the Omega (ω) values representing overall rate of selection (gray scaled for visualization of selection intensity) for the subretinal genes in each category of expression pattern in mouse and human (NS., not significant; $**P < 0.01$, $****P < 0.0001$ (Wilcoxon test)). (c) Co-expression network for DEGs with consistent subretinal expression pattern in both mouse and human.

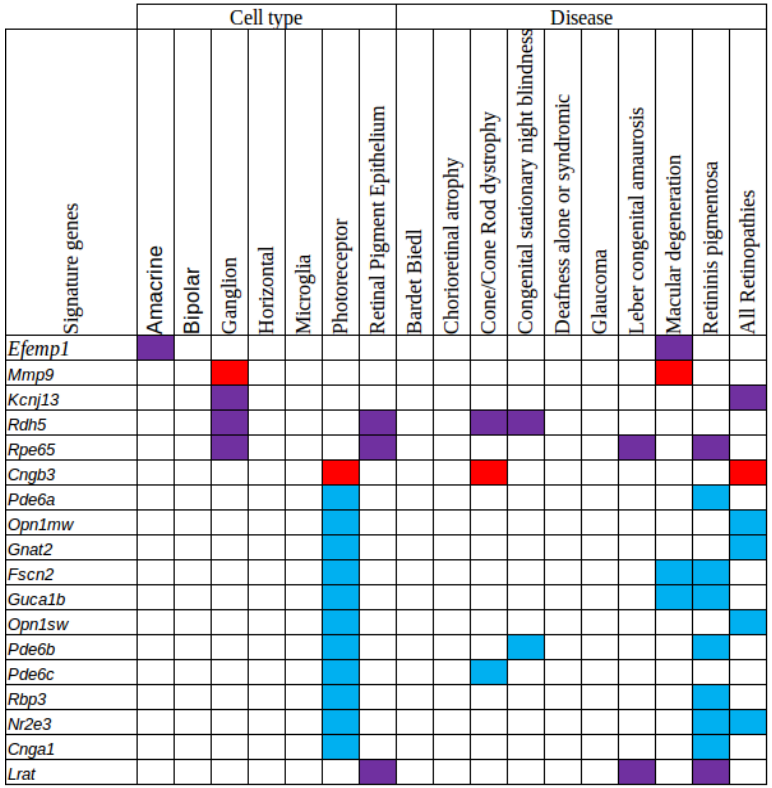

Figure 3: Differentially expressed genes with respect to their neural retina and RPE cell type specificity and to their association to retinal disease. Color codes: Species-specific expression pattern in mice (violet); Species-specific expression pattern in humans (red); Consistent expression pattern in both species (cyan).

Expression pattern, cell-type and disease signature genes highlights possible genes of importance in retinal physiology

Disease phenotypes are most likely caused by mutations in the genes involved in the structure, function, and maintenance of certain retinal cell types. By cross-checking retinal cell-type specific genes associated with retinal diseases from published literature with the DEGs identified in the present analysis, the relationship between retinal cell-type specificity, disease association and individual DEGs status was determined. This study identified sixteen genes with cell-type specificity, linked to various retinal diseases, and exhibiting a consistent pattern of differential expression between the retina and the RPE in mice and humans (Figure 3). Eleven of these genes (*Pde6a, Opn1mw, Gnat2, Fscn2, Guca1b, Opn1sw, Pde6b, Pde6c, Rbp3, Nr2e3* and *Cnga1*) showed retinal cell-types specificity while five genes (*Lrat, Rdh5, Rpe65, Kcnj13* and *Efemp1*) showed RPE specificity. A few more genes with cell-type specificity linked to retinal disease were also identified; however, their retina/RPE expression status differs between mice and humans. *Mmp9* and *Cngb3*, for example, are specifically upregulated in the neural retina in comparison to the RPE in mice (Figure 1b). Such genes may be involved in the species-specific physiological process of retinal pigment epithelialization, and are prospective candidates for pathological differences in disease phenotypes between mice and humans.

Discussion

Characterizing subretinal gene expression attributes in both mice and humans is central to revealing general and specific factors underlying mammalian vision processes. This study has demonstrated that the proportion of subretinal genes showing a species-specific expression pattern in mice and human is much higher than those showing a consistent pattern of expression between the two mammalian species. These findings demonstrate the long-term effects of the molecular evolutionary changes that have occurred between mice and humans, some of which are evident at the gene expression level. Neurological genes tend to be evolutionarily conserved at the structural level (Huang et al. 2004; Invergo et al. 2013), however this study found that this conservation extends to gene expression patterns at both tissue and species levels (Oldham et al. 2006). For instance, when subretinal DEGs are categorized based on tissue and expression conservation patterns, it becomes evident that selective constraint is significantly stronger in DEGs showing conserved expression pattern of upregulation than those DEGs displaying species-specific upregulation patterns. The conservation of gene expression patterns between these species is especially important as these may suggest common evolutionary paths in the physiology of the retina and associated tissues, allowing for the use of mice models in the exploration of pathological processes relevant to human retinal visual functions.

DEGs can also be used to identify specific genes with the most notable variations in expression in the context of individual genes, because highly expressed genes are more likely to participate in critical functions. However, as part of the system, evolutionary pressure is expected to act not only on one gene at a time, but also on the entire pathway in which these genes are involved (He and Zhang 2006). For example, two of the genes with the most significant expression differential between retina and RPE in mice, according to this study, is a gene that encodes the cyclic-GMP (cGMP)-specific phosphodiesterase 6A alpha subunit (*Pde6a*), expressed in cells of the retinal rod outer segment and a cyclic nucleotide gated channel subunit alpha 1 (*Cnga1*), which contributes to the formation of a cGMP-gated cation channel in the plasma membrane, allowing depolarization of rod photoreceptors. We note that these two genes have undergone accelerated molecular evolution in the context of the evolution of mammals, pointing to a potential remodeling function for these two genes in the mammalian rod phototransduction system (Figure 2c) (Lagman et al. 2016; Tian et al. 2022). In humans, two of the most significant genes

with higher expression in human retina are *CACNG5* (log-fold = ~7), which encodes a γ-subunit of a voltage-dependent calcium channel regulating neurotransmitter trafficking, or gating, and *ONECUT1* (log-fold = ~6), which encodes a transcription factor specific for horizontal cells. *ONECUT1* overexpression in human retinas may be associated with the occurrence of three kinds of horizontal cells, as opposed to only one subtype in mice (Kolb et al. 1994, Peichl and González-soriano 1994). These findings point to a probable link between cellular composition and gene expression as a source of cross-species diversity in retinal characteristics.

For some neurological disorders, there is a clear relationship between the causative gene and its cell-type specificity, in terms of its expression (Xu et al. 2014). This study assessed whether neural retina/RPE expression pattern conservation, combined with cell-type specificity, could be a reliable predictor of particular gene associated with disease. The findings indicate that cell-type specific genes associated with retinal diseases tend to have a common expression pattern in humans and mice and are enriched more in rods and cones than in any other retinal cell-types. This is expected given the centrality of photoreceptors and their constituent genes in the phototransduction system. If this conclusion is valid, it is also plausible to predict that retinal cell-type specific genes associated with disease, but with species-specific expression pattern, may participate in molecular pathways leading to the species-specific disease features. For example, *Cngb3*, which is specifically expressed in photoreceptors and is differentially expressed in mouse but not in human in this analysis (Figure 1), is a promising gene therapy candidate in the treatment of achromatopsia. However, manifestations of achromatopsia in humans include total lack of cone function from birth which is not the case with *Cngb3* deficient mice in which some functional cones are retained (Xu et al. 2011). While this disparity might arise due to the heterogeneity of the achromatopsia expression, the existence of a species-specific *Cngb3* pathway linked to its differential regulation within the cells of the retina also remains a possibility.

Conclusion

The objective of this research – to understand commonalities between human and mouse visual functions at gene expression details – is also inexorably linked to understanding their differences. The present analysis provides clues to common and unique gene expression patterns prevailing in the retina of humans and mice. The approach described here could provide a framework to help identifying the key drivers essential for visual functions in mice, humans and mammals in general. Given that mice are an indispensable model in the study of retinal diseases, comparative transcriptomic studies, such as this, can contribute to the understanding of the disparity of retinal diseases between humans and mammalian models used for their investigation.

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Declaration of interests

The authors declare to have no competing interests.

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