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Identification of rod- and cone-specific expression signatures to identify candidate genes for retinal disease

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ABSTRACT

Recent advances in technology have greatly increased our ability to identify genetic variants in individuals with retinal disease. However, determining which are likely to be pathogenic remains a challenging task. Using a transgenic coneless (*cl*) mouse model, together with rodless (*rd/rd*) and rodless/coneless (*rd/rd cl*) mice, we have characterised patterns of gene expression in the rod and cone photoreceptors at a genome-wide level. We examined the expression of >27,000 genes in the mice lacking rods, cones or both and compared them with wild type animals. We identified a list of 418 genes with highly significant changes in expression in one or more of the transgenic strains. Pathway analysis confirmed that expected Gene Ontology terms such as phototransduction were over-represented amongst these genes. However, many of these genes have no previously known function in the retina. Gene set enrichment analysis further demonstrated that the mouse orthologues of known human retinal disease genes were significantly enriched amongst those genes with decreased expression. Comparison of our data to human disease loci with no known causal genetic changes has highlighted genes with significant changes in expression making these strong candidates for further screening. These data add to the current literature through the utilisation of the specific *cl* and *rd/rd cl* models. Moreover, this study identifies genes that appear to be implicated in photoreceptor function thereby providing a valuable filter for variants identified by high-throughput sequencing in individuals with retinal disease.

1. Introduction

Inherited retinal dystrophies, leading to either partial or total blindness, affect approximately 1 in 2500–3500 people. Many more suffer from age-related macular degeneration (AMD) in later life with 25% of people over the age of 70 affected by AMD (Van Newkirk et al., 2000). Over the last 15 years the number of published loci associated with human retinal disease has shown a dramatic four-fold increase, from 55 in 1995 to 252 as of March 2014. While the underlying gene has been identified in a significant number of cases (212, as of March 2014), the proportion of unknown causal variants has remained relatively steady at approximately 15–20% (RetNet, http://www.sph.uth.tmc.edu/RetNet) (Daiger et al., 1998). Traditionally, causal genes have been cloned via a process of linkage analysis followed by candidate gene screening in large families (for example (Akahori et al., 2010; Li et al., 2004, 2010)). However, this has not always been successful, not least because the linkage regions may be large and contain many genes (Chaib et al., 1997; Ismail et al., 2006; Yang et al., 2006). In addition, while the emergence of new sequencing technologies is making it increasingly accessible to identify all variants in the exome or genome of a patient, the process of locating the underlying variant(s) remains a laborious process, especially with patients for whom there is little or no family data (Chiang et al., 2012; Falk et al., 2012; Koenekoop et al., 2012; Perrault et al., 2012). As such, it is increasingly important to utilise complementary

Abbreviations: CORD, Cone-Rod Retinal Dystrophy; FDR, False Discovery Rate; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; LCA, Leber Congenital Amaurosis; LGDM2C, Limb-Girdle Muscular Dystrophy Type 2 C; miRNA, MicroRNA; NES, Normalised Enrichment Score; qPCR, Quantitative Polymerase Chain Reaction; RAR, Retinoic Acid Receptor; RXR, Retinoid X Receptor; SLR, Signal Log Ratio; UCSC, University of California Santa Cruz.

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approaches to help identify novel retinal disease genes, both directly and in combination with methods such as high throughput sequencing. One such approach is still the use of microarrays; a technology first developed in the late 1980's (Augenlicht and Kobrin, 1982) which enables the simultaneous analysis of thousands of transcripts.

Dysfunction or death of rod and cone photoreceptors is the primary cause of blindness in the vast majority of retinal degenerative diseases. Many of the disease genes already identified are abundantly expressed in the retina and have well defined and specific functions in the photoreceptors. Therefore, a catalogue of the specific gene expression profiles of rods and cones will provide a comprehensive database of candidates for retinal degenerations. We have therefore utilised microarray technology and three mouse models, *rd/rd*, *cl* and *rd/rd cl* to investigate the differences in gene expression in their retinae compared to wild type animals.

The *rd* mutation is an autosomal recessive cause of retinal degeneration in mice and occurs in the gene encoding the β subunit of cGMP phosphodiesterase (*Pde6b*) (Bowes et al., 1990; Pittler and Baehr, 1991). The mutation consists of a C to A transversion in exon 7, resulting in a stop codon which eliminates both a membrane binding domain, and a domain critical for protein function (Pittler and Baehr, 1991). Mice carrying the *Pde6b*^{rd1} mutation (*rd/rd*) have rapid degeneration of rods in all regions of the retina. In the posterior region of the eye, there is approximately 98% loss of rods by postnatal day 17, and total loss in all regions by day 65. The cone cells are also affected, although to a much lesser extent, only 25% are lost in the posterior region of the eye by day 17 and approximately 1.5% still remain after 18 months.

Cone photoreceptors can be specifically lesioned using the *cl* transgene, which is comprised of a partial human red cone opsin promoter combined with an attenuated diphtheria toxin gene (Soucy et al., 1998). The resulting mice lose >95% of UV cones and have a total ablation of green cones, <1% remain in some retinae, while in contrast the rods remain unaffected (Freedman et al., 1999). Mice carrying both the *rd/rd* mutation and *cl* transgene show loss of virtually all rod and cone cells in the eye. By 80 days of age the retinae lack an outer nuclear layer and show no immunoreactivity to rods and both green and UV cones (Lucas et al., 1999).

There have been a number of previous studies of gene expression in the mouse retina using both custom microarrays and Affymetrix arrays using a variety of models including *rd*, *rd7* and *nrl*$^{-/-}$ (Chen et al., 2005; Corbo and Cepko, 2005; Dorrell et al., 2004; Hackam et al., 2004a, 2004b; Yoshida et al., 2004; Yu et al., 2004). More recently Gamsiz and colleagues have used RNA-seq to produce a map of the retinal transcriptome (Gamsiz et al., 2012). All of which have generated extremely useful lists of genes that are expressed in the retina. However, the unique aspect of this study is that we use the *cl* transgene alone and in combination with *rd* to identify genes involved in both rod and cone photoreceptor function. We determined the genome-wide expression profile of four biological replicates of each transgenic strain and compared them to those of wild type mice in order to identify those genes with significant alterations in expression in the mutant animals. The advantages of this whole genome approach utilising the specific lesioning of photoreceptors in these mice models is that it will allow clearer insights into the role of genes in each cell type, including those not previously implicated in retinal function.

2. Experimental procedures

2.1. Animals

All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 (PPL 30/2812) and the University of Oxford Policy on the Use of Animals in Scientific Research and approved by the Home Office (UK) Animals Scientific Procedures Department (ASPD). Animals were sacrificed by Schedule 1 methods. Mouse lines used included *rd/rd cl* mice (C3H/HeNPde6b$^{rd1/rd1}$/Tg(OPN1LW-DT)1Mame$^{+/-}$) and *rd/rd* mice (C3H/HeNPde6b$^{rd1/rd1}$) kept on a C3H genetic background and non-*rd* (C3H/HeN Pde6b$^{+/+}$)and non-*rd/cl* (C3H/HeN Pde6b$^{+/+}$/Tg(OPN1LW-DT)1Mame$^{+/-}$) which do not carry the rd mutation (Sanyal and Bal, 1973; Soucy et al., 1998). Animals were housed under a 12:12 LD cycle with food and water *ad libitum*. Eyes were removed and the retina was dissected from 3 month old mice from all mouse lines used.

2.2. RNA extraction

Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, UK) following the manufacturer's protocol, including the optional on-column DNase digestion steps. Total RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, UK) and quality confirmed using a Bioanalyser (Agilent) according to manufacturer's instructions.

2.3. Affymetrix probe generation/arrays

cDNA was generated using the Ambion® WT Expression Kit (Ambion Life Technologies, Paisley, UK) following the manufacturer's protocol. Briefly, 200 ng of total RNA was used to synthesise first strand cDNA, which in turn was used as template for second strand cDNA synthesis. cRNA was then generated by *in vitro* transcription, purified and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, UK). cDNA was then generated from 10 μg of cRNA, and the template cRNA hydrolysed using RNase H. The cDNA was purified and quantified. Probes were produced from the cDNA using the GeneChip® WT Terminal Labeling Kit (Affymetrix, High Wycombe, UK) following the manufacturer's protocol. Probes were used on the GeneChip® Mouse Exon 1.0 ST arrays (Affymetrix) and processed on a GeneChip® Fluidics Station 450 (Affymetrix) and GeneChip® Scanner 3000 7G (Affymetrix).

2.4. RT-PCR

cDNA was generated from total RNA using the QuantiTect Reverse Transcription kit (Qiagen, UK), following manufacturer's protocols. Reactions were performed using 300 ng of total RNA as template, and with extended genomic DNA wipeout (10 min) and reverse transcription (30 min) steps. Reactions without reverse transcriptase were performed as negative controls to ensure no genomic DNA contamination. Test PCRs were performed using primers for β-actin to confirm the presence of cDNA and no genomic DNA contamination.

PCRs were performed using BioMix (Bioline, UK) typically with a total volume of 25 μl, 35 cycles, final concentration of $MgCl_2$ of 2 mM, 60 °C annealing temperature. Either 50 ng of genomic DNA or 1 μl of 1:10 diluted cDNA was used as template. PCR products were resolved on 2% agarose gels and visualised with ethidium bromide under UV fluorescence.

2.5. Primers

Primers for standard PCR were designed by eye. Those for qPCR were designed using Primer3 (http://primer3.ut.ee/) and specified to have final products of 90–140 bp. Primer sequences are available upon request.