From disease genes to cellular pathways: a progress report

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Abstract. Mutations in a large number of retinal and retinal pigment epithelium (RPE) expressed genes can lead to the degeneration of photoreceptors and consequently the loss of vision. The genetic and phenotypic heterogeneity of retinal dystrophies poses a complex problem with respect to rational development of therapeutic strategies. Delineation of physiological functions of disease genes and identification of pathways that lead to disease pathogenesis represent essential goals towards developing a systematic and global approach to gene-based treatments. We are interested in identifying cellular pathways that are involved in photoreceptor differentiation, function and degeneration. We are, therefore, generating comprehensive gene expression profiles of retina and RPE of humans and mice using both cDNA- and oligonucleotide-based (Affymetrix) microarrays. Because of the under-representation of retinal/ RPE genes in the public databases, we have constructed several unamplified cDNA libraries and produced almost twenty thousand expressed sequence tags (ESTs) that are being printed onto glass slides ('*I-Gene*' microarrays). In this presentation, we will report the microarray analysis of the rodless (and cone-enhanced) retina from the *Nrl*-knockout mouse as a paradigm to initiate the identification of cellular pathways involved in photoreceptor differentiation and function.

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Background and basic concepts

Retinal dystrophies (RD) comprise a group of clinically and genetically heterogeneous retinal disorders, which typically result in the degeneration of

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photoreceptors followed by the impairment or loss of vision. To date, the online retinal information network (RetNet, *http://www.sph.uth.tmc.edu/Retnet*) has listed over 130 loci associated with retinal dystrophies. RD is a major cause of blindness in the industrialized world and is, for the most part, currently untreatable. Retinitis pigmentosa (RP) primarily causes rod photoreceptor degeneration and early symptoms include night blindness and loss of peripheral vision. The prevalence of RP is approximately 1/3000, with a total of over 1.5 million people affected worldwide (Saleem & Walter 2002). In contrast, cone dysfunction occurs early during the progression of cone or cone-rod dystrophies (CRD), thereby affecting visual acuity and colour vision. Leber congenital amaurosis (LCA) is the most common cause of congenital visual impairment with age of onset in infants or children. LCA accounts for 5–10% of all retinal dystrophies and is perhaps the most severe RD. Age-related macular degeneration (AMD) is highly prevalent in the elderly population, accounting for 22% of monocular blindness and 75% of legal blindness in adults over age 50 in the USA (Klein et al 1995). It preferentially affects the macular region, leading to loss of central vision and visual acuity. Unlike other forms of RD, AMD is the culmination of a complex interplay of genetic and non-genetic components. The complexity afforded by the considerable genetic heterogeneity in RD has greatly hindered the application of gene-based therapies; nonetheless, all of these diseases result in the same fate, i.e. the death of the photoreceptors.

A number of innovative strategies have been employed with the objectives of slowing down, preventing, or even reversing photoreceptor cell death in RD. One approach of circumventing the heterogeneity of RD is symptom-based disease treatments without correcting underlying genetic defects. To restore sight in highly visually handicapped individuals, several research groups are working on the development of electronic photoreceptor prosthesis (Zrenner et al 2001, Hammerle et al 2002) and cell/tissue transplantations (Otani et al 2002, Radner et al 2002, Semkova et al 2002). However, these strategies are currently limited due to issues regarding biocompatibility, stability and longevity of transplants. Another generic approach involves the use of growth or survival factors (LaVail et al 1998). In any event, the need for understanding both the physiological function of disease genes and the cellular processes leading to photoreceptor degeneration is inescapable.

Gene-based therapy seeks to rescue retinal diseases by correcting the underlying genetic defect or a consequent physiological deficiency. Over 80 genes have been associated with retinal dystrophies (Bessant et al 2001, Saleem & Walter 2002), including the neural retinal leucine zipper (*NRL*) gene, *NR2E3* (nuclear receptor subfamily 2, group E, member 3), *PDE6B* (phosphodiesterase 6B, cGMP-specific, rod, beta), *CRX* (cone-rod homeobox) and *RHO* (rhodopsin). To rescue RD, numerous researchers have attempted to deliver a functional copy

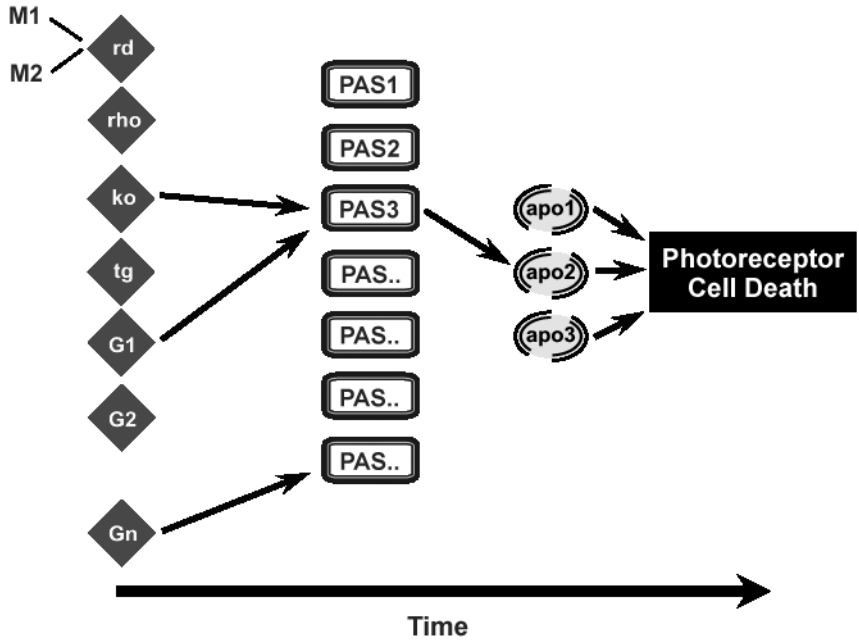

FIG. 1. The progression of disease in retinal dystrophies: from genes to pathways. This schematic representation shows our approach for gene-based therapy that focuses on the convergence of different pre-apoptotic cellular pathways in time, in order to develop novel therapeutic targets for several forms of RD. In a majority of retinal dystrophies, the photoreceptors die by apoptosis. Mutations in hundreds of genes may disrupt the cellular homeostasis and selected signalling pathways. M1, M2 represent different mutations in the same gene (*rd*), and the blue squares indicate various 'disease' genes. The response of photoreceptors to the presence of a mutation is predicted to converge on a few pre-apoptotic signalling pathways (PAS1,2,3 . . . indicates pre-apoptic signals) that lead eventually to photoreceptor cell death via apoptotic pathways (apo1–3). In this model, various pre-apoptotic signals (PAS) would be ideal targets for drug discovery.

of the mutant gene into photoreceptor cells using viral-based vectors (Bennett et al 1998, Cheng et al 2002). However, gene transfer technology faces a number of hurdles, including the sheer number of distinct targets that need to be addressed due to the heterogeneity of RD, and issues regarding the safety and efficacy of such vectors.

An alternative approach that we advocate is a therapeutic design based on the understanding of the cellular pathways leading to photoreceptor cell death (Fig. 1). Although a large number of retinal and retinal pigment epithelium (RPE) expressed genes can lead to RD, studies have shown that only a few common cellular pathways are involved in disease progression and the photoreceptor cells

in many, if not all, forms of RD die via apoptosis (Travis 1998). Pharmacological approaches have been advanced to slow photoreceptor degeneration through the introduction of growth and survival factors (LaVail et al 1998, Liang et al 2001, Tao et al 2002). Unfortunately, most experiments were only able to slow cell death for a week to a month, possibly due to the irreversible stage of disease by the time apoptotic pathways are induced. In order to devise a therapeutic strategy that targets multiple forms of RD prior to the induction of massive photoreceptor cell death, we are elucidating the common pathways of photoreceptor degeneration at a pre-apoptotic stage of disease. As illustrated in Fig. 1, pathways of disease pathogenesis initiated by different mutant gene products (or the lack thereof) must converge over time and follow limited routes to cell death. Therefore, temporal profiling of gene expression in normal developing, mature and ageing retinas and in retinal degeneration mouse models should lead to the identification of common pre-apoptotic signals (PAS) that can be targeted for drug discovery. A crucial aspect of this approach is the understanding of normal differentiation and function of rods and cones since it serves as the baseline against which abnormal changes may be recognized.

We propose that the adaptive response of the retinal neurons or RPE to disease or ageing is reflected by modulation of specific cellular pathways and, consequently, changes in gene expression. Profiling of diseased or ageing retina or RPE from humans and mice will facilitate the identification of these pathways. In this manuscript, we will primarily focus on the regulatory networks of photoreceptor development and function in the context of the transcription factor *Nrl*, using the $Nrl^{-/-}$ mouse as a paradigm.

Nrl: an essential transcription factor for rod development and function

The *Nrl* gene, encoding a basic motif leucine zipper protein of Maf-subfamily, was initially identified from a subtracted retinal library (Swaroop et al 1992). It showed a highly restricted pattern of expression, primarily in rod photoreceptors (Farjo et al 1993, Swain et al 2001). Six phosphorylated isoforms of *Nrl* have been identified in rod but not cone photoreceptor nuclei (Swain et al 2001). The *Nrl* protein can positively regulate rhodopsin gene expression by binding to an extended AP-1-like sequence element (called NRE) in the upstream promoter region (Kumar et al 1996, Rehemtulla et al 1996). Further studies indicated that *Nrl* regulates several other rod genes, and can interact with other transcriptional factors, such as *Crx*, in the regulation of retinal expressed genes (Chen et al 1997, Mitton et al 2000, Lerner et al 2001). Mutations in the human *NRL* gene have been associated with autosomal dominant RP (Bessant et al 1999, 2000, Martinez-Gimeno et al 2001, DeAngelis et al 2002). Interestingly, 5 of the 6 currently identified mutations

alter the residues S50 and P51, resulting in possibly hypermorphic alleles of NRL and suggesting their functional importance.

To define the role of *Nrl* in photoreceptor development and function, the *Nrl* gene was deleted in mice by homologous recombination (Mears et al 2001). Since *Nrl* plays a key role in the regulation of rod-specific genes, it was anticipated that the deletion of *Nrl* would affect rod photoreceptors. Surprisingly, the $Nrl^{-/-}$ mouse retina is functionally rodless. The knockout retina has abnormal histology, with rosettes and whorls within the outer nuclear layer. Only 20% of photoreceptors elaborate outer segments, most of which have abnormal disk morphology. Electroretinogram (ERG) recording revealed no scotopic response and detected a light-adapted b-wave of two to three times larger amplitude in knockout than that of wild-type retina, demonstrating the absence of rod function and an enhanced cone function. Using monochromatic stimuli of 400 nm or 530 nm, this large b-wave amplitude is explained by increased S-cone activity. Preliminary gene expression analysis revealed an absence of rod-specific transcripts, and an increase in the expression of cone-specific genes (Mears et al 2001). Dramatic retinal changes observed in this mouse establish it as an excellent model for expression profiling corresponding to different pathways associated with rod and cone development and function. We propose genes with reduced expression in the $Nrl^{-/-}$ retina relative to normal would be associated with rod-signalling pathways, while those with augmented expression relate to cone function.

Microarray analysis

High-throughput technologies, including cDNA microarrays and Affymetrix GeneChips have made large-scale gene expression studies of retinal tissues readily achievable (Farjo et al 2002, Yoshida et al 2002, Swaroop & Zack 2002). Microarrays allow us to investigate changes in expression at a genome scale in a single experiment. This approach is limited only by the number and types of genes represented on the arrays. In addition to being a powerful gene-discovery tool in the identification of candidate genes, microarrays may shed considerable light on the cellular pathways of the tissue under study (Livesey et al 2000, 2002). A schema of microarray analysis is presented in Fig. 2. Although Affymetrix technology is relatively well developed, with appropriate quality controls, standard data pre-processing and ready-to-use data analysis software, its application to our studies is limited by the under-representation of retinal expressed genes on their GeneChips. For comprehensive profiling, customized *I-Gene* cDNA microarrays were also utilized. These arrays were generated by printing retina/eye-expressed genes and expressed sequence tags (ESTs) obtained from a variety of cDNA libraries (*www.umich.edu/~igene/*; Yu et al 2003) onto glass

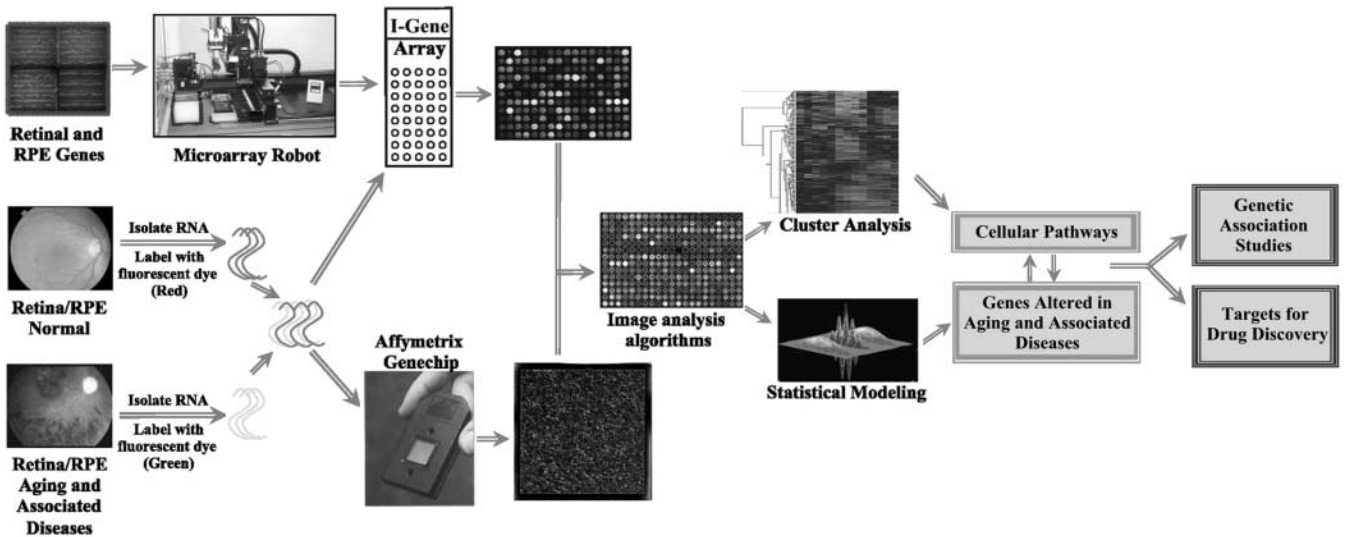

FIG. 2. Comprehensive gene profiling of control and mutant retinas using Affymetrix GeneChips and custom *I-Gene* microarrays. Temporal expression profiling followed by statistical modelling and cluster analysis can lead to the identification of pathways and molecular targets.

slides using a robotic micro-arrayer (Farjo et al 2002, Yu et al 2002). For these high-throughput studies, total RNA was isolated from either control (normal) or experimental (diseased or ageing) retinas, labelled with fluorescent dyes and hybridized to either Affymetrix GeneChips or *I-Gene* microarrays (Fig. 2). Image analysis and statistical modelling were employed to identify differentially expressed genes between control and experimental samples. Clustering algorithms were used to group co-expressed genes under different experimental conditions, which might lead to the identification of functional/regulatory networks and pathways (Fig. 2). We have used gene profiling of retinas from the normal and *Nrl*-knockout mice as a paradigm and to establish the proof of principle.

Affymetrix GeneChip study

Gene profiling of postnatal day 2 (PN2), PN10 and 2 month-old retinas from the control and *Nrl*-knockout mice using mouse GeneChips showed approximately equal number of up- or down-regulated genes at each time point (data not shown). At PN2, only 6 genes are found to be differentially expressed, compared with 74 at PN10 and 136 at 2 months. As predicted, several rod photoreceptor-specific genes, including rhodopsin (*Rho*) and rod transducin alpha (*Gnat1*), were found to be greatly under-expressed in the knockout mouse, while cone genes, such as S-opsin (*Opn1sw*) and cone transducin alpha (*Gnat2*), are up-regulated. Quantitative real-time PCR (qRT-PCR) analyses of almost 50 genes have validated gene expression changes revealed by GeneChips; qRT-PCR profiles of four genes are shown in Fig. 3. More than 20% of differentially expressed transcripts were unknown ESTs. These are of considerable interest, as they may represent novel retinal dystrophy candidate genes or lead to the elucidation of specific cellular pathways associated with photoreceptor differentiation and function. Clusters of differentially expressed genes may also provide insights into pathways and functional networks (Fig. 4).

I-Gene micoarray study

Gene expression of wild type and $Nrl^{-/-}$ mice retinas were compared at five developmental time points: PN0, PN2, PN6, PN10 and PN21. Custom *I-Gene* microarrays containing over 6500 eye/retina expressed genes and ESTs printed in duplicate were generated for hybridization (Figs 5A,B). Five replicates were performed for each stage utilizing labelled targets from different mice to reduce individual variance. Density plots of the log-ratios of gene expression in PN21 $Nrl^{+/+}$ and $Nrl^{-/-}$ mice retinas detected by five independent replicated experiments showed similar patterns of distribution. Log-ratios of all replicates are centred at 0, with most genes lying within –1 and +1 (Fig. 5C), suggesting

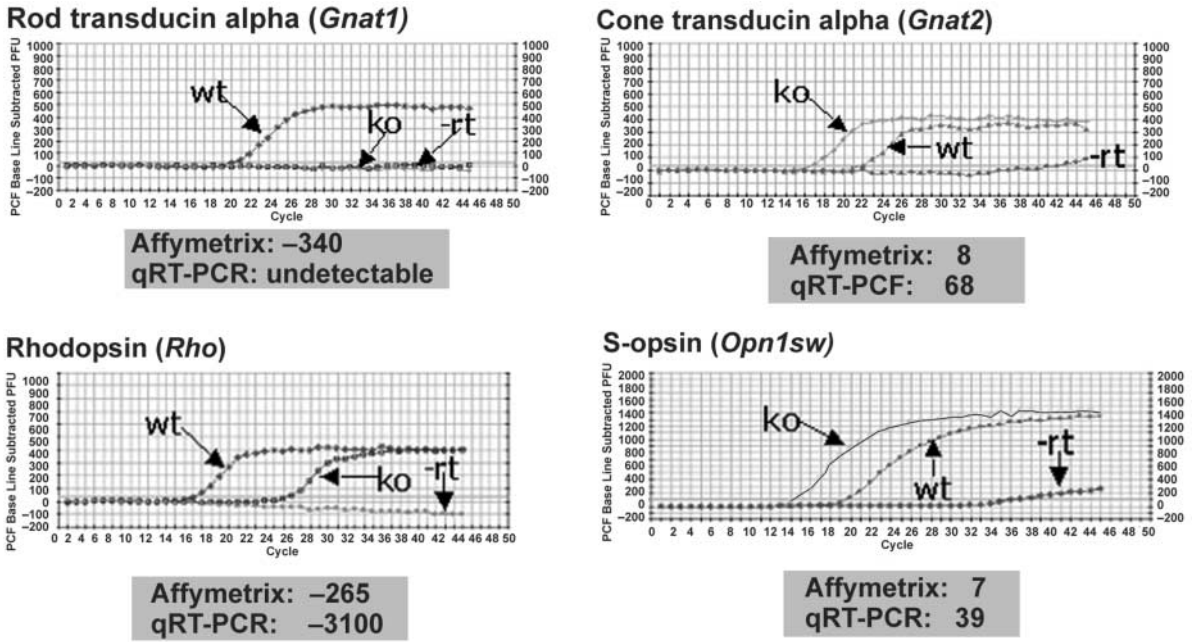

FIG. 3. qRT-PCR analyses of four differentially-expressed genes identified by Affymetrix GeneChip analysis. Total RNA from wild-type (wt) and *Nrl*$^{-/-}$ (ko) mice retinas were first reverse transcribed either with or without (−rt) reverse transcriptase, and then subjected to real-time PCR. The negative control (−rt) experiments were utilized to demonstrate that RNA samples are free from genomic contamination. qRT-PCR profiles of wt, ko and −rt were shown for four genes, *Gnat1*, *Rho*, *Gnat2* and *op1sw*. The fold difference was calculated as 2 to the power of the difference in threshold cycles (Ct) between wild-type and knockout samples. Affymetrix chips and qRT-PCR showed high concordance for all genes, with qRT-PCR generally being more sensitive.

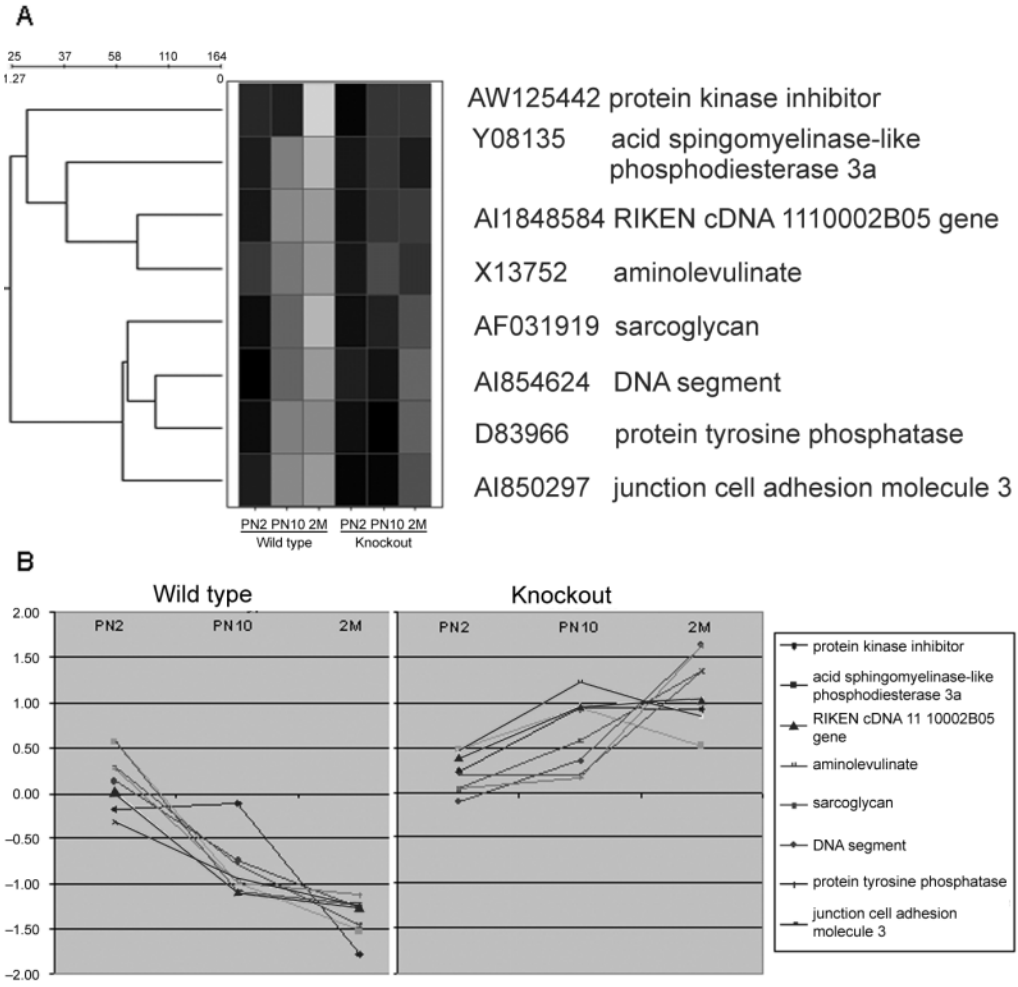

FIG. 4. Cluster analysis of the temporal expression profiles generated from Affymetrix GeneChips. (A) Representation of clustering analysis of differentially expressed genes. The data matrix was first standardized to z-score and hierarchical clustering analysis performed using the 'Euclidean distance' method. Colour-coding indicates relative expression: green being low, red high (this appears as grey scale on this black and white reproduction). Eight genes shown are clustered based on their similarity of expression profile, which is also graphically represented in (B), where z-scores (Y-axis) are plotted against time-point (X-axis).

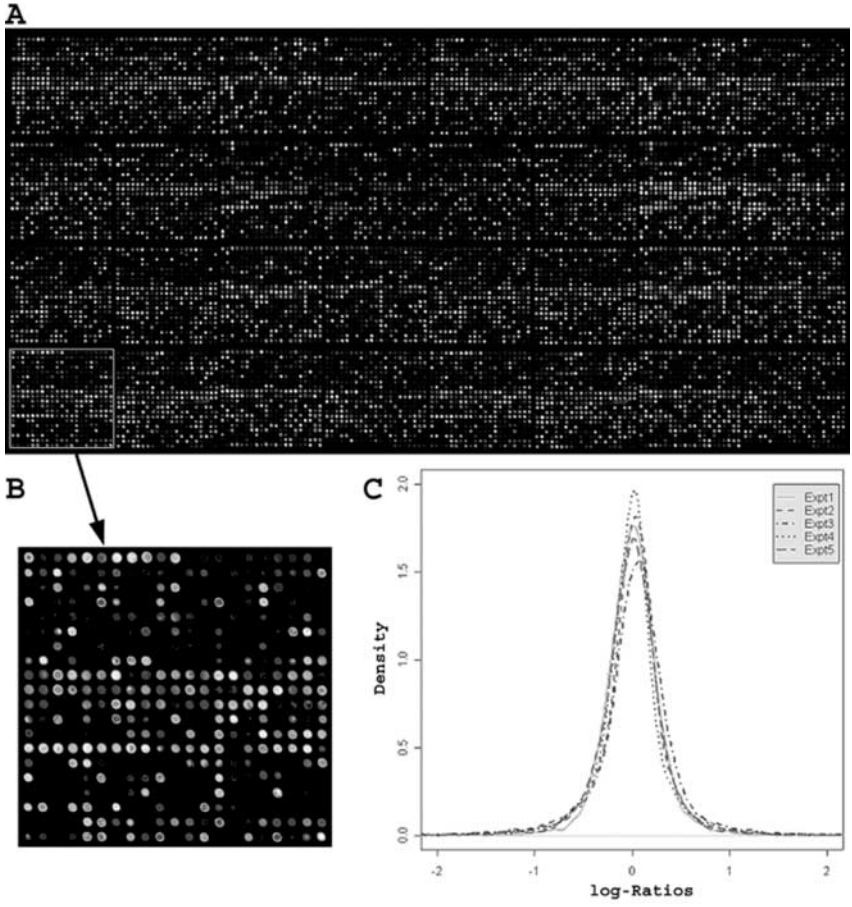

FIG. 5. *I-Gene* microarray and density plots of log-ratios in five replicate experiments. (A) A TIFF image of the Cy3 channel of an *I-Gene* microarrays containing over 6500 genes or ESTs printed in duplicate. False colour has been applied to indicate the intensity of hybridization, with black having no signal, blue low, red high, and white saturated (these appear as grey scales on this black and white reproduction). (B) Enlargement of the left lower corner grid of the array, showing uniform spot diameter, clear hybridization and low background signal. (C) Ratios of gene expression indicate the abundance of each gene in $Nrl^{-/-}$ mice retinas relative to $Nrl^{+/+}$ retinas. Smooth density plots of log-ratios shows that, in all replicates (expt1–expt5), log-ratios are centred at 0, with majority of spots lying between −1 and +1.

that the expression of a majority of genes is unaltered or minimally altered between the control and *Nrl*-knockout retinas. Microarrays tend to underestimate the true biological change and perhaps a log ratio threshold of less than 1 needs to be established. Statistical analysis of PN21 expression data identified 52 cDNAs, representing 39 unique genes, with the highest possibility of differential

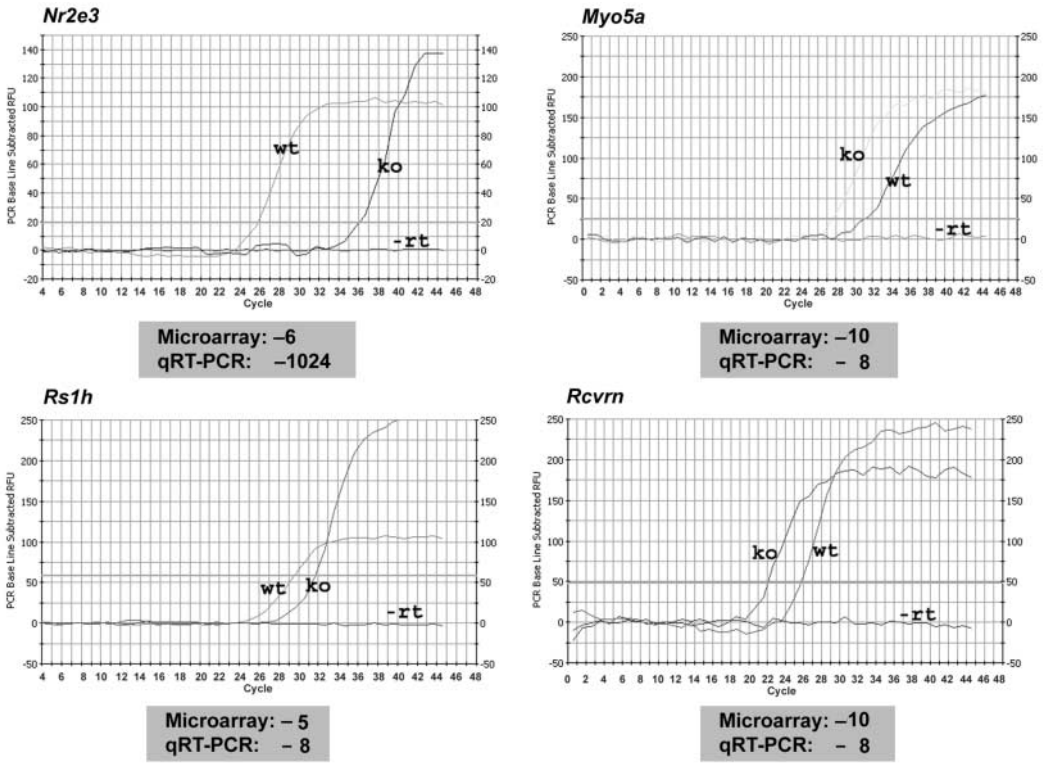

FIG. 6. qRT-PCR validation of *I-Gene* microarray results: analysis of *Nr2e3*, *Rs1h*, *Myo5a*, and *Rcvrn* expression in retinas of wild-type (wt) and *Nrl*-knockout (ko) mice. Total RNA from wild-type and knockout mice retinas were first reverse transcribed either with or without (−rt) reverse-transcriptase, and then subjected to real-time PCR. The negative control (−rt) experiments were utilized to demonstrate that RNA samples are free from genomic DNA contamination. qRT-PCR tends to be more sensitive than the hybridization-based microarray experiments.

expression. Over 30% of these genes are known to play important roles in the retina; these include *Rho*, *Opn1sw*, *Gnat1*, *Gnat2*, *Nr2e3*, Retinoschisis 1 homolog (*Rs1h*), myosin 5a (*Myo5a*) and Recoverin (*Rcvrn*). qRT-PCR analyses validated these expression alterations (Fig. 6). Further examination of these differentially expressed genes suggests a bias in the utilization of the bone morphogenetic protein (Bmp) signalling pathway, *Wnt*/Ca^{2+} signalling pathway and the retinoic acid pathway between rods and cones (J. Yu, A.J. Mears and A. Swaroop, unpublished data).

Pathway consolidation

Affymetrix GeneChip studies, presented here, showed differential gene expression from PN2, PN10 to 2-month-old retinas, whereas *I-Gene* cDNA microarray data indicated alterations of signalling pathways in the PN21 knockout mice retinas. Systematic examination of gene expression levels at PN0, PN2, PN6, PN10 and PN21 followed by statistical analysis should further assist in the identification of genes that are downstream of *Nrl* in regulatory hierarchy and play key roles in photoreceptor differentiation and/or function. Clustering based on temporal expression profiles may identify coordinately regulated genes involved in rod and cone photoreceptor development. Since hypermorphic alleles of *Nrl* are predicted to cause retinal degeneration, the signalling pathways downstream of *Nrl* may also be studied in the context of other retinal degenerative mouse models.

Conclusions

Delineation of cellular pathways involved in photoreceptor differentiation and disease pathogenesis presents an attractive approach to identify targets for treatment of RD. In this presentation, we have used a single paradigm to illustrate our research approach and the focus on cellular pathways downstream of an important retinal gene. *Nrl* is a rod-specific transcription factor that is required for rod differentiation and regulation of rod-specific gene expression. Mutations in the human *NRL* gene have been identified in patients with autosomal dominant RP. The $Nrl^{-/-}$ mouse retina is rodless, with an increased number of functional S-cones. Using Affymetrix GeneChips and custom *I-Gene* cDNA microarrays, we have so far identified over 150 genes that are differentially expressed in the *Nrl*-knockout mouse retina as compared to controls. Several of these cDNAs represent novel genes that are attractive candidates for RD. Further characterization of differentially-expressed cDNAs should reveal direct or indirect targets of *Nrl* and assist in developing transcriptional regulatory hierarchy downstream of *Nrl*. Initial studies also suggest differential utilization of signalling pathways in rods and cones. Our investigations provide an initial

framework for establishing pathway-based treatment strategies for retinal and macular diseases.

Acknowledgements

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DISCUSSION

McInnes: When is *Nrl* turned on developmentally?

Swaroop: By RT-PCR we can detect it around E16.5 in embryonic mouse retina, but by Northern analysis it is more like E18.5. The antibodies we have currently pick up another protein called p45, which is present in all developing neurons. We are currently generating additional, more specific antibodies.

McInnes: Is p45 a product of the *Nrl* gene?

Swaroop: No, it is encoded by a different gene expressed probably in all neural cells. It is antigenically similar.

Hauswirth: In the *Nrl* knockout mouse, is the enhanced photopic ERG amplitude due to the presence of more cones? Or is there a higher response from the cones that are there?

Swaroop: The ERG studies show a higher response but there is more S opsin. The outer segments of Cods (cone–rod hybrids) that are there have S opsin.

Hauswirth: So is the extra amplitude coming from 'Cods', not from a conversion of rods to real cones?

Swaroop: We don't know what these Cods are. We use this term because we don't want to call them cones. It is too early to say whether they are rods converted to cones. We are working on it.

Hauswirth: What about the rest of the phototransduction cycle in cones?

Swaroop: It is all present in these Cod outer segments. All rod-specific genes have been switched off. None of the rod-specific proteins are expressed, whereas every cone-specific phototransduction protein that we have looked at is expressed at high levels.

Hauswirth: So if you want to preserve cone function in humans you just need to knock out *NRL*!

Bok: Anand Swaroop, did you say that the photoreceptors in this knockout mouse do not die?

Swaroop: The function of these cones is bizarre, and we do not see any large-scale change in the thickness of the outer nuclear layer at least for 6 months. So there is minimal cell loss during this period.

Bok: I presume the reason that Ed Stone and others looked at S-cone enhanced syndrome was because there is some sort of disease process in those retinas. Is there a cell loss, or is it just bizarre physiology?

Swaroop: I think there may be cell death in the *rd7* mouse.

Farber: The *rd7* mouse has a mutation in the *PNR/Nr2e3* gene. *NR2E3* mutations in humans cause enhanced S-cone syndrome (ESCS).

Bok: Do those humans lose cells?

Farber: No.

Bird: They have a restricted form of retinitis pigmentosa, and different mutations in the same gene cause a very severe form of retinal dystrophy.

Swaroop: My understanding is that there is some degeneration of photoreceptors in ESCS.

Dryja: They are different mutations. These are knockout mice, and all the humans are dominant missense mutations.

Bok: So are you talking about a gain of function in humans?

Swaroop: No. The human mutations in *NR2E3* are also loss of function. Why do *NR2E3* mutations lead to retinal degeneration, whereas we don't see this in the *Nrl* knockout mice? We have only looked up to six months. The mice are now two years old and we are working with Dr Paul Sieving to examine the retina of older mice by histology and ERG to figure out whether there has been any loss of cones. We have done some histology in mice older than 6 months and the outer nuclear layer is thinner. I don't know whether there is slow loss of cone function, but we are working on it. I must state that these may not be real cones because they do express some rod markers. Our collaborator, Dr David Hicks, has two antibodies, Ret P3 and L1, which appear to specifically recognize rods, not cones. These two antibodies recognize antigens in the knockout retina. In addition, Dr Enrica Strettoi has observed that the synaptic connections these Cods are forming are also apparently different from the normal rod and cone connections. According to Dr Ed Pugh, the Cods function as cones.

Farber: Many years ago we worked a lot with ground squirrels, and found that they had some cells that were intermediate between rods and cones. They all happened to be S cone cells. It might be worth looking here.

Swaroop: Maybe they don't have NRL, and that is why they are all S cones.

Zack: Have you used arrays on the *Rd7* mouse to complement these?

Swaroop: Yes. We have done two time points but the data have not been analysed yet.

Bhattacharya: I have a general question about the microarray data. What is your feeling about the level of variability seen from one experiment to another?

Swaroop: The correlation coefficient we get with Affymetrix GeneChips is over 0.99. If the same person dissects the retina and at the same time of the day the variability is minimum. If you take another knockout mice you see a little more variability. In slide microarrays we get closer to 0.98, so there is a little bit more variability in these. Even in slide microarrays there are ways to normalize the data. We are working with data-driven normalization. Rather than a global normalization of signals over the whole slide we do this on the basis of the data on each slide. This helps a great deal.

Aguirre: In an earlier talk Donald Zack showed a variability that was mainly patient related rather than age related. What are the prospects for looking at microarray data on patients?

Swaroop: I was talking primarily about mouse, where the data are very clean. We have done human studies with eight Affymetrix chips for young and eight Affymetrix chips with old retina, and there is a huge amount of variability within

the samples. You have to throw away many of the data that may be real but we can't be confident. I tend to be very conservative. This variability could be because of inherent variations in humans or because of many other factors, including tissue collection time and tissue preservation.

Cremers: There was a recent paper in *Science* showing the variance of different genes (Yan et al 2002). They showed that in families expression levels could vary in normal individuals two- to fourfold.

Swaroop: That is why we chose to work in mice.

Cremers: Why do you think it is different in mice?

Swaroop: Because we are working with isogenic strains and we are controlling the sample preparation more carefully. With mice the variation is very low if the same strains are used. In humans there are many confounding factors.

Thompson: Are you using gender matching in your analysis?

Swaroop: Yes. My feeling is that there will be some genes that will show large variation, but most of the genes do not change. Once we define the baseline expression profile of all genes it will be easier.

Bok: You would do a service to all of us to figure out what the gender differences are so we can subtract these out.

Swaroop: We are working on many aspects of microarray data with respect to retinal biology. We are also working on the development of databases and web-based sharing of information. We hope to make all of our data available on our website.

Bhattacharya: If we identify a mutation in a novel gene in humans, we may want to look at the disease biology (the impact of the mutation and how it might lead to cell death) through microarray techniques. If there is a huge amount of variation, would it be worthwhile generating a mouse model for each human gene and then studying the disease biology in the mouse?

Swaroop: From my own experience, the human work with microarrays has been very frustrating. Dr Shigeo Yoshida, a Japanese postdoc in my lab, had worked 16 h days for two years and produced few useful data, ultimately. For the postdoc's sake it might be better to use mice than humans, at least initially. I would also advise people not to do just one time point: I would look at the progression of disease in the mouse model and pick four or five time points. It is a lot of work, but the information gained is very valuable.

Thompson: In terms of the human data, once we can get a line on more pathways affected in retinal degeneration, so we are not looking at everything but just focusing on one pathway at a time, then it will be easier to see important changes.

Swaroop: Once you have defined the pathways then you can fit in the data you get from human studies. It is much easier.

Zack: In terms of variability in different genes, I agree. In our experiments it turns out that rhodopsin is one of the most variable genes in the retina. Because

rhodopsin is expressed at such a high level it is very easy to measure accurately on an array, but the level can vary by sixfold just at the RNA level in age-matched individuals.

Bird: Does this vary by time of day?

Zack: We have too few data to answer that. But in mouse models rhodopsin is not one of the genes that is subject to significant circadian regulation.

Swaroop: That is a good point. With all our mice we dissect them between 12 and 2 pm because we are not sure whether this is a significant factor or not. I think it probably does matter. As long as everything is kept the same the variation is lower.

Reference

Yan H, Yuan W, Velculescu VE, Vogelstein B, Kinzler KW 2002 Allelic variation in human gene expression. Science 297:1143