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 rod-less (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.
Background and Basic Concepts

Retinal dystrophies (RD) comprise a group of clinically and genetically heterogeneous retinal disorders, which typically result in the degeneration of 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 world-wide (Saleem & Walter 2002). In contrast, cone dysfunction occurs early during the progression of cone or cone-rod dystrophies (CRD), thereby affecting visual acuity and color 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 Unites States (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 defect. 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 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 (Figure 1). Although a large number of retinal and 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 Figure 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 aging 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 aging is reflected by modulation of specific cellular pathways and, consequently, changes in gene expression. Profiling of diseased or aging retina or RPE from humans and mice will facilitate in
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; Bessant et al 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*<sup>−/−</sup> mouse retina is functionally rod-less. 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 530nm, 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 at the *Nrl*<sup>−/−</sup> retina relative to normal would be associated with rod signaling 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; Livesey 2002). A schema of microarray analysis is presented in Figure 2. Although Affymetrix technology is relatively well developed, with appropriate quality controls, standard data preprocessing 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 ESTs obtained from a variety of cDNA libraries onto glass 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 aging) retinas, labeled with fluorescent dyes and hybridized to either Affymetrix GeneChips or I-Gene microarrays (Figure 2). Image analysis and statistical modeling 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 (Figure 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 4 genes are shown in Figure 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 (Figure 4).

**I-Gene Microarray Study**

Gene expression of wild type and Nrl−/− mice retinas were compared at 5 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 (Figure 5A, B). Five replicates were performed for each stage utilizing labeled 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 centered at 0, with most genes lying within −1 and +1 (Figure 5C), suggesting 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 lower threshold instead of 1 needs to be established. Statistical analysis of PN21 expression data identified 52 cDNAs, representing 39 unique genes, with highest possibility of differential expression. Over 30% of these genes are known to play important roles in the retina; these include \textit{Rho}, \textit{Opn1sw}, \textit{Gnat1}, \textit{Gnat2}, \textit{Nrg2e3}, \textit{Retinoschisis 1 homolog (Rs1h)}, \textit{myosin 5a (Myo5a)}, and \textit{Recoverin (Rcvrn)}. qRT-PCR analyses validated these expression alterations (Figure 6). Further examination of these differentially expressed genes suggests a bias in the utilization of the Bone Morphogenetic Protein (Bmp) signaling pathway, \textit{Wnt/Ca}^{2+} signaling pathway and the retinoid acid pathway between rods and cones (Yu, Mears and Swaroop, unpublished data).

**Pathway Consolidation**

Affymetrix GeneChip studies, presented here, showed differential gene expression from PN2, PN10 to 2-month old retinas, whereas \textit{l-Gene} cDNA microarray data indicated alterations of signaling 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 \textit{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 \textit{Nrl} are predicted to cause retinal degeneration, the signaling pathways downstream of \textit{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. \textit{Nrl} is a rod-specific transcription factor that is required for rod differentiation and regulation of rod-specific gene expression. Mutations in the human \textit{NRL} gene have been identified in patients with autosomal dominant RP. The \textit{Nrl}\textsuperscript{−/−} mouse retina is rod-less, with an increased number of functional S-cones. Using Affymetrix GeneChips and custom \textit{I-Gene} cDNA microarrays, we have so far identified over 150 genes that are differentially expressed in the \textit{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 \textit{Nrl} and assist in developing transcriptional regulatory hierarchy downstream of \textit{Nrl}. Initial studies also suggest differential utilization of signaling pathways in rods and cones. Our investigations provide an initial framework for establishing pathway-based treatment strategies for retinal and macular diseases.
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Figure Legends

Figure 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 signaling 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 signaling pathways (PAS1…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.

Figure 2

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

Figure 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 4 genes, *Gnat1*, *Rho*, *Gnat2* and *oplsw*. The fold difference was calculated as $2^{\Delta \text{Ct}}$ between wt and ko samples. Affymetrix chips and qRT-PCR showed high concordance for all genes, with qRT-PCR generally being more sensitive.

**Figure 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. Color-coding indicates relative expression: green being low, red high. 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).

**Figure 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 color has been applied to indicate the intensity of hybridization, with black...
having no signal, blue low, red high, and white saturated. (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 Nrt\(^{+/−}\) mice retinas relative to Nrt\(^{+/+}\) retinas. Smooth density plots of log-ratios shows that, in all replicates (expt1-expt5), log-ratios are centered at 0, with majority of spots lying within −1 and +1.

**Figure 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 wt and 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 DNA contamination. qRT-PCR tends to be more sensitive than the hybridization-based microarray experiments.
Figure 1
Figure 2
Figure 3

Rod transducin alpha (Gnat1)

Affymetrix: 340
qRT-PCR: undetectable

Cone transducin alpha (Gnat2)

Affymetrix: 8
qRT-PCR: 63

Rhodopsin (Rho)

Affymetrix: 265
qRT-PCR: -3100

S-opsin (Opulsw)

Affymetrix: 7
qRT-PCR: 39
Figure 4

A

AW125442  protein kinase inhibitor
Y08135  acid sphingomyelinase-like phosphodiesterase 3a
A1849584  RIKEN cDNA 1110002B05 gene
X13752  aminolevulinate
AF031919  sarcoglycan
AI854624  DNA segment
D83955  protein tyrosine phosphatase
AI050297  junction cell adhesion molecule 3

B

Wild type  Knockout

-2.00  -1.50  -1.00  -0.50  0.00  0.50  1.00  1.50  2.00

PN2  PN10  2M  PN2  PN10  2M
Figure 5
Figure 6

Nuclear receptor subfamily 2, group E, member 3 (Nr2e3)  
Myosin 5a (Myo5a)

Retinoschisis 1 homolog (Rslh)  
Recoverin (Rcvrn)

Microarray: -6  
qRT-PCR: -1024

Microarray: 10  
qRT-PCR: 8

Microarray: -5  
qRT-PCR: -8

Microarray: 8  
qRT-PCR: 10
REFERENCES


