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Transcriptome profiling of NIH3T3 cell lines expressing opsin and the P23H opsin mutant identifies candidate drugs for the treatment of retinitis pigmentosa

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ABSTRACT

Mammalian cells are commonly employed in screening assays to identify active compounds that could potentially affect the progression of different human diseases including retinitis pigmentosa (RP), a class of inherited diseases causing retinal degeneration with compromised vision. Using transcriptome analysis, we compared NIH3T3 cells expressing wildtype (WT) rod opsin with a retinal disease-causing single P23H mutation. Surprisingly, heterologous expression of WT opsin in NIH3T3 cells caused more than a 2-fold change in 783 out of 16,888 protein coding transcripts. The perturbed genes encoded extracellular matrix proteins, growth factors, cytoskeleton proteins, glycoproteins and metalloproteases involved in cell adhesion, morphology and migration. A different set of 347 transcripts was either up- or down-regulated when the P23H mutant opsin was expressed suggesting an altered molecular perturbation compared to WT opsin. Transcriptome perturbations elicited by drug candidates aimed at mitigating the effects of the mutant protein revealed that different drugs targeted distinct molecular pathways that resulted in a similar phenotype selected by a cell-based high-throughput screen. Thus, transcriptome profiling can provide essential information about the therapeutic potential of a candidate drug to restore normal gene expression in pathological conditions.

1. Introduction

Small molecules are the mainstay of pharmacotherapeutics for the treatment of human diseases. Such agents often are easy to administer, well tolerated by patients, and relatively inexpensive. Nonetheless, complex diseases remain difficult to manage, and typically worsen with age. In infectious diseases or cancer, poly-pharmacology aimed at diverse and unrelated targets can effectively deal with the primary problem but often with adverse side effects. This risk can be acceptable for treating terminal diseases or chronic life-threatening infections but not for slowly progressive diseases that are not fatal or overly burdensome during a patient's normal lifespan. To minimize such drug side effects, recent approaches in systems biology can be used to characterize the molecular features of disease models used in both the early and later stages of drug development [1,2]. Moreover, these technologies can be adapted to improve our understanding of the

Abbreviations: ad, autosomal dominant; BP, biological process; CHOP, C/EBP homologous protein; CRE, the cAMP-responsive element; CREB, the cAMP-responsive element-binding protein; CREM, the cAMP-responsive element modulator; ATF-1, activating transcription factor-1; DAVID, the database for annotation visualization and integrated discovery; DE, differential expression; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; FDA, Food and Drug Administration; FDR, false discovery rate; FPKM, fragments per kilobase of transcript per million mapped reads; GFP, green fluorescence protein; GPCR, G protein-coupled receptor; GO, gene ontology; HTS, high-throughput screen or screening; NGS, next-generation sequencing; qPCR, quantitative real-time polymerase chain reaction; RP, retinitis pigmentosa; WT, wild-type.

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pharmacology of a starting molecule with respect to its possible off-target effects and therapeutic potential.

Retinitis pigmentosa (RP) is a progressive retinal degenerative disease associated with mutations in more than 50 genes [3,4]. Effective treatments of RP are unavailable, even though gene therapy [5,6] and pharmacological intervention with valproic acid [7–11] are currently undergoing clinical trials. The rhodopsin pigment, comprised of the protein opsin bound to a vitamin A chromophore that is regenerated in the endoplasmic reticulum and outer segment disc membranes, is an essential component of rod photoreceptor cells due to its pivotal role in phototransduction and its abundance in the photosensitive outer segments [12]. Thus, it is not surprising that *Rho* encoding the rod opsin, is the most frequent causal gene among autosomal dominant (ad) RP patients [3,13]. The P23H mutation, observed in 10% of adRP cases, is a representative Class II mutation that causes opsin misfolding due to its thermal instability [3,13–16]. Instability of P23H opsin leads to its progressive massive degradation in the rod photoreceptors of P23H knock-in mice [17,18]. To rescue these photoreceptors, we hypothesized that improving P23H opsin stability could decrease photoreceptor cell death and improve vision. Alternatively, increasing the degradation of mutant opsin and leaving the WT rhodopsin allele to preserve visual function could be an equally viable therapeutic strategy for adRP. Thus, we developed and performed two sets of cell-based, small-molecule high-throughput screens (HTSs) to identify compounds that either improve the stability of the P23H opsin mutant or enhance its degradation [19]. Even though a valid photoreceptor cell line is unavailable, mammalian cells have been commonly used to study the biosynthesis of rhodopsin and to screen for drug candidates because the pre-ciliary biosynthesis of rhodopsin is generally shared by mammalian cells and rod photoreceptor cells [14,20,21]. In mammalian cell cultures, heterologously expressed WT opsin is located on the plasma membrane whereas P23H opsin accumulates in the endoplasmic reticulum (ER) due to its structural instability [14–16,19–21].

For drug discovery, lead compounds identified from HTS in cell models are then tested in an animal model that represents the genetic defect and exhibits pathological signs seen in the corresponding human disease. The challenge of developing drug candidates showing efficacy in both cell and animal models sometimes lies in the dramatic difference of the two model systems. To improve our success rate of drug development, we need a better understanding of our disease models and the lead compounds' mechanisms of action. Advances in microarray and next generation sequencing (NGS)-based transcriptome profiling (RNA-seq) have already identified novel molecular pathways or key genes associated with the development of disease states in the mouse retina [22–30]. Although transcriptome studies have been applied to low-dose pharmacological treatments in disease models to evaluate drug efficacy and side effects [30–34], RNA-seq studies have rarely been used in the early stages of drug discovery to characterize the cell models used for high throughput screening and to investigate a lead compound's mechanism of action [30,35,36].

Here, using high-throughput RNA-seq technology, we profiled the transcriptomes of three stable cell lines expressing either opsin/green fluorescent protein (GFP), P23Hopsin/GFP or GFP alone, under different treatment conditions. This study addresses three questions related to drug discovery: (1) what are the general transcriptome changes in an established mammalian cell line due to heterologous expression of rod opsin; (2) what transcriptome changes arise from the expression of the P23H opsin mutant; and (3) what genes and associated molecular pathways are affected by treatment with active compounds selected from a HTS?

2. Materials and methods

2.1. Cells

NIH3T3 (WTopsin/GFP), NIH3T3 (P23Hopsin/GFP) and NIH3T3 (GFP) stable cell lines were generated by viral infection with pMiLRO, pMiLRO23 and pMXs-IG constructs, as previously published [19]. Mouse opsin or P23H opsin was co-expressed with GFP. When single clones were selected, no significant differences were observed between them with respect to cell shape, GFP fluorescence or localization of WT opsin (on the plasma membrane) or P23H opsin (in the ER) suggesting the defect of P23H opsin transport is not due to a clonal difference.

2.2. Immunostaining and fluorescence imaging

Immunostaining and fluorescence imaging followed published procedures [19]. Briefly, cells were seeded in a 384-well plate at 5000 cells/well on day 1. These cells were treated with active compounds or a DMSO control on day 2. Cells were fixed with 4% paraformaldehyde 24 h after treatment. Rod opsin was immunostained with 1D4 monoclonal anti-rhodopsin antibody [37] followed by secondary labeling with Cy3-conjugated goat anti-mouse antibody (Jackson Immuno Research, West Grove, PA, USA). Nuclei were stained with DAPI. Fluorescence images were captured with an Operetta High Content Imaging System (PerkinElmer, Waltham, MA, USA). Five images were taken from each well of the 384-well plate containing a total of 600–1000 cells. Three channels of fluorescence emission were used, Alexa488 (GFP), DAPI, and Cy3. Representative images of immunostained cells are shown in Fig. 1A–F.

2.3. Image analysis

Only intact cell images were selected for analysis. The cytoplasm was defined by the GFP fluorescence of each cell with the cell boundary as the 0% line. Nuclei were visualized by DAPI fluorescence, and each nucleus, as defined by its edges, was assumed to comprise 50% of each cell. The membrane region was defined within 5% around the cell boundary, and the ER region was defined within 25–50% at the perinuclear region. Opsin staining on the plasma membrane was represented by the ratio of Cy3 intensity in the membrane region to that in the entire cell (MEM-total). Opsin staining in the ER region was denoted by the ratio of Cy3 intensity in the ER region to that in the entire cell (ER-total). MEM-total and ER-total were averaged from all the imaged intact cells in each well. Each condition was repeated in 3 or 8 wells, for treatments or controls, respectively. MEM-total and ER-total were then averaged from those biological repeats and presented as data points in Fig. 1G–N. Error bars were standard deviations from those biological repeats.

2.4. Identification of active compounds by HTS

Compound 1 (an isoquinoline-2(3*H*)-hexanamide) and compound 2 (4-(5-chlorothiophen-2-yl)furan-2(5H)-one) were selected by a cell-based HTS of small molecules from the University of Cincinnati 2.5 K Diversity Set of Small-Molecules Library [19]. To identify small molecular compounds which rescue the P23H opsin mutant from ER retention, we performed a HTS with a β-galactosidase fragment complementation assay as described in reference [19]. Briefly, two complementary subunits of beta-galactosidase were individually fused with a plasma membrane-anchored peptide, the PH domain of phospholipase C delta (PLC), and the mouse opsin P23H mutant, respectively. A U2OS stable cell line was generated that consistently expresses