Modulation of Aerobic Glycolysis Genes During the Progression of Retinitis Pigmentosa

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Purpose. Photoreceptors are retinal cells with a high glucose metabolism and retinal degeneration, specifically retinitis pigmentosa (RP), affects glycolysis. We aimed to evaluate changes in the expression of genes related to glucose metabolism in rod photoreceptors at different stages of retinal degeneration in murine models and human retinal organoids.

Methods. RNA sequencing (RNA-seq) analysis was performed on a photoreceptor-like cell line induced to undergo degeneration and validated by real-time qPCR analysis of retinas from two murine models and one human organoid model of RP. Bioinformatic analysis was performed on published RNA-seq datasets from three murine RP models. Real-time qPCR analysis was also performed on retinas treated with an adeno-associated virus type 2 vector carrying the neurotrophic H105A peptide, derived from the pigment epithelium-derived factor.

Results. The aerobic glycolysis genes, *Hk2*, *Pkm1*, *Pkm2*, *Ldha*, and *Slc6a6* and other glucose metabolism genes were found downregulated in the in vitro model of photoreceptor degeneration and in the in vivo $Rho^{P23H/+}$, *rd1*, and *rd10* models at early stages of the disease. The decreased expression of the aerobic glycolysis genes, except for *PKM2*, was confirmed in human organoids with mutations in the *USH2A* gene associated with RP. Expression was partially recovered in $Rho^{P23H/+}$ retinas after treatment with the adeno-associated virus type 2 vector expressing the neurotrophic H105A peptide.

Conclusions. Glucose metabolism gene expression was found altered during the progression of RP in murine and human models of the disease. Expression was partially recovered in a molecular response to the treatment with the neurotrophic factor H105A.

Keywords: glucose, photoreceptor metabolism, retinitis pigmentosa, human retinal organoids, PEDF

Photoreceptors are extremely metabolically active cells with high adenosine triphosphate (ATP) consumption to maintain a depolarized membrane potential in the dark and for phototransduction in the light.[1] Glucose is the primary fuel that meets the photoreceptors' energetic needs because it can generate large amounts of ATP. Although the majority of neurons use oxidative phosphorylation, photoreceptors have been suggested to mostly use aerobic glycolysis, also known as Warburg metabolism, that is important for their outer segment biogenesis and generates lactate to be secreted and used for energy production.[2–4] The enzymes LDHA and PKM2 have been reported to drive aerobic glycolysis in photoreceptor cells and to support outer segment biogenesis.[2] Glucose is delivered to photoreceptors through apical and basal transporters of the RPE. Accordingly, RPE-specific knock-out of the glucose transporter 1 has a strong impact on photoreceptor metabolism with shortening of photoreceptor outer segments followed by photoreceptor degeneration.[5] In turn, the progressive shortening of the outer segments and loss of connections to the RPE affect glucose transport to photoreceptor cells.[6] A recent study, based on a retinal explant setup, suggested that rod photoreceptors rely on oxidative phosphorylation under healthy conditions, but that the absence of the RPE leads to photoreceptor degeneration and to a metabolic shift toward aerobic glycolysis.[7] This apparent contradiction might be related to the fact that glucose metabolism in photoreceptors is regulated by several mechanisms, including taurine. Taurine is a nonprotein amino acid mainly localized in photoreceptor inner segments and synapses[7] and has an important function in photoreceptor differentiation.[8] In highly metabolic cells, taurine drives glycolysis and favors tumor malignancy in leukemia.[9] Interestingly, the expression of the taurine transporter SLC6A6 is finely regulated in the retina.[10] The loss of *Slc6a6* in mice causes photoreceptor cell death[11] and variants in the *SLC6A6* gene in humans have been linked to a syndromic form of retinal degeneration.[12] Taken together, these data suggest that defects in taurine transport are detrimental to photoreceptors, possibly owing to impaired glycolysis.

Metabolic dysregulation can contribute to photoreceptor degeneration, as has been observed in several models of

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retinitis pigmentosa (RP). RP is an inherited form of retinal degeneration, characterized by progressive rod degeneration and a secondary loss of cones. It has been proposed that the secondary cone degeneration might also be linked to metabolic imbalance. Glucose uptake by cones is stimulated by the rod-derived cone viability factor, which is released by rods and would decrease with the progressive loss of rods in RP.[13]

Although several studies have suggested that photoreceptor cells rely heavily on glucose metabolism and a transcriptomic study indicated a decrease in glycolysis in degenerating photoreceptors,[14] a systematic characterization of changes in the expression of glucose metabolism genes in rod photoreceptors at different stages of degeneration is not available. The aim of this study was to assess the expression of genes linked to glucose metabolism in RP mutant retinas at early and advanced stages of degeneration. To this end, we performed a transcriptomic analysis on an in vitro model simulating photoreceptor degeneration and identified dysregulated expression of genes encoding glycolysis enzymes, as well as related transporters. These gene expression changes were validated in (i) two murine models of RP with different kinetics of photoreceptor degeneration; (ii) publicly available datasets from the same and additional murine RP models; and (iii) a human induced pluripotent stem cell–derived retinal organoid (hRO) model of RP. Our study indicates that the downregulation of aerobic glycolysis genes correlates with early stages of the disease and represents a potential target for neuroprotective interventions. This finding was further validated by a proof-of-concept study following treatment with H105A, a neurotrophic peptide derived from the pigment epithelium–derived factor (PEDF).[15]

Materials and Methods

Cell Culture

The 661W-A11 photoreceptor-like cell line,[16] derived from 661W cells by stable expression of the NRL rod-specific transcription factor,[17] was cultured and stressed for 24 hours using the phosphodiesterase 6 (PDE6) inhibitor zaprinast (ZAP, Sigma-Aldrich, St. Louis, MO, USA) as previously described.[16]

Transcriptomic Analysis on 661W-A11 Cells

Transcriptomic analysis was performed on four biological replicates of cells treated either with ZAP or vehicle (1% DMSO). RNA was extracted from cell pellets using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and the quality of the purified RNA was analyzed with Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). The samples used for RNA sequencing (RNA-seq) had an RNA integrity number of greater than 9 and were sequenced at Next Generation Diagnostics srl (Pozzuoli, Italy) to a depth of 4 million reads per sample. Data were analyzed by ROSALIND (ROSALIND, San Diego, CA, USA) and individual sample counts were normalized via relative log expression using the DESeq2 R library. DESeq2 was also used to calculate fold changes and *P* values and to perform optional covariate correction. Genes with an adjusted *P* value of 0.05 or less ($-\log_{10}(\text{padj}) = 1.3$) and fold changes of 1.5 ($\log_2\text{FC} = \pm 0.5$) were considered as differentially expressed. ShinyGO 0.83[18] was used for pathway enrichment analyses of Kyoto Encyclopedia of Genes & Genomes (KEGG) and Gene Ontology (GO) biological processes.

Human Retinal Organoids and Transcriptomic Analysis

The human induced pluripotent stem cell–derived hROs from a healthy individual[19] and from an individual with autosomal-recessive RP (arRP) (USH2A-RP-1[20]) were generated as previously reported.[21] The USH2A-RP-1 hROs were compound heterozygous for the c.2299delG and c.2276G > T variants in the *USH2A* gene.

RNA-seq was performed on control and USH2A-RP-1 hROs at days 150 and 225 of differentiation ($n = 3$–4 per condition). Total RNA from individual hROs was extracted using the NucleoSpin RNA Plus XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. cDNA libraries were prepared by IntegraGen SA (Evry, France) using the Watchmaker mRNA Library Prep Kit for Illumina, following the supplier's recommendations. Sequencing was performed on an Illumina NovaSeq X Plus sequencer as 100 base paired-end read, to a depth of 40 million reads per sample. Reads quality was assessed using FastQC. Mapping to the genome assembly GRCh38, annotation and differential gene expression analysis were conducted as previously described.[22] Filtering criteria included a false discovery rate *P* value of 0.05 or less, fold change of 2, and a minimum transcript-per-million value of 5 in at least one sample.

Experimental Animals and Treatments

Two animal models for RP were employed for this study and age-matched wild-type (WT) C57BL6/J mice were used as controls. The *Rho*$^{P23H/+}$ knock-in murine model, on a C57BL6/J background, is heterozygous for the P23H variant in the *Rhodopsin* (*Rho*) gene associated with autosomal-dominant RP.[23] The *rd1* model (C3H/HeNCrl, Charles River Laboratories, Wilmington, MA, USA) is homozygous for loss-of-function variants in the *Pde6b* gene and a model for arRP.[24]

Mice were bred and housed in the animal facility CSSI (Centro Servizi Stabulario Interdipartimentale), on normal chow diet, in a 12-hour light/dark cycle and with free access to food and water. All procedures were approved by the Ethical Committee of University of Modena and Reggio Emilia and by the Italian Ministero della Salute (authorization number 150/2021-PR) and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The adeno-associated virus type 2 (AAV2)-derived vectors carrying the H105A peptide (AAV2-H105A) or green fluorescent protein (AAV2–green fluorescent protein), as a control, were intravitreally injected in *Rho*$^{P23H/+}$ mice at postnatal (PN) day 5, as previously described.[25] Expression of H105A and green fluorescent protein was confirmed in all retinal samples, as previously published.[25]

Real-Time qPCR

Real-time qPCR was performed as previously described.[26] RNA was extracted from 661W-A11 cell pellets obtained from three independent experiments; for murine retinal samples, the two retinas of each animal were pooled before

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RNA extraction, and samples were obtained from at least three animals per experimental group; for hROs, RNA was extracted from pools of three organoids. Primers used are listed in Supplementary Table S1. Relative gene expression was calculated as ΔΔCt by setting the value of WT equal to 1. *Rps26* or *RPS26* and *GAPDH* were used as reference genes for murine or human samples, respectively. Statistical significance was assessed by two-tailed Student *t* test and a value of *P* of 0.05 or lower was considered significant.

Public RNA-Seq Dataset Analysis

Transcriptomic data from prior publications on *Rho*$^{P23H/+}$, *rd1*, and *rd10* vs. WT murine retinas at different ages were analyzed for differential expression of the preselected genes (Supplementary Table S2). When feasible, our empirical data were processed with fold change values. Otherwise, we acquired publicly available RNA-seq datasets from the Gene Expression Omnibus database. Raw gene counts from the datasets GSE169527[27] and GSE270758[28] were normalized by removing genes with low-expression levels and converting the remaining counts to counts per million, followed by a $\log_2$ transformation. Only $\log_2$ transformation was applied to GSE152474 and GSE156533[29] datasets, provided as fragments per kilobase of transcript per million mapped reads, and to GSE281959,[30] provided as prenormalized counts per million. Differential expression analysis of interest was evaluated by R software with a permutation *t* test.

Results

Transcriptomic Analysis of Stressed Rod-Like Photoreceptor Cells

With the aim of identifying candidate pathways involved in photoreceptor cell death, we took advantage of the photoreceptor-like cell line 661W-A11 and mimicked rod degeneration by treating the cells with the PDE6 inhibitor ZAP, that causes intracellular increase in cGMP and calcium.[16] RNA-seq analysis identified 1312 differentially expressed genes, 697 of which were significantly downregulated and 615 significantly upregulated upon ZAP treatment (Fig. 1A and Supplementary Table S3). Gene enrichment of KEGG pathways and Gene Ontology terms

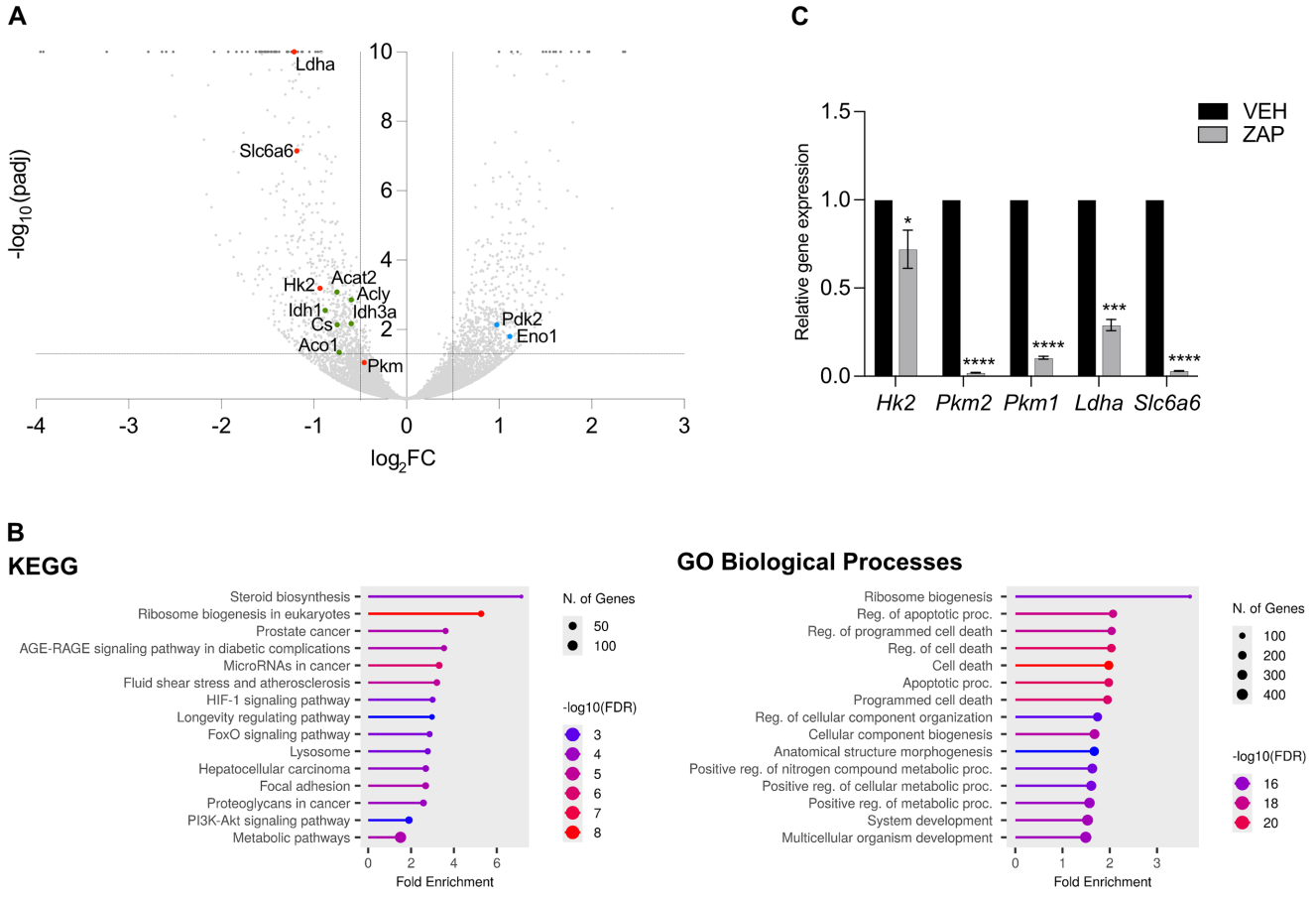

Figure 1. Differentially expressed genes in ZAP-treated 661W-A11 cells. (**A**) The RNA-seq data, four biological replicates of cells treated with ZAP and four biological replicates of control cells treated with vehicle (VEH), are represented as a volcano plot that shows the differentially expressed genes with each dot symbolizing a gene. Thresholds of significance ($\log_{10}$(padj) and $\log_2$FC) are indicated by horizontal and vertical lines. Downregulated genes related to aerobic glycolysis or TCA cycle are highlighted with *red and green dots*, respectively. Upregulated genes related to glucose metabolism are highlighted in *blue*. (**B**) The first 15 enriched KEGG pathways and Gene Ontology (GO) biological processes were ranked by fold enrichment. The size of dots correlates with the number of genes found in each pathway and the colors represent the false discovery rate of enrichment. (**C**) Real-time qPCR analysis of *Hk2*, *Pkm2*, *Pkm1*, *Ldha* and *Slc6a6* gene expression in ZAP-treated 661W-A11 cells ($n = 3$) compared with control (VEH, DMSO-treated) cells ($n = 3$). Relative gene expression is represented as $2^{-\Delta\Delta Ct}$ and VEH was set as 1. Student's unpaired two-tailed *t* test *$P \leq 0.05$, ***$P \leq 0.001$, and ****$P \leq 0.0001$.

highlighted cell death and cell proliferation pathways, as expected, as well as metabolic processes in ZAP-treated cells (Fig. 1B). Notably, differentially expressed genes associated with metabolic pathways were predominantly downregulated, indicating that photoreceptor degeneration was accompanied by an early failure of essential metabolic functions. These pathways included impaired membrane maintenance and steroid biosynthesis homeostasis genes, as well as loss of anabolic capacity, decreased ATP production, and disrupted redox balance, all of which can accelerate degeneration. KEGG enrichment allowed the identification of several genes related to glucose metabolism, including the hypoxia inducible factor 1 pathway, which plays essential roles in modulating glucose uptake, storage, and use during stress adaptation,[31] and the AGE-RAGE signaling pathway, which is linked to glucose-related damage.[32] Moreover, we found that genes involved in aerobic glycolysis were downregulated after ZAP treatment (red dots in Fig. 1A), including *Hk2*, *Ldha*, *Slc6a6*, and *Pkm*. Upregulation was observed in two glycolysis-related genes: *Eno1* and *Pdk2* (blue dots in Fig. 1A). ENO1 is a key enzyme in glycolysis. It also interacts with Arrestin in photoreceptors, suggesting a possible role in the visual process, thus extending beyond its metabolic function.[33] PDK2 regulates glycolysis by inhibiting pyruvate dehydrogenase and the tricarboxylic acid (TCA) cycle.[34] Additionally, several genes functionally connected to the TCA cycle were downregulated upon ZAP treatment (green dots in Fig. 1A), including *Cs*, *Idh1*, *Idh3a*, *Aco1*, *Acly*, and *Acat2*.

In this study, we focused on the aerobic glycolysis enzymes that showed high levels of downregulation, and validated RNA-seq results by real-time qPCR in unstressed and ZAP-stressed 661W-A11 cells (Fig. 1C). We confirmed the downregulation of the aerobic glycolysis genes *Hk2* and *Ldha*, and of the taurine transporter *Scl6a6*. This analysis also allowed us to distinguish between the two isoforms of the pyruvate kinase M enzyme (PKM), PKM1 and PKM2, derived by alternative splicing, that was not possible using RNA-seq. Interestingly, *Pkm1* but also *Pkm2*, the PKM isoform promoting aerobic glycolysis, were found to be downregulated.[35]

Validation of Changes in the Expression of Glycolysis Genes in Murine Models of RP

To better understand the implication of the aerobic glycolysis metabolic pathway at different stages of the photoreceptor degenerative process, we evaluated changes in expression of the key genes related to the pathway, *Hk2*, *Pkm2*, *Pkm1, Ldha*, and *Slc6a6* in models for RP.

We first assessed gene expression in the $Rho^{P23H/+}$ model, which displays a slow progressing retinal degeneration and closely resembles the disease course in patients.[23] In line with the in vitro data *Hk2*, *Pkm2*, and *Slc6a6* were downregulated in $Rho^{P23H/+}$ retinas at PN19, which is the peak of photoreceptor cell death in this murine model, as well as at 3 and 6 months of age, which represent advanced disease stages with only a few photoreceptors remaining (Fig. 2A). Notably, only the *Pkm2* isoform that drives aerobic glycolysis,[35] rather than *Pkm1*, was downregulated in this murine model, suggesting that disruption of aerobic glycolysis itself may contribute to retinal degeneration.

Gene expression was also assessed in the fast-degenerating *rd1* murine model of RP at timepoints before the peak of photoreceptor cell death (PN8, PN9, and PN10) and at the peak of degeneration itself (PN11). The expression of *Pkm2*, *Pkm1*, *Ldha*, and *Slc6a6* was decreased in the mutant retina at the early timepoint (PN8), but not later (Fig. 2B).

Changes in Genes Linked to the Glycolysis Pathways in Publicly Available Datasets

We took advantage of publicly available RNA-seq datasets from $Rho^{P23H/+}$ and *rd1* mutant retinas to further assess expression of glycolysis-related genes at different degeneration timepoints (Supplementary Table S2). We extended the analysis to the *rd10* mutant mouse, which carries a mutation in the *Pde6b* gene, but has a slower progression of degeneration than the *rd1* mouse.

In agreement with our findings, genes directly involved in aerobic glycolysis, such as *Hk2, Pkm*, and *Ldha*, were generally downregulated (Table). Among them, *Hk2* was the gene most consistently downregulated at early timepoints in all the models analyzed. Notably, single cell RNA-seq (scRNA-seq) analysis performed by Karademir et al.[36] revealed that *Hk2* downregulation in *rd10* mice occurred specifically in photoreceptor cells, both rods and cones, at PN21. At the later PN28 timepoint, *Hk2* expression was no longer significantly dysregulated in rod cells, further suggesting that its downregulation correlates with early stages of photoreceptor degeneration. Consistent with our observations, a similar trend was reported in *rd1* retinas, with *Hk2* downregulated at PN8, but not at PN10.[37] In the same study, HK2 protein levels were found reduced at PN10, indicating that post-translational regulation of this enzyme may contribute to the control of HK2 levels.

Pkm and *Ldha* were also found dysregulated in the slower degenerating models, although less consistently. Notably, in the *rd10* retinas at PN21 *Pkm* and *Ldha* were found to be significantly decreased only in cone cells, and not in rods.[36] In studies on total retinas from $Rho^{P23H/+}$ and *rd10* mutant mice *Pkm* and *Ldha* were significantly downregulated[29,38,39] or showed trends toward downregulation at various timepoints. Similarly, in line with our observations, *Slc6a6* was downregulated in retinas from *rd10*[27,28,39] and in $Rho^{P23H/+}$ mutants.[29,30]

Effects of a Neuroprotective Treatment on the Expression of Glycolysis Genes

To validate the importance of aerobic glycolysis imbalance in retinal degeneration, we evaluated changes in the expression levels of *Hk2, Pkm2*, *Ldha*, and *Scl6a6* genes in response to a neuroprotective agent. We chose the PEDF-derived H105A peptide, which we previously demonstrated to be neuroprotective for the $Rho^{P23H/+}$ retina.[25] H105A was delivered by a single intravitreal injection of AAV2-H105A in $Rho^{P23H/+}$ eyes at PN5 and aerobic glycolysis genes were analyzed at PN19 and at 1 month of age. Using this delivery protocol, H105A is secreted by retinal ganglion and Müller glia cells to reach photoreceptors, where the PEDF receptor localizes, and expression is maintained for at least 6 months post injection.[25]

Ectopic expression of H105A resulted in a significantly increase in *Hk2* expression at PN19 and at the age of 1 month (Fig. 3A), when compared with retinas transduced with the control AAV2–green fluorescent protein vector.

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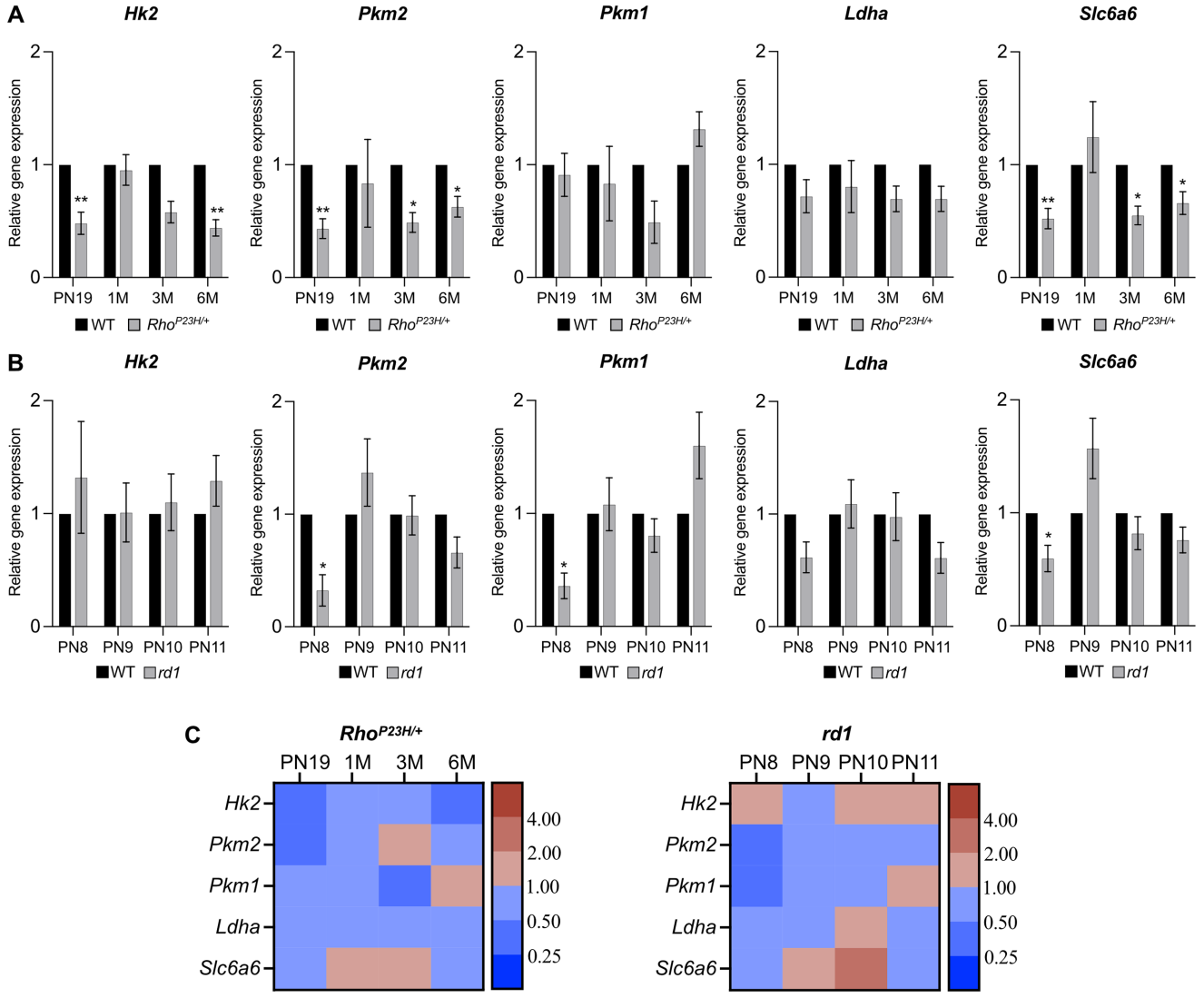

Figure 2. Relative expression of genes related to aerobic glycolysis in $Rho^{P23H/+}$ and *rd1* retinas. Real-time qPCR analysis of *Hk2*, *Pkm2*, *Pkm1*, *Ldha*, and *Slc6a6* gene expression on murine models of RP. (**A**) Analyses of $Rho^{P23H/+}$ retinas and age-matched WT controls ($n = 4$). (**B**) Analyses of *rd1* retinas and age-matched WT controls ($n = 3$). Relative gene expression is represented as $2^{-\Delta\Delta Ct}$ and WT values were set as 1. Student's unpaired two-tailed *t* test $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. (**C**) Representation of results as heatmap. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Nevertheless, *Hk2* expression in AAV2-H105A treated retinas did not reach the WT levels at PN19. Interestingly, a significant upregulation of *Scl6a6* was observed in the treated retinas at the age of 1 month, whereas a higher but not significant level was observed for *Pkm2* and *Ldha* (Fig. 3B–D). These data suggest that elevated levels of *Hk2* at early degeneration timepoints and, subsequently, increased levels of *Scl6a6*, might contribute to the neuroprotective action of H105A.

Changes in Genes Linked to the Glycolysis Pathways in the Human Retina

To assess whether glycolysis-related alterations observed in murine models could be relevant to the human pathology, we analyzed expression of the candidate genes in a hRO model for arRP with mutations in the *USH2A* gene. We previously characterized hROs derived from isolated RP patients with *USH2A* variants and observed defects in rod photoreceptor differentiation with disorganized outer segments.[21]

We compared expression in the RP hROs to control hROs at 150 days of differentiation (D150), a timepoint before complete maturation, and at D225, when organoids were fully mature. Based on transcriptomic analyses, D150 corresponds with approximately PN5 in the mouse retina and D225 with approximately PN8.[40] RNA-seq analysis showed that the expression levels of glycolysis genes were decreased in RP organoids. Indeed, *HK2* and *SLC6A6* displayed a trend toward downregulation at both timepoints, whereas *LDHA* was significantly downregulated at D225 (Fig. 4A). *PKM* showed only a slight decrease in expression, but RNA-seq did not distinguish between the two PKM isoforms. Therefore, real-time qPCR analysis was performed to specifically investigate the *PKM2* isoform, which was significantly increased in RP hROs compared with control hROs (Fig. 4B).

Table. Changes in Expression of Aerobic Glycolysis Genes

Mutant and Age	*Hk2*	*Pkm*	*Ldha*	*Slc6a6*
rd1 PN8[37]	*	†	*	†
rd1 PN10[37]	‡	†	‡	†
rd10 PN15[28]	§	‡	‡	‡
rd10 PN21 (rods and cones)[36]	Rods/cones*	Cones only*	Cones only*	†
rd10 PN28 (rods)[36]	†	†	†	†
rd10 PN24[27]	§	‡	§	§
rd10 PN24[39]	*	‡	‡	‡
rd10 PN45[39]	*	*	*	*
rd10 PN61[38]	*	*	*	†
$Rho^{P23H/+}$ 1 month[29]	*	*	*	‡
$Rho^{P23H/+}$ 3 months[29]	*	*	*	‡
$Rho^{P23H/+}$ 1 month[30]	§	‡	§	‡
$Rho^{P23H/+}$ 3 months[30]	§	‡	§	‡
$Rho^{P23H/+}$ 6 months[30]	§	§	§	§

* Downregulated Significant.
† Not available data.
‡ Unchanged gene expression.
§ Downregulated trend.

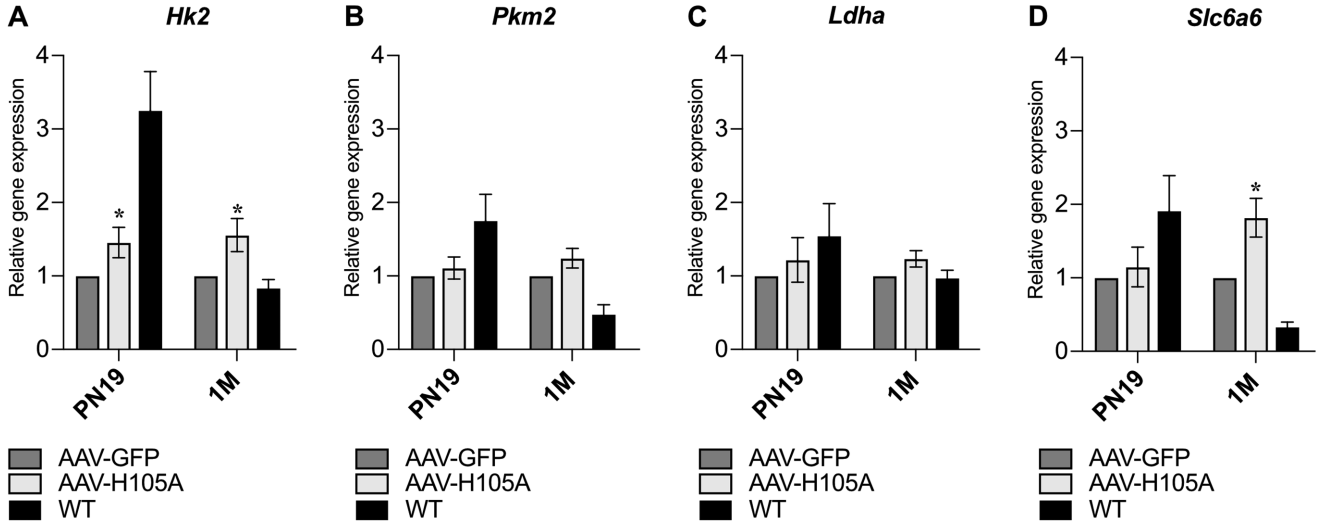

Figure 3. Relative expression of genes related to aerobic glycolysis in $Rho^{P23H/+}$ retinas transduced with the AAV2-H105A vector. Gene expression of *Hk2* (**A**), *Pkm2* (**B**), *Ldha* (**C**), and *Slc6a6* (**D**) was analyzed by real-time qPCR in retinas at PN19 ($n = 8$) and at 1 month ($n = 3$), thus 14 days and 25 days after transduction with either AAV2-H105A or AAV2–green fluorescent protein (control). Relative gene expression is represented as $2^{-\Delta\Delta Ct}$ and AAV2–green fluorescent protein values were set as 1. Student's unpaired two-tailed *t* test, $^*P \leq 0.05$.

These data suggest that similar transcriptomic changes occur at early degeneration timepoints in both mouse and human RP models, demonstrating the relevance for patients.

Discussion

The disruption of glucose homeostasis is a converging feature in RP. Diverse studies have shown that, in RP models, photoreceptors experience glucose deprivation that accelerates cell death, and that therapeutic approaches enhancing the aerobic glycolysis pathway hold promise for slowing down or even preventing vision loss.[41]

In this study, we used the photoreceptor-like 661W-A11 cell line to simulate the acute phase of photoreceptor cell death characterized by high levels of cGMP and Ca^{2+}.[42] A strong association with cell death and metabolic processes suggested that the cellular stress disrupts energy homeostasis and biosynthetic activity and results in significant metabolic alterations. Among the genes downregulated, several were involved in glucose metabolism, including various genes related to the TCA cycle, results that are in accordance with the existing literature.[34,43,44] In support of a diminished TCA cycle, *Pdk2* was found to be upregulated. PDK2 phosphorylates and inhibits the pyruvate dehydrogenase (PDH) complex, thereby reducing pyruvate entrance into the TCA cycle. Interestingly, PDK2 upregulation was also reported by Kanan et al.[34] in *rd10* retinas at PN35, an advanced degeneration timepoint, along with increased phosphorylation of the PDH component PDHE1-A. At the same time, they observed decreased total levels and increased inhibitory phosphorylation of PKM2 and LDHA, pointing to an overall decrease in the TCA cycle and glycolytic activity, also in line with our find-

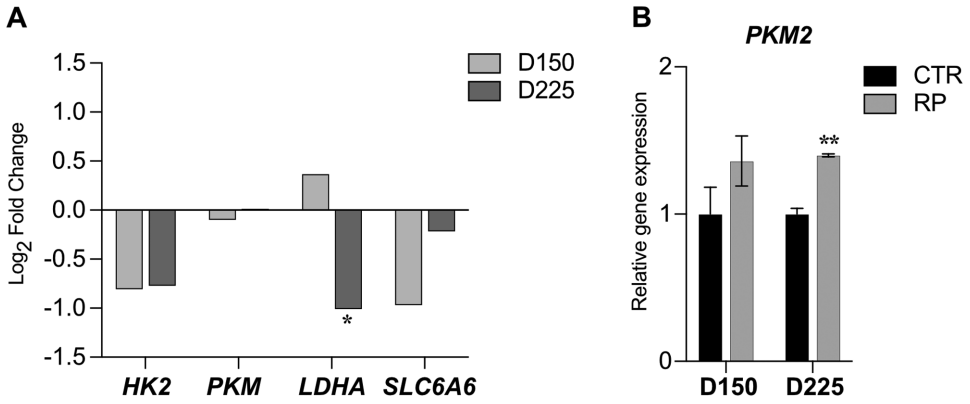

Figure 4. Expression of genes related to aerobic glycolysis in hROs. (**A**) Log_2 fold change in RP hROs at differentiation time 150 (D150) and at D225 compared with control (CTR) hROs at the same stages. * False discovery rate ≤ 0.05. (**B**) Real-time qPCR analysis of *PKM2* gene expression in RP hROs compared with CTR hROs ($n = 3$). Expression values of CTR samples were set as 1. Student's unpaired two-tailed *t* test, $^{**}P \leq 0.01$.

ings. The expression changes described here partially align with a scRNA-seq study performed on *rd1* mutant retinas that reported unchanged levels of *Hk2* and increased *Pkm2* in mutant rods at PN11.[14] The differences may stem from the fact that the scRNA-seq study examined metabolic changes at later stages of degeneration, that is, P11 to P17, compared with the earlier ages that we studied, and focused on glycolysis-related genes that were different from the ones that we analyzed. Nevertheless, in agreement with our findings, the authors reported dysregulation of glycolysis-associated genes during the degeneration process.[14]

We found that the glycolysis-related genes *Hk2*, *Pkm1*, *Pkm2*, and *Ldha* were generally downregulated in vivo in murine RP models at early timepoints and at corresponding timepoints in a human RP model, underlining relevance for patients as well. Additionally, we observed a downregulation of the taurine transporter *Slc6a6*, which may exacerbate glucose dysregulation. Although not directly involved in glycolysis, the function of SLC6A6 is closely linked to metabolic homeostasis. Taurine supports mitochondrial function, decreases reactive oxygen species, and regulates glucose metabolism, as shown by *Slc6a6* knockout, which negatively affects the glycolysis/TCA pathways and metabolites in leukemia cells.[9] In photoreceptors, where high energy demands are met primarily through aerobic glycolysis as in tumor cells, proper SLC6A6 levels are required to sustain glycolytic activities and mitigate metabolic stress in the retina.

In contrast, in hROs we observed an upregulation of *PKM2*. PKM is a key glycolysis enzyme and the PKM2 isoform possesses very complex and still not completely characterized biological effects by acting as tetramer or dimer.[35] Environmental stress favors the tetramer form, with high pyruvate kinase activity and PKM2 activation can protect photoreceptors from cell death.[45] Notably, a study performed on *rd10* retinal explants showed that PKM2 protein levels were decreased in the mutant compared with the WT retinas at PN9, but increased with the progression of degeneration.[45] Alternatively, the difference in *PKM2* expression between hROs and murine retinas may reflect the absence of RPE in hROs. The upregulation of *PKM2* in hROs is suggestive of activated aerobic glycolysis, which has been reported in murine retinal cultures without RPE.[7]

Although imbalanced glucose metabolism had been linked to secondary cone degeneration, the scRNA-seq study showed that *Hk2* is dysregulated in *rd10* retinas both in cones and rods, underscoring the critical role of HK2 levels in RP starting from the onset of degeneration.[36] HK2 might play different roles during the degenerative process. In addition to the enzymatic role that is central for glucose metabolism, mitochondrial HK2 exerts protection against cell death through nonenzymatic functions.[46] Moreover, HK2 was shown to confer a survival advantage to photoreceptors under conditions of metabolic stress.[47–49] Because rods are the first cell type affected during RP, early HK2 downregulation might compromise their ability to manage stress, thus contributing to retinal degeneration, including indirect exacerbation of metabolic stress in cones.[50] Importantly, H105A-mediated restoration of *Hk2* expression levels may initiate a positive response to support long-term photoreceptor survival through a currently uncharacterized pathway. We previously demonstrated that H105A treatment activates the PEDF receptor encoded by *Pnpla2*.[15] Based on reports that a PEDF-derived peptide overlapping the H105A domain can act via AKT and ERK,[51,52] we can hypothesize that H105A regulates *Hk2* expression through AKT and ERK signaling. In fact, AKT and ERK promote glycolysis and can drive the Warburg effect, including the induction of HK2.[53] Furthermore, these same pathways have been shown to mediate PEDF neurotrophic effects.[54]

Conclusions

The present study, although based on transcriptomic analyses that need to be validated at the protein level, highlights decreased glucose metabolism as a common feature of photoreceptor degeneration and suggests that hROs may represent a useful tool to test therapeutic interventions aimed at restoring glucose metabolism.

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Investigative Ophthalmology & Visual Science

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