nature portfolio

Peer Review File

Multi-omics profiling of retinal pigment epithelium reveals enhancer-driven activation of RANK-NFATc1 signaling in traumatic proliferative vitreoretinopathy

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript the authors conducted a comprehensive epigenomic analysis of RPE cells isolated from control and PVR mouse eyes. They discovered that RPE cells undergo profound epigenetic changes that were associated with changes in chromatin accessibility and gene expression. Inhibition BET or preventing some of the gene changes associated with PVR attenuated the severity of the disease.

The strength of this manuscript is the elegant and comprehensive analysis of EMT of RPE cells in an in vivo model of PVR. In addition, the authors identify new therapeutic targets and demonstrate their efficacy.

The major shortcoming is the lack of evidence that the findings that emerged using the animal model relate to human PVR. While Fig 5D shows that Nfat is in the nucleus in an RPE-containing PVR membrane from a patient, these data do not address if this level of Nfat is high or low. Fig 5E attempts to addresses this issue, however it is not clear that the ILM (an avascular membrane) is a suitable control for PVR membranes, which presumably contained cells, including RPE cells.

Additional comments
1. Please explain how this dispase model reflects trauma.

2. The description of this PVR model in the Results section should state that it is or is not the same as what has been published by other investigators.

Reviewer #2:
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This paper outlines a relationship between PVR progression and the RANK-NFATC1 pathway. The story is interesting and for the most part logically laid out. The wet work approach seems to be on point and the potential data from the dry experiments could be useful to the community. However, a lack of details about the methodology as well as results accessible by non-bioinformaticians dampers my excitement for this study.
Highlights
• The OPG-FC experiment is immediately clinically relevant
• The generation of the eNano-JQ1 could lead to some interesting experimental approaches in the future.
Major Issues
1. All of the analysis for the sequencing-based experiments are not described in sufficient detail for another bioinformatician to replicate the analysis with the same data. Ideally, a github or a supplementary text file with the code used for the analysis would be provided. This should include the version numbers for each package used. Without this it is basically impossible for someone

else to derive your results using your data.
2. Within the results sections you do not provide enough information to judge the bioinformatic approach. For example, you state you did GO enrichment, but not the package or methodology. Did you use the hypeR, goseq, or any of the other 100 R packages available for go analysis? The results nor methods stated how it was done so it is left to the imagination. You did state you used DESEQ2 for the differential genes analysis for the RNA-seq in the methods, but it would be nice to include those details in the relevant results section. The common approach for the bioinformatic experiments is to state the results and provide little to no detail on how they were derived. I have read the paper thoroughly I am still unsure of your analysis pipeline or any assumptions made in the analysis. I imagine a reader that has a vague familiarity with the analysis methods for the sequencing experiments will be unable to understand how your results support your conclusions with more details and a clearer walk through of the results.
3. For non-bioinformaticians you should provide at minimum supplementary tables for each analysis ran. You say you have identified ~600 ATAC-seq peaks, but provide no supplementary table for the peak locations, in the RNA-seq you found ~2400 genes, but no table and no way for a non-bioinformatician to explore this data. I could run your analysis (if you had provided the code), but without excel tables for all of your bioinformatic analysis (GO enrichment, etc.) your data is useless for 95% of readers. Sequencing studies should not be published without those excel compatible tables. The lack of these tables greatly reduces the utility of the work and raises the question of why you would seek to publish without providing these key data.
4. For no experiment did you report the number of samples. The phrase n= is not in this manuscript. I assume you did two samples per condition for the RNA-seq based on the heatmaps, but that is an assumption I should not have to make. Nor should your readers have to count the dots on the various charts throughout the figures to see how many samples were tested per experiment.
5. Several of the figures have aspects that are unseeable when printed.

Minor Issues

• Figure 2F: Why these genes, why not genes in the abstract or something related to the rest of the story. I personally do not see what this panel adds to the manuscript.
• Figure 3B: had to look up zeta potentials it seems to be specific to engineering and the eye audience is unlikely to know what it is or means.
• Figure 3G: couldn’t see the yellow dotted line in printed document, but it was in pdf (this happens for every yellow line, maybe increased the weight)
• Figure 4C: This heatmap is super confusing, you need to better walk the reader through it in the text or figure legend.
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• Figure 7: no figure legend
• Most of the microscopy images could be bigger and easier to see if you reduce some of the white space in those figures

Reviewer #3:

Remarks to the Author:
In this study, Liao et al. used multi-omics profiling of RPE cells and many other methods to study the mechanisms underlying PVR. To this end, the authors used a popular PVR mouse model in which the proteolytic enzyme dispase is injected into the vitreous body of mice. Using this animal model, the authors suggested that the RANK-NFATc1 signaling cascade plays a role in the pathogenesis and progression of PVR. While the data appears sound, the study suffers from multiple flaws:
1) Experimental design is unclear. Did the authors treat both eyes with the enzyme or just one using the other eye as a control? How many animals were used in the control and experimental groups? How many animals were used to obtain one sample of RPE cells? Why were only the C57BL/6 male mice used in the study? Etc. The lack of detailed experimental design makes it difficult to read the manuscript.
2) Could the RPE cell isolation procedure used affect the expression and epigenome of these cells? The authors should demonstrate that this impact is minimal.
3) The manuscript does not indicate how many times the ATAC-seq, ChIP-seq and RNA-seq experiments were repeated. At least three biological replicates ($n \geq 3$) are needed to establish the statistical significance of the results. Reading the manuscript, it seems that the authors used only one ($n=1$) biological replicate in the ATAC-seq and ChIP-seq experiments, while in the RNA-seq analysis they used only two biological replicates ($n=2$). If this is the case, then the results obtained are not statistically significant.
4) The authors should provide the secure token to allow reviewers access to the GEO GSE244812 data.
5) Statements like this (line 126: "In the PVR group, the promoter region of the Mdm2 gene displayed enhanced chromatin accessibility (fig. S2A).") must be accompanied by actual measured values (including p-value).
6) In their drug study, the authors do not indicate the size of the animal groups treated. The statement in the "Methods/Statistics" section that a minimum of three independent experiments were carried out does not allow us to determine the statistical significance of the results obtained. The authors must indicate the exact number of animals in each group for each treatment. In general, the authors nowhere provide the results of a power analysis to determine the minimum size of groups of animals and samples necessary to obtain statistically significant results. Therefore, it is unclear whether the approach/data is reproducible.
7) It is difficult to understand from the images presented in Figures 3, 5, and 6 whether there is an improvement after treatment with drugs. All that remains is to trust the authors that there was such an improvement. The authors should provide more convincing images.
8) It is impossible to understand anything from the results of the TUNEL apoptosis assay. Authors should use positive controls to show that the assay works in their hands.
9) There is a significant difference between the Western blots shown in Figure 3H and Figure 5J. While Figure 5J shows a clear difference between the treated and control samples, this difference is not visible in Figure 3H. It is proposed to revise the Western blot presented in Figure 3H. The authors should also provide Western blots containing protein ladders.
10) Although injection of the proteolytic enzyme dispase is the most popular mouse PVR model, this model only partially mimics the pathogenesis and progression of PVR in humans. In Figure 5D, the authors presented the results of the funduscopic examination as well as H&E staining and IHC

of samples acquired from one PVR patient. However, it is quite difficult to interpret the presented images. Thus, the findings still need to be confirmed in eye tissues from several PVR patients. Otherwise, it is not clear whether the findings have anything in common with the real disease.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript the authors conducted a comprehensive epigenomic analysis of RPE cells isolated from control and PVR mouse eyes. They discovered that RPE cells undergo profound epigenetic changes that were associated with changes in chromatin accessibility and gene expression. Inhibition BET or preventing some of the gene changes associated with PVR attenuated the severity of the disease.

The strength of this manuscript is the elegant and comprehensive analysis of EMT of RPE cells in an in vivo model of PVR. In addition, the authors identify new therapeutic targets and demonstrate their efficacy.

The major shortcoming is the lack of evidence that the findings that emerged using the animal model relate to human PVR. While Fig 5D shows that Nfat is in the nucleus in an RPE-containing PVR membrane from a patient, these data do not address if this level of Nfat is high or low. Fig 5E attempts to addresses this issue, however it is not clear that the ILM (an avascular membrane) is a suitable control for PVR membranes, which presumably contained cells, including RPE cells.

Response: We are grateful to the reviewer for highlighting this crucial concern. In response, using donor eyeball sections as controls, we have further analyzed the expression of NFATc1 in human eyeballs to directly address this issue (Methods, Page 27, lines 573-574). Our findings indicate an elevated level of NFATc1 when comparing the mean fluorescent intensity between these donor eyeball sections and PVR membranes (N=4/group). This data, now included in the Results section of our revised manuscript, strengthens the connection between our animal model findings and human PVR. Please see Fig.5D on Page 14, lines 294-297 for this updated information.

Additional comments

1. Please explain how this dispase model reflects trauma.

Response: In our animal model, we created a puncture postierior to the level of the equator, resulting in a full-layer injury of the retina, to more accurately replicate a penetrating wound observed in patients with open globe injuries. The injection of dispase further exacerbates tissue damage within the eye, promoting the formation of PVR. This procedural detail has been incorporated into the 'Results' section of the revised manuscript (Page 6, line 104-107).

2. The description of this PVR model in the Results section should state that it is or is not the same as what has been published by other investigators.

Response: We thank the reviewer for pointing this out. The morphological characteristics of our PVR model are consistent with those described in prior studies utilizing the same mouse model (Cantó et al., *Exp Eye Res.*, 2002. PMID: 12457862; Szczesniak et al., *Neuropharmacology*, 2017. PMID: 27569993; Yoo et al., *Mol Vis.*, 2017. PMID: 29296073; Iribarne et al., *Am J Pathol.*, 2008. PMID: 18310504.) We have added this information in the Results (Page 6, line 114-116).

Reviewer #2 (Remarks to the Author):

This paper outlines a relationship between PVR progression and the RANK-NFATC1 pathway. The story is interesting and for the most part logically laid out. The wet work approach seems to be on point and the potential data from the dry experiments could be useful to the community. However, a lack of details about the methodology as well as results accessible by non-bioinformaticians dampers my excitement for this study.

Highlights

• The OPG-FC experiment is immediately clinically relevant

• The generation of the eNano-JQ1 could lead to some interesting experimental approaches in the future.

Major Issues

1. All of the analysis for the sequencing-based experiments are not described in sufficient

detail for another bioinformatician to replicate the analysis with the same data. Ideally, a github or a supplementary text file with the code used for the analysis would be provided. This should include the version numbers for each package used. Without this it is basically impossible for someone else to derive your results using your data.

Response: Following the reviewer's suggestion, we have included the analysis code in the Source Data file. We have also specified the version numbers for each software package used: bowtie2 (v2.3.5.1), deepTools (v3.4.3), SAMtools (v1.9), HISAT2 (v2.1.0), and featureCounts (v1.6.0). These details can be found in the Source Data file for reference.

2. Within the results sections you do not provide enough information to judge the bioinformatic approach. For example, you state you did GO enrichment, but not the package or methodology. Did you use the hypeR, goseq, or any of the other 100 R packages available for go analysis? The results nor methods stated how it was done so it is left to the imagination. You did state you used DESEQ2 for the differential genes analysis for the RNA-seq in the methods, but it would be nice to include those details in the relevant results section. The common approach for the bioinformatic experiments is to state the results and provide little to no detail on how they were derived. I have read the paper thoroughly I am still unsure of your analysis pipeline or any assumptions made in the analysis. I imagine a reader that has a vague familiarity with the analysis methods for the sequencing experiments will be unable to understand how your results support your conclusions with more details and a clearer walk through of the results.

Response: Thank you for highlighting this aspect. We have employed Metascape for Gene Ontology (GO) enrichment analysis, a detail now included in the figure legend (Page 38, lines 816-817). Further descriptive information has also been added to the Results section. This includes the utilization of HOMER for identifying differential ATAC-seq peaks and the DESEQ2 algorithms for analyzing differentially expressed genes. These additions have been made to the revised manuscript (Page 7, lines 126-128 and Page 9, lines 184-188), providing a clearer understanding of our bioinformatic approaches.

3. For non-bioinformaticians you should provide at minimum supplementary tables for each analysis ran. You say you have identified ~600 ATAC-seq peaks, but provide no supplementary table for the peak locations, in the RNA-seq you found ~2400 genes, but no

table and no way for a non-bioinformatician to explore this data. I could run your analysis (if you had provided the code), but without excel tables for all of your bioinformatic analysis (GO enrichment, etc.) your data is useless for 95% of readers. Sequencing studies should not be published without those excel compatible tables. The lack of these tables greatly reduces the utility of the work and raises the question of why you would seek to publish without providing these key data.

Response: Thank you for the suggestion. In response to the reviewer's advice, we have supplemented our manuscript with the addition of ATAC-seq peak locations and the list of differentially expressed genes from the RNA-seq in the Source Data file. Specifically, through HOMER analysis, we identified 9,819 peaks with increased accessibility in the PVR group, and 1,454 peaks with decreased accessibility (Page 7, lines 126-128). Additionally, using DESEQ2 algorithms, we discovered significant differences in the transcriptomic profiles, identifying 2,004 genes as upregulated and 543 genes as downregulated in PVR compared to the normal control (Page 9, lines 184-188). This added information enhances the utility and accessibility of our data for a broader audience, including those without a bioinformatics background.

4. For no experiment did you report the number of samples. The phrase n= is not in this manuscript. I assume you did two samples per condition for the RNA-seq based on the heatmaps, but that is an assumption I should not have to make. Nor should your readers have to count the dots on the various charts throughout the figures to see how many samples were tested per experiment.

Response: We appreciate the reviewer's attention to detail and acknowledge the oversight. In response, we have now explicitly stated the number of samples used in our experiments within the 'Materials and Methods' section and the figure legends. We have also added additional replicates for our ATAC-seq, ChIP-seq, and RNA-seq experiments, ensuring that each now includes three biological replicates. This information has been detailed in the revised manuscript (Page 22, lines 460-464 and Page 38, lines 813-814).

5. Several of the figures have aspects that are unseeable when printed.

Response: We are grateful to the reviewer for highlighting this issue. In response, we have revised the figures across the manuscript, utilizing high-resolution images or graphics (at least 300 dpi) to guarantee that finer details remain visible upon printing. Additionally, for figures that incorporate lines or borders, we have increased the line thickness to ensure it is sufficiently visible in printed form.

Minor Issues

• Figure 2F: Why these genes, why not genes in the abstract or something related to the rest of the story. I personally do not see what this panel adds to the manuscript.

Response: We agree with the reviewer's observation and have accordingly removed this panel from the manuscript.

• Figure 3B: had to look up zeta potentials it seems to be specific to engineering and the eye audience is unlikely to know what it is or means.

Response: We appreciate the reviewer's concern regarding the specificity of zeta potentials to our intended audience. Zeta potential is a key physical property of particles in suspension, reflecting the surface charge that significantly affects their stability and interactions. To ensure our findings are accessible to all readers, including those without a background in bioengineering, we have added a brief explanation of zeta potential and its relevance in nanoparticle characterization in the revised manuscript, specifically on Page 11, lines 226-230.

• Figure 3G: couldn't see the yellow dotted line in printed document, but it was in pdf (this happens for every yellow line, maybe increased the weight)

Response: We thank the reviewer for this suggestion and have adjusted the line color and weight to ensure its visibility. Furthermore, we conducted a print test of the revised figure to verify that it can be clearly seen in the printed document.

• Figure 4C: This heatmap is super confusing, you need to better walk the reader through it in the text or figure legend.

Response: We are grateful for the reviewer's insightful feedback on the heatmap presented in Figure 4C. To clarify, the triangular heatmap illustrates the co-occurrence of transcription factors (TFs) within the same ATAC-peak regions. The 'Fraction of overlap' metric quantifies the extent of shared elements between pairs of TFs, with a value of 0 indicating no overlap and a value of 1 signifying complete overlap. Additionally, 'Set size' denotes the total number of genomic regions containing each TF's motif. To assist readers in understanding this complex data, we have enriched the figure legend with more detailed descriptions, thereby facilitating a clearer interpretation of the heatmap's implications (Figure legend, Page 40, line 861-864).

• Figure 4D: when printed lines are hard to see, especially gray lines (maybe increased the weight, darken a few shades)

Response: We thank the reviewer for this suggestion and have increased the line weight for this panel to enhance visibility.

• Figure 7: no figure legend

Response: We thank the reviewer for pointing out this issue. We have accordingly added the figure legend in the revised manuscript as the following:

Fig.7 Working Model. A schematic illustration presents the epigenetic mechanisms activating RANK-NFATc1 signaling during the progression of PVR, and outlines the strategies for targeted intervention (Figure legend, Page 43, lines 925-927).

• Most of the microscopy images could be bigger and easier to see if you reduce some of the white space in those figures

Response: We thank the reviewer for this suggestion and have accordingly adjusted the layout to reduce the white space around these figures, ensuring that the images are now larger

and easier to examine.

Reviewer #3 (Remarks to the Author):

In this study, Liao et al. used multi-omics profiling of RPE cells and many other methods to study the mechanisms underlying PVR. To this end, the authors used a popular PVR mouse model in which the proteolytic enzyme dispase is injected into the vitreous body of mice. Using this animal model, the authors suggested that the RANK-NFATc1 signaling cascade plays a role in the pathogenesis and progression of PVR.

While the data appears sound, the study suffers from multiple flaws:

1) Experimental design is unclear. Did the authors treat both eyes with the enzyme or just one using the other eye as a control? How many animals were used in the control and experimental groups? How many animals were used to obtain one sample of RPE cells? Why were only the C57BL/6 male mice used in the study? Etc. The lack of detailed experimental design makes it difficult to read the manuscript.

Response: We are thankful for the reviewer's suggestion to provide a more comprehensive outline of our experimental design. In our experimental setup, we treated only one eye of each animal with the enzyme, designating the untreated contralateral eye as the normal control. This approach ensured that each animal served as its own control, thereby reducing variability. This information has been detailed in the revised manuscript (Page 21, lines 434-436).

Due to the singular layer structure of the retinal pigment epithelium (RPE) and the consequent challenge in isolating a sufficient quantity of cells for sequencing, it was necessary to aggregate RPE cells from multiple animals. Specifically, for sequencing analyses such as ATAC-seq and RNA-seq, cells from three animals were pooled for each replicate. For the ChIP-seq experiments aimed at investigating a range of histone modifications, a larger sample size was required, with nine animals contributing to each replicate. This information has been detailed in the revised manuscript (Page 22, lines 460-464).

To ensure clarity and accessibility of this information for our readers, we have clearly indicated the number of animals used for each experiment within our figures and figure

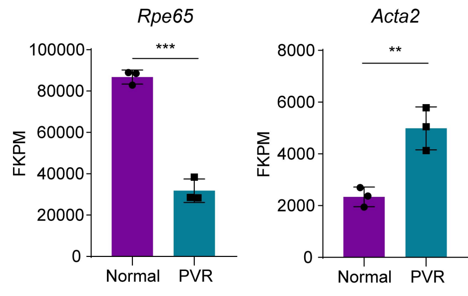
legends in the revised manuscript.

In addressing the aspect of animal use in our study, we referred to a systematic review and meta-analysis by Xiang et al. (*PLoS One.*, 2023. PMID: 37903162), which showed no significant difference in the progression of PVR between male and female patients. Furthermore, our decision to use exclusively male mice aligns with the predominant practice in this research field, as evidenced by several key studies (Cantó et al., *Exp Eye Res.*, 2002. PMID: 12457862; Wada et al., *Antioxidants (Basel)*, 2022. PMID: 36290802; Szczesniak et al., *Neuropharmacology*, 2017. PMID: 27569993; Iribarne et al., *Am J Pathol.*, 2008. PMID: 18310504.) We have also included this information in the abstract (Page 3, line 48) and Methods section (Page 20, line 428).

2) Could the RPE cell isolation procedure used affect the expression and epigenome of these cells? The authors should demonstrate that this impact is minimal.

Response: We appreciate the reviewer's thoughtful concerns. Indeed, when isolating RPE cells from tissues, there is a possibility for changes in their expression profiles and epigenetic landscapes. To mitigate such effects, we have simplified the isolation process, notably reducing its complexity and duration. By avoiding in vitro culture, our approach aims to more closely approximate the cells' native in vivo state, offering advantages over the use of immortalized cell lines or primary cells cultured in vitro for the study of gene expression and epigenetic regulation.

To verify that our isolation method accurately reflects in vivo conditions, we performed immunofluorescence staining on a selection of genes identified by RNA-seq as differentially expressed. The alignment between our immunofluorescence and RNA-seq findings offers support to our method's reliability.



Rpe65
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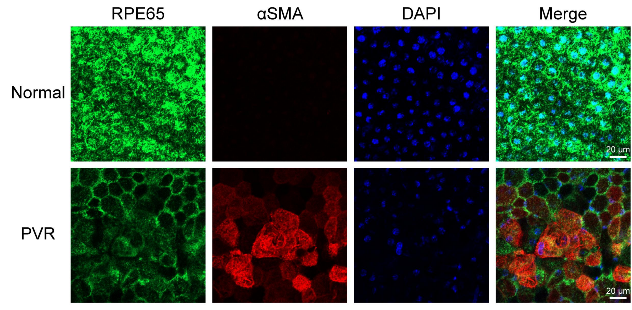

RPE65
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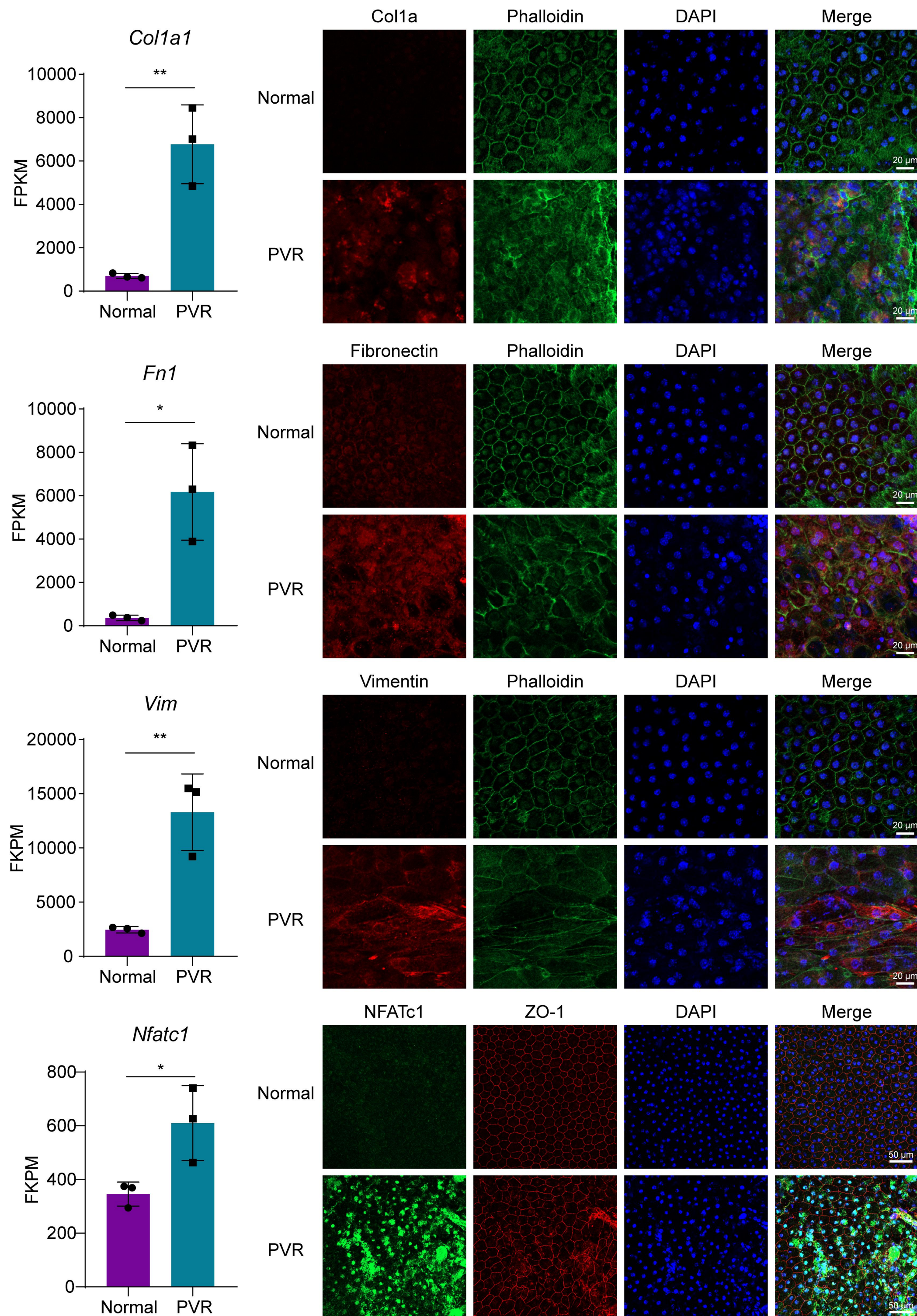

Our epigenomic analysis highlights changes in specific chromatin, rather than broad, global alterations. Unfortunately, there are currently no established methods for in situ analysis and visualization of chromatin states in specific DNA regions. This limitation prevents us from directly validating changes in chromatin accessibility and histone modifications within tissues samples.

3) The manuscript does not indicate how many times the ATAC-seq, ChIP-seq and RNA-seq experiments were repeated. At least three biological replicates ($n \geq 3$) are needed to establish the statistical significance of the results. Reading the manuscript, it seems that the authors used only one ($n=1$) biological replicate in the ATAC-seq and ChIP-seq experiments, while in the RNA-seq analysis they used only two biological replicates ($n=2$). If this is the case, then the results obtained are not statistically significant.

Response: We concur with the reviewer's insights regarding the necessity for statistical robustness in our analysis. In response, we have augmented our dataset to include three biological replicates for each of the RNA-seq, ATAC-seq, and ChIP-seq experiments involving all histone modifications, ensuring our results now meet the statistical significance criteria. This information has been detailed in the revised manuscript (Page 22, line 464 and Page 38, lines 813-814).

4) The authors should provide the secure token to allow reviewers access to the GEO GSE244812 data.

Response: We have provided a token (gpwlisycfxuddgh) for our GEO GSE244812 data. This information has been added in the "Data Availability" section of the revised manuscript (Page 29, line 615).

5) Statements like this (line 126: "In the PVR group, the promoter region of the Mdm2 gene displayed enhanced chromatin accessibility (fig. S2A).") must be accompanied by actual measured values (including p-value).

Response: We have expanded our biological replicates and now include the *P* value information for all differentially accessible chromatin regions. These details have been added to the Source Data file.

6) In their drug study, the authors do not indicate the size of the animal groups treated. The statement in the "Methods/Statistics" section that a minimum of three independent experiments were carried out does not allow us to determine the statistical significance of the

results obtained. The authors must indicate the exact number of animals in each group for each treatment. In general, the authors nowhere provide the results of a power analysis to determine the minimum size of groups of animals and samples necessary to obtain statistically significant results. Therefore, it is unclear whether the approach/data is reproducible.

Response: We are grateful for the reviewer's detailed attention regarding the specification of animal group sizes and the importance of conducting a power analysis to establish statistical significance. In our revised manuscript, we have detailed the number of animals used in each treatment group, ensuring this information is displayed in both the figures and the figure legends.

We also conducted a Power analysis (Charan et al., *J Pharmacol Pharmacother.*, 2013. PMID: 24250214) to ensure the statistical robustness of our animal experiments. This information has been added to the 'Methods' section of our revised manuscript to provide clarity on our experimental design and validation (Page 28, lines 602-603).

7) It is difficult to understand from the images presented in Figures 3, 5, and 6 whether there is an improvement after treatment with drugs. All that remains is to trust the authors that there was such an improvement. The authors should provide more convincing images.

Response: We appreciate the reviewer's suggestion. To enhance clarity and facilitate understanding, we have added arrows to indicate pathological changes in fundus photography images (Fig. 3F, 5F, 6H) and lines to mark specific areas of change in optical coherence tomography (OCT) scans (Fig. 3F, 5F, 6H). Furthermore, we have performed statistical analyses on all quantifiable results, including PVR grade scores based on H&E stain, gray value of WB, and mean fluorescence intensity of αSMA to visually demonstrate these findings more effectively (Fig. 3G-I, 5H-J, 6G, 6J, 6L).

8) It is impossible to understand anything from the results of the TUNEL apoptosis assay. Authors should use positive controls to show that the assay works in their hands.

Response: We thank the reviewer for pointing out this issue and have added the positive

controls in Figure S3E and Figure S5E, and Methods of the revised manuscript (Page 28, lines 592-593).

9) There is a significant difference between the Western blots shown in Figure 3H and Figure 5J. While Figure 5J shows a clear difference between the treated and control samples, this difference is not visible in Figure 3H. It is proposed to revise the Western blot presented in Figure 3H. The authors should also provide Western blots containing protein ladders.

Response: We appreciate the reviewer's constructive suggestion. The updated results are presented in the new Figure 3H in the revised manuscript. Additionally, we have included all Western blot images that feature protein ladders in Supplementary Fig.9.

10) Although injection of the proteolytic enzyme dispase is the most popular mouse PVR model, this model only partially mimics the pathogenesis and progression of PVR in humans. In Figure 5D, the authors presented the results of the funduscopic examination as well as H&E staining and IHC of samples acquired from one PVR patient. However, it is quite difficult to interpret the presented images. Thus, the findings still need to be confirmed in eye tissues from several PVR patients. Otherwise, it is not clear whether the findings have anything in common with the real disease.

Response: We agree. In acknowledgment of the concerns raised, we have expanded our investigation to include the examination of NFATc1 expression in human eyeballs (Methods, Page 27, lines 573-574). This enhanced analysis revealed an elevated level of NFATc1 when comparing the mean fluorescent intensity between donor eyeball sections with PVR membranes across groups (N=4/group). These updated findings are now shown in the new Fig.5D on Page 14, lines 294-297 in the revised manuscript, providing a more direct correlation with the pathology observed in human PVR patients.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have successfully addressed all of the previously raised points.

Reviewer #2:
Remarks to the Author:
My critiques based on the transparency of the bioinformatic methodology have been satisfactorily addressed. Additionally, the tables added have greatly increased the value of this study by making the results accessible to the wider community. Overall this rework represents a substantial improvement over the original submission.

Reviewer #4:
Remarks to the Author:
The authors. reported an interesting manuscript entitled “Multi-omics profiling of retinal pigment epithelium reveals enhancer-driven activation of RANK-NFATc1 signaling in traumatic proliferative vitreoretinopathy”. In addition, Liao et al, have responded in great detail to previous reviewers' comments. The work has been significantly improved but I still have some doubts. PRV is a complication with a huge inflammatory component, in this sense I doubt whether the authors have explored the activation of the NF-kB pathway because its activation is related to the activation of RANK.

On the other hand, I am curious about the animal model used and its relation to the pathophysiology in humans. In reference to the control group, is it the contralateral eye? Or are they eyes with OGI but no PVR? I think this needs to be explained very well in material and methods section.

Reviewer #5:
Remarks to the Author:
This study is very interesting and important, addresing an important and difficult clinical question in ophthalmology - traumatic proliferative vitreoretinopathy. The study is very well designed and conducted. The data analyses are valid and results solid. The study findings can elucidate the epigenetic basis underlying the activation of PVR-associated genes during RPE cell fate transitions and offer promising therapeutic targets.

In this revised manuscript, which is well written, the authors have fully and satisfactorily addressed all the comments from the reviewers. I have no further comments.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have successfully addressed all of the previously raised points.

Response: Thank you for your positive feedback. We appreciate your thorough review and are glad to have satisfactorily addressed all your concerns.

Reviewer #2 (Remarks to the Author):

My critiques based on the transparency of the bioinformatic methodology have been satisfactorily addressed. Additionally, the tables added have greatly increased the value of this study by making the results accessible to the wider community. Overall this rework represents a substantial improvement over the original submission.

Response: Thank you for your constructive feedback. We are pleased that the revisions have substantially enhanced the quality of our submission.

Reviewer #4 (Remarks to the Author):

The authors. reported an interesting manuscript entitled "Multi-omics profiling of retinal pigment epithelium reveals enhancer-driven activation of RANK-NFATc1 signaling in traumatic proliferative vitreoretinopathy". In addition, Liao et al, have responded in great detail to previous reviewers' comments. The work has been significantly improved but I still have some doubts. PRV is a complication with a huge inflammatory component, in this sense I doubt whether the authors have explored the activation of the NF-kB pathway because its activation is related to the activation of RANK.

Response: We thank the reviewer for the insightful comments on NF-κB signaling pathway activation in PVR. In our study, we specifically examined this pathway's activation. We observed elevated phosphorylation levels of NF-κB (p65) in a PVR mouse model, as shown in Figure 6D (Page 16, lines 334-336).

On the other hand, I am curious about the animal model used and its relation to the pathophysiology in humans. In reference to the control group, is it the contralateral eye? Or are they eyes with OGI but no PVR? I think this needs to be explained very well in material and methods section.

Response: The control group in our study consists of the untreated contralateral eyes, allowing each animal to serve as its own control. We have detailed this methodology in the revised manuscript under the 'Methods' section (Page 21, lines 434-436).

Reviewer #5 (Remarks to the Author):

This study is very interesting and important, addresing an important and difficult clinical question in ophthalmology - traumatic proliferative vitreoretinopathy. The study is very well designed and conducted. The data analyses are valid and results solid. The study findings can elucidate the epigenetic basis underlying the activation of PVR-associated genes during RPE cell fate transitions and offer promising therapeutic targets.

In this revised manuscript, which is well written, the authors have fully and satisfactorily addressed all the comments from the reviewers. I have no further comments.

Response: Thank you for your positive remarks. We are pleased that the revisions have met your approval.

Reviewers' Comments:

Reviewer #4:
Remarks to the Author:
The authors have successfully addressed all of the previously raised points.

REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

The authors have successfully addressed all of the previously raised points.

Response: We thank the reviewer for this positive appraisal of our revision.