nature portfolio

Peer Review File

Deficient RPE Mitochondrial Energetics Leads to Subretinal Fibrosis in Age-related Neovascular Macular Degeneration

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This report explores how subretinal fibrosis and a change toward a mesenchymal phenotype is induced by RPE cells when stimulated by TGFbeta. The investigators make several major advances in this set of experiments. They show that the VLDL receptor protein can suppress the effects of TGFbeta though a mechanism that probably does not involve lipoprotein transport. They also use transcript analysis to identify transcripts whose levels change in response to TGFbeta. They choose to focus on one of them, from the CPT1A gene, and find that expression of CPT1A has a strong influence on RPE cell metabolism and on the mesenchymal phenotype linked to fibrosis. This is a very important set of findings, the experiments for the most part include the appropriate controls and the paper is well written and clearly conveys the findings and their importance.

There are some important issues that I would like the authors to address.

1. Fig. 4E: What fuel was used in the experiment? Probably glucose, but that needs to be stated. Also, in this set of experiments, unlike in the other respirometry experiments, the OCR with uncoupler has not reached a steady OCR. I think this one needs to be repeated, because it is not clear if the TGF experiments might have ended up at the same maximal OCR as the controls if the experiments had gone on for a longer time.

2. For supplementary fig. 3: Please provide the raw data for this. Extrapolating the actual data to “ATP consumption” requires assumptions which may or may not be correct for these cells and the types of mitochondria, transporters, etc. that they have. But the actul OCR data would contain the real information and knowing that information and the conditions used would be very informative for readers.

3. Lines 185-188: Please provide more information about how the cells respond to 2-DG. Is it toxic to the cells? Do they die? How does it affect O2 consumption?

4. Is it really correct to conclude on lines 187-188 "indicating that VLDLR suppressed TGFb-induced fibrosis by blocking the TGF-b induced Warburg-like effect."? It seems like preventing the use of glucose altogether and depleting cells of ATP (by using it non-productively to phosphorylate non-metabolizable glucose could have many effects on RPE cells beyond its effect on glycolysis.

5. Line 152. Please describe why the authors decided to focus on CPT1A vs any of the other transcripts they identified. If it was based on the previous findings in kidney that should be stated. Otherwise, it comes across as kind of a random decision.

6. Fig. 5A: Please describe more clearly what the numbers on the y-axis mean.

7. The significance of red vs black markers in Fig. 5G should be defined in the legend.

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9. line 231: should refer to supplementary figure 4 panels, not 3.

10. It is very hard to see the CPT1A data in Fig. 7B. Please include that in a separate graph with a different y axis range so that readers can see the extent to which CPT1A levels are diminished.

11. Please clarify the use of controls in Fig. 8. It seems to me like an important control is missing. In each case the FLAG-tagged TGFBR protein is present only in the fourth lane. Isn't it most appropriate as a control to have a lane showing FLAG-tagged TGFBR without the myc-tagged VLDLR? That would show that the very strong signal from the tagged TGFBR only comes down when the tagged VLDLR is present. Unless I am misinterpreting this I think this is an important control that is needed to make these results convincing.

Review by James B. Hurley, March 3, 2024

Reviewer #2 (Remarks to the Author):

The manuscript by Ma et al. presents a valuable study that reveals the link between disturbed metabolism in retinal pigment epithelium (RPE) and subretinal fibrosis, as well as the roles of VLDLR and CPT1A in this process. These findings provide new targets and strategies for treating subretinal fibrosis in patients with neovascular age-related macular degeneration. The authors used single-cell RNA sequencing to identify the downregulation of genes involved in mitochondrial fatty acid oxidation in the RPE of Vldlr-/- mice, which are a model of subretinal fibrosis. They further demonstrated that overexpression of CPT1A, the rate-limiting enzyme of this pathway, could suppress epithelial-to-mesenchymal transition and fibrosis in the RPE. They also elucidated the mechanism by which TGFβ2 induced fibrosis by activating a Warburg-like effect, i.e. increased glycolysis and decreased mitochondrial respiration, through ERK-dependent CPT1A degradation. Moreover, they showed that VLDLR could block the formation of the TGFβ receptor I/II complex by interacting with unglycosylated TGFβ receptor II, thereby attenuating TGFβ2-induced metabolic reprogramming and fibrosis.
The article is well-written and organized, with clear aims, methods, results, and conclusions. The authors used appropriate references to support their arguments and data presentation. The scientific reasoning is sound and logical, and the implications are discussed and speculated. The article is of high quality and significance for the field of subretinal fibrosis and its treatment. This is a very thorough and well executed study. I think it will make an excellent addition to Communications Biology.

Reviewer #3 (Remarks to the Author):

It has been reported that subretinal fibrogenesis is associated with nAMD and VLDLR gene variants are associated with the risk of AMD. This manuscript described the mechanism of subretinal fibrosis in Vldlr-/- mice. Their evidences suggested that VLDLR blocked TGFβ ligand-induced formation of a TGFβ receptor heterotetramer. This block downregulated the CPT1A and increased the (α-SMA), CTGF and Fibronectin at protein levels, which may lead to subretinal fibrosis in nAMD. These findings are interesting and of novelty, which may provide a potential therapeutic target for subretinal fibrosis. However, before acceptance of publication, some concerns are suggested to answer.

1) This study setup a nAMD model in Vldlr-/- mice. Here, VLDR is key elements. No VLDR expression in RPE cells from Vldlr-/- mice and from AMD patients should be confirmed by immnuo staining and WB analysis. Also, in Fig. 5, VLDR expression in sections from AMD patients should be checked.
2) Also, it is interesting and important to investigate if nAMD happens when VLDR expression is rescued in Vldlr-/- mice.
3) TGFb/TGFR pathway contributes to EMT and tissue or organ fibrosis. Since evidences demonstrated that VLDLR blocked TGFβ ligand-induced formation of a TGFβ receptor heterotetramer, it will be interesting to investigate if fibrosis occurs in other organs or cells besides RPE cells in Vldlr-/- mice..
4) In Fig.6 K, immune staining of α-SMA and CTGF are suggested also, besides collagen I.
5) How did reduction of CPT1A expression upregulate α-SMA, CTGF and fibronectin expression? Or, was reduction of CPT1A expression not associated with α-SMA, CTGF and fibronectin expression? The direct evidences are suggested to give.
6) Also, single-cell RNA sequencing revealed downregulated fatty acid β-oxidation and electron transport chain in RPE of Vldlr-/- mice. But, in RPE of Vldlr-/- mice, how did metabolism reprogramming or suppressed FAO and ETC, contribute to subretinal fibrosis in nAMD? No detailed mechanism was given. Further investigation or some deep discussion is highly suggested.

Response to Comments from Reviewer 1

Comment: *This report explores how subretinal fibrosis and a change toward a mesenchymal phenotype is induced by RPE cells when stimulated by TGFbeta. The investigators make several major advances in this set of experiments. They show that the VLDL receptor protein can suppress the effects of TGFbeta though a mechanism that probably does not involve lipoprotein transport. They also use transcript analysis to identify transcripts whose levels change in response to TGFbeta. They choose to focus on one of them, from the CPT1A gene, and find that expression of CPT1A has a strong influence on RPE cell metabolism and on the mesenchymal phenotype linked to fibrosis. This is a very important set of findings, the experiments for the most part include the appropriate controls and the paper is well written and clearly conveys the findings and their importance.*

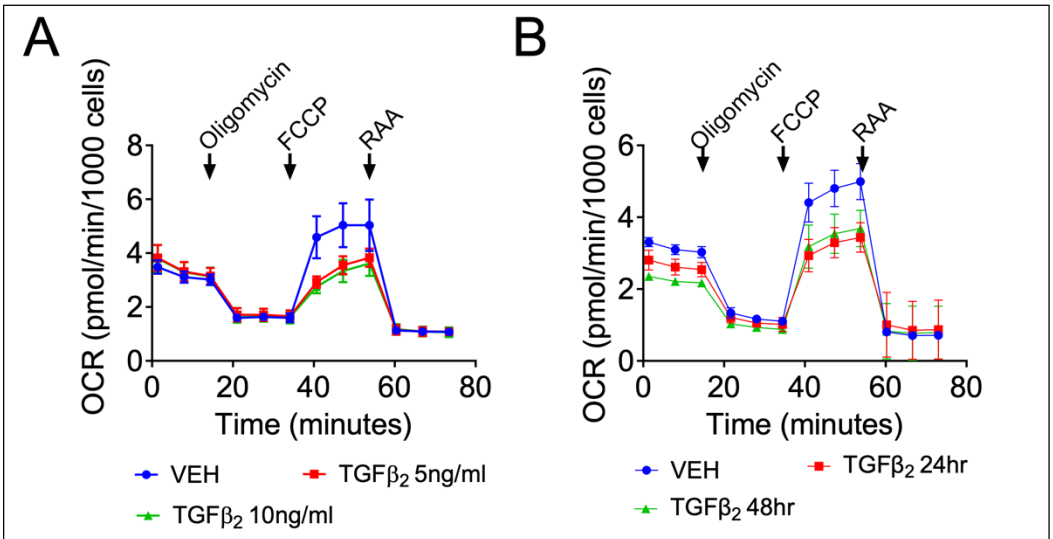
There are some important issues that I would like the authors to address.

Response: Thank you for the positive comments. We sincerely appreciate the reviewer's time and effort in reviewing our manuscript. The reviewer's comments and suggestions are constructive for improving the quality of this manuscript. The followings are detailed responses and revisions.

Comment 1. *Fig. 4E: What fuel was used in the experiment? Probably glucose, but that needs to be stated. Also, in this set of experiments, unlike in the other respirometry experiments, the OCR with uncoupler has not reached a steady OCR. I think this one needs to be repeated, because it is not clear if the TGF experiments might have ended up at the same maximal OCR as the controls if the experiments had gone on for a longer time.*

Response 1. We apologize for missing the information. In Figure 4E, the mitochondrial stress test was performed using the standard protocol from the Seahorse XF Cell Mito Stress Test (https://www.agilent.com/cs/library/usermanuals/Public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf). The fuels included glucose, pyruvate, and glutamine in this experiment. According to the manufacturer's protocol, the basic Seahorse XF DMEM medium was supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. As suggested, the experimental media and fuels are stated in the revised figure legend (page 37, Line 815-816).

We have observed clear evidence that TGFβ_2 significantly downregulates the OCR in RPE cells in the mitochondrial stress test (Online Figure 1). Here, we demonstrated our previous data regarding the response of ARPE-19 cells to different doses of TGFβ_2 (Online Figure 1A) and the response of human primary RPE cells to a different duration of TGFβ_2 treatment (Online Figure 1B).

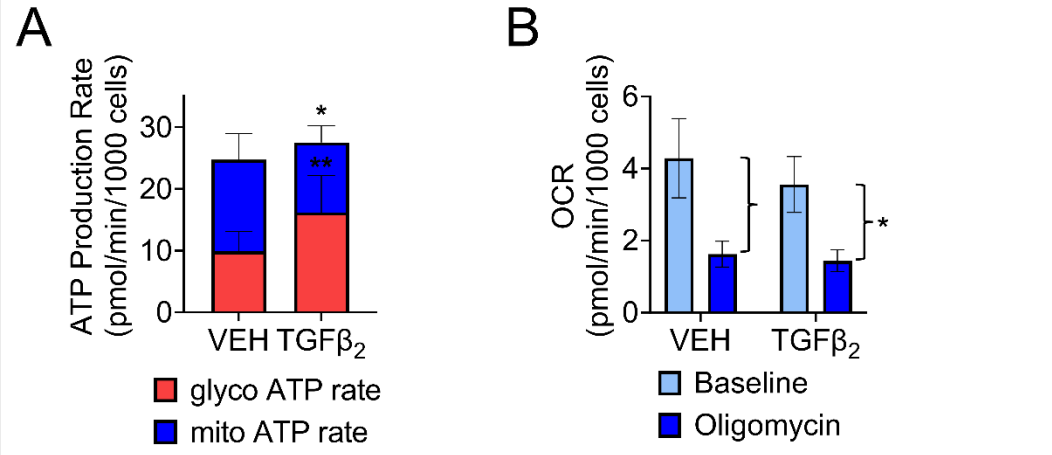


Online Figure 1. The TGFβ effect on OCR in RPE cells in the mitochondrial stress test.
A: Mitochondrial stress test in ARPE-19 cells treated with the indicated dose of $TGF\beta_2$ for 24 hr. **B:** Mitochondrial stress test in human primary RPE cells treated with 5 ng/ml $TGF\beta_2$ for indicated times (n = 8-12). Data are presented as mean ± SD.

We appreciate the reviewer's insightful comments regarding the TGFβ effect on OCR when different energy sources are provided. We hypothesize that TGFβ downregulates OCR through the inhibition of fatty acid transport into mitochondria (CPT1A route) because we observed that TGFβ reduces OCR in response to palmitate in the FAO test (Figure 4I). Meanwhile, as pointed out by the reviewer, fatty acids are not used as energy fuel in the mitochondrial stress test (Figure 4E) as the basic Seahorse XF DMEM medium does not contain fatty acid. This indicates that TGFβ reduces the OCR through other routes. Recently, others have reported that TGFβ may suppress the TCA cycle metabolism through the reduction of the transcription factor PGC-1α, which regulates the expression of TCA cycle enzymes (PMCID: PMC8663777) [1]. Another recent work reported that TGFβ downregulates OCR through induction of mitochondrial fission, and the knockdown of fission protein Drp1 alleviates TGFβ-induced mitochondrial dysfunction (PMCID: PMC6965618) [2]. All evidence suggests that TGFβ-medicated metabolic changes occur at the downstream of the TGFβ receptors. In this study, we proved that VLDLR interacts with TGFβ signaling at the receptor level. Thus, although we propose that VLDLR alleviates TGFβ-induced metabolic dysfunction through the CPT1A route, we cannot rule out molecular mechanisms other than mitochondrial lipid transport. This is a potential limitation of our hypothesis on molecular mechanisms. We stated it in the discussion section (page 21, lines 412-417).

Comment 2. *For supplementary fig. 3: Please provide the raw data for this. Extrapolating the actual data to "ATP consumption" requires assumptions which may or may not be correct for these cells and the types of mitochondria, transporters, etc. that they have. But the actul OCR data would contain the real information and knowing that information and the conditions used would be very informative for readers.*

Response 2. Thank you for your constructive comment. As suggested, the actual OCR data used for the calculation of ATP production rate was provided in the revised Supplementary Figure 3B.



Online Figure 2. The ATP production rate and raw oxygen consumption rate (OCR) data in TGF β_2-treated RPE cells.

A: ATP production rate in human primary RPE cells treated with 5 ng/ml TGFβ_2 or vehicle (VEH) for 24 hr (n = 11-13). **B:** The actual OCR data used for the calculation of ATP production rate (n = 11-13). Data are presented as mean ± SD.

According to the manufacturer's protocol, injection of oligomycin results in an inhibition of mitochondrial ATP synthesis and results in a decrease in OCR. The rate of oxygen consumption is coupled to ATP production during oxidative phosphorylation. Thus, OCR_{ATP} (OCR difference between the baseline and the addition of oligomycin) can be used to calculate the mitoATP (mitochondrial ATP production rate). The followings are the equations.

(Equation 1) *OCR_{ATP} (pmol O_2/min) = OCR (pmol $_{O2}$/min) – OCR_{Oligo} (pmol O_2/min)*

(Equation 2) *mitoATP Production Rate (pmol ATP/min) = OCR_{ATP} (pmol O_2/min) * 2 (pmol O/pmol O_2) * P/O (pmol ATP/pmol O)*

P/O value is set as 2.75 by manufacturer.

Comment 3. *Lines 185-188: Please provide more information about how the cells respond to 2-DG. Is it toxic to the cells? Do they die? How does it affect O2 consumption?*

Response 3. Thank you for your comments on 2-deoxy-d-glucose (2-DG) toxicity and RPE response. We used 2-DG with the concentration of 2 g/L (12 mM) as an intervention for 24 hr (Figure 4O). The glucose level in the culture medium was 1 g/L (5.5 mM). In this condition, we did not observe obvious toxicity or cell death at the concentration of 2 g/L 2-DG for 24 hr. The RPE cells maintained a confluent monolayer. In another setting, 2-DG with concentration of 8.2 g/L (50 mM) was used as the last injection of glycolysis stress test according to manufacturer's protocol. The glucose level in the culture medium was 1 g/L (5.5 mM). We did not observe obvious cell death within 1 hr after addition of 8.2 g/L (50 mM) 2-DG. According to the publication, 2-DG induces cell death in a dose- and time-dependent manner (PMCID: PMC6411866) [3]. Within 24 hr, the dose of 2-DG of 6.6g/L (40mM) can induce obvious cell death in renal tubular epithelial cells (PMCID: PMC6411866) [3].

The effect of 2-DG on oxygen consumption could be determined by cell metabolic status, and the dose and duration of 2-DG treatment. In the present study, we did not observe the OCR difference at least for 30 min after the addition of 2-DG (Figure 6E).

Comment 4. *Is it really correct to conclude on lines 187-188 "indicating that VLDLR suppressed TGFb-induced fibrosis by blocking the TGF-b induced Warburg-like effect."? It seems like preventing the use of glucose altogether and depleting cells of ATP (by using it non-productively to phosphorylate non-metabolizable glucose could have many effects on RPE cells beyond its effect on glycolysis.*
Response 4. Thank you very much for the constructive comment. We reconstructed the conclusion as "indicating that VLDLR suppressed TGFβ-induced fibrosis by blocking the TGFβ-mediated downregulation of oxidative phosphorylation and upregulation of glycolysis (page 11, lines 185-187)."

Comment 5. *Line 152. Please describe why the authors decided to focus on CPT1A vs any of the other transcripts they identified. If it was based on the previous findings in kidney that should be stated. Otherwise, it comes across as kind of a random decision.*

Response 5. Thank you! We decided to focus on CPT1A for 3 major reasons. 1) CPT1A is screened out from our scRNA-seq dataset, and we validated it using traditional methods, including real-time PCR and western blotting (Figure 5A-5D). 2) CPT1A is the rate-limiting enzyme of fatty acid utilization. It indicates that the rescue of CPT1A may boost the entire lipid metabolism pathway. 3) As mentioned by the reviewer, we were excited to see the supportive literature that CPT1A plays an anti-fibrotic role in kidney fibrosis (PMCID: PMC7919728) [4]. As suggested, we added the description in the result section and cited the reference (page 11, lines 192, 195-196, 201-203).

Comment 6. *Fig. 5A: Please describe more clearly what the numbers on the y-axis mean.*

Response 6. Sorry for missing an essential information for interpreting the single cell RNA sequencing data (scRNA-seq). As suggested, we have added the information in the figure legend (page 37, line 831). The y-axis is the expression level of a specific gene (e.g. *Cpt1a*) and the numbers of y-axis are normalized counts of this gene transcript performed by *cellranger aggr* function in R.

In the scRNA-seq dataset, the expression level of a specific gene is measured by the observed unique molecular identifier (UMI) count for this gene. UMI counts are absolute numbers of observed transcripts that should be normalized for sequencing depth, technical variation, or RNA content per cell. After normalization, UMI counts can be compared between samples.

The following links may provide more detailed information and explanations.

1. Definition of UMI (https://www.10xgenomics.com/support/software/cell-ranger/latest/resources/cr-glossary).

2. Are UMI counts comparable between samples? (https://kb.10xgenomics.com/hc/en-us/articles/218169603-Are-UMI-counts-comparable-between-samples#:~:text=UMI%20counts%20represent%20the%20absolute,or%20RNA%20content%20per%20cell).

3. How are the UMI counts normalized before PCA and differential expression? (https://kb.10xgenomics.com/hc/en-us/articles/115004583806-How-are-the-UMI-counts-normalized-before-PCA-and-differential-expression).

Comment 7. *The significance of red vs black markers in Fig. 5G should be defined in the legend.*

Response 7. Sorry for missing the information. The significance of red vs black markers in the revised Fig. 5H has been defined in the legend (page 38, lines 840-842).

Comment 8. *line 225: "collage" should be "collagen".*

Response 8. Sorry for the typo. The typo in line 225 has been corrected.

Comment 9. *line 231: should refer to supplementary figure 4 panels, not 3.*

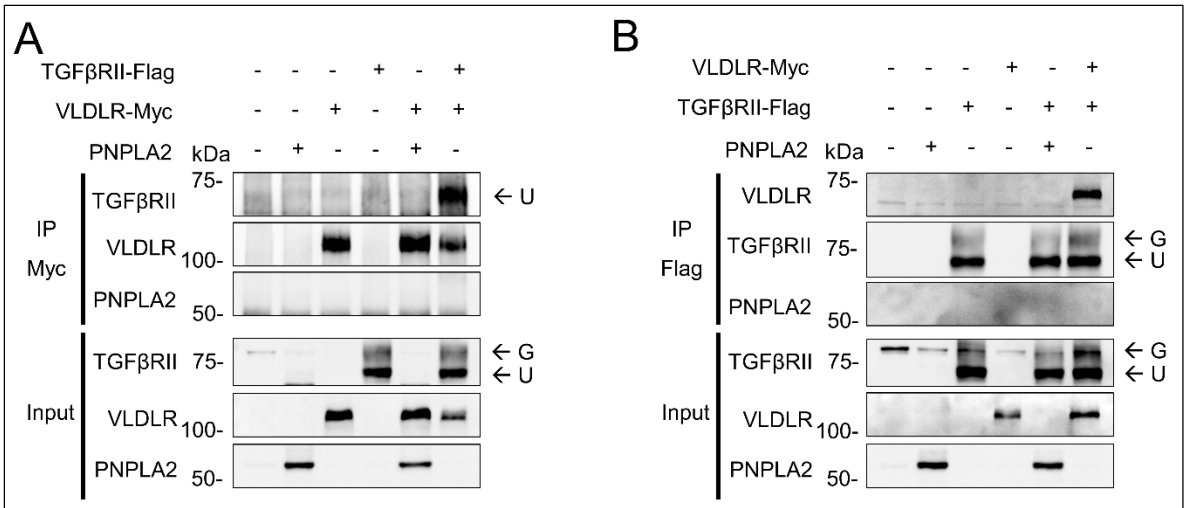
Response 9. Sorry for the error. The figure number has been corrected.

Comment 10. *It is very hard to see the CPT1A data in Fig. 7B. Please include that in a separate graph with a different y axis range so that readers can see the extent to which CPT1A levels are diminished.*
Response 10. Thank you for the suggestion! We generated separate graphs with a more suitable y-axis range for CPT1A so that bars of CPT1A levels can be seen clearly. Figure 7B was revised accordingly.

Comment 11. *Please clarify the use of controls in Fig. 8. It seems to me like an important control is missing. In each case the FLAG-tagged TGFBR protein is present only in the fourth lane. Isn't it most appropriate as a control to have a lane showing FLAG-tagged TGFBR without the myc-tagged VLDLR? That would show that the very strong signal from the tagged TGFBR only comes down when the tagged VLDLR is present. Unless I am misinterpreting this I think this is an important control that is needed to make these results convincing.*

Response 11. We appreciate your insightful comments regarding the negative control in Figure 8. We agree with your interpretation. Following the recommendation, we reperformed the assay. In the revised Figure 8A, the group of Flag-tagged TGFβRII alone without Myc-tagged VLDLR was added as a key negative control of the co-immunoprecipitation experiment for pulling down the Myc-tag. As shown by Western blot, the robust signal of TGFβRII was only shown in the precipitated sample from the last group of Flag-tagged TGFβRII with Myc-tagged VLDLR. Also, Flag-tagged TGFβRII alone did not precipitate by Myc resins without VLDLR-Myc. This result suggests that the TGFβRII signal in the precipitated sample is due to the interaction between TGFβRII and VLDLR protein, instead of residual TGFβRII in the immunoprecipitation buffer.

In addition, we have confirmed the TGFβRII and VLDLR interaction by reciprocal co-immunoprecipitation. As shown in Fig. 8B, the group of Myc-tagged VLDLR alone without Flag-tagged TGFβRII was added as a key negative control of the co-immunoprecipitation experiment for pulling down the Flag-tag.



Online Figure 3. VLDLR interacts with unglycosylated TGFβ receptor II.

A: Interaction between VLDLR and TGFβ receptor II (TGFβRII) by Myc-tag pulldown. Membrane protein PNPLA2 was used as the negative control. G: glycosylated TGFβRII, U: unglycosylated TGFβRII. **B:** Interaction between VLDLR and TGFβ receptor II (TGFβRII) by Flag-tag pulldown. Membrane protein PNPLA2 was used as the negative control. G: glycosylated TGFβRII, U: unglycosylated TGFβRII.

Review by James B. Hurley, March 3, 2024

Reviewer #2 *(Remarks to the Author):*

Comment. *The manuscript by Ma et al. presents a valuable study that reveals the link between disturbed metabolism in retinal pigment epithelium (RPE) and subretinal fibrosis, as well as the roles of VLDLR and CPT1A in this process. These findings provide new targets and strategies for treating subretinal fibrosis in patients with neovascular age-related macular degeneration. The authors used single-cell RNA sequencing to identify the downregulation of genes involved in mitochondrial fatty acid oxidation in the RPE of Vldlr-/- mice, which are a model of subretinal fibrosis. They further demonstrated that overexpression of CPT1A, the rate-limiting enzyme of this pathway, could suppress epithelial-to-mesenchymal transition and fibrosis in the RPE. They also elucidated the mechanism by which TGFβ2 induced fibrosis by activating a Warburg-like effect, i.e. increased glycolysis and decreased mitochondrial respiration, through ERK-dependent CPT1A degradation. Moreover, they showed that VLDLR could block the formation of the TGFβ receptor I/II complex by interacting with unglycosylated TGFβ receptor II, thereby attenuating TGFβ2-induced metabolic reprogramming and fibrosis. The article is well-written and organized, with clear aims, methods, results, and conclusions. The authors used appropriate references to support their arguments and data presentation. The scientific reasoning is sound and logical, and the implications are discussed and speculated. The article is of high quality and significance for the field of subretinal fibrosis and its treatment. This is a very thorough and well executed study. I think it will make an excellent addition to Communications Biology.*

Response: Thank you for the positive comments. We sincerely appreciate the reviewer's time and effort in reviewing our manuscript.

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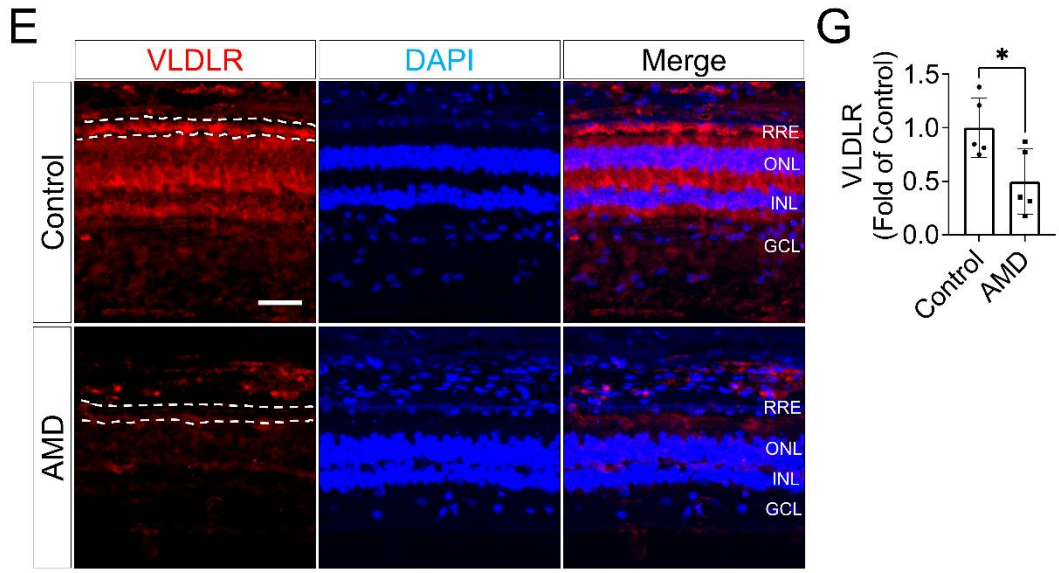
Response: Thank you for the positive comments. We sincerely appreciate the reviewer's time and effort in reviewing our manuscript. The reviewer's comments and suggestions are constructive for improving the quality of our study. The detailed responses and revisions are summarized as the followings.

Comment 1. *This study setup a nAMD model in Vldlr-/- mice. Here, VLDR is key elements. No VLDR expression in RPE cells from Vldlr-/- mice and from AMD patients should be confirmed by immnuo staining and WB analysis. Also, in Fig. 5, VLDR expression in sections from AMD patients should be checked.*

Response 1. Thank you for your comments on the validation of VLDLR knockout in the RPE cells from *Vldlr*$^{-/-}$ mice. We used global VLDLR knockout mice (*Vldlr*$^{-/-}$) that were generated and characterized in 1995 (PMCID: PMC41175) [5]. Since 2003, *Vldlr*$^{-/-}$ mice has been reported to manifest neovascular AMD (nAMD) phenotypes with choroidal neovascularization by our group and others (PMID: 12972764, PMID: 17890782) [6,7]. In 2008, Dr. Xiaoxi Qiao's lab confirmed the absence of VLDLR RNA and protein in RPE cells of *Vldlr*$^{-/-}$ mice (Figures. 1 & S1, PMID: 18172119) [8]. Thus, we took it as an established foundation for current research.

We appreciate your suggestion to measure the VLDLR expression in retinal sections from AMD patients. We observed lower levels of VLDLR in the RPE layer of AMD patients compared to age-matched healthy controls, which supported our central hypothesis. The result was incorporated in the revised version of Figure 5E, and figure legend was revised accordingly.



Online Figure 4. The level of VLDLR is decreased in the RPE layer of AMD patients.

E: Representative immunostaining of VLDLR (red) in the retinal sections of nAMD patients and controls. Scale bar: 30 μm. **G:** Quantification of VLDLR intensity in the RPE layer of nAMD patients compared to age-matched controls (n = 5 for nAMD patients, n = 5 for non-AMD controls). Data are presented as mean ± SD. * $P < 0.05$.

Comment 2. *Also, it is interesting and important to investigate if nAMD happens when VLDR expression is rescued in Vldlr-/- mice.*

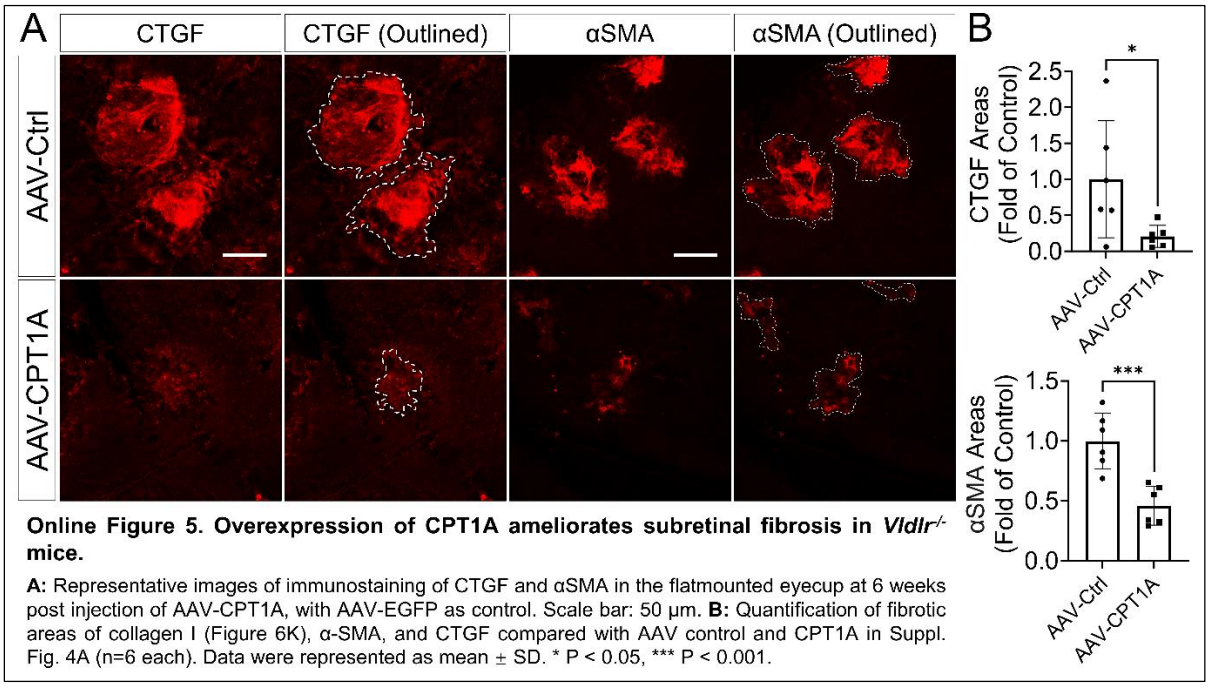
Response 2. Thank you! We are working on generating a new line of inducible VLDLR overexpression mice. We hope to answer this question in future study using the transgenic mice. The limitation on delivery efficiency and consistency of intravitreal/subretinal delivery of VLDLR plasmids/AAV may not guarantee the right levels of VLDLR expressing at the right time and right location in *Vldlr*$^{-/-}$ mice. It creates the obstacle to answer if the restoration of VLDLR expression rescues neovascularization and fibrosis in *Vldlr*$^{-/-}$ mice.

Comment 3. *TGFb/TGFR pathway contributes to EMT and tissue or organ fibrosis. Since evidences demonstrated that VLDLR blocked TGFβ ligand-induced formation of a TGFβ receptor heterotetramer, it will be interesting to investigate if fibrosis occurs in other organs or cells besides RPE cells in Vldlr-/- mice.*

Response 3. Thank you! It is an interesting question worth investigating in the future.

Comment 4. *In Fig.6 K, immune staining of α-SMA and CTGF are suggested also, besides collagen I.*

Response 4. Thank you! As suggested, we re-performed the animal experiments with subretinal injection of AAV-Best1-CPT1A and harvested the eyecups for immunostaining of CTGF and α-SMA in flatmounted eyecups. We found that overexpression of human CPT1A in RPE tissues significantly reduced the CTGF areas and α-SMA areas compared to those treated with control virus. The result was incorporated in the revised version of Supplementary Figure 4A-4B, and figure legend was revised accordingly.



Online Figure 5. Overexpression of CPT1A ameliorates subretinal fibrosis in *Vldlr*$^{-/-}$ mice.

A: Representative images of immunostaining of CTGF and αSMA in the flatmounted eyecup at 6 weeks post injection of AAV-CPT1A, with AAV-EGFP as control. Scale bar: 50 μm. **B:** Quantification of fibrotic areas of collagen I (Figure 6K), α-SMA, and CTGF compared with AAV control and CPT1A in Suppl. Fig. 4A (n=6 each). Data were represented as mean ± SD. * $P < 0.05$, *** $P < 0.001$.

Comment 5. *How did reduction of CPT1A expression upregulate α-SMA, CTGF and fibronectin expression? Or, was reduction of CPT1A expression not associated with α-SMA, CTGF and fibronectin expression? The direct evidences are suggested to give.*

Response 5. Thank you! Under TGFβ$_2$ treatment, CPT1A was downregulated, and fibrosis markers were upregulated (Figure 7A). We also observed that the reduction of CPT1A was associated with the upregulation of α-SMA in RPE layers of AMD patients (Figure 5H). The direct evidence indicated that reduction of CPT1A is correlated with upregulation of fibrosis markers under fibrotic stress or disease stress.

Mechanistically, we think that under profibrotic stress, the reduction of CPT1A downregulates oxidative phosphorylation and upregulates glycolysis. This may provide not only more ATP but also metabolic byproducts for fibrogenesis.

Comment 6. *Also, single-cell RNA sequencing revealed downregulated fatty acid β-oxidation and*

electron transport chain in RPE of Vldlr-/- mice. But, in RPE of Vldlr-/- mice, how did metabolism reprogramming or suppressed FAO and ETC, contribute to subretinal fibrosis in nAMD? No detailed mechanism was given. Further investigation or some deep discussion is highly suggested.
Response 6. Thank you! As suggested, we discussed how metabolic reprogramming in the RPE contributes to fibrosis in the revised discussion section (pages 20-21, lines 400-410).

Reference

1 Nam, H. *et al.* The TGF-β/HDAC7 axis suppresses TCA cycle metabolism in renal cancer. *JCI Insight* **6** (2021). https://doi.org:10.1172/jci.insight.148438

2 Wang, Y. *et al.* Drp1-mediated mitochondrial fission promotes renal fibroblast activation and fibrogenesis. *Cell Death Dis* **11**, 29 (2020). https://doi.org:10.1038/s41419-019-2218-5

3 Zhao, J. *et al.* Low-dose 2-deoxyglucose and metformin synergically inhibit proliferation of human polycystic kidney cells by modulating glucose metabolism. *Cell Death Discov* **5**, 76 (2019). https://doi.org:10.1038/s41420-019-0156-8

4 Miguel, V. *et al.* Renal tubule Cpt1a overexpression protects from kidney fibrosis by restoring mitochondrial homeostasis. *J Clin Invest* **131** (2021). https://doi.org:10.1172/jci140695

5 Frykman, P. K., Brown, M. S., Yamamoto, T., Goldstein, J. L. & Herz, J. Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor. *Proc Natl Acad Sci U S A* **92**, 8453-8457 (1995). https://doi.org:10.1073/pnas.92.18.8453

6 Heckenlively, J. R. *et al.* Mouse model of subretinal neovascularization with choroidal anastomosis. *Retina* **23**, 518-522 (2003). https://doi.org:10.1097/00006982-200308000-00012

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors satisfactorily addressed each of my previous concerns.

My only additional suggestion (that does not require me to review it) would be to include a panel C with the data for ECAR in addition to the panel B that shows the OCR data in Online Fig. 2.

Review by James Hurley June 22, 2024.

Reviewer #2 (Remarks to the Author):

The author has answered all my questions.

Reviewer #3 (Remarks to the Author):

Authors have replied all comments from reviewers.

But, Response to reviewer 3's comment 1 didn't give the VLDLR immnuo staining and WB analysis results in Fig 1C, Fig.1F and S1 A.

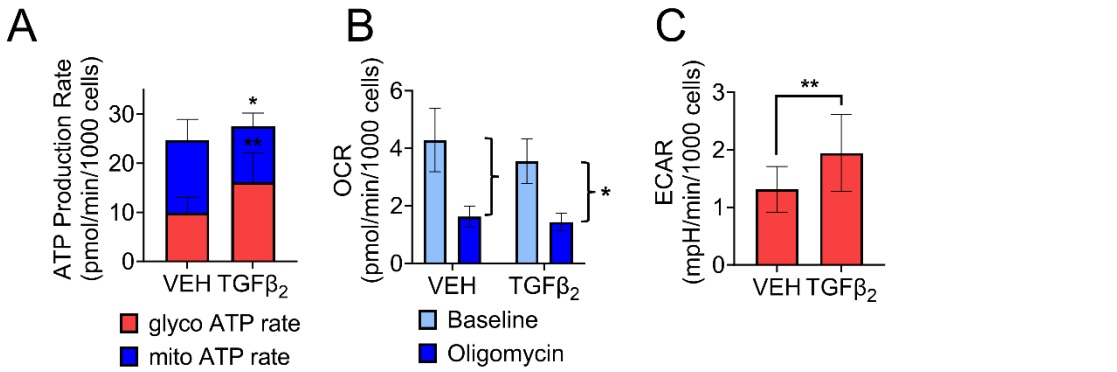
Authors mentioned that in 2008, Dr. Xiaoxi Qiao's lab confirmed the absence of VLDLR RNA and protein in RPE cells of Vldlr-/- mice (Figures. 1 & S1, PMID: 18172119). However, confirmations of VLDLR expressions are still needed in this study.

In S1 D, insert area of Vimentin and Collagen 1 are suggested to modify to include RRE, ONL,INL and GCL.

Response to Comments from Reviewer 1

Comment: *The authors satisfactorily addressed each of my previous concerns. My only additional suggestion (that does not require me to review it) would be to include a panel C with the data for ECAR in addition to the panel B that shows the OCR data in Online Fig. 2.*

Response: Thank you for the positive comments. We sincerely appreciate the reviewer's time and effort in reviewing our manuscript. Online Figure 2 in the first round revision is Supplementary Figure 3 in the manuscript (or Online Figure 1 in this second round revision). The ECAR data were added into panel C as shown below and Supplementary Figure 3 was revised accordingly.



Online Figure 1. ATP-rate assay of TGFβ-treated RPE cells.

A: ATP production rate in human primary RPE cells treated with 5 ng/ml $TGF\beta_2$ or vehicle (VEH) for 24 hr (n = 11-13). The ATP production rate was measured using a Seahorse analyzer. **B:** The actual oxygen consumption rate (OCR) data used for the calculation of ATP production rate (n = 11-13). **C:** The actual extracellular acidification rate (ECAR) data used for the calculation of ATP production rate (n = 11-13). Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Response to Comments from Reviewer 2

Comment. *The author has answered all my questions.*

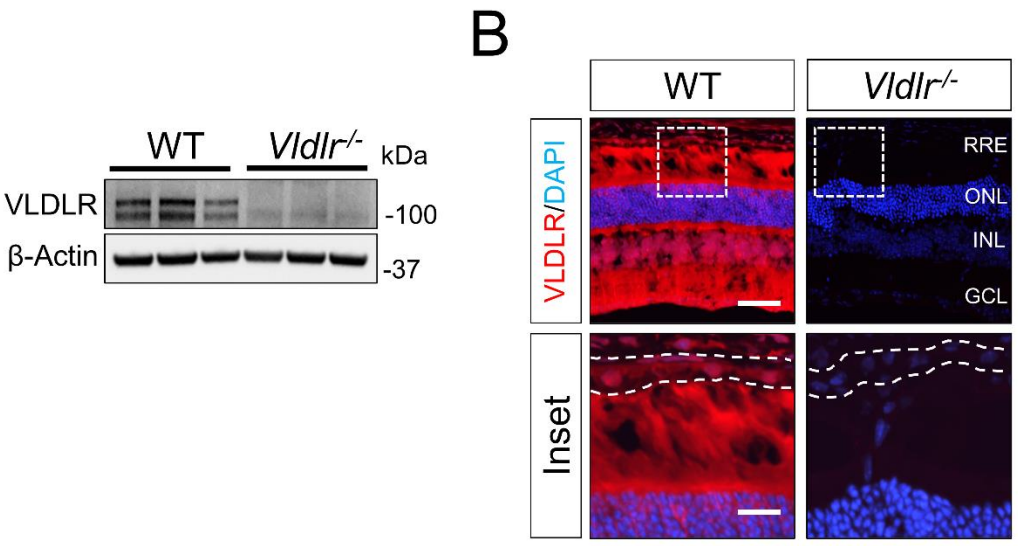
Response: Thank you for the positive comments. We sincerely appreciate the reviewer's time and effort in reviewing our manuscript.

Response to Comments from Reviewer 3

Comment 1. *Authors have replied all comments from reviewers. But, Response to reviewer 3's comment 1 didn't give the VLDLR immnuo staining and WB analysis results in Fig 1C, Fig.1F and S1*

A. Authors mentioned that in 2008, Dr. Xiaoxi Qiao's lab confirmed the absence of VLDLR RNA and protein in RPE cells of Vldlr-/- mice (Figures. 1 & S1, PMID: 18172119). However, confirmations of VLDLR expressions are still needed in this study.

Response: Thank you for your comments on the validation of VLDLR knockout in the RPE cells from *Vldlr*$^{-/-}$ mice. As suggested, we performed Western blotting and immunostaining to confirm the absence of VLDLR in RPE cells of *Vldlr*$^{-/-}$ mice (Online Figure 2). Results of Western blot result (Online Figure 2A) and immunostaining (Online Figure 2B) were incorporated into the revised Figure 1F and into the revised Supplementary Figure 1A, respectively. Also figure legends were revised accordingly.



Online Figure 2. The absence of VLDLR protein in *Vldlr*$^{-/-}$ mice.

One-month-old *Vldlr*$^{-/-}$ mice and age-matched WT C57BL/6J mice were used to confirm the absence of VLDLR expression in *Vldlr*$^{-/-}$ mice. **A:** Representative images of Western blotting in RPE tissues from WT and *Vldlr*$^{-/-}$ mice. **B:** Representative immunostaining of VLDLR in ocular cryosection of WT and *Vldlr*$^{-/-}$ mice. Scale bar: 50 μm (15 μm in inset).

Comment 2. *In S1 D, insert area of Vimentin and Collagen 1 are suggested to modify to include RRE, ONL,INL and GCL.*

Response: Thank you for the suggestion! All layers of RPE, ONL, INL, and GCL were labeled in both WT and *Vldlr*$^{-/-}$ sections in the revised version.

REVIEWERS' COMMENTS:

Reviewer #3 (Remarks to the Author):

Authors have satisfactorily answered all comments from reviewer.