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TITLE: Targeting Metabolic Reprogramming for the Prevention and Treatment of Proliferative Vitreoretinopathy

PRINCIPAL INVESTIGATOR: Dr. Magali Saint-Geniez

CONTRACTING ORGANIZATION: Schepens Eye Research Institute of Mass. Eye and Ear, 20 Staniford St, Boston, MA, 02114

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6. AUTHOR(S)
Magali Saint-Geniez, Daisy Shu

Betty Diamond

E-Mail: Magali_SaintGeniez@meei,harvard.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT This is our second year on the project, and we have published our findings identifying the key metabolic reprogramming traits associated with proliferative vitreoretinopathy (PVR) using our in-vitro model of epithelial-mesenchymal transition (EMT) of human retinal pigment epithelial cells (RPE). We have identified promising metabolic drugs which effectively block retinal EMT in vitro to be tested in our in vivo rabbit ocular surgery model of PVR. Since re-opening of the lab, we have now collected a total of 34 human vitreous samples. We have sent through primary human RPE cells for metabolomics processing and will compare these results with our ARPE-19 cell line data. We have identified another cytokine that induces EMT of RPE, namely, tumor necrosis factor-alpha, and found that it exerts opposite effects to metabolic reprogramming compared to transforming growth factor-beta and found a novel metabolic drug, dimethyl fumarate (DMFu) that blocks to activity of tumor necrosis factor-alpha.

15. SUBJECT TERMS
Proliferative vitreoretinopathy, retinal pigment epithelial cells, epithelial-mesenchymal transition, metabolism, mitochondria, transforming growth factor-beta

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1. INTRODUCTION:

Proliferative vitreoretinopathy (PVR) is a common and serious complication of retinal detachment surgery and retinal trauma. PVR is characterized by the uncontrolled proliferation and migration of retinal cells, predominantly retinal pigment epithelial (RPE) cells, resulting in the formation of a fibrotic membrane at the retinal surface. A key established mechanism in PVR is conversion of RPE cells into mesenchymal cells through epithelial-mesenchymal transition (EMT). The purpose of our project is to investigate the role of metabolic rewiring and mitochondrial dysfunction during EMT of RPE. In doing so, we aim to find novel drug targets to combat PVR. The scope of our research involves 1) using an in vitro human RPE model to understand the metabolic changes and identify drug candidates to inhibit PVR formation and 2) validation of the top candidate drugs using an in vivo rabbit model of PVR.

2. KEYWORDS:

- Proliferative vitreoretinopathy
- Epithelial-mesenchymal transition
- Wound healing
- Transforming growth factor-beta
- Retina pigment epithelial cells
- Metabolism
- Mitochondria

3. ACCOMPLISHMENTS**:**

- **What were the major goals of the project?**

Specific Aim 1	Timeline (months)	Completion (%)
Major Task 1: To characterise the metabolic reprogramming involved in EMT of RPE and PVR		
Subtask 1: characterization of the metabolic traits characteristic of RPE mesenchymal transition by metabolic and gene expression profiling	1-12	100
Subtask 2: Untargeted metabolic analysis of extracellular and intracellular compartments of RPE cells following EMT	6-12	95
Milestone: identification of the precise metabolic changes associated with human RPE mesenchymal conversion	8-12	95
Major Task 2: To determine the key metabolic changes associated with PVR by metabolic profiling of human vitreous samples		
Subtask 3: Collection of vitreous samples from PVR and control patients	1-18	30
Milestone: HRPO Approval	6	100
Subtask 4: Metabolomics profiling by Mass Spectrometry of human PVR samples and control patients	18-24	0
Milestone: Identification of potential metabolic biomarkers of PVR progression and severity	20-24	0
Specific Aim 2	**Timeline (months)**	**Completion (%)**
Major Task 3: To evaluate the efficiency of pharmacological metabolic regulation in preventing and treating PVR		
Subtask 5: Submission of Animal Protocol to IACUC	1-3	100
Milestone: Local IACUC approval	6	100
Milestone: ACURO approval	9	100
Subtask 6: Selection of metabolic drugs able to prevent/revert RPE mesenchymal transition in vitro	12-24	100
Subtask 7: Testing the top 3 pre-screened metabolic drugs in the rabbit model of PVR	24-36	20

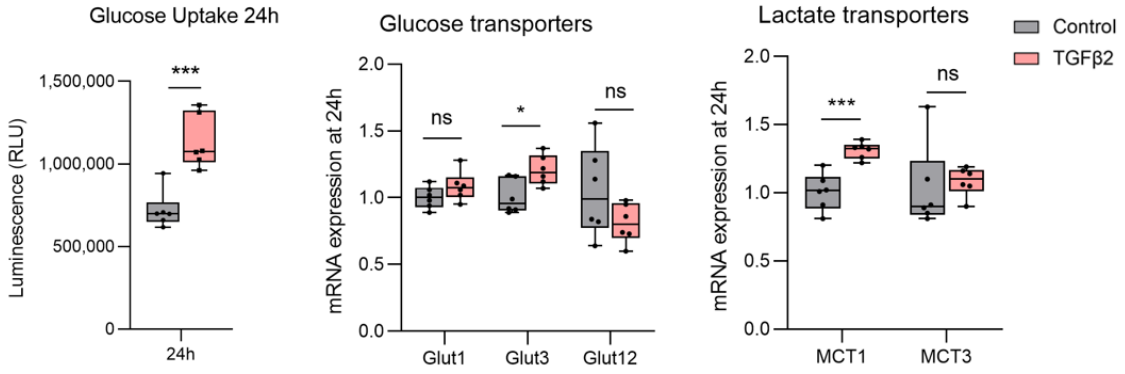
- **What was accomplished under these goals?**

Major Activities

1. Published our characterization of the metabolic changes associated with TGFβ2-induced EMT in RPE and the novel ability of ZLN005 to block this EMT response: Shu DY, Butcher ER, Saint-Geniez M. Suppression of PGC-1α Drives Metabolic Dysfunction in TGFβ2-Induced EMT of Retinal Pigment Epithelial Cells. International Journal of Molecular Sciences. 2021; 22(9):4701.
2. IACUC and ACURO approval for in vivo rabbit PVR model has been obtained and we are now in the process of ordering all the reagents/consumables/animals required for our animal experiments.
3. IRB and HRPO approval for human sample collection for metabolomics study has been obtained and we have now collected a total of 34 human vitreous samples.
4. Due to validation issues with our first round of metabolomics data, we sent through another set of metabolomics samples for processing and have obtained the data and identified various metabolic pathways impacted during TGFβ2-driven EMT of RPE. Since we are now working with human primary RPE cells (H-RPE), we have also sent through another set of metabolomics samples on H-RPE and are awaiting the data to be processed by the metabolomics core facility.
5. We have identified that tumor necrosis factor-alpha (TNFα) also induces EMT in H-RPE cells but induces differing metabolic reprogramming effects compared to TGFβ2. We identified a novel metabolic drug, dimethyl fumarate (DMFu), can block the inflammatory and metabolic changes induced by TNFα in H-RPE and will be testing this drug in our in vivo rabbit PVR model.

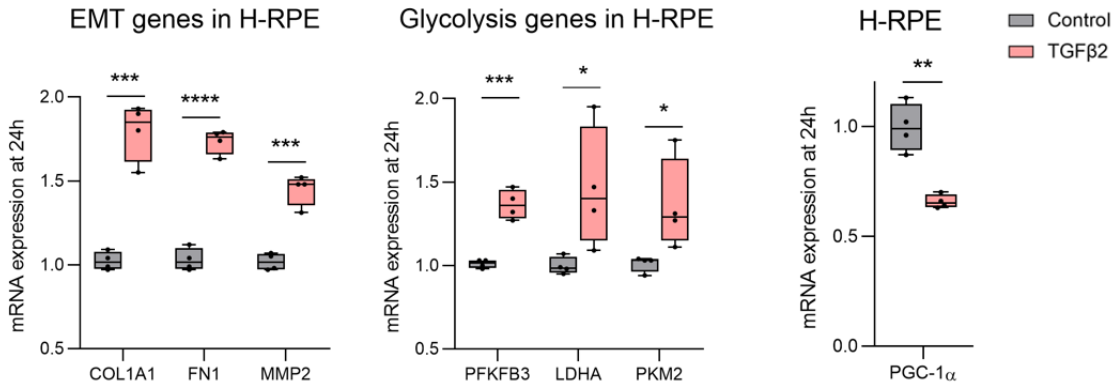
<u>Characterization of metabolic reprogramming in TGFβ-induced EMT in RPE</u>

For our manuscript revisions, we conducted additional metabolic characterization experiments. We found that the increased glycolysis observed with TGFβ2 treatment in ARPE-19 was associated with an increase in glucose uptake using the Glucose uptake Glo Assay (Promega #J1342). We also found an increase in the Glut3 (glucose transporter) and MCT1 (lactate transporter) following stimulation with TGFβ2 for 24 hours in ARPE-19 on qPCR.

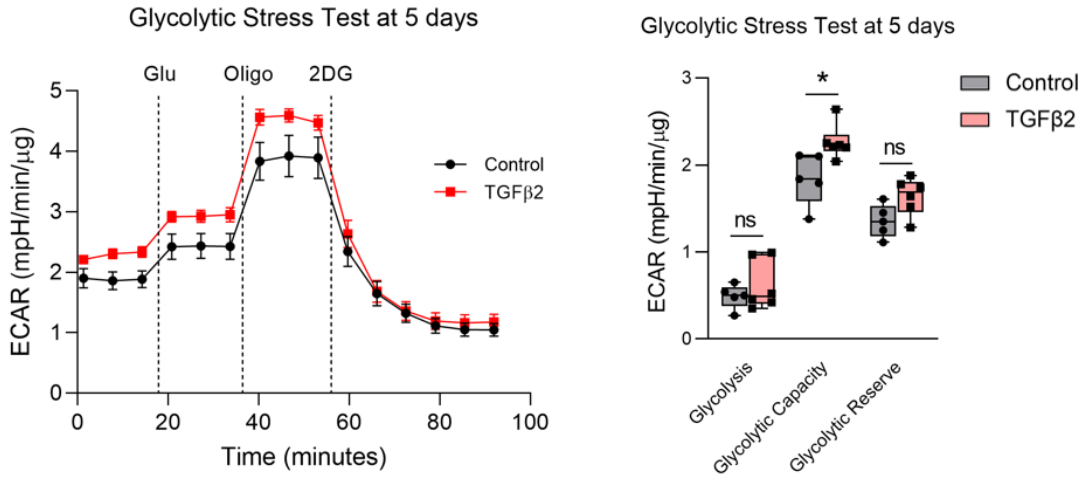


Based on reviewers' comments for our submitted manuscript on Aim 1 in characterizing the metabolic and mitochondrial changes involved in TGFβ-induced EMT in ARPE-19, we were requested to validate our data in primary human RPE cells. Thus, we have been culturing primary human RPE cells purchased from Lonza (H-RPE, Catalog #: 00194987) and have treated the cells with 10ng/ml of TGFβ2.

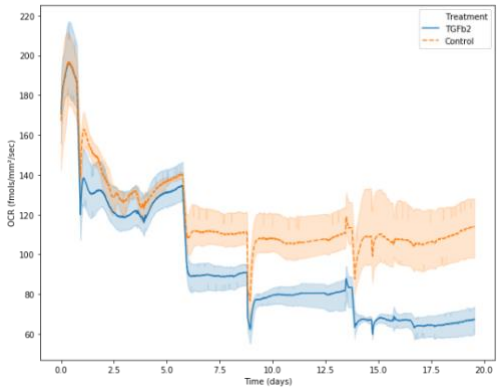
We confirmed that EMT markers (collagen 1A1, fibronectin 1 and matrix metalloproteinase 2) were upregulated in H-RPE after 24h of treatment with 10ng/ml of TGFβ2. We also showed that this EMT response was associated with an increase in glycolytic enzyme gene expression (PFKFB3, LDHA and PKM2) and a suppression of the mitochondrial biogenesis gene PGC-1α which all validate the findings we have from our ARPE-19 model.



H-RPE treated with TGF-β2 for 5 days showed an increase in glycolytic capacity using the Seahorse XFe96 Glycolytic Stress Test.



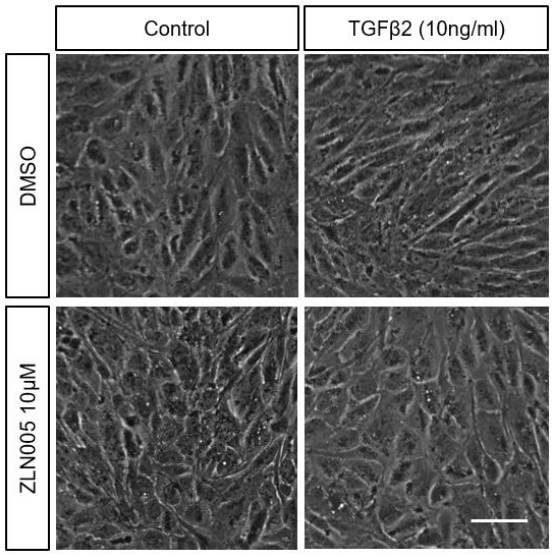
Excitingly, we have recently utilized new metabolic flux technology (called the Resipher by Lucid Scientific) to further track changes in oxygen consumption of our cell cultures in real-time whilst in the incubator. We showed that TGFβ2 is able to reduce oxygen consumption in H-RPE and with each successive dose of TGFβ2, a further reduction in oxygen consumption is observed.



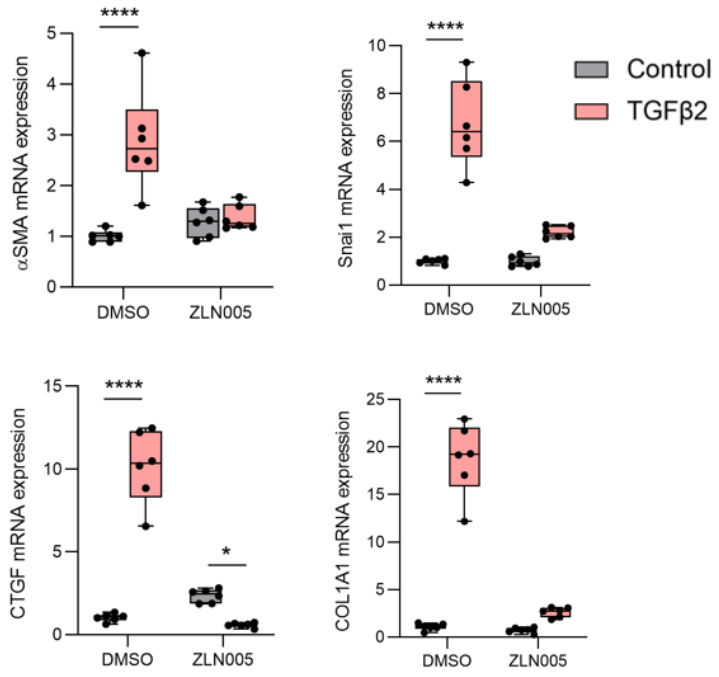
Efficacy of ZLN005 in blocking retinal EMT

ZLN005, a drug known to promote PGC-1α, a master regulator of mitochondrial health and function. We have now shown that ZLN005 is able to block TGFβ2-driven EMT in both ARPE-19 and H-RPE.

Phase-contrast microscopy showed that ZLN005 suppressed the spindle morphology induced by TGFβ2 in ARPE-19 as cells appear to retain a regular polygonal monolayer (scale bar is 50 μm).

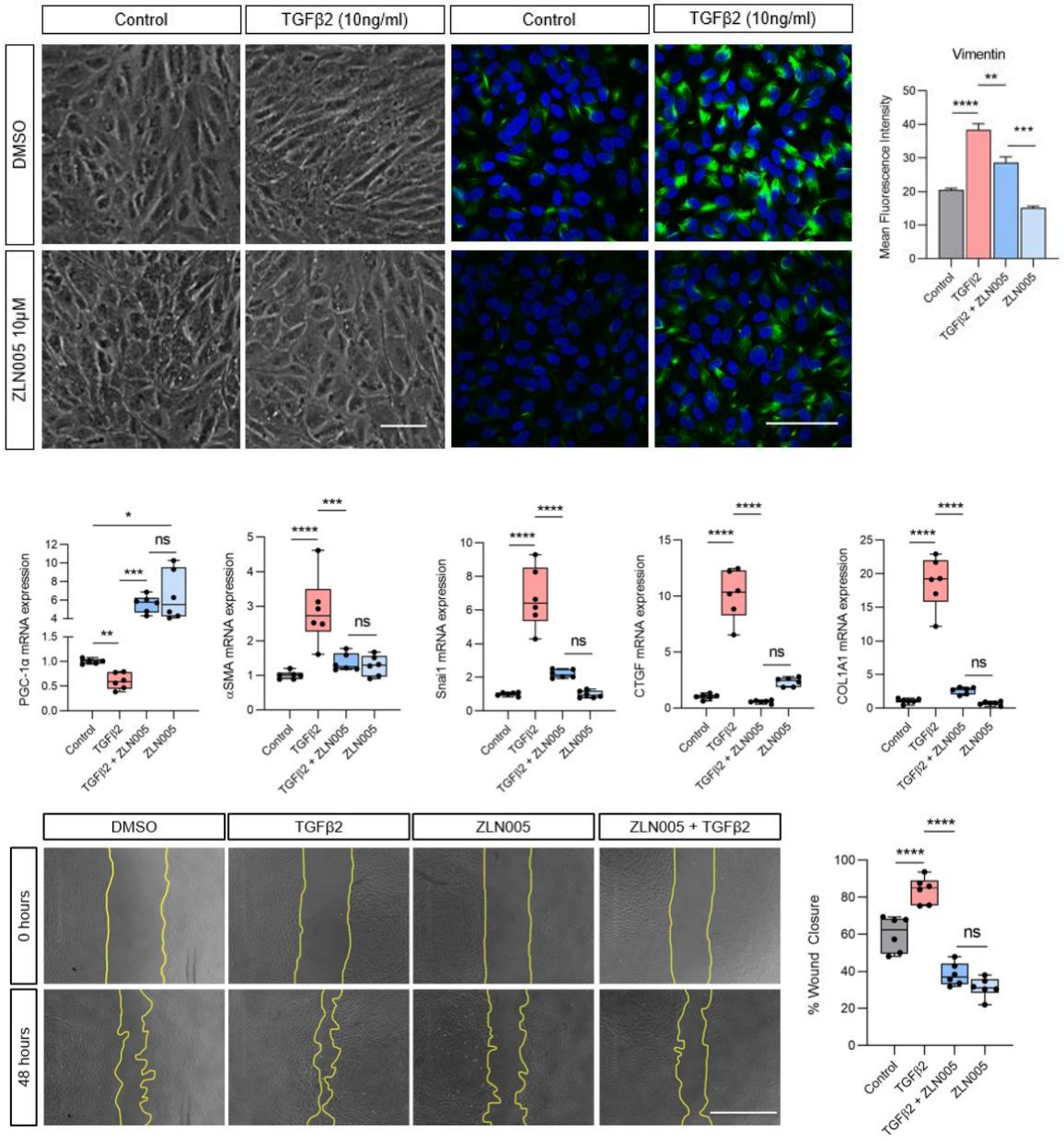


Moreover, ZLN005 robustly blocked TGFβ2-induced upregulation of mesenchymal gene expression, specifically α-SMA, Snai1, CTGF and COL1A1 in ARPE-19.

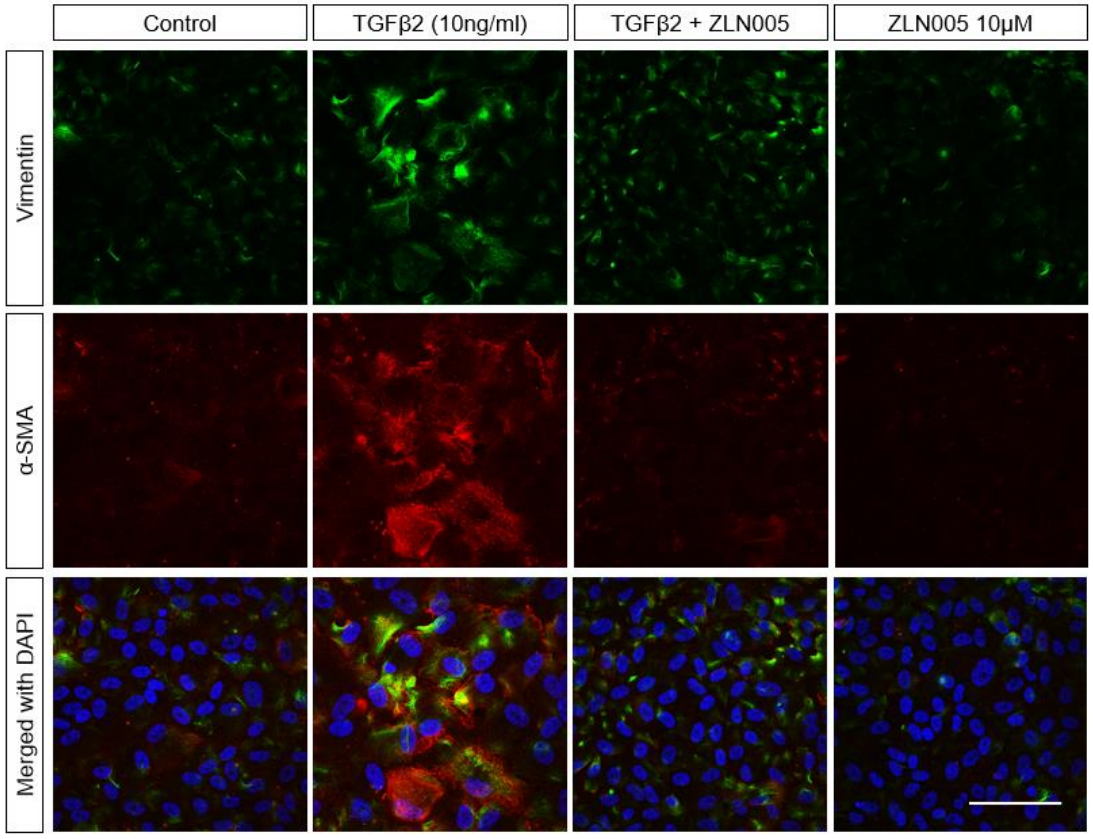


We showed that the ability of the metabolic drug, ZLN005, in effectively blocking EMT in RPE in both ARPE-19 and H-RPE cell cultures. In ARPE-19, ZLN005 blocked TGFβ2-induced morphological elongation, vimentin protein expression and gene expression of EMT markers (α-SMA, Snai1, connective tissue growth factor, collagen 1A1). ZLN005 increased PGC-1α expression and blocked

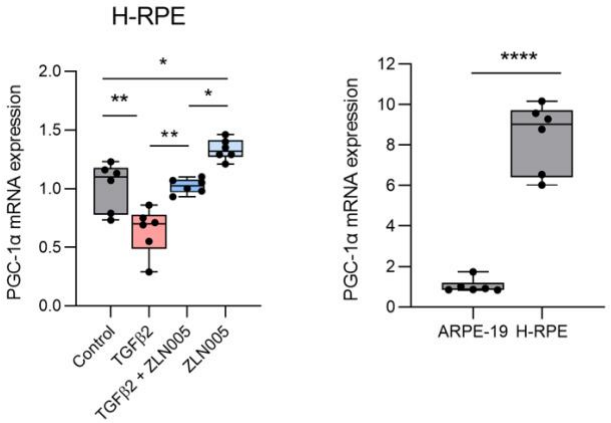
TGFβ2-induced suppression of PGC-1α, maintaining it at basal levels. ZLN005 also blocked TGFβ2-induced cell migration on scratch wound assay.



Similarly in H-RPE, ZLN005 blocked TGFβ2-induced upregulation of both vimentin and alpha-smooth muscle actin on immunofluorescence confocal imaging.

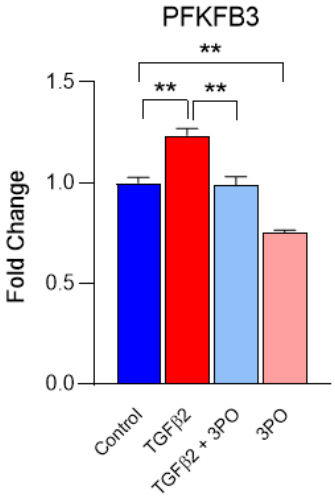


We also confirmed that TGFβ2 suppressed PGC-1α in H-RPE and ZLN005 blocked TGFβ2-induced suppression of PGC-1α gene expression, maintaining it at basal control levels. ZLN005 itself increased PGC-1α gene expression compared to basal control levels in H-RPE. Notably, the levels of PGC-1α were 8-fold higher in H-RPE compared to ARPE-19.

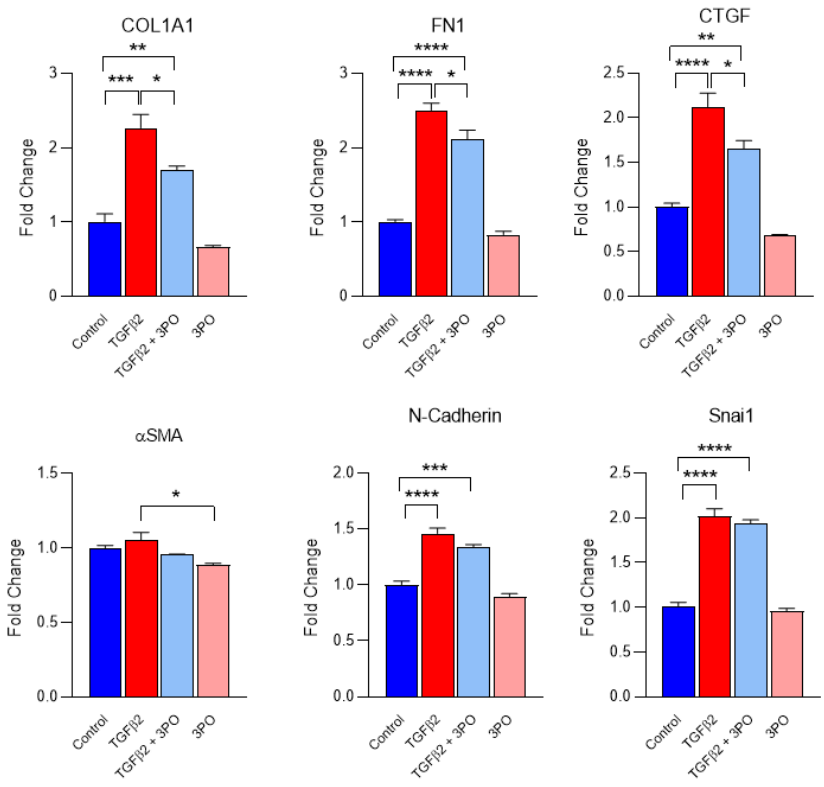


Efficacy of 3PO in blocking retinal EMT

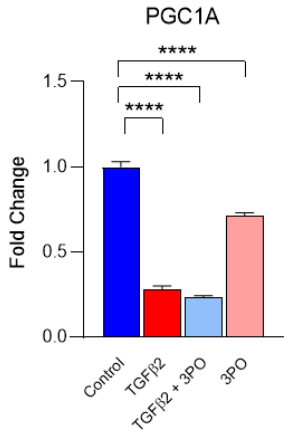
We next performed a qPCR for gene expression of PFKFB3 and found that 3PO did indeed block TGFβ2-induced upregulation of PFKFB3 gene expression in ARPE-19.



3PO was effective in blocking some EMT markers induced by TGFβ2 including COL1A1, FN1, CTGF but not N-Cadherin or Snai1

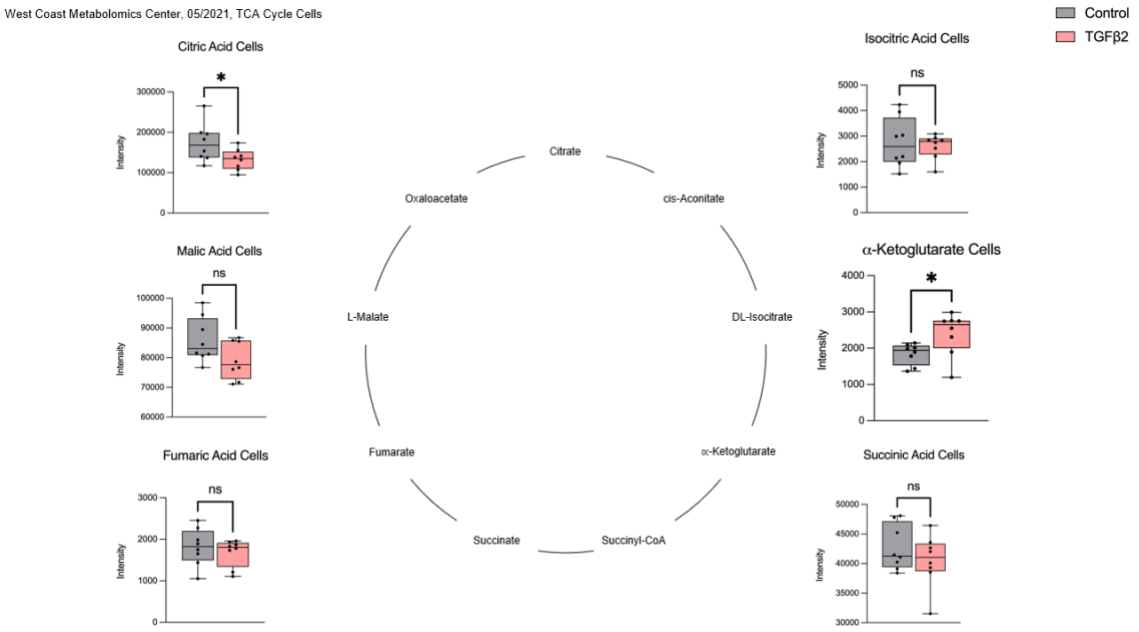


Intriguingly, 3PO is unable to block TGFβ2-induced suppression of PGC-1α levels, indicating that its ability to block aspects of EMT is independent of changes in PGC-1α activity. This opens a potential novel therapeutic avenue for combination therapy with both 3PO and ZLN005 which we will investigate in our future studies.

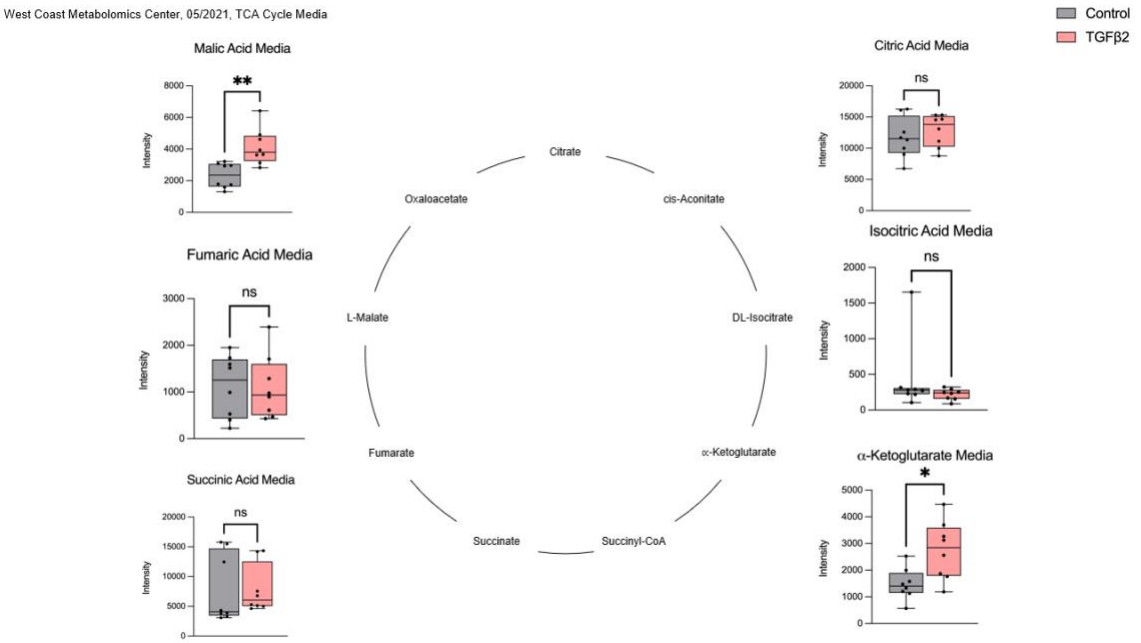


Metabolomics Analysis

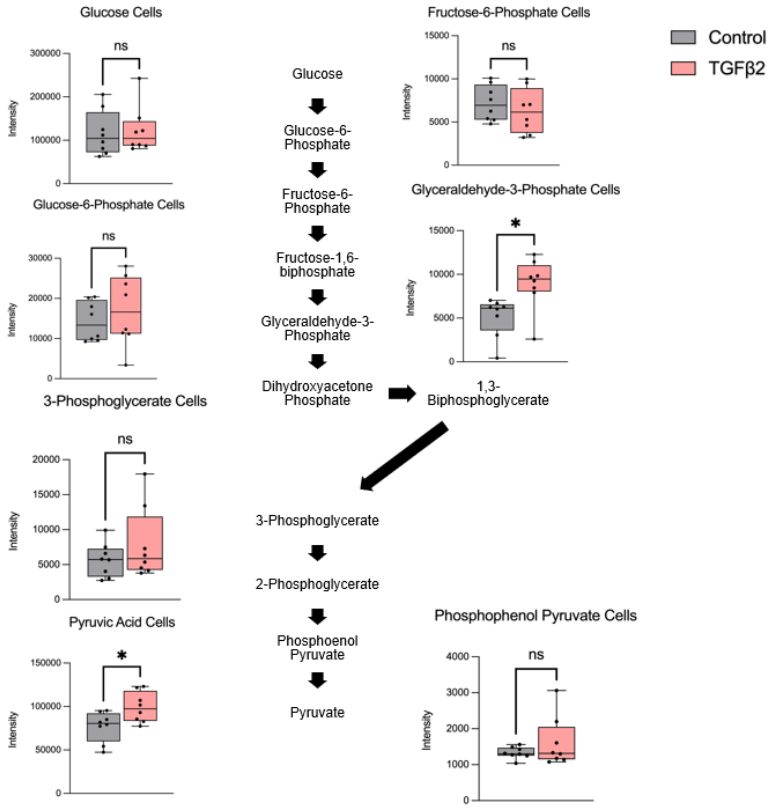
We are currently analyzing the untargeted metabolomics data from West Coast Metabolomics Center. In terms of Krebs cycle metabolites, TGFβ2 significantly reduced citric acid and increases α-ketoglutarate in ARPE-19 cells.



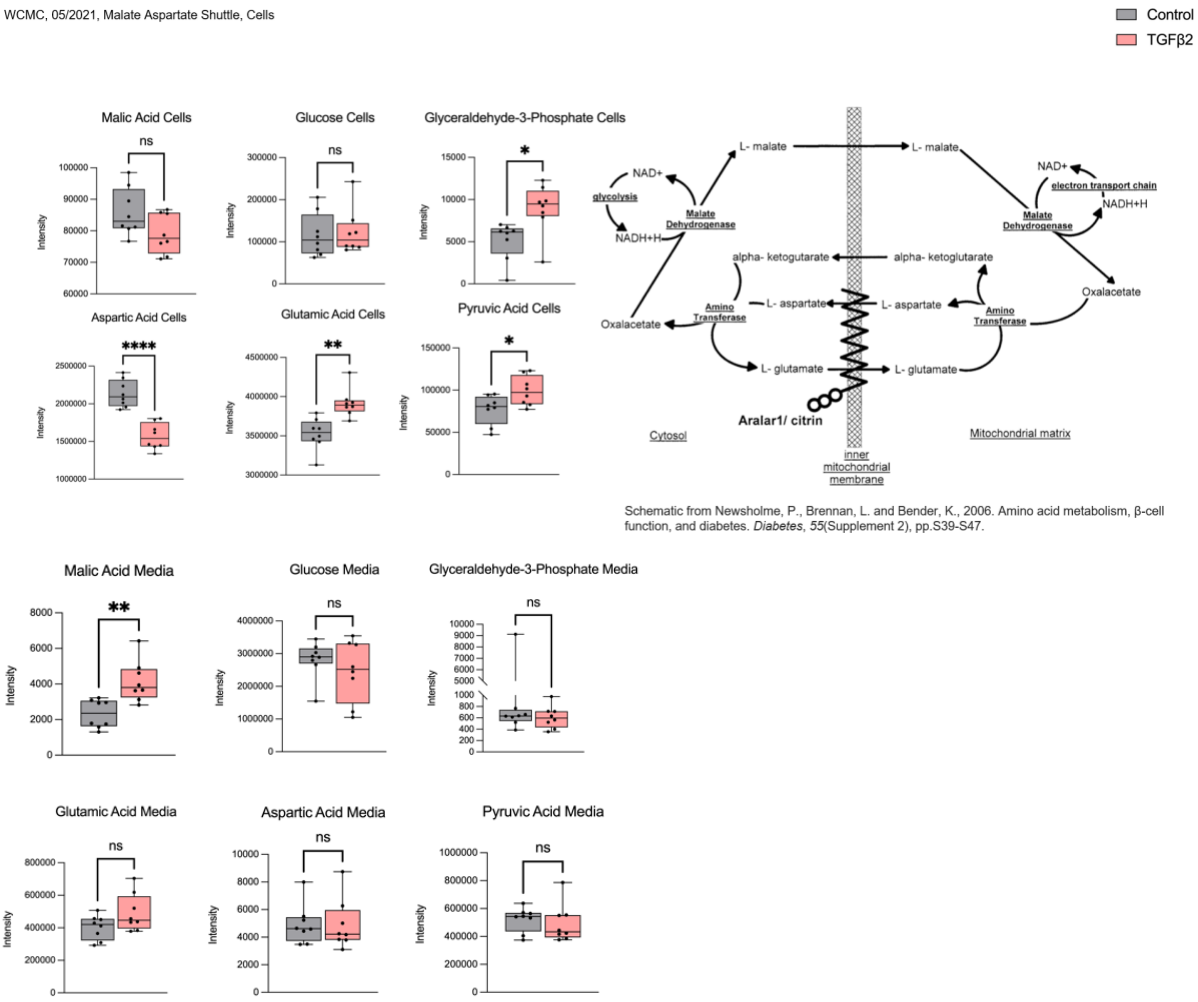
In terms of secreted metabolites, TGFβ2 significantly increased malic acid and α-ketoglutarate in ARPE-19.



TGFβ2 increased cellular metabolites in the glycolytic pathway including glyceraldehyde-3-phosphate and pyruvic acid.



TGFβ2 significantly impacted the levels of cellular metabolites in the malate-aspartate shuttle, specifically, TGFβ2 increased glyceraldehyde-3-phosphate, glutamic acid and pyruvic acid and decreased aspartic acid. Notably, in the secreted media metabolites, malic acid was increased with TGFβ2.

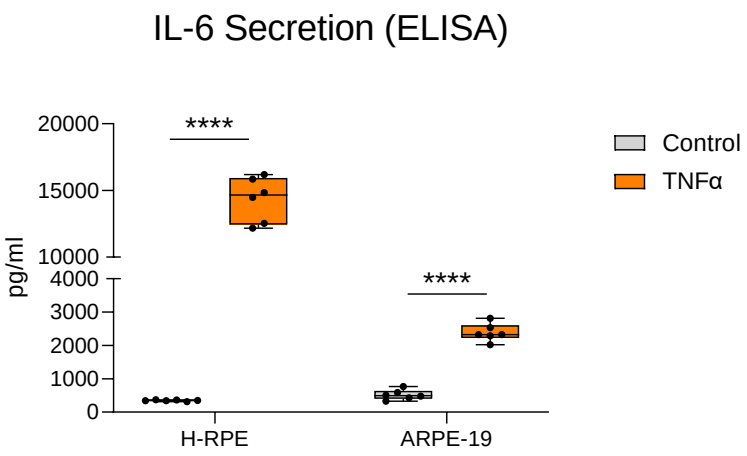


Schematic from Newsholme, P., Brennan, L. and Bender, K., 2006. Amino acid metabolism, β-cell function, and diabetes. *Diabetes*, *55*(Supplement 2), pp.S39-S47.

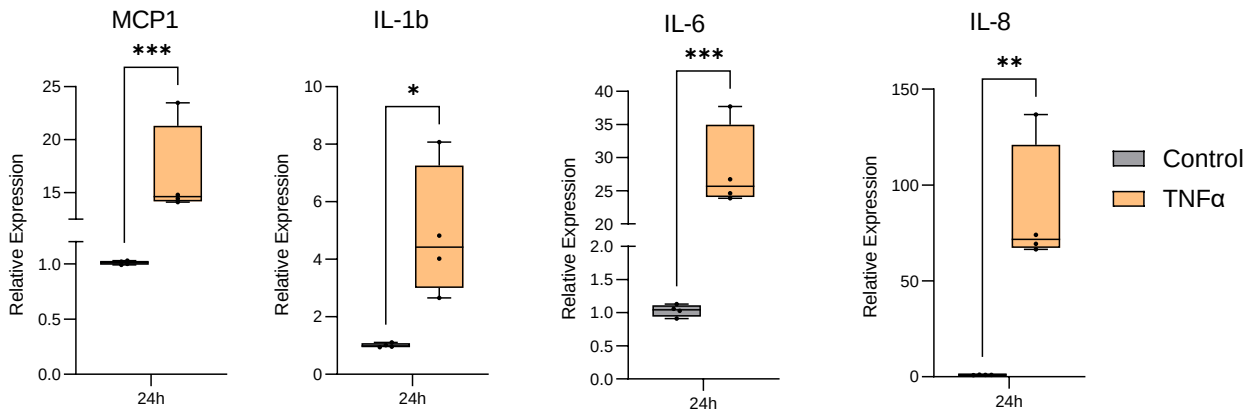
Effect of TNFα in inducing inflammation and EMT in RPE

Since tumor necrosis factor-alpha (TNFα) is also implicated in the pathogenesis of PVR (Dai et al. 2020), we also explored the metabolic changes associate with TNFα. Firstly, we confirmed that TNFα induced an inflammatory response in both ARPE-19 and H-RPE with robust increases in secretion of interleukin-6 (IL-6) into the media, more pronounced for H-RPE cells.

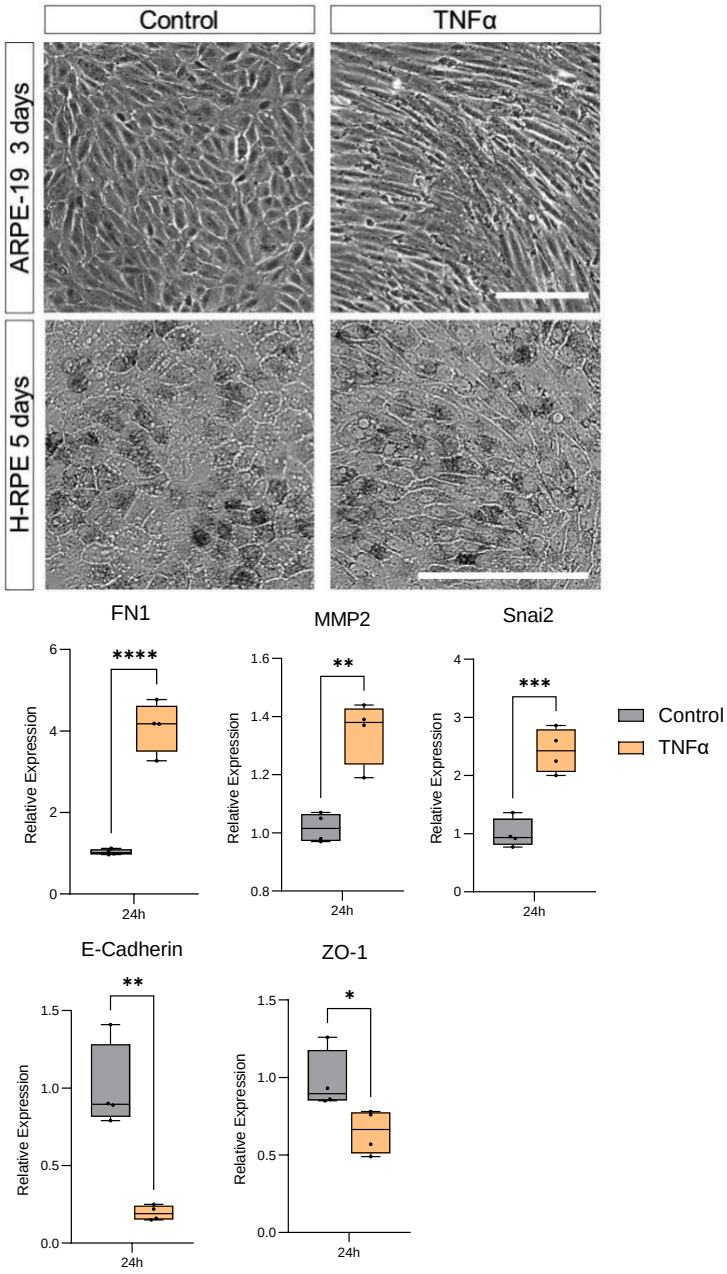
Reference: Dai, Y., Dai, C. and Sun, T., 2020. Inflammatory mediators of proliferative vitreoretinopathy: Hypothesis and review. International ophthalmology, 40(6), pp.1587-1601.



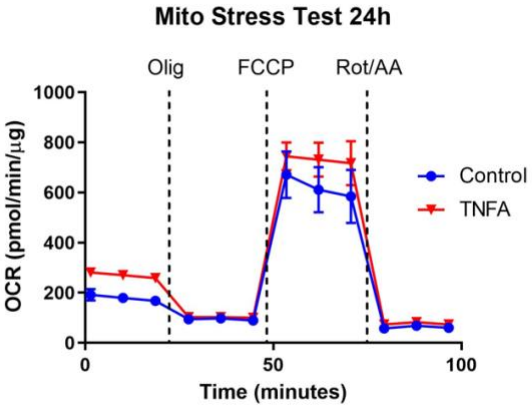
Treatment of H-RPE with TNFα for 24 hours induced an increase in inflammatory genes including IL-6, IL-8, IL-1b and MCP1.

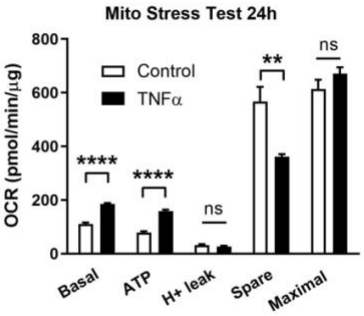


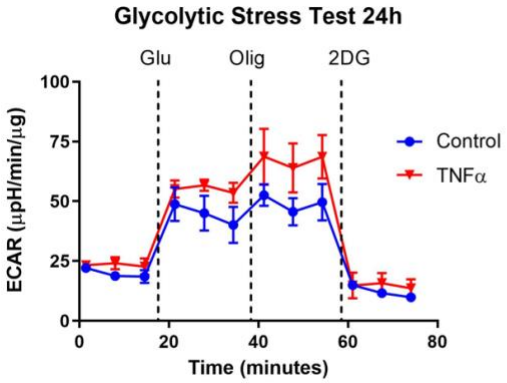
TNFα induced an elongation of both ARPE-19 and H-RPE cells (scale bar is 100μm). This elongation was associated with an acquisition of a mesenchymal phenotype with increased gene expression of mesenchymal markers (fibronectin 1, matrix metalloproteinase 2 and Snai2) and a reduction in epithelial markers (E-cadherin and ZO-1).

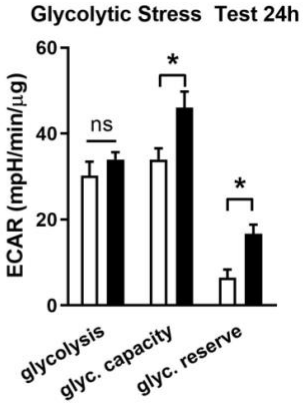


Intriguingly, we found differing effects compared to TGFβ2. TNFα increased both mitochondrial respiration and glycolysis on Seahorse metabolic flux analysis in ARPE-19.

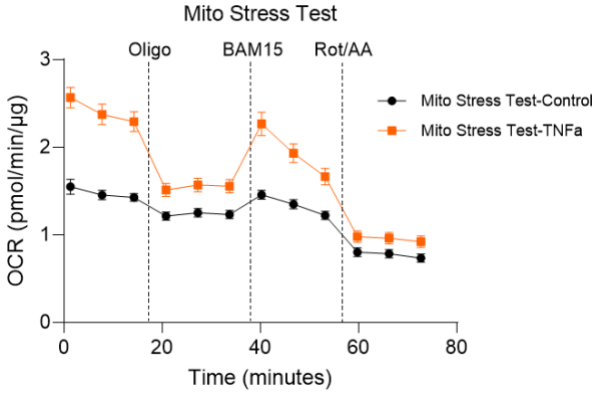


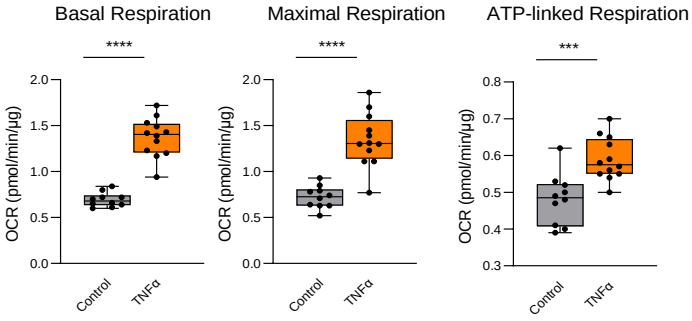




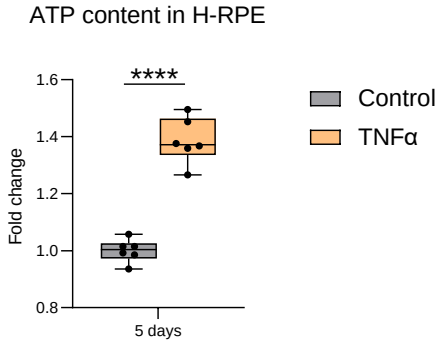


More recently, we have replicated the increased mitochondrial OXPHOS with TNFα treatment in H-RPE with robust increases in basal respiration, maximal respiration, and ATP-linked respiration.

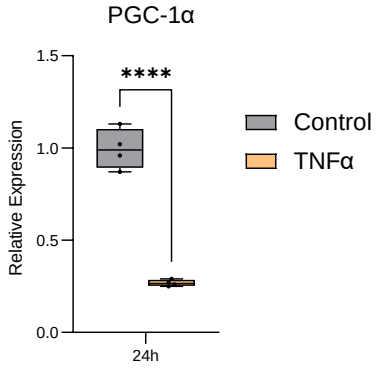


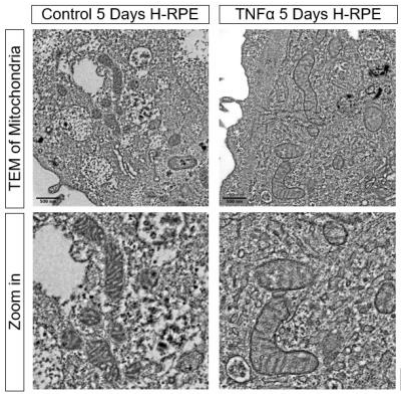


We found that TNFα increased total cellular ATP content in H-RPE.

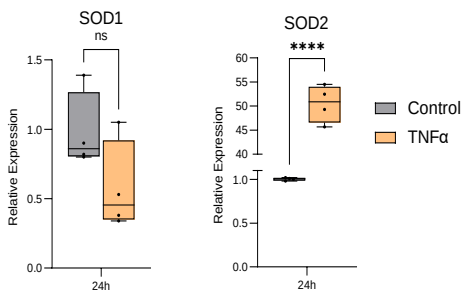


Surprisingly, this enhanced mitochondrial OXPHOS was accompanied by a reduction in PGC-1α levels in H-RPE. Ultrastructural analysis of mitochondrial in H-RPE using transmission electron microscopy (TEM) revealed that mitochondria in cells treated with TNFα resulted in enlarged mitochondria with lack of cristae structure, suggesting that TNFα was inducing mitochondrial dysfunction.



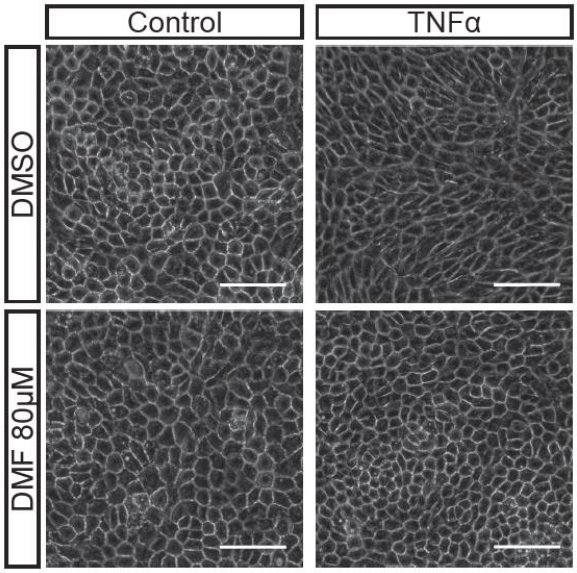


TNFα disrupted normal redox homeostasis in H-RPE with increasing gene expression of the mitochondrial antioxidant SOD2, but also enhanced expression of the professional reactive oxygen species (ROS) generator, Nox4. Overall, the mitochondrial superoxide levels in H-RPE were significantly elevated.

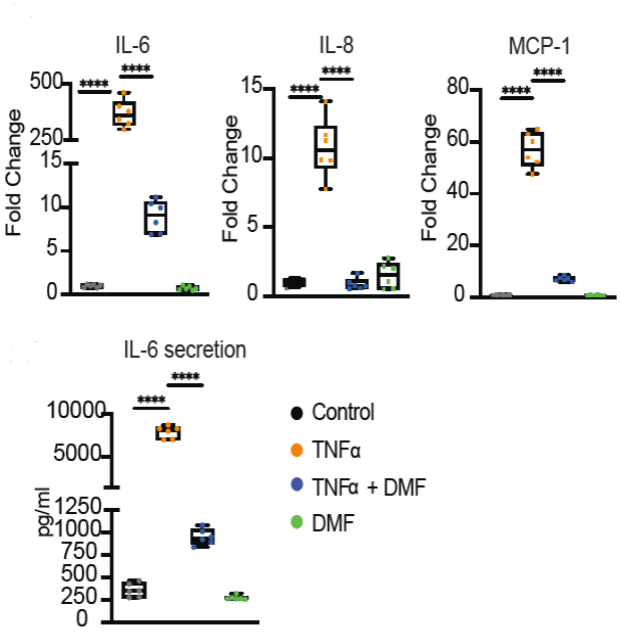


Dimethyl Fumarate (DMFu) blocks TNFα activity

Phase-contrast micrography reveals that DMFu block the morphological elongation induced by TNFa in H-RPE and restored the regular hexagonal pattern (scale bar is 100 μm).



DMFu also blocked the inflammatory effects of TNFα in H-RPE by blocking IL-6 secretion into the media and gene expression of key inflammatory cytokines including IL-6, IL-8 and MCP-1.



Human vitreous samples collection

Currently, we have collected a total of 34 human vitreous samples as listed in the table below. We are still actively seeking PVR samples to perform quantitative metabolomics.

	Control (e.g. ERM)	Retinal Detachment	PVR
1	VPVR_006	VPVR_002	VPVR_001
2	VPVR_008	VPVR_003	VPVR_009
3	VPVR_011	VPVR_004	VPVR_013
4	VPVR_014	VPVR_005	VPVR_033
5	VPVR_021	VPVR_007	
6	VPVR_022	VPVR_010	
7	VPVR_024	VPVR_012	
8	VPVR_025	VPVR_015	
9	VPVR_027	VPVR_016	
10	VPVR_028	VPVR_017	
11	VPVR_030	VPVR_018	
12	VPVR_031	VPVR_019	
13	VPVR_032	VPVR_020	
14		VPVR_023	
15		VPVR_026	
16		VPVR_029	
17		VPVR_034	

- **What opportunities for training and professional development has the project provided?**

Training activities for both Dr. Saint-Geniez and Dr. Shu include the hands-on rabbit handling training and rabbit ocular surgery training at the Schepens Eye Research Institute Animal Facility. Dr. Shu has also participated in the Schepens Eye Research Institute Postdoctoral Training

Fellowship which include workshops such as the Molecular Basis of Eye Diseases Lecture Series, Responsible Conduct of Research (RCR) Lectures and Data Analysis Workshop.

- **How were the results disseminated to communities of interest?**

Publications, posters, and talks are listed below.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Over the next year, we plan to:

1. Finalize analysis of metabolomics data and publish the findings.
2. Finalize the manuscript on the metabolic effects of TNFα and the efficacy of DMFu in blocking TNFα activity.
3. Collect human vitreous samples from normal, PVR and retinal detachment patients for metabolomics analysis.
4. Test metabolic drugs for their efficacy in blocking EMT of RPE and designate the top 3 candidates to be selected for the in vivo rabbit PVR model

4. IMPACT**:**

- **What was the impact on the development of the principal discipline(s) of the project?**

To date, our findings reveal that proliferative vitreoretinopathy (PVR) is associated with dramatic changes to the metabolic pathways and mitochondria of retinal pigment epithelial cells. This is an exciting finding as it presents a potential novel therapeutic window for treatment of PVR by targeting specific metabolic pathways and promoting mitochondrial health.

- **What was the impact on other disciplines?**

Nothing to Report

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS**:**

- **Changes in approach and reasons for change**

Nothing to Report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The lab shutdown due to the COVID-19 pandemic has delayed our progress in performing experiments and collecting human vitreous samples. Our lab is now open and fully operational and thus, we are working diligently to account for these delays.

- **Changes that had a significant impact on expenditures**

Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report

- **Significant changes in use or care of human subjects**

Nothing to Report

- **Significant changes in use or care of vertebrate animals.**

Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

Nothing to Report

6. PRODUCTS**:**

- **Publications, conference papers, and presentations**

1st year

1. Departmental Presentation by postdoctoral fellow Daisy Shu presented on November 4th 2019 titled "TGFβ and the retinal pigment epithelium: integrating metabolic reprogramming with epithelial-mesenchymal transition (EMT)"
2. Conference abstract accepted for a talk at the ARVO annual meeting in Baltimore 2020 titled "Metabolic alterations during TGFβ2-induced EMT in retinal pigment epithelial (RPE) cells". Note that ARVO has been cancelled due to public health concerns regarding COVID-19 pandemic.
3. Review article accepted for publication: Shu DY, Butcher E, Saint-Geniez M. EMT and EndMT: Emerging Roles in Age-Related Macular Degeneration. International Journal of Molecular Sciences. 2020; 21(12), 4271.

2nd year

Peer Reviewed Publications:

1. Shu DY, Butcher ER, Saint-Geniez M. Suppression of PGC-1α Drives Metabolic Dysfunction in TGFβ2-Induced EMT of Retinal Pigment Epithelial Cells. International Journal of Molecular Sciences. 2021; 22(9):4701.

Talks (virtually presented)

1. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Gollapalli D, Saint-Geniez M. Suppression of PGC-1α drives metabolic dysfunction in TGFβ2-induced EMT of retinal pigment epithelial cells. [Abstract]. AOPT XV Meeting on March 6th 2021 in the session on "Novel therapeutic targeting for age-related macular degeneration – overcoming the challenging path to success" moderated by Goldis Malek and Aprana Lakkarju, Virtually presented and Daisy Shu was awarded the BrightFocus Foundation Honorable Mention Paper Award at the XV AOPT meeting on line.
2. Dr. Saint-Geniez presented "Targeting metabolic dysfunction for the treatment of retinal degeneration and fibrosis" at the New England Eye Center Grand Rounds, Boston, MA. May 20th 2021
3. Shu DY, Saint-Geniez M. Suppression of PGC-1α drives metabolic dysfunction in TGFβ2-induced EMT of retinal pigment epithelial cells. [Abstract for Departmental Seminar]. Schepens Eye Research Institute Trainees' Work in Progress Seminar, April 2021, Virtual
4. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Gollapalli D, Saint-Geniez M. Differential effects of TNFα on mitochondrial function and metabolic activity in the retinal pigment epithelium. [Abstract] ARVO Annual Meeting, May 5th 2021, Virtual
5. Shu DY, Saint-Geniez M. Integrating metabolic reprogramming and epithelial-mesenchymal transition (EMT): insights from the retina. [Abstract]. ASIP Young Investigator Keynote Seminar Series, June 16th 2021 12pm EST, Virtual

6. Shu DY, Saint-Geniez M. EMT, mitochondria and metabolic reprogramming: insights from the retinal pigment epithelium. [Abstract]. AOIP Young Investigator Seminar Series, September 10th 2021 3pm EST, Virtual. Followed by sharing career development tips in the AOIP Trainee Roundtable.
7. Shu DY, Butcher ER , Nnuji-John E, Frank S, Shah R, Cai S, Gollapalli D, Saint-Geniez M. Interplay Between TNFα-Induced Inflammation and Metabolic Dysfunction in Retinal Pigment Epithelial Cells [Abstract]. PISA 2021 (Pathobiology for Investigators, Students, and Academicians Young Investigators Virtual Meeting by the American Society for Investigative Pathology) on October 6th 2021 4:45-5pm, Virtual

Posters (virtually presented)

1. Virtual Conference poster presentation at the American Society for Investigative Pathology PISA Meeting 9-13 November 2020 entitled “Metabolic Rewiring and Mitochondrial Dysfunction in Transforming Growth Factor-Beta 2-Induced Retinal Epithelial-Mesenchymal Transition”.
2. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. [Abstract]. Metabolic Rewiring and Mitochondrial Dysfunction in Transforming Growth Factor-Beta 2-Induced Retinal Epithelial-Mesenchymal Transition. MGH Scientific Advisory Committee (SAC) 2021 Virtual Poster Session on April 7th 2021
3. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. [Abstract]. Metabolic Reprogramming of the Retinal Pigment Epithelium Drives TGFβ2-Induced Epithelial-Mesenchymal Transition. Experimental Biology 2021 Virtual Poster Session April 27-30th 2021
4. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. [Abstract]. Metabolic Reprogramming of the Retinal Pigment Epithelium Drives TGFβ2-Induced Epithelial-Mesenchymal Transition. Experimental Biology 2021 Virtual Poster Session April 27-30th 2021
5. Shu D, Butcher E, Cai S, Frank S, Nnuji-John E, Gollapalli D, Saint-Geniez M. [Abstract]. Role of metabolic dysfunction in TNFαinduced inflammation in retinal pigment epithelial cells. XIX International Symposium on Retinal Degeneration, RD2021 Hybrid Format, Attended virtually. Sept. 27 – Oct. 2, 2021 Online and in person at the Sonesta Nashville Airport Hotel, Nashville, TN

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**:**

- **What individuals have worked on the project?**

Name:	*Dr. Magali Saint-Geniez*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0001-9897-138X*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Saint-Geniez led the conceptualization of the experiments, assisted with data curation, data analysis, project administration and helped complete and submit the forms for local IACUC and HRPO submission.*
Funding Support:	*NA*

Name:	*Dr. Daisy Shu*
Project Role:	*Postdoctoral Research Fellow*

Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-5382-6450*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Shu has performed the experiments and data analysis and helped complete the forms for local IACUC and HRPO submission.*
Funding Support:	*Salary is funded by this DoD grant*

Name:	*Dr. Leo Kim*
Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9106-6416*
Nearest person month worked:	*0.00** **Dr. Kim contributed 1% effort to the project for Year 2*
Contribution to Project:	*Dr. Kim coordinates the collection of vitreous samples from patients with retinal detachment epiretinal membranes*
Funding Support:	*NA*

Name:	*Dr. Kip Connor*
Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9106-6416*
Nearest person month worked:	*0.00** **Dr. Connor contributed >1% effort to the project for Year 2*
Contribution to Project:	*Dr. Connor assisted in the design and analysis of the metabolomics study.*
Funding Support:	*NA*

Name:	*Dr. Deviprasad Gollapalli*
Project Role:	*Research technician*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Deviprasad Gollapalli provided technical support specific to this project by maintaining cell cultures, assisting the fellow with in vivo*

	and in vitro experimentation, masked data collection and analysis, collecting and storing vitreous samples, coordinating reagent orders and storage, and performing primary human cells genotyping.
Funding Support:	*NA*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Dr. Kip Connor has left academia in June 2021 and thus is no longer part of our project. This should not impact on our research progress.

- **What other organizations were involved as partners?**

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: N/A

9. APPENDICES:

Conference Abstracts

Experimental Biology Abstract 2021 Virtual Conference

Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. Metabolic Reprogramming of the Retinal Pigment Epithelium Drives TGFβ2-Induced Epithelial-Mesenchymal Transition. Experimental Biology 2021 Virtual Poster Session April 27-30th 2021

Abstract: Transforming growth factor-beta 2 (TGFβ2) is a key orchestrator of retinal wound healing through induction of epithelial-mesenchymal-transition (EMT) in retinal pigment epithelial cells (RPE). Here we describe a previously unrecognized function of TGFβ2 in modulating mitochondrial morphology and metabolic function in human RPE cells. Treating ARPE-19 (human RPE cell line) with TGFβ2 (10 ng/ml) induced defects in mitochondrial network integrity with increased sphericity and fragmentation. Correspondingly, TGFβ2 reduced expression of genes regulating mitochondrial dynamics, reduced citrate synthase activity and intracellular ATP content. High-resolution respirometry showed that TGFβ2 reduced mitochondrial oxidative phosphorylation (OXPHOS) levels consistent with reduced expression of NDUFB5, a key gene of Complex I of the electron transport chain. The reduced mitochondrial respiration was associated with a compensatory increase in gene expression of glycolytic enzymes (PFKFB3, PKM2, LDHA) and glycolytic reserve. TGFβ2 induced a severe suppression of PGC-1α gene expression and treatment with the selective small molecule activator of PGC-1α, ZLN005, blocked TGFβ2-induced upregulation of mesenchymal genes (αSMA, Snai1, CTGF, COL1A1) and TGFβ2-induced migration using the scratch wound assay. Our data show that EMT is accompanied by mitochondrial dysfunction and a profound metabolic shift towards reduced OXPHOS and increased glycolysis that may be driven by PGC-1α suppression. The PGC-1α promoter, ZLN005, effectively blocks EMT in RPE and thus serves as a novel therapeutic avenue for treatment for subretinal fibrosis.

ARVO Abstract 2021 Virtual Meeting

Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Gollapalli D, Saint-Geniez M. Differential effects of TNFα on mitochondrial function and metabolic activity in the retinal pigment epithelium. ARVO Annual Meeting, May 5th 2021, Virtual

Abstract: The retinal pigment epithelium (RPE) acts as a metabolic gatekeeper between photoreceptors and the choroidal vasculature to maintain healthy retinal function. RPE dysfunction is a key feature of age-related macular degeneration (AMD), the leading cause of blindness in developed countries. Tumor necrosis factor-alpha (TNFα), a potent pro-inflammatory cytokine, has been implicated in the pathogenesis of AMD. Growing evidence supports metabolic dysfunction as another key mechanism driving AMD. To date, there is no literature on the metabolic effects of TNFα on RPE and thus, this study sheds light on the impact of TNFα on mitochondrial morphology and metabolic function in RPE.
Methods: Matured ARPE-19 were treated with TNFα (10 ng/ml) for up to 72h. Glycolysis and oxidative phosphorylation (OXPHOS) were examined by high-resolution respirometry on the Seahorse XF24. Gene expression of EMT and metabolic markers were assessed by qPCR. Mitochondrial morphology was assessed by confocal imaging and ImageJ processing of MitoTracker Orange-stained ARPE-19.
Results: TNFα induced ARPE-19 to elongate into spindle-shaped cells, reminiscent of epithelial-mesenchymal transition (EMT). However, qPCR showed that TNFα reduced EMT genes expression (Col1A1: 1.2 vs 0.39, p=0.015; α-SMA: 1.01 vs 0.66 p=0.001) indicating that the elongated cells were not mesenchymal. TNFα increased basal OXPHOS levels (OCR = 2.2 vs 3.7 pmol/min/µg, p=0.042) and increased glycolytic capacity (ECAR = 0.68 vs 0.92 mpH/mol/µg, p=0.03). Paradoxically, TNFα induced a reduced expression of OXPHOS genes (COX4I1: 1.02 vs 0.16, p < 0.0001; COX5B: 1.02 vs 0.24, p<0.0001). TNFα significantly upregulated expression levels of the mitochondrial antioxidant SOD2 (1.14 vs 14.8, p<0.0001) and disrupted mitochondrial network morphology exhibiting increased sphericity and fragmentation.
Conclusions: Taken together, we find that TNFα robustly disrupts mitochondrial function and morphology in RPE, although shifting the bioenergetic profile in a paradoxical manner, i.e. TNFα raised the levels of basal respiration and glycolysis despite the suppression of genes regulating OXPHOS. These findings highlight the potential of targeting metabolic pathways in RPE as a promising therapeutic avenue for AMD. Further research is required to elucidate the mechanisms underlying the intriguing TNFα-driven metabolic changes.

PISA Abstract 2021 Virtual Meeting

Shu DY, Butcher ER , Nnuji-John E, Frank S, Shah R, Cai S, Gollapalli D, Saint-Geniez M. Interplay Between TNFα-Induced Inflammation and Metabolic Dysfunction in Retinal Pigment Epithelial Cells [Abstract]. PISA 2021 (Pathobiology for Investigators, Students, and Academicians Young Investigators Virtual Meeting by the American Society for Investigative Pathology) on October 6th 2021 4:45-5pm, Virtual. Awarded the Postdoctoral Award for Outstanding Research

Abstract. Purpose: Dysfunction of the retinal pigment epithelial cells (RPE) is a key feature of age-related macular degeneration (AMD), the leading cause of blindness in developed countries. Inflammation is a known pathogenic mechanism in AMD through complement system activation, upregulation of inflammatory cytokines and macrophage recruitment. A critical cytokine mediating inflammatory responses is tumor necrosis factor-alpha (TNFα), which has been implicated in the pathogenesis of AMD. Growing evidence supports metabolic dysfunction as another key mechanism driving AMD. To date, little is known about the metabolic effects of TNFα on RPE. Here we bridge the gap between the interplay of inflammation and metabolic dysfunction in RPE showing that the pro-inflammatory effects of TNFα are associated with a dramatic change in mitochondrial morphology, function, and a rewiring of the metabolic pathways.
Methods: Matured ARPE-19 and primary human RPE cells (H-RPE, Lonza) were treated with TNFα (10 ng/ml). Glycolysis and oxidative phosphorylation (OXPHOS) were examined by high-resolution respirometry. Gene expression of EMT and metabolic markers were assessed using qPCR. The pro-inflammatory effect of TNFα was assessed by measuring IL-6 secretion using ELISA. ATP content was measured using a bioluminescent assay. Cells were stained with MitoTracker Orange and imaged using confocal microscopy to visualize changes in mitochondrial morphology. Ultrastructural features of mitochondria were assessed using transmission electron microscopy (TEM).

Results: TNFα significantly upregulated the expression of pro-inflammatory cytokines and IL-6 secretion and induced RPE to elongate into spindle-shaped cells, reminiscent of epithelial-mesenchymal transition (EMT). However, gene expression analysis showed that TNFα repressed numerous EMT genes (Col1A1, α-SMA) indicating that the elongated cells were not mesenchymal in nature. Metabolic profiling by high-resolution respirometry revealed an increase in basal OXPHOS levels and increased glycolytic capacity, associated with increased ATP content. TNFα significantly upregulated expression levels of the mitochondrial antioxidant SOD2 and induced defects in both the mitochondrial network morphology and ultrastructure with loss of cristae integrity.
Conclusions: Taken together, we show that TNFα-induced pro-inflammatory activation of RPE is associated with robust disruption of mitochondrial function, mitochondrial ultrastructure, and bioenergetic rewiring towards higher mitochondrial respiration and ATP production. Understanding the interplay between TNFα-induced inflammation and metabolic dysfunction opens new therapeutic avenues for druggable targets in treating AMD.

Review Article (attached at the end of the document)
Shu DY, Butcher E, Saint-Geniez M. EMT and EndMT: Emerging Roles in Age-Related Macular Degeneration. International Journal of Molecular Sciences. 2020; 21(12), 4271.

Original Research Paper (attached at the end of the document)
Shu DY, Butcher ER, Saint-Geniez M. Suppression of PGC-1α Drives Metabolic Dysfunction in TGFβ2-Induced EMT of Retinal Pigment Epithelial Cells. International Journal of Molecular Sciences. 2021; 22(9):4701.