2018

# Investigating the functions of PGC-1 isoforms in retinal pigment epithelia metabolism and their implications on age-related macular degeneration

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

# INVESTIGATING THE FUNCTIONS OF PGC-1 ISOFORMS IN RETINAL PIGMENT EPITHELIA METABOLISM AND THEIR IMPLICATIONS ON AGE-RELATED MACULAR DEGENERATION

by

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B.S., Boston University, 2016

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2018

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## ACKNOWLEDGMENTS

I would like to express my utmost gratitude and appreciation to Dr. Magali Saint-Geniez for providing me with the opportunity to work in her laboratory and giving me the guidance needed to complete this thesis. I would also like to thank Dr. Mariana Rosales and members of the D'Amore, Kim, Arboleda, Lei, and Ng Lab at the Schepens Eye Institute for assisting me. Finally, I would like to extend my deepest gratitude to Dr. Louis Toth for mentoring me and taking the time out to assist me with my thesis.

**INVESTIGATING THE FUNCTIONS OF PGC-1 ISOFORMS IN RETINAL PIGMENT EPITHELIA METABOLISM AND THEIR IMPLICATIONS ON AGE-RELATED MACULAR DEGENERATION**

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ABSTRACT

**INTRODUCTION:**

Retinal Pigment Epithelia (RPE) degeneration is a key event in the development of age-related macular degeneration (AMD). RPE dysfunction in AMD is thought to occur through the accumulation of reactive oxygen species (ROS) and oxidative damage. The transcriptional co-activators, PGC-1α and PGC-1β, are important regulators of mitochondrial biogenesis and anti-oxidant capacity. Our group has previously shown that the PGC-1α protein promotes RPE oxidative metabolism and that overexpression of the *PGC-1α* gene protects cells from AMD-associated pro-oxidants. On the other hand, *PGC-1β* gene expression has been found to be upregulated in patients with neovascular AMD, and in-vitro overexpression of *PGC-1β* damages cells and induces pro-oxidant conditions.

**OBJECTIVE:**

Given the divergence of PGC-1α and PGC-1β functions in RPE and their clinical relevance in AMD pathogenesis, this project will seek to investigate the impact of the upregulation of PGC-1α and PGC-1β in RPE metabolism. PGC-1α will be upregulated through treatment with compound ZLN005. A new methodology for

PGC-1β expression will be developed to closely modulate in-vitro PGC-1β induction.

**METHODS:**

In-vitro experiments were performed on the ARPE-19 cell line. Cells were treated with 10μM of ZLN005 for 24 hours. Oxidative stress was induced by exposure to $H_2O_2$ and $NaIO_3$ under serum-free conditions. Lactate dehydrogenase (LDH) levels were used to quantify cell death. Quantitative PCR (qPCR) and Western Blot were performed to measure changes in gene and protein expression respectively. Superoxide production by the mitochondria was measured to evaluate ROS levels within the cell. Intravitreal injections of 20μM ZLN005 were performed on eight-week old male C57BL/6J mice. After 24 and 72 hours of treatment, the mice were euthanized and the enucleated eyes were dissected to obtain the RPE and neural retina layers. Total RNA was extracted from these layers and qPCR was performed to measure gene expression. A tetracycline-inducible PGC-1β plasmid was designed and transfected into ARPE-19 cells. The cells were exposed to 0.01-100μg/ml doxycycline for 48-hours and qPCR was used to measure gene expression. Transfected cells were treated with ZLN005 and cell death upon exposure to oxidative stress was quantified.

**RESULTS:**

Gene expression analysis on ARPE-19 cells treated with ZLN005 showed robust upregulation of *PGC-1α, PGC-1β* and their associated transcription factors and enzymes. Induction of PGC-1α at the protein level was also confirmed. ZLN005 efficiently protected ARPE-19 cells from $H_2O_2$ and $NaIO_3$ cytotoxicity and its protection was negated in PGC1α-silenced cells. Treatment with ZLN005 also decreased mitochondrial superoxide production. ZLN005 intravitreal injections were safely administered to the animals and did not cause cataracts or other damage to the ocular tissues. While statistical significance in gene expression changes was limited due to the small sample size, anti-oxidants *GPX1* and *TXN2*, and electron transport chain gene, *ATP50*, were found to be potentially induced in the neuro-retina, while *FOXO3* was found to be downregulated. Evaluation of our novel tetracycline-inducible PGC-1β adenoviral vector showed that upregulation of *PGC-1β* was efficiently controlled by the addition of doxycycline to transfected cells. Upon exposure to $H_2O_2$, transfected cells treated with doxycycline experienced greater cell death than transfected cells not exposed to doxycycline. ZLN005 treatment was able to decrease cell death in both conditions.

**CONCLUSION:**

The present study shows that ZLN005 efficiently protects RPE cells from oxidative damage through selective induction of *PGC-1α*. While still preliminary,

the in-vivo study indicates that ZLN005 is safe to be injected into the eye and may be able to increase the expression of mito-protective and anti-oxidant genes in the neuronal retina. In addition, our design of the tetracycline inducible PGC-1β plasmid allows for tight control over *PGC-1β* expression through doxycycline addition. Upregulation of *PGC-1β* at levels similar to those observed in clinical conditions caused increased pro-oxidant induced cell death and treatment with ZLN005 was able to protect against cell death.

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## LIST OF ABBREVIATIONS

AMD .....Age-Related Macular Degeneration

AmpR .....Ampicillin Resistance

AREDS.....Age-Related Eye Disease Study

ATCC ..... American Type Culture Collection

BCA..... Bicinchoninic Acid Assay

CAT.....Catalase

cDNA.....Complimentary Deoxyribonucleic Acid

CNV ..... Choroidal Neovascularization

DMEM/F12.....Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 media

DMSO ..... Dimethyl Sulfoxide

Dox.....Doxycycline

EGFP .....Enhanced Green Florescent Protein

ESRRA..... Estrogen Related Receptor Alpha

FBS ..... Fetal Bovine Serum

GPX .....Glutathione Peroxidase

HBSS ..... Hank’s Buffered Salt Solution

HRP .....Horseradish Peroxidase

IgG .....Immunoglobulin G

IRES..... Internal Ribosome Entry Sequence

LDH.....Lactate Dehydrogenase

Mt ..... Mitochondrial

NF-kB ..... Nuclear Factor kappa-light-chain-enhancer of activated B cells

NRF ..... Nuclear Respiratory Factor

OD ..... Optical Density

ox-LDL ..... Oxidized Low Density Lipoprotein

PBS-T ..... Phospho-Buffed Saline, 0.1% (vol/vol) Tween-20

PenStrep ..... Penicillin-Streptomycin

PEDF ..... Pigment Epithelium-Derived Factor

PGC-1α .... Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGC-1β ..... Peroxisome proliferator-activated receptor gamma coactivator 1-beta

PIGF ..... Placenta Derived Growth Factor

PMSF ..... Phenylmethanesulfonyl Fluoride

POS ..... Photoreceptor Outer Segments

qPCR ..... Quantitative Polymerase Chain Reaction

RNA ..... Ribonucleic Acid

ROS ..... Reactive Oxygen Species

RPE ..... Retinal Pigment Epithelia

SOD ..... Superoxide Dismutase

TBS-T ..... Tris-Buffed Saline, 0.1% (vol/vol) Tween-20

TK ..... Total Kill

THBS1 ..... Thrombospondin-1

VEGF ..... Vascular Endothelial Growth Factor

veh ..... Vehicle

# INTRODUCTION

## Characterization of Age-Related Macular Degeneration

Age-Related Macular Degeneration (AMD) is a multifactorial degenerative disease of the retina, retinal pigment epithelia (RPE), Bruch's membrane, and choroidal capillaries in the central, posterior region of the eye called the macula (Fig. 1A, 1B). Damage to the macula is largely responsible for the loss of photopic vision and visual acuity in AMD patients (Hageman et al. 1995). Affecting 8.7% of the world (Wong et al. 2014), AMD is the third leading cause of vision loss in the geriatric population (Resnikoff et al. 2004). By 2020, AMD is projected to impact 196 million individuals globally (Wong et al. 2014). Risk factors of AMD include age (Klein, Klein, and Linton 1992; Mitchell et al. 2002; Jonasson et al. 2014;

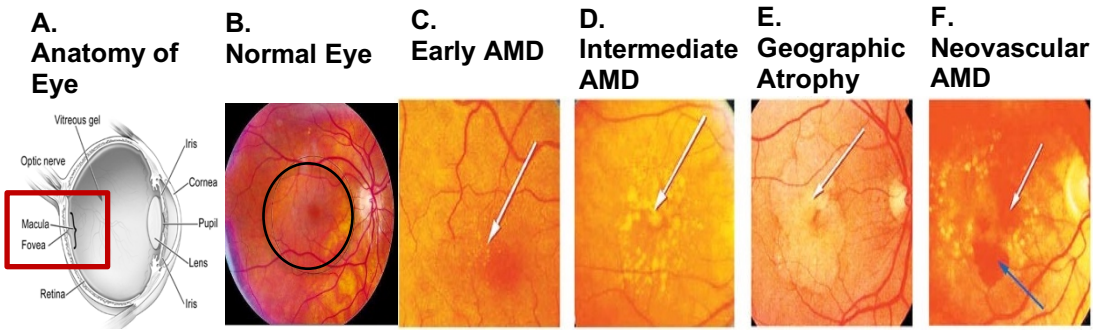


**Figure 1**: **Stages of AMD observed at the fundus of the eye during ophthalmic examination, as proposed by the Age-Related Eye Disease Study (AREDS)** (A) Anatomy of the eye: the red box indicates the macula, which is an area on the posterior retina rich in photoreceptors (National Eye Institute 2018). The center of the macula is called the fovea. (B)-(F) Funduscopic images of (B) Normal Eye: the circle marks the macula of the eye. (C) Early AMD: the arrow points to the presence of medium sized (63-123 μm) drusen. (D) Intermediate AMD: the arrow points to the presence of a large druse (greater than 124 μm) and many medium drusen. (E) Geographic Atrophy (Non-neovascular Advanced AMD): the arrow points to the presence of soft confluent drusen. (F) Neovascular Advanced AMD: the white arrow shows choroidal neovascularization and the blue arrow shows sub-retinal hemorrhage (Jager, Mieler, and Miller 2008)

Yasuda et al. 2009; Evans 2001), smoking (Smith et al. 2001; Klein et al. 2008; Thornton et al. 2005; Christen 1996; Chakravarthy et al. 2007), and genetic variants (Seddon, Ajani, and Mitchell 1997; Weeks et al. 2004; Despriet et al. 2006; Jabbarpoor Bonyadi et al. 2017).

Another important risk factor for AMD is the presence of drusen. Drusen are debris-like extracellular deposits that aggregate with age between the RPE and the Bruch's membrane in the peripheral retina. The accumulation of drusen in the macula is a major risk factor and bio-indicator for AMD. Drusen can be observed as yellow lesions in the funduscopic examination of the eye and is characterized by size, margin and number. This characterization of drusen is an important measure in the staging of AMD as seen in Figure 1. There are many different methods for staging AMD (Bird et al. 1995; Klein et al. 1991; Age-Related Eye Disease Study Research Group 2001), but the method defined by the Age-Related Eye Disease Study Research group (AREDS) is most commonly used. AREDS categorizes AMD into four levels, with Level 1 describing features in a normal eye (Fig. 1B, Fig. 2A). Level 2 or early AMD (Fig. 1C) is characterized by changes in RPE pigmentation and the presence of medium sized drusen. Excessive drusen accumulation and further RPE dysfunction can cause the disease to progress to Level 4 or advanced AMD (Fig. 1E and 1F), which can present as non-neovascular/dry AMD or neovascular/wet AMD. Dry AMD (Fig. 2B) is characterized by the geographic atrophy of photoreceptors and RPE in the macula which leads to a gradual loss of central

vision. Wet AMD (Fig. 2C) is the aberrant growth of choroidal blood vessels in the retina, called choroidal neovascularization (CNV), and leads to sudden and significant vision loss due to sub-retinal edema and hemorrhage.

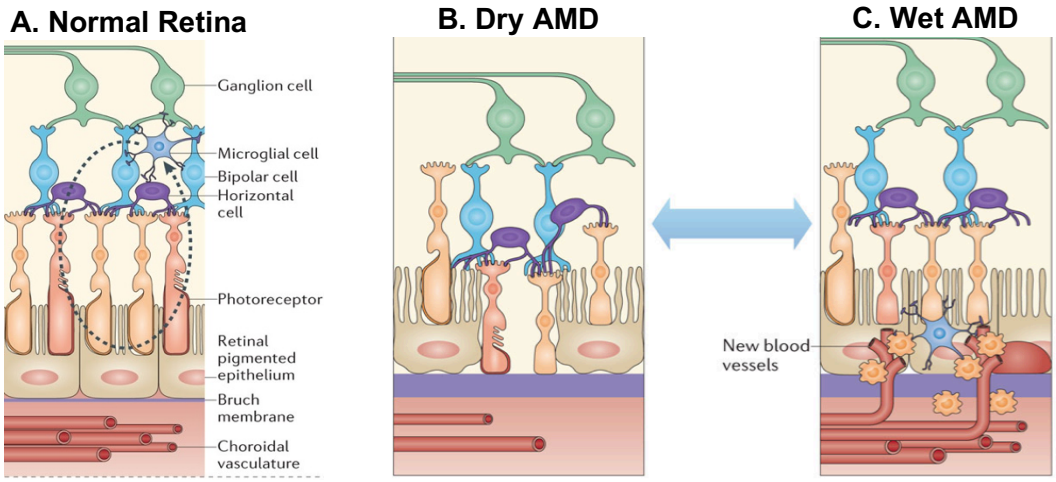


**Figure 2: Main forms of advanced AMD, geographic atrophy (dry AMD) versus neovascularization (wet AMD).** (A) The normal retina consists of ganglion cells, bipolar cells, horizontal cells, and photoreceptors. The RPE, Bruch's membrane and the choroidal blood supply lie below the neural retina. The microglial cell migrates around the retina as denoted by the dashed line. (B) In dry AMD, geographic atrophy of the RPE and overlying photoreceptors occurs. In addition, the Bruch's membrane thickens and the choroidal vasculature constricts. These changes lead to the gradual loss of central vision. (C) Wet AMD is characterized by the growth of new leaky blood vessels through breaks in the Bruch's membrane, also known as choroidal neovascularization (CNV). The leaky vessels cause edema in the sub-retinal space and can hemorrhage in the retina, releasing systemic macrophages into the immune privileged eye and causing sudden and extreme vision loss. The arrow between dry and wet AMD indicates that one form can progress into the other (Ambati, Atkinson, and Gelfand 2013).

Dry AMD, which affects 90% of AMD patients, currently has no established effective treatment. For wet AMD, anti-vascular endothelial growth factor (anti-VEGF) agents are the main therapeutics used to manage CNV (Rein 2009). While effective, sustained use of anti-VEGF agents can lead to adverse local and systemic side effects, such as retinal atrophy and systemic thrombotic complications (Ventrice et al. 2013). Alternatives such as anti-oxidant

supplements (Rein 2009) and photodynamic therapy (for the V. S. Group et al. 1999; Arnold et al. 2001) help decrease the rate of disease progression (Bressler et al. 2003), but they tend to be less efficacious and pose their own unfavorable effects (Olli P. Heinonen and Demetrius Albanes 1994; Eva Lonn, MD 2005), such as causing an increased risk of lung cancer. Therefore, there exists a need for a better understanding of the biochemical pathways involved in the development and progression of AMD in order to innovate new pharmacological therapies to manage, and potentially cure, AMD.

**Retinal Pigment Epithelia dysfunction is integral for AMD development**

The RPE is a polarized monolayer of cells that is integral for the survival of photoreceptors and the function of the eye. As seen in Figure 3, microvilli on the apical surface of the RPE surround the photoreceptor outer segments (POS) in the retina, while the basal surface sits on a specialized membrane (called the

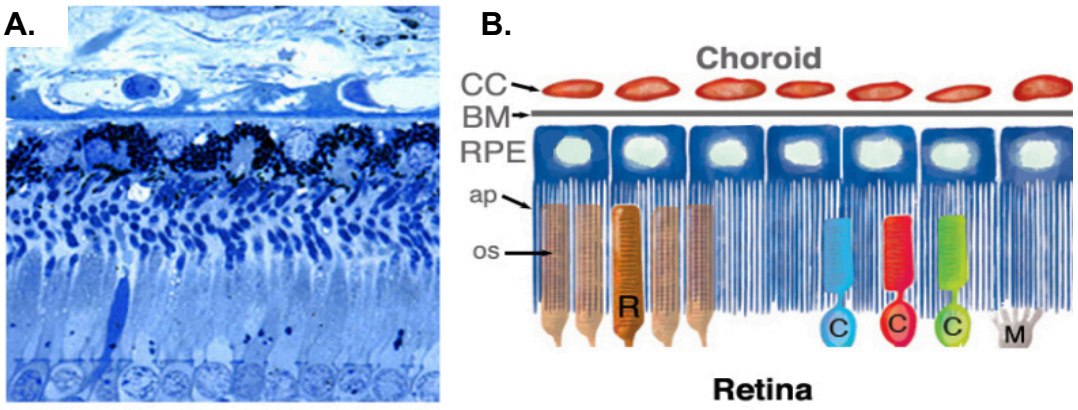


**Figure 3**: **Location of the RPE** (A) Light micrograph of the RPE, choroid (above) and photoreceptors (below) in a human eye (B) Corresponding carton depiction of the structures viewed in the light micrograph CC: choriocapillaries, BM: Bruch's Membrane, RPE: Retinal Pigment Epithelium, ap: apical microvilli processes, os: outer segments of photoreceptors, R: Rods, C: Cones (Strauss 1995)

Bruch's membrane) and is in close contact with the choroidal vasculature, the main blood supply of the photoreceptors. The RPE plays an important role in shuttling ions, metabolites and macromolecules between the blood and retina (Miller and Edelman 1990; Quinn et al. 1992) and maintains the composition of the sub-retinal space (Adijanto et al. 2009; deS Senanayake P et al. 2001). In addition, the tight junctions and complex junctional attachments in the RPE help prevent passive diffusion of substances from the fenestrated choroidal capillaries to the retina (Rizzolo et al. 2011), thereby forming the outer blood-retinal barrier by separating the retina from the rich blood supply in the choroid.

The RPE also directly participates in photo transduction by regenerating the 11-*cis*-retinal chromophore (Fongs et al. 1984; Bridges 1976) responsible for the light sensitivity of the photoreceptors. Apically located melanosomes in the RPE aid in absorbing scattered light thereby preventing reflections within the eye. Since the eye is an immune privileged space, the RPE is responsible for secreting immunosuppressive factors (Taylor, Dixit, and Yu 2015) and carrying out phagocytosis of POS that have undergone photo-oxidative damage (Chiang et al. 2017; Young and Bok 1969). The RPE also secretes growth and neurotrophic factors, such as VEGF and pigment epithelium-derived factor (PEDF), to maintain stability of the surrounding vascular and neuronal tissues (Adamis et al. 1993).

Because of its location between the rich oxygen supply in the choroid and the high energy source in the retina, the RPE is exposed to high levels of

reactive oxygen species (ROS). Further ROS is generated by the electron transport chain (ETC) in respiration and during phagocytosis of POS (Miceli, Liles, and Newsome 1994). To protect themselves from oxidative stress, the RPE employ two separate mechanisms. The first is the use of melanosomes and other light absorbing pigments to uptake excess energy. The second mechanism is the use of anti-oxidant enzymes, such as superoxide dismutase (SOD1, SOD2), catalase (CAT), and glutathione peroxidase (GPX) (Lu et al. 2009; Zou et al. 2012), and small molecules, such as glutathione. Both are controlled by the nuclear respiratory factor 2 (NRF2 or NRE2L2) transcription factor (Plafker, O'Mealey, and Szweda 2012; Iacovelli et al. 2016).

With age, this balance between ROS generation and removal can be disrupted. Auto-fluorescent granules, called lipofuscin, are a byproduct of photoreceptor phagocytosis and begin occupying large volumes in the RPE with age. Irradiation of lipofuscin deposits break down its bisretinoid, lipid, and protein components causing photo-oxidative damage to the RPE (Wassell et al. 1999; Rózanowska et al. 1995; Boulton et al. 1993). The age-related loss of anti-oxidant enzymes decrease the RPE's defense against oxidative stress (Frank, Amin, and Puklin 1999). This loss plays a significant role in AMD pathogenesis as animal models with anti-oxidant enzyme knock-outs have shown development of several AMD features (Justilien et al. 2007; Mao et al. 2014; Zhao et al. 2011). Smoking, a major AMD risk factor, induces further oxidative stress through the addition of ROS and free radicals (Bertram et al. 2009). This rising oxidative

stress causes mitochondrial damage (Karunadharma et al. 2010; H. Lin et al. 2011; Feher et al. 2006) which leads to significant RPE dysfunction, inducing pathological secretion of growth factors promoting CNV or cell death and geographic atrophy.

This increase in oxidative stress also dysregulates lipid metabolism, causing the production of oxidized low-density lipoproteins (ox-LDL). Oxidized LDL can be produced during phagocytosis of POS (Picard et al. 2010; Huang, Curcio, and Johnson 2008; Holz et al. 1994) or obtained exogenously from the blood through the CD36 receptor in RPE (Yin et al. 2012; Picard et al. 2010). Once inside the cell, ox-LDL is sequestered in lysosomes, and causes dysregulation of the phagocytosis function of the RPE and buildup of lipofuscin (Gnanaguru et al. 2016; Hoppe et al. 2004). In addition, ox-LDL has been found to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Ebrahimi et al. 2014; Stewart et al. 2010; Joffre et al. 2007; Hu et al. 2014; Larrayoz et al. 2010; Rodriguez, Alam, and Lee 2004; Dasari et al. 2010), which has been shown to play a role in neovascularization of the retina (Yoshida et al. 1999).

**PGC-1α and PGC-1β play divergent roles in RPE function**

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and beta (PGC-1β) are transcriptional co-activators that regulate mitochondrial function and metabolism in many tissues (J. Lin et al. 2002; Wu et

al. 1999; Puigserver et al. 1998), including the retina (Saint-Geniez et al. 2013; Guo et al. 2014; Egger et al. 2012). To mediate their functions, the PGC-1 isoforms interact with transcription factors, such as estrogen- related receptor alpha (ESRRA)(Mootha et al. 2004) and nuclear respiratory factor 1 and 2 (NRF1 and NRF2)(Wu et al. 1999) to control respiration, mitochondrial biogenesis, and expression of anti-oxidants (Iacovelli et al. 2016).

While PGC-1α and PGC-1β share numerous gene targets and have sequence homology (J. Lin et al. 2002), they can carry out different functions in a tissue-specific manner. For instance, in brown adipose tissue, PGC-1α promotes thermogenesis (Puigserver et al. 1998) and PGC-1β is involved in energy expenditure (Kamei et al. 2003). In the RPE, epithelial maturation is correlated with *PGC-1α* upregulation and *PGC-1β* repression (Iacovelli et al. 2016). Over-expression of *PGC-1α* in RPE was also found to downregulate *PGC-1β* expression. Therefore, there is a need to elucidate the specific functions of both genes in the RPE.

So far, PGC-1α has been found to regulate expression of VEGF, control phagocytic and lysosomal function, enhance respiration and mitochondrial biogenesis, upregulate anti-oxidant genes in the RPE, and protect RPE from oxidative stress (Saint-Geniez et al. 2013; Iacovelli et al. 2016; Roggia and Ueta 2015). Due to its impact on oxidative stress, PGC-1α is being investigated as a therapeutic for numerous degenerative diseases, including AMD. A novel compound, ZLN005, was found to upregulate *PGC-1α* and its associated factors

in skeletal muscle (Zhang et al. 2013). Since ZLN005's effects in RPE have not been evaluated, this project will focus on examining the impact of ZLN005 on RPE function and metabolism in-vitro and evaluating its therapeutic efficiency in a mouse model of acute RPE oxidative damage.

Meanwhile, the function of PGC-1β in RPE metabolism and homeostasis remains unclear. Preliminary data from our group has shown that forced expression of *PGC-1β* increases mitochondrial respiration like *PGC-1α*, but downregulates the expression of anti-oxidant genes, causing increased RPE cell death in basal and pro-oxidative conditions (Charles and Saint-Geniez 2017). In ARPE-19 cells, *PGC-1β* levels are upregulated upon exposure to ox-LDL, a pathological lipoprotein that is present in the RPE and drusen. Clinical data has shown a significant increase in *PGC-1β* (> 2-fold), but not *PGC-1α*, expression in the RPE/choroid layer of patients with advanced neovascular AMD compared to age-matched controls and intermediate AMD subjects (Courtesy of Dr. Margaret DeAngelis, John A. Moran Eye Center, Salt Lake City, UT). Given *PGC-1β* upregulation in neovascular AMD, the effect of *PGC-1β* on angiogenic factors was tested in-vitro. *PGC-1β* upregulation was found to induce expression of Placenta-derived Growth Factor (PlGF), a potent pro-angiogenic factor, while decreasing the expression of anti-angiogenic factors, such as thrombospondin-1 (THBS1) and PEDF. All together, these findings strongly suggest that pathological induction of *PGC-1β* in RPE cells may participate in AMD pathogenesis by inducing RPE oxidative damage and promoting

neovascularization. However, it is important to note that the adenovirus-based *PGC-1β* augmentation method used led to high and uncontrolled overexpression of the gene in RPE, potentially triggering aberrant cellular responses. Therefore, functional analysis with a tightly controlled expression system should be performed to address this issue and confirm the deleterious functions of *PGC-1β* in RPE cells.

**Specific Aims**

Given the divergence of PGC-1α and PGC-1β functions in RPE and their clinical relevance in AMD pathogenesis, this project will seek to investigate the impact of the upregulation of *PGC-1α* and *PGC-1β* in RPE metabolism. The specific aims of the project are:

1. To investigate the protective effects of compound ZLN005, a selective PGC-1α up-regulator, on RPE gene expression and susceptibility to oxidative stress.
2. To develop a new methodology for controlled *PGC-1β* expression in RPE and to analyze the effect of physiological *PGC-1β* induction in RPE oxidative metabolism.

## METHODS

### Cell culture

The human retinal pigment epithelia ARPE-19 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). ARPE-19 cells were expanded in growth medium, consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 media (DMEM/F12, Thermo Fisher Scientific, Wilmington, DE, USA) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Lawrenceville, GA), and 1% penicillin and streptomycin (PenStrep, Lonza, Walkersville, MD, USA), at 37°C and 10% $CO_2$. Once cells reached confluency, they were maintained in differentiation medium consisting of DMEM/F12, 1% FBS and 1% PenStrep. Unless otherwise specified, quiescent ARPE-19 cells were grown to confluency in growth medium and then maintained for 24 hours in differentiation medium. For all experiments involving differentiated ARPE-19, cells were grown to confluency and then maintained in differentiation medium for one week before further treatment.

### RNA collection and quantitative PCR

Cells were maintained in serum free conditions for 24 hours and then treated for 48 hours with 10μM ZLN005 (Cayman Chemicals, Ann Arbor, MI, USA) or a similar dilution of dimethyl sulfoxide (DMSO, D8418-Sigma, St.Louis, MO, USA) vehicle (veh), prepared in serum free DMEM/F12. Total RNA was collected using RNA-Bee (AMSbio, Lake Forest, CA, USA) and re-suspended in

distilled water. The concentration and purity of each sample was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

Reverse transcription was performed with 1µg of RNA to produce the associated complimentary DNA (cDNA) using the IV Vilo Mastermix with EZDNASE (Thermo Fisher Scientific). To measure the changes in gene expression, a quantitative polymerase chain reaction (qPCR) was performed using 3 µl of cDNA template, the Fast Universal SYBR Green Master Mix, and the Lightcycler 480 (Roche Life Sciences, Indianapolis, IN, USA). Data was normalized to the mean expression of housekeeping genes (*CRYAB, PPIH, β-ACTIN,* and *HPRT1* for the human ARPE-19 cell line, *PPIA, B2M, and HPRT1* for the in-vivo mouse retina and RPE/choroid*)* and quantified using the $2^{-\Delta\Delta CT}$ method. The primer sequences used are listed in Table 1 below.

**Table 1: Sequence of primers used for qPCR**

| Gene Code | Gene Name | Primer Sequence | |
|---|---|---|---|
| *qhCRYAB* | Human Crystallin Alpha B | Forward (5'-3') | GTTCTTCGGAGAGCACCTGTT |
| | | Reverse (5'-3') | GAGAGTGCAGTGTCAAACCAG |
| *qhPPIH* | Human Peptidylprolyl Isomerase H | Forward (5'-3') | CCCCAACAATAAGCCCAAG |
| | | Reverse (5'-3') | CACCACCAAGAAGAAGGGAA |
| *qhHPRT1* | Human Hypoxanthine Phosphoribosyltransferase 1 | Forward (5'-3') | CCTGGCGTCGTGATTAGTGAT |
| | | Reverse (5'-3') | AGACGTTCAGTCCTGTCCATAA |
| *qhβactin* | Human Beta-Actin | Forward (5'-3') | CTGTCTGGCGGCACCACCAT |
| | | Reverse (5'-3') | GCAACTAAGTCATAGTCCGC |
| *qhPGC1α* | Human Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha | Forward (5'-3') | GTCACCACCCAAATCCTTAT |
| | | Reverse (5'-3') | ATCTACTGCCTGGAGACCTT |

| | | | |
|---|---|---|---|
| *qhPGC1β* | Human Peroxisome proliferator-activated receptor gamma, coactivator 1 beta | Forward (5'-3') | CCACATCCTACCCAACATCAAG |
| | | Reverse (5'-3') | CACAAGGCCGTTGACTTTTAGA |
| *qhESRRA* | Human Estrogen Related receptor alpha | Forward (5'-3') | TATGGTGTGGCATCCTGTG |
| | | Reverse (5'-3') | GTCTCCGCTTGGTGATCTC |
| *qhNRF2* | Human Nuclear factor, erythroid 2-like 2 | Forward (5'-3') | TCCAGTCAGAAACCAGTGGAT |
| | | Reverse (5'-3') | GAATGTCTGCGCCAAAAGCTG |
| *qhFOXO3* | Human Forkhead box O3 | Forward (5'-3') | CTTCAAGGATAAGGGCGACA |
| | | Reverse (5'-3') | AGTTCCCTCATTCTGGACCC |
| *qhATP50* | Human ATP Synthase Subunit O, Mitochondrial | Forward (5'-3') | TTTGAATCCCTATGTGAAGCGTT |
| | | Reverse (5'-3') | CCTTGGGTATTGCTTAATCGACC |
| *qhSOD2* | Human Superoxide Dismutase 2 | Forward (5'-3') | CAGACCTGCCTTACGACTATGG |
| | | Reverse (5'-3') | CGTTCAGGTTGTTCACGTAGG |
| *qhFIS1* | Human Fission, Mitochondrial 1 | Forward (5'-3') | TGACATCCGTAAAGGCATCG |
| | | Reverse (5'-3') | CTTCTCGTATTCCTTGAGCCG |
| *qhMFN1* | Human Mitofusin 1 | Forward (5'-3') | TGCCCTTCACATGGACAAAG |
| | | Reverse (5'-3') | CTCTGTAGTGACATCTGTGCC |
| *eGFP* | Enhanced Green Fluorescent Protein | Forward (5'-3') | AAGCAGCACGACTTCTTCAAGTC |
| | | Reverse (5'-3') | TCGCCCTCGAACTTCACCTC |
| *qmPPIA* | Mouse Peptidylprolyl Isomerase A | Forward (5'-3') | GAGCTGTTTGCAGACAAAGTTC |
| | | Reverse (5'-3') | CCCTGGCACATGAATCCTGG |
| *qmHPRT1* | Mouse Hypoxanthine Phosphoribosyltransferase 1 | Forward (5'-3') | TCAGTCAACGGGGGACATAAA |
| | | Reverse (5'-3') | GGGGCTGTACTGCTTAACCAG |
| *qmB2M* | Mouse Beta-2-Microglobulin | Forward (5'-3') | TTCTGGTGCTTGTCTCACTGA |
| | | Reverse (5'-3') | CAGTATGTTCGGCTTCCCATTC |
| *qmPGC1α* | Mouse Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha | Forward (5'-3') | AGCCGTGACCACTGACAACGAG |
| | | Reverse (5'-3') | GCTGCATGGTTCTGAGTGCTAAG |
| *qmPGC1β* | Mouse Peroxisome proliferator-activated receptor | Forward (5'-3') | CCCAGCGTCTGACGTGGACGAGC |

| | | | |
|---|---|---|---|
| | gamma, coactivator 1 beta | Reverse (5'-3') | CCTTCAGAGCGTCAGAGCTTGCTG |
| *qmESRRA* | Mouse Estrogen Related receptor alpha | Forward (5'-3') | GGAGGACGGCAGAAGTACAAA |
| | | Reverse (5'-3') | GCGACACCAGAGCGTTCAC |
| *qmFOXO3* | Mouse Forkhead box O3 | Forward (5'-3') | AGTGGATGGTGCGCTGTGT |
| | | Reverse (5'-3') | CTGTGCAGGGACAGGTTGT |
| *qmNRF1* | Mouse Nuclear Respiratory Factor 1 | Forward (5'-3') | GACAAGATCATCAACCTGCCTGTAG |
| | | Reverse (5'-3') | GCTCACTTCCTCCGGTCCTTTG |
| *qmGPX1* | Mouse Gluthathione Peroxidase 1 | Forward (5'-3') | CACCGAGATGAACGATCTG |
| | | Reverse (5'-3') | CAGGTCGGACGTACTTGAG |
| *qmTXN2* | Mouse Thioredoxin 2 | Forward (5'-3') | CACACAGACCTTGCCATTGA |
| | | Reverse (5'-3') | ATCCCCACAAACTTGTCCAC |
| *qmATP50* | Mouse ATP Synthase Subunit O, Mitochondrial | Forward (5'-3') | AGGCCCTTTGCCAAGCTT |
| | | Reverse (5'-3') | TTCTCCTTAGATGCAGCAGAGTACA |

**Protein Collection and Western Blot Analysis**

Prior to 48-hour treatment with 10μM ZLN005 or vehicle, the cells were kept in serum free conditions for 24 hours. The vehicle and 10μM ZLN005 were prepared in serum free DMEM/F12. Protein lysates were collected on ice using 1× Cell Lysis Buffer (Cell Signaling, Danvers, MA, USA) containing 2 mM phenylmethanesulfonyl fluoride (PMSF), sonicated and then quantified using the bicinchoninic acid assay (BCA) method (Thermo Scientific Pierce, Rockford, IL, USA). Electrophoresis was carried out on 10% Mini-PROTEAN® TGX gels (Bio-Rad Laboratories, Hercules, CA) using 80μg of protein and transferred to Immobilon-FL polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Dilutions of primary antibodies were made in 5% non-fat dry milk prepared in Phospho-Buffed Saline, 0.1% (vol/vol) Tween-20 (PBS-T) or Tris-

Buffed Saline, 0.1% (vol/vol) Tween-20 (TBS-T). The concentrations of primary antibodies used were: mouse anti-human PGC-1α –1:250 in 5% milk PBS-T, mouse anti-human α-tubulin – 1:1000 in 5% milk TBS-T. Following overnight incubation with primary antibodies at 4°C, the membranes were washed three times in PBS-T or TBS-T and then incubated with the secondary antibody at room temperature for 1 hour. The concentration of secondary antibody used was: Goat anti-mouse IgG20-HRP – 1:1000. Images were acquired using the ECL machine and the signal density of each band was measured using ImageJ. In each lane, the signal density of the PGC-1α band was normalized to the associated α-tubulin band signal density. Then, the ZLN005 α-tubulin normalized PGC-1α signal densities were normalized to that of the vehicle.

**Quantification of mitochondria superoxide production**

Prior to 24-hour treatment with 10μM ZLN005 or vehicle, the cells were serum starved for 24 hours. The vehicle and 10μM ZLN005 were prepared in serum free DMEM/F12. After treatment, cells were washed with Hanks Buffered Salt Solution (HBSS, Thermo Fisher) and removed from the adherent surface using Trypsin-EDTA. Cells were re-suspended at a concentration of 300,000 cells in 1.8 ml of 5μM MitoSox Red mitochondrial superoxide indicator (Thermo Fisher) in HBSS and incubated at 37°C, 5% $CO_2$ for 30 minutes. The suspended cells are plated in a 96-well plate and the associated fluorescence was measured as a function of superoxide levels produced by the mitochondria.

**Cell Death Assay**

Quiescent ARPE-19 cells were grown to confluency in growth medium and then maintained for 24 hours in serum free DMEM/F12. Differentiated cells were kept in serum free conditions after one week in differentiation medium. Cells were pre-treated with 10μM ZLN005 prepared in serum free, phenol free DMEM for 24 hours before treatment with 500-1000μM hydrogen peroxide ($H_2O_2$) or 2 - 3 mg/ml sodium iodate ($NaIO_3$). After 18 and 24 hours of pro-oxidant exposure respectively, the supernatant media was collected and centrifuged to remove cells and debris. Cell death was quantified by the release of lactate dehydrogenase (LDH) from the cytoplasm of damaged cells into the media using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corp, Madison, WI, USA) which quantifies LDH levels as an optical density (OD) at 490nm. Background levels of LDH present in the medium alone were measured, averaged and subtracted from all other samples. Basal levels of LDH (0% cell death), measured in the supernatant of vehicle only treated cells, and maximal levels of LDH (100% cell death, total kill), measured by inducing complete cell death with 1x Lysis Buffer, were averaged and used to calculate percentage cell death using the following equation.

$$\%\ cell\ death = \frac{OD_{490_{sample}} - OD_{490_{basal\ LDH}}}{OD_{490_{maximal\ LDH}}}$$

**Animal Study**

All in-vivo experimental procedures were approved by the Schepens Eye Research institutional animal care and use committee. Eight-week old male C57BL/6J mice (Jackson Laboratory, Bar Harbour, ME) were housed in standard laboratory environment and maintained on a 12-hour light dark cycle at 21°C. Intravitreal injections of 20μM ZLN005/vehicle were performed on anesthetized animals in a 1μL volume using a 33-gauge needle. After 24 and 72 hours, mice were euthanized and the eyes were enucleated. RNA was extracted from the RPE/choroid and neural retina, and gene expression was analyzed through qPCR.

**Design of PGC-1β Tetracycline Inducible (Tet-On) Adenovirus**

To control the upregulation of *PGC-1β*, an all-in-one tetracycline inducible PGC-1β adenoviral vector was designed (Fig. 4). The plasmid contains the Tet-On-3G gene that is transcribed and translated into a protein trans-activator. This trans-activator undergoes a conformational change in the presence of doxycycline (Dox) and activates the transcription of the TRE3G promoter. This allows the transcription of the human *PGC-1β* gene, fused to the tag protein Myc for protein detection, and the enhanced green florescent protein (*EGFP*) gene to be tightly modulated by doxycycline concentration. An internal ribosome entry sequence (IRES) is inserted between the PGC-1β-Myc and EGFP sequences to allow for independent translation of the two proteins. Before adenoviral production, the tetracycline inducible PGC-1β plasmid was tested for sensitivity

to doxycycline. The plasmid and adenovirus were produced by Vector Builder (Vector Builder, Santa Clara, CA) using synthetic gene synthesis.

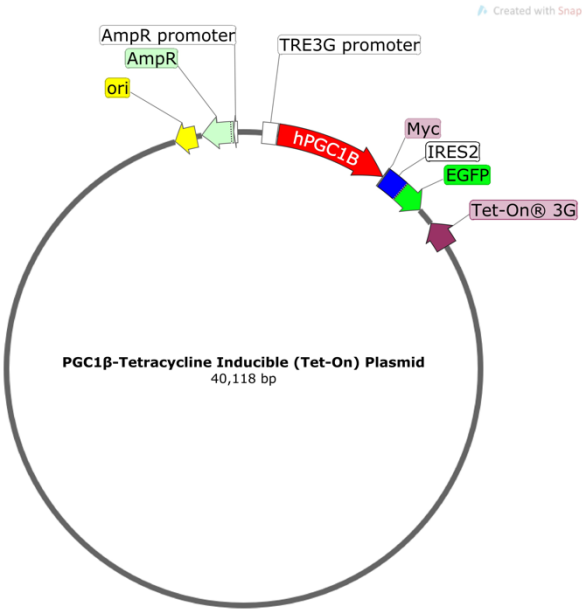


**Figure 4: PGC-1β Tetracycline Inducible (Tet-On) Plasmid**. The plasmid has the Tet-On 3G transactivator gene that controls the transcription of PGC-1β, Myc and EGFP under the TRE3G promoter. Ampicillin Resistance (AmpR) is added to help select bacteria cells containing the plasmid. Ori is the origin of replication and the grey backbone represents the adenovirus genome

### Transfection of ARPE-19 cells with PGC-1β Tet-On plasmid

The PGC-1β Tet-On plasmid was created by Vector Builder. The plasmid was collected using the Qiagen Maxi-prep protocol (Qiagen, Valencia, CA, USA) and resuspended in Tris-EDTA buffer. The concentration and purity of the DNA was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

ARPE-19 cells were seeded at 25,000 cells/$cm^2$ in a 24-well plate. At 70% confluency, ARPE-19 cells were transfected with 2µg of plasmid DNA using 4 µl of Optifect transfection reagent (Thermo Fisher Scientific) in OptiMEM media, containing reduced serum and no antibiotics (Thermo Fisher Scientific), for 24 hours. Doxycycline (Sigma) concentrations of 0.01µg/ml, 1µg/ml and 100µg/ml were prepared in serum free DMEM/F12 and added to the cells for 48 hours before RNA extraction. For LDH quantification, cells were seeded at 15,000 cells/$cm^2$ in a 48-well plate. Transfection was carried out as described above and cells were exposed to 0.01µg/ml Dox, prepared in serum free DMEM/F12, for 48-hours before 24-hour treatment with 10µM ZLN005/veh in phenol free DMEM. Then, cells were exposed to 1000µM $H_2O_2$ for 18 hours, after which LDH levels were quantified.

**Statistical Analysis**

Statistical analysis was carried out by GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Data is presented in graphs as mean ± SEM. The Student's t-test or the two-way ANOVA followed by Tukey's multiple comparison test was used to calculate statistical significance. Statistical significance is denoted by the following: ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

## RESULTS

### ZLN005 upregulates PGC-1α and its associated targets in ARPE-19 cells

ZLN005 upregulates *PGC-1α* in skeletal muscle myotubes, but not in hepatocytes (Zhang et al. 2013). Because ZLN005's action appears cell-specific, the effect of ZLN005 on ARPE-19 cells was investigated in both quiescent and differentiated cells. Endogenous PGC-1 expression in RPE is indeed tightly regulated by the cell epithelialization status and our lab has previously shown that *PGC-1α* is induced in ARPE-19 cells only after differentiation for one week (Iacovelli et al. 2016).

First, qPCR analysis was used to investigate whether ZLN005 was able to upregulate PGC-1 isoforms and their main targets in the quiescent ARPE-19 monolayer, which has low basal expression of *PGC-1α*. Total RNA was collected from cells that were treated for 48 hours with 10μM ZLN005 or vehicle. Treatment with ZLN005 increased the expression of *PGC-1α* ($p < 0.0001$), *PGC-1β* ($p < 0.0001$), *SOD2* ($p = 0.0001$), and *ATP50* ($p < 0.0001$) compared to the vehicle (Fig. 5A, 5B). These results confirmed that ZLN005 increases gene expression of PGC-1 isoforms in RPE and potentially promotes RPE anti-oxidant activity through the upregulation of *SOD2*, the mitochondrial superoxide dismutase. ZLN005 may also affect oxidative phosphorylation through the upregulation of *ATP50*, a component of the mitochondrial ATPase involved in the ETC. ZLN005's impact on mitochondrial function was examined by measuring the amount of mitochondrial superoxide produced during respiration. A 24-hour

treatment with ZLN005 decreases mitochondrial superoxide production, a major source of intracellular ROS (Fig. 5C).

Second, to examine if ZLN005 could upregulate *PGC-1α* in cells already expressing high basal levels, differentiated cells were treated with ZLN005. An increase in *PGC-1α* ($p < 0.0001$), *PGC-1β* ($p < 0.0001$), *FOX03* ($p = 0.0004$), *ESRRA* ($p = 0.04$) and *NRF2* ($p = 0.01$) expression was observed above the vehicle control (Fig. 5D). Mitochondrial respiration gene, *ATP50* ($p = 0.0007$), and mitochondrial dynamic gene, *FIS1* ($p < 0.0001$) were also upregulated with ZLN005 treatment (Fig. 5E). While *ATP50* was also induced in the ARPE-19 monolayer, the impact on mitochondrial dynamic gene, *FIS1* was not observed, potentially due to low basal mitochondrial dynamic gene expression in the quiescent monolayer (Iacovelli et al. 2016). Although ZLN005 was able to increase the expression of key anti-oxidant transcription factors, a significant increase in *SOD2* was not observed. However, the decrease in mitochondrial superoxide production was maintained in differentiated cells treated for 24 hours with ZLN005. (Fig. 5F)

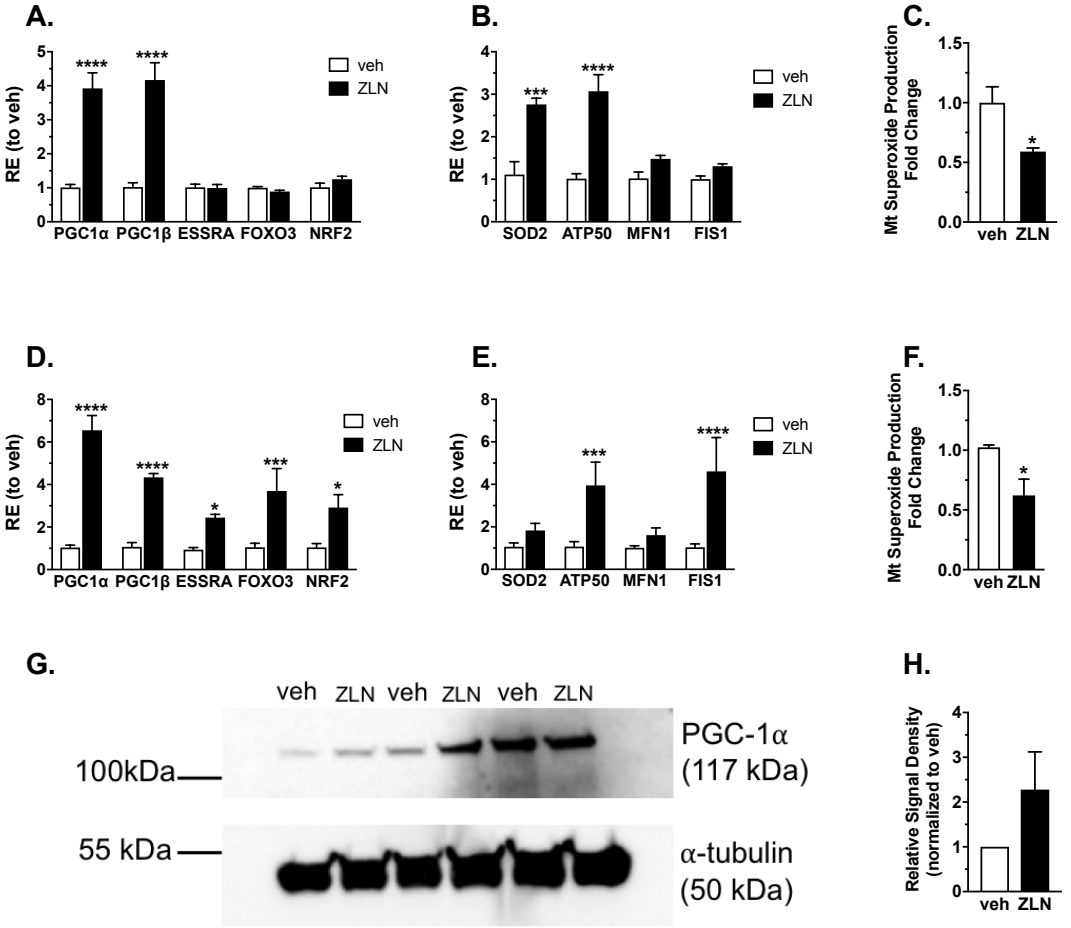


**Figure 5: ZLN005 upregulates PGC-1α in ARPE-19 cells** (A) Relative gene expression (RE) of anti-oxidant transcription factors in ARPE-19 monolayer treated for 48-hours with ZLN005 compared to vehicle (veh). ZLN005 causes the upregulation of *PGC-1α* and *PGC-1β* (n = 4 for each gene). (B) ZLN005 upregulates expression of anti-oxidant enzyme, *SOD2* and mitochondrial ETC enzyme, *ATP50* (n = 3 for each gene). (C) Mitochondrial (Mt) superoxide production is measured as fold change to veh. ZLN005 decreases superoxide production in the ARPE-19 monolayer (n = 5 for both conditions). (D) Differentiated cells treated with ZLN005 for 48 hours upregulate expression of *PGC-1α*, *PGC-1β*, *FOX03*, *ESRRA* and *NRF2* (n = 4 for each gene). (E) ZLN005 upregulates *ATP50* and mitochondrial gene, *FIS1* in differentiated cells (n = 4 for each gene). (F) Mitochondrial superoxide production is decreased in the differentiated ARPE-19 after 24-hour treatment with ZLN005 (n = 4 for veh, n = 5 for ZLN005). (G) Western blot of PGC-1α and α-tubulin protein bands from the ARPE-19 monolayer treated with veh/ZLN005. (H) Western blot quantification where the α-tubulin normalized signal density of ZLN005 PGC-1α bands is normalized to the veh PGC-1α bands (n = 3 for both conditions). All gene expression data was analyzed by two-way ANOVA followed by Tukey's multiple comparison test. Mitochondrial superoxide production was analyzed using the Student's T-test. Statistical significance is represented as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

The impact of ZLN005 on PGC-1α expression at the protein level was examined by Western blot analysis of protein lysates from the ARPE-19 monolayer. An increase in protein levels was observed with 48-hours of ZLN005 treatment compared to the vehicle control (Fig. 5G). To quantify the western blot, the PGC-1α signal densities were first normalized to the corresponding α-tubulin signal densities. Then, the α-tubulin normalized PGC-1α ZLN005 signal densities were normalized to that of the vehicle control (Fig. 5H). The increase observed in the western blot was found to be non-significant, using a Student's T-test, due to the low number of replicates and further work will focus on reproducing these results.

**ZLN005 anti-oxidant effect on RPE is PGC-1α-dependent**

ZLN005 treatment upregulates key anti-oxidant transcription factors and decreases ROS levels in ARPE-19 cells, potentially creating an environment for these cells to withstand oxidative stress. To determine if ZLN005 was able to protect cells in pro-oxidative conditions, ARPE-19 cells were exposed to hydrogen peroxide ($H_2O_2$) and sodium iodate ($NaIO_3$). Hydrogen Peroxide is a form of ROS that is produced endogenously in RPE during respiration and phagocytosis of POS (Miceli, Liles, and Newsome 1994). ARPE-19 cells pre-treated for 24 hours with 10μM ZLN005 or vehicle were exposed to 500μM or 1000μM $H_2O_2$ for 18 hours. To measure cell death, lactate dehydrogenase (LDH) released from the cytoplasm of apoptotic or necrotic cells was quantified as an optical density at 490nm ($OD_{490}$). Cells pre-treated with ZLN005 experienced a

decrease in cell death (Fig. 6A and 6B) caused by 500μM (monolayer: $p = 0.02$, differentiated: $p = 0.002$) and 1000μM $H_2O_2$ (monolayer: $p = 0.0001$, differentiated: $p<0.0001$). To determine if ZLN005's protection against oxidative stress is dependent on PGC-1α, ARPE-19 cells silenced for PGC-1α (shPGC-1α) and the associated control (shControl), previously generated by the laboratory and provided by Dr. Rosales (Saint-Geniez lab, Schepens Eye Research Institute), were exposed to 1000μM $H_2O_2$. As expected, ZLN005 is no longer able to protect shPGC-1α cells ($p = 0.34$) from $H_2O_2$ mediated cytotoxicity, but decreased cell death in the shControl cells ($p < 0.0001$) (Fig. 6C). This indicates that PGC-1α is required for ZLN005 anti-oxidant function.

While the exogenous addition of $H_2O_2$ is commonly used to induce oxidative stress on cultured cells, it does not accurately mimic the RPE dysfunction caused in AMD. Therefore, the effect of ZLN005 on cell death induced by sodium iodate, a stable oxidant that is used in animal models (Redfern et al. 2011; Franco et al. 2009) and cell culture (Wang et al. 2014) to cause retinal degeneration and RPE dysfunction similar to that in geographic atrophy in AMD patients, was examined. Pre-treatment with ZLN005 protects both the ARPE-19 monolayer (2mg/ml: $p = 0.0009$, 2.5mg/ml: $p = 0.0002$, 3mg/ml: $p<0.0001$) and differentiated cells ($p<0.0001$ for all concentrations) from $NaIO_3$-mediated cytotoxicity (Fig. 6D and 6E). With differentiated ARPE-19 cells, pre-treatment with ZLN005 decreased LDH levels below basal levels, measured from cells exposed to vehicle only. This could be due to the induction of

additional anti-oxidant transcription factors in differentiated cells with ZLN005 treatment (Fig. 5).

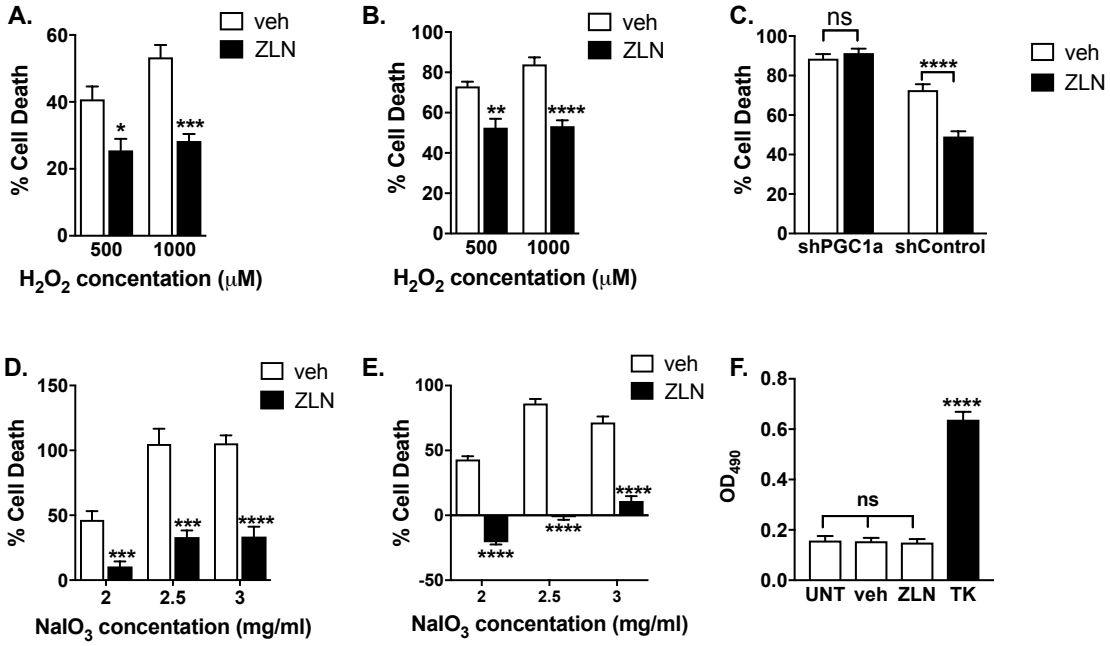


**Figure 6: ZLN005 protects ARPE-19 cells from pro-oxidant induced cell death through the upregulation of PGC-1α.** Cell death induced by 18-hour exposure to 500μM and 1000μM $H_2O_2$ decreases significantly with pre-treatment with 10μM ZLN005 in the (A) ARPE-19 monolayer (n = 6 for all conditions) and (B) Differentiated ARPE-19 (n = 6 for all conditions). (C) Cells lacking PGC-1α (shPGC-1α) show a loss of the protective effect of 10μM ZLN005 upon exposure to 1000μM $H_2O_2$ (n=6), while the effect is maintained in the associated control cells (shControl) (n=12). 24-hour pre-treatment with 10μM ZLN005 protects against $NaIO_3$ induced cell death in (D) the quiescent ARPE-19 monolayer (n = 6 for all conditions) and (E) Differentiated ARPE-19. Pre-treatment with ZLN005 in differentiated cells decreased LDH levels below basal LDH levels when exposed to 2 and 2.5mg/ml $NaIO_3$ (n = 5 for veh, n = 6 for ZLN005) (F) 48-hours of exposure to ZLN005 (n = 6) does not increase LDH levels, measured as $OD_{490}$, compared to untreated cells (UNT, n = 6) and vehicle only treatment (veh, n = 6). Total Kill (TK) of cells is achieved by treatment with 1x Lysis Buffer for 30 min. All data sets were analyzed using the Student's T-test (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

$OD_{490nm}$ measured from cells treated with vehicle ($p = 0.90$) or ZLN005 ($p = 0.74$) alone for 48 hours were not significant compared to untreated ARPE-19 cells not exposed to any additives. Therefore, treatment with vehicle or ZLN005

alone was not cytotoxic (Fig. 6F). The $OD_{490nm}$ of cells exposed to total kill (TK) conditions was significant when compared to the untreated, vehicle and ZLN005 conditions ($p<0.0001$).

**ZLN005 increases expression of certain anti-oxidants in the neural retina**

Due to the effectiveness of ZLN005 in protecting ARPE-19 cells from oxidative stress, ZLN005 was administered to mice through intravitreal injections. At 20μM, ZLN005 was deemed safe as it caused no cataract or damage to the ocular tissue. After 24 and 72 hours, the animals were euthanized and the neural retina and RPE were extracted from the eyes. Total RNA was extracted and gene expression of PGC-1 isoforms and their downstream targets were measured.

The neural retina expresses high levels of *PGC-1β* basally. While a slight increase in *PGC-1β* was observed 72 hours after the injection, this change was found to be nonsignificant (Fig. 7A). Anti-oxidants, *GPX1* ($p = 0.0285$) and *TXN2* ($p = 0.0442$), and ETC gene, *ATP50* ($p = 0.0058$) were upregulated by ZLN005 treatment 72 hours after injection (Fig. 7B), while expression of anti-oxidant transcription factor *FOXO3* ($p = 0.0112$) decreased (Fig. 7C). No significant change in *ESRRA* and *NRF1* expression was observed.

In the RPE, no change in *PGC-1α* expression was observed. *PGC-1β* levels moderately decreased 24 hours after treatment and slightly increased 72 hours after treatment (Fig. 7D). *FOXO3, NRF1* and *ATP50* had slight increases

in expression 24 hours after treatment (Fig. 7E). All these changes in the RPE/choroid layers were found to be non-significant. No change in *ESRRA*, *GPX1* and *TXN2* expression was observed (Fig. 7F).

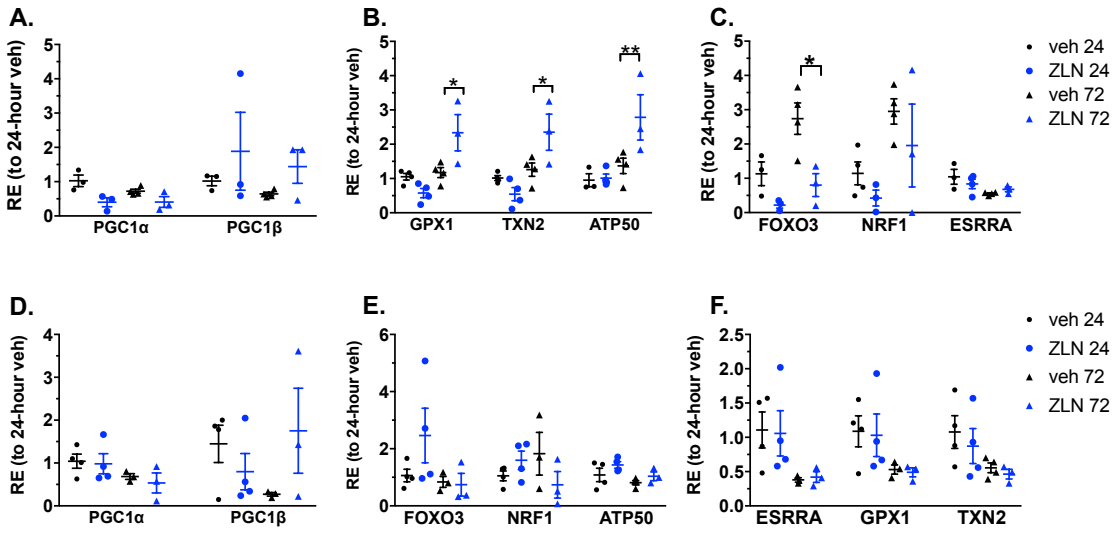


**Figure 7: ZLN005 increases expression of anti-oxidants in the neural retina.** (A) Gene expression of PGC-1 isoforms in the neural retina at 24 (n = 4 for all conditions) and 72 hours (n = 4 for veh, n = 3 for ZLN005) after treatment (B) Anti-oxidants *GPX1* and *TXN2* had a significant increase in expression in the neural retina 72 hours after treatment. ETC gene, *ATP50*, was also upregulated. (C) *FOX03* expression significantly decreased 72 hours after treatment (D) PGC-1 isoform expression in the RPE at 24 (n = 4 for all conditions) and 72 hours (n = 4 for veh, n = 3 for ZLN005) after ZLN005 was administered. (B) Slight upregulation in *FOX03*, *NRF1* and *ATP50* was observed 24 hours after treatment in the RPE. (C) No change in *ESRRA*, *GPX1* and *TXN2* was observed in the RPE. All data sets were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to 24-hour veh)

## Evaluation of a novel doxycycline dependent PGC-1β expression vector system

The tetracycline inducible PGC-1β adenovirus plasmid (Fig. 4) was designed to modulate the level of *PGC-1β* upregulation in cell culture in order to mimic the induction levels observed in AMD patients.

To test the sensitivity of the tetracycline inducible system to doxycycline induction prior to adenoviral production, the plasmid was isolated and transfected into ARPE-19 cells. *PGC-1β* expression (Fig. 8A) was increased in the presence of 0.01 µg/ml (p = 0.0012) and 100 µg/ml (p = 0.0261) doxycycline compared to transfected cells not exposed to doxycycline. There was a significant rise in *PGC-1β* expression with 100 µg/ml compared with 0.01 µg/ml (p = 0.03). *EGFP* expression (Fig. 8B) was also increased with 0.01 µg/ml Dox (p = 0.0024). An increase was observed with 100 µg/ml doxycycline however this increase was non-significant due to high variability between the data points.

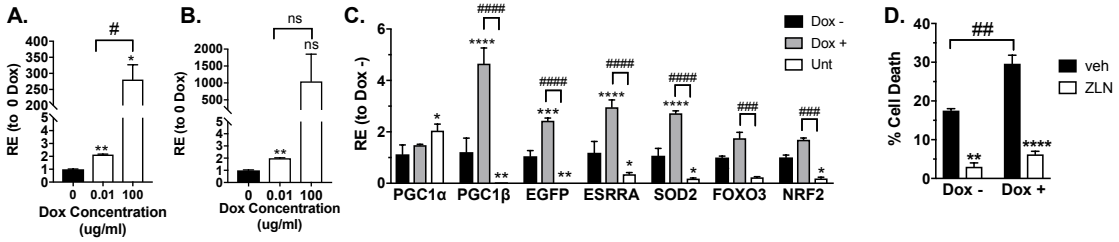


**Figure 8: PGC-1β expression is sensitive to doxycycline concentration.** (A) Induction of *PGC-1β* expression with different concentration of doxycycline (n = 2 per group). (B) *EGFP* expression induced by doxycycline addition (n = 2 per group). (A)-(B) Data was analyzed using the Student's T-test (* P < 0.05, ** P < 0.01, *** P < 0.001 compared to 0 µg/ml Dox, # P< 0.05 compared to 0.01 µg/ml ) (C) Expression of downstream targets of PGC-1β in un-transfected cells (Unt, n = 3), transfected cells exposed to no doxycycline (Dox-, n = 3), and transfected cells treated with 1 µg/ml doxycycline (Dox+, n = 3) for 48-hours. Data was analyzed using two-way ANOVA followed by Tukey's multiple comparison test (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared to Dox-, ### P< 0.001, #### < 0.0001 compared to Unt) (D) 1000µM $H_2O_2$-mediated cell death increases in cells treated with 0.01 µg/ml doxycycline (n = 6) for 48 hours compared to transfected cells not exposed to doxycycline (n = 3). ZLN005 protects cells in both conditions (n = 3 for Dox-, n = 6 for Dox+) from cell death. Data was analyzed using two-way ANOVA followed by Tukey's multiple comparison test (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared to veh, ## P< 0.01 compared to Dox- veh)

To measure the expression of downstream targets of *PGC-1β*, 1 µg/ml Dox was added to transfected cells for 48 hours (Fig. 8C). Gene expression of un-transfected cells and transfected cells treated with doxycycline (Dox+) was normalized to that of transfected cells not exposed to doxycycline (Dox-). Un-transfected cells were found to have a significant reduction in *PGC-1β* ($p = 0.0024$), *ESRRA* ($p = 0.04$), *SOD2* ($p = 0.03$) and *NRF2* ($p = 0.045$), and an increase in *PGC-1α* ($p = 0.02$) expression when compared to the Dox- cells. This unexpected induction of *PGC-1β* (Fig. 8C) in Dox- cells is potentially due to vector leakiness or the presence of tetracycline in the cell media and further evaluations are on-going. As expected, no basal expression of *EGFP* ($p = 0.0082$) was measured in un-transfected cells.

Doxycycline addition upregulated *PGC-1β* 4-fold ($p < 0.0001$) and EGFP 2-fold ($p < 0.0001$) above the Dox- control. Dox+ cells were found to have increased expression of *ESRRA* ($p < 0.0001$) and *SOD2* ($p < 0.0001$). When compared to un-transfected cells, Dox+ cells upregulated *PGC-1β* ($p < 0.0001$), *EGFP* ($p < 0.0001$), *ESRRA* ($p < 0.0001$), *SOD2* ($p < 0.0001$), *FOXO3* ($p = 0.0006$) and *NRF2* ($p = 0.0002$).

Dox+ cells were found to have significantly higher expression of transcription factor, *ESRRA*, and anti-oxidant enzyme, *SOD2*. To examine if this upregulation conferred protection to Dox+ cells upon exposure to oxidative stress, Dox- and Dox+ cells were treated with 1000µM $H_2O_2$ with or without pre-treatment with 10µM ZLN005 (Fig. 8D). Dox+ cells were treated with 0.01µg/ml

Dox, which caused a 2-fold rise in *PGC-1β* expression (Fig. 8A). Dox+ cells experienced more cell death in the presence of 1000μM $H_2O_2$ compared to Dox- cells ($p = 0.009$), indicating that a 2-fold induction of *PGC-1β* makes cells more susceptible to oxidative stress. ZLN005 was able to protect both Dox- ($p = 0.009$) and Dox+ ($p<0.0001$) cells from $H_2O_2$ mediated cytotoxicity.

## DISCUSSION

The purpose of this study was to evaluate the role of PGC-1 isoforms in RPE metabolism through two specific aims. Through Aim 1, compound ZLN005 was found to effectively upregulate the PGC-1 isoforms and confer protection against oxidative stress in the ARPE-19 cell line. In Aim 2, a novel vector for doxycycline dependent PGC-1β induction was designed and validated.

In Aim 1, treatment with 10 μM ZLN005 was found to effectively upregulate gene expression of both PGC-1 isoforms and their associated targets in the ARPE-19 cell line. However, this induction was dependent on whether cells were quiescent or differentiated, as not all downstream targets of the isoforms were found to be upregulated in all conditions. For instance, *SOD2* was only found to be induced in quiescent cells while *ESRRA, NRF2* and *FOXO3* were upregulated in differentiated cells. This selective induction could point to specific pathways activated by ZLN005 treatment in response to the cell metabolic and/or redox status. Differentiation could be a factor that can change the oxidative status of cells, as quiescent cells are transitioning from glycolytic to oxidative metabolism and oxidative phosphorylation is a major source of endogenous ROS in differentiated cells (Iacovelli et al. 2016, Agathocleous et al., 2012). Despite the potentially different mechanisms utilized in quiescent and differentiated cells, ZLN005 decreased mitochondrial superoxide production in both conditions. Furthermore, protein expression of ARPE-19 monolayer lysate showed an increase in PGC-1α expression that was not statistically significant due to the low

number of repeats. Future work will focus on repeating these experiments and quantifying upstream and downstream target protein expression.

ZLN005 also provided protection against $H_2O_2$ and $NaIO_3$ mediated cytotoxicity. Interestingly, differentiated cells exposed to $NaIO_3$ showed a decrease in LDH levels below basal levels, leading to a negative percentage cell death. This phenomenon was not observed in the quiescent ARPE-19 monolayer exposed to $NaIO_3$ and could be indicative of the different mechanisms utilized by ZLN005 as mentioned above. By testing the effect of ZLN005 in PGC-1α silenced cells, it was determined that PGC-1α plays an integral role in mediating the cytoprotective effect of ZLN005. However, *PGC-1α* is not the only PGC-1 isoform upregulated. *PGC-1β*, which is normally repressed in ARPE-19, is also induced with ZLN005 treatment. Through previous work done by our group, *PGC-1β* upregulation was found to increase $H_2O_2$ mediated cell death (Charles and Saint-Geniez 2017). Despite inducing PGC-1β, ZLN005 is able to protect cells from $H_2O_2$ induced oxidative damage and has no basal cytotoxic effect on ARPE-19 cells. This suggests that the ratio of *PGC-1α : PGC-1β* expression plays an important role in RPE function and this observation is supported by the cellular dysfunction observed when this ratio is decreased. To elucidate the role *PGC-1β* expression plays in ZLN005 anti-oxidant function, the effect of ZLN005 on oxidative stress should be tested in PGC-1β silenced cells.

Intravitreal injections of ZLN005 was not found to have significant effect on PGC-1 isoform expression in the neural retina and RPE/choroid layers in mice.

As a small sample size of animals were used for this study, further work will involve measuring the effect of higher ZLN005 concentrations in a larger sample size of animals. It is important to note that the intravitreal injection of ZLN005 did not cause any obvious deleterious changes to the structure of the eye or toxic responses within the animals. Histological sectioning of the eye will be performed to confirm that ZLN005 does not cause structural changes in the retinal and RPE layers. As ZLN005 action may be dependent on the oxidative environment of the cells, future work will involve testing an appropriate ZLN005 concentration on a mouse model of RPE dysfunction. While further work is needed, this current study has introduced ZLN005 as a potential therapeutic against AMD pathogenesis.

Aim 2 of this study focused on designing a novel vector for doxycycline dependent PGC-1β induction. The effect of increasing doxycycline concentration on *PGC-1β* expression was demonstrated in ARPE-19 cells transfected with the vector. With 1µg/ml doxycycline, *PGC-1β* levels were upregulated 4 –times above the induction in transfected cells not treated with doxycycline, mirroring the fold changes observed in patients with choroidal neovascularization. Expression of downstream targets, *ESRRA* and *SOD2*, was increased with *PGC-1β* expression and is consistent with previous studies from our group (Charles and Saint-Geniez 2017). The work done in this study with transfected ARPE-19 cells has demonstrated the successful design of a doxycycline dependent PGC-1β vector. However, future studies with this vector will involve adenoviral delivery as

the ARPE-19 cell line is not able to be easily transfected and is modified upon transfection. The increased efficiency and decreased stress of adenoviral delivery will allow for a well-modulated induction of *PGC-1β* in response to doxycycline concentration.

Despite challenges faced during transfection, this modulated increase in *PGC-1β* expression was found to increase pro-oxidant induced cell death. This rise in cell death is seen with a 2-fold increase in *PGC-1β* expression and models the rise seen in CNV patients. Most importantly, ZLN005 was found to protect cells with increased *PGC-1β* expression against pro-oxidant mediated cell death. This demonstrates that ZLN005's protective effect is maintained in cells with upregulated *PGC-1β* expression which suggests that this compound could be beneficial for clinical use.

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