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## MicroRNA Expression Signature of Retinal Pigmented Epithelial Cells Associated with the Phenotype of Age-Related Macular Degeneration

Mark Anthony Gutierrez  
*Dominican University of California*

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**MicroRNA Expression Signature of Retinal Pigmented Epithelial Cells Associated with the  
Phenotype of Age-Related Macular Degeneration**

A Thesis Submitted to the Faculty of

Dominican University of California

&

The Buck institute for Research on Aging

In Partial Fulfillment of the Requirements for the Degree

Master of Science

In

Biology

By:

Mark Anthony Gutierrez

San Rafael, California

May 2014

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## **Certification of Approval**

I certify that I have read *MicroRNA Expression Signature of Retinal Pigmented Epithelial Cells Associated with the Phenotype of Age-Related Macular Degeneration* by Mark Anthony Gutierrez, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and the Buck Institute for Research on Aging.

Dr. Deepak Lamba,

Graduate Research Advisor,

Assistant Professor

Dr. Mohammed El Majdoubi,

2<sup>nd</sup> Reader,

Associate Professor

Dr. Mary Sevigny,

MS Biology Graduate Program Director

## **Abstract**

Age-related macular degeneration is a debilitating condition that manifests as a loss of the central portion of an individual's field of vision, affecting 1 in 3 people over the age of 80. With regards to the retina, the organ primarily responsible for an individual's sense of vision, this condition is associated with the degeneration of the central point in the retina, known as the macula, which contains the highest concentration of light-sensitive photoreceptors. It is currently known that the human retina exhibits a gradual decrease in protective detoxifying factors such as glutathione-S-transferase-1 and catalase as well as increased lipid peroxidation, all of which resulting in the age-related increase in autofluorescent lipofuscin between the retina and the choroid. This prevents the proper deliverance of nutrients to the foundational layer of cells in the retina, leading to oxidative stress-related apoptosis of first the retinal pigmented epithelium (RPE) and then the photoreceptors.

MicroRNAs (miRNAs) are a group of endogenous noncoding RNA molecules that originate from parts of the genome that were originally speculated to be "junk" DNA. However, it is now known that these small RNA molecules are responsible for gene silencing on the post-transcriptional level via the degradation or transcription inhibition of specific mRNA transcripts. In the context of age-related macular degeneration, certain miRNAs may be upregulated to induce the downregulation of apoptotic genes. On the other hand, certain miRNAs may become downregulated to promote enhanced expression of protective factors. We hypothesize that miRNAs play a role in regulating oxidative response of RPE cells, which may then contribute to cell survival by the upregulation of endogenous pro-survival antioxidant elements.

## **Acknowledgments**

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## Table of Contents

	<u>Page Number</u>
<b>Introduction</b> .....	1
Anatomy of the Retina .....	1
Age-Related Macular Degeneration: Symptoms and Treatments.....	3
Oxidative Stress as a Key Factor in the Progression of AMD .....	7
Basic Biology of miRNAs.....	9
miRNAs in RPE Cells.....	11
Research Focus.....	12
<b>Materials and Methods</b> .....	13
Cell Culture and RPE Determination Conditions.....	13
Stress Treatment of RPEs .....	14
RNA Extraction, Reverse Transcription, and PCR Analysis.....	15
Dichlorofluorescein Assay for ROS Generation.....	17
Lentiviral Transduction of RPE Cells with miRNA Decoys .....	17
<b>Results and Discussion</b> .....	19
Establishment of Oxidative Stress Phenotype Reflective of AMD .....	19
Correlation of Oxidative Stress Phenotype with NRF2/KEAP1 Activity and miRNA Analysis ...	22
Modulation of miRNAs Associated with Oxidative Stress and its Effect on Stress Phenotype	25
<b>Conclusion</b> .....	29
<b>References</b> .....	32

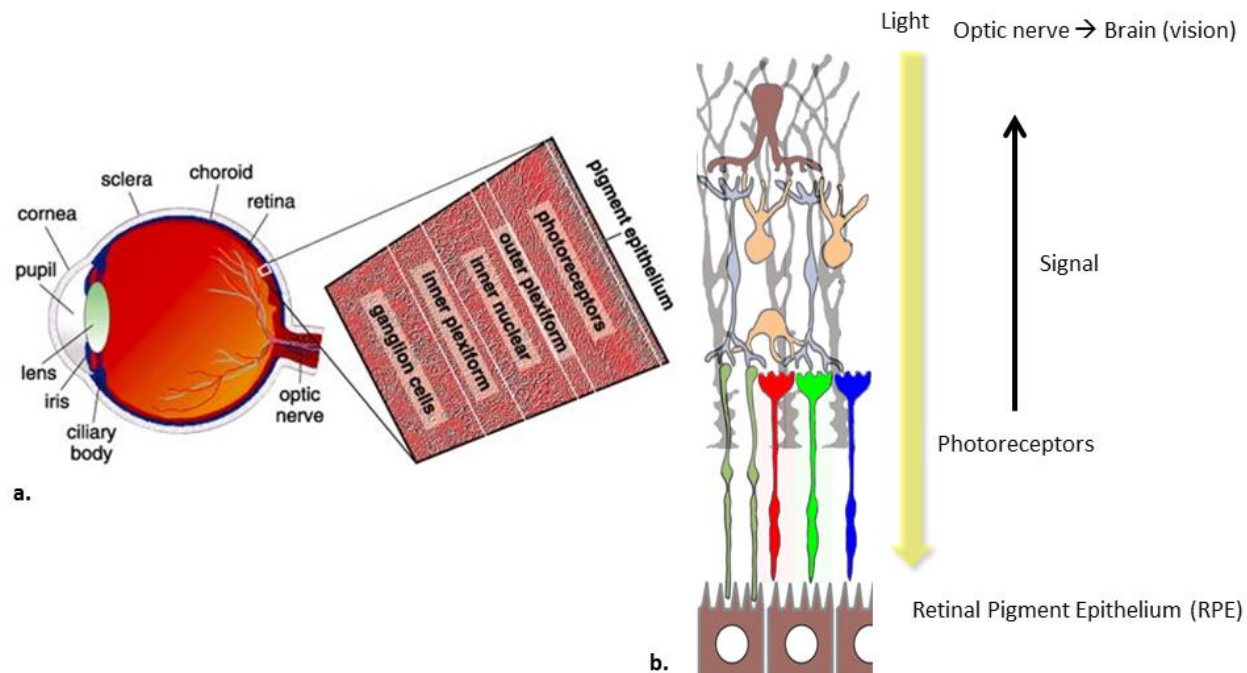
## **Introduction**

### Anatomy of the Retina

The vertebrate retina is a multilayered structure composed of multiple cell types, including rod and cone photoreceptors, ganglion and amacrine cells, and retinal pigmented epithelium cells (RPEs). These cells are arranged in layers at the back of the eye, forming a complex neural network that allows for the sense of sight.[1] As shown in Figure 1, this multilayered structure lines the back of the eye between a vascular layer, known as the choroid, and the vitreous gel. The retina itself is known to be made up of two distinct regions. The neuroretina is composed of various neural cell types such as the rod and cone photoreceptors, amacrine cells and ganglion cells, all of which are collectively responsible for relaying signals generated by light coming into the eye. In this process, light enters the eye through the lens and excites the light-sensitive photoreceptors. These cells then send signals via the amacrine cells to the retinal ganglion cells, whose axons form the optic nerve by which these signals are sent to the brain.

The second region of the retina is known as the pigmented epithelium, which is a layer of cells known as retinal pigmented epithelial cells (RPEs). This layer of cells plays multiple important roles in maintaining the intercellular homeostasis of the neuroretina, including the phagocytosis of outer segments shed by the photoreceptors,[2] maintaining the separation between the neuroretina and the blood supply by the formation of tight junctions,[3] the transfer of ions and other nutrients from the blood supply to the neuroretina,[4] and the conversion and storage of retinoid as part of the visual transduction cycle.[5]





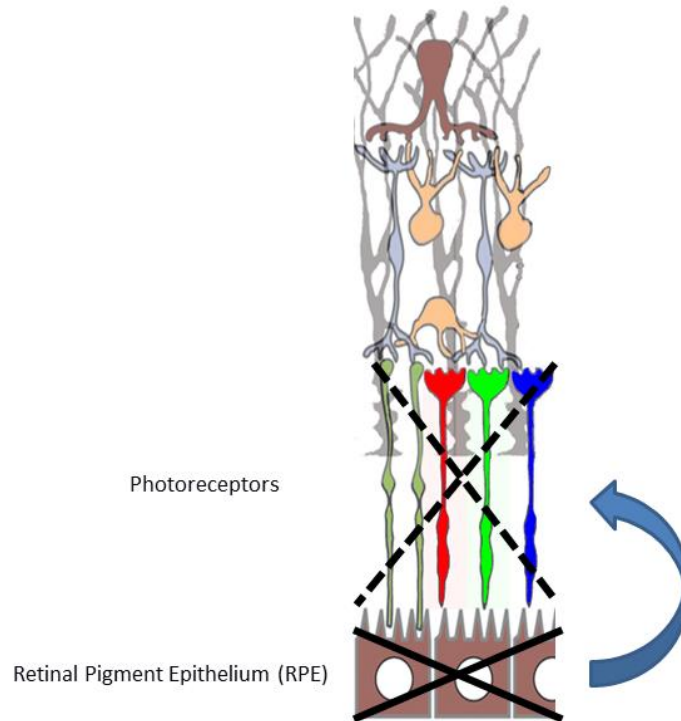
**Figure 1: Anatomy and Functional Schematic of the Vertebrate Retina.** Modified from “How the Retina Works,” *American Scientist*, (91) 2003. a. Anatomical diagram of the eye with emphasis on the retina and the cellular layers it is composed of. b. Functional schematic of the retina.

Visual impairment can result from damage to the retina from various sources that one may encounter during his or her lifespan. Age-related macular degeneration (AMD) is an example of the lifelong progression in which the accumulation of damage to the retina can coalesce in the loss of sight, which can be a problematic issue with regards to the quality of life in the aging population. There are two types of cells currently under investigation in the context of age-related retinal degeneration: the light sensitive rod and cone photoreceptors found in the neuroretina and the RPE cells that form the foundation of the retina. Functionally, as previously mentioned, the RPE cells provide structural and nutrient support for the photoreceptors. Therefore, the pigmented epithelium plays a vital role in the viability of the retina, the

degeneration of which would inevitably lead to the degeneration of downstream cells such as the photoreceptors.[6]

### Age-Related Macular Degeneration: Symptoms and Treatments

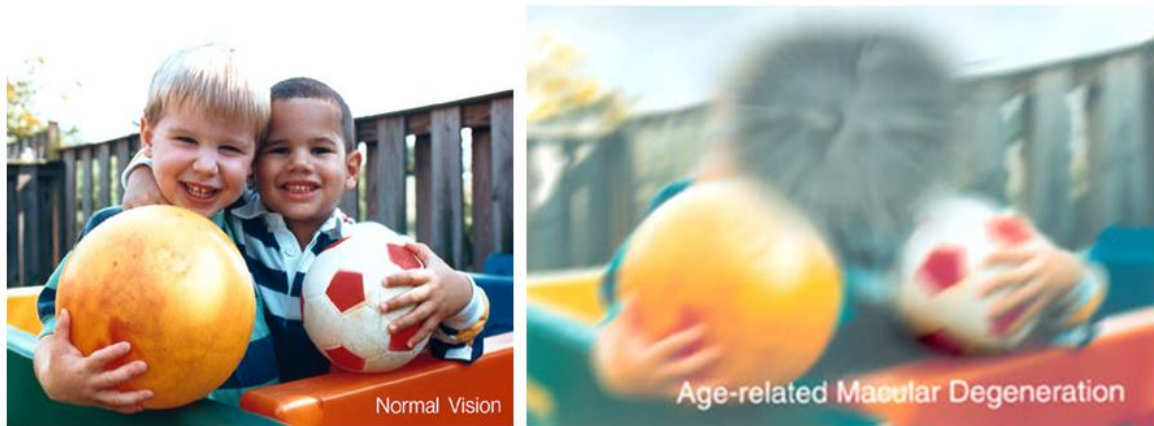
Age-related macular degeneration (AMD) is a prime example of a neurodegenerative phenomenon, displaying an age-related progression of degeneration in the retina. AMD is the leading cause of vision loss in people over the age of 50 years, affecting approximately 15 million people in the U.S. The condition is characterized by the deterioration of the macula, a portion of the retina that contains the largest concentration of cone photoreceptor cells and is responsible for high resolution vision. There are two main forms of AMD: dry and wet. Wet AMD, the more rare form of AMD, is associated with the resulting retinal detachment due to the formation of new blood vessels in the retina. Dry AMD, which accounts for over 90% of AMD cases, is associated with the age-related build-up of cellular debris between Bruch's membrane and the RPE layer. This accumulation continues with age, leading to the atrophy and eventual death of the pigmented epithelium, ultimately disrupting the cellular homeostasis of downstream retinal cells and eventually cell death of cells in the neuroretina.[7] This progression is outlined in Figure 2.



**Figure 2: Progression of Cell Death Exhibited in AMD.** As cellular debris builds up between Bruch's membrane and the RPE layer, cells in the RPE layer eventually undergo cell death. As more RPE cells die due to cellular stress, intercellular homeostasis in the neuroretina is disrupted, leading to the cell death of photoreceptors and other neural cells.

Symptoms of AMD include the perceived distortion of straight lines, diminished or changed color perception, and dark blurry areas or white-outs that appear at the center of vision. As a result, individuals affected by AMD will present with "reversed tunnel-vision," where they have a blind spot at the center of their field of vision that grows outward with age, as described in the simulation from the National Eye Institute in Figure 3. Eventually the blind spot will gradually progress outward with age as more retinal cells succumb to the degenerative process. In the more advanced form of this degeneration, affected individuals will develop the condition known as geographic atrophy (GA), at which point the deterioration completely disrupts the

layer of pigmented epithelial cells, leading to legal or complete blindness if a clinical intervention isn't administered.



**Figure 3: Field of Vision Perceived by an Individual Affected by AMD Compared to an Unaffected Individual.**

Compared to an individual unaffected by AMD, an AMD patient experiences a blind spot at the center of their field of vision. With age, as retinal cell death accumulates due to increasing cellular stress, this blind spot grows outward. Eventually, without intervention, this phenomenon will result in legal or complete blindness.

To date, there are no treatments that have been shown to effectively halt the degeneration of the retina or rescue vision once cells in the retina have been lost. Therefore, current aims in scientific research are focused on the prevention of this degeneration. With regards to therapeutics, drugs used in the treatment of AMD are VEGF-inhibitors which attempt to halt retinal degeneration through the neovascularization process associated with wet AMD.[8] However, this has no use in the treatment of the dry form of AMD, which doesn't manifest through the neovascularization process. Additionally, VEGF-inhibitor treatments have not been shown to completely rescue the vision of such an affected individual once retinal cells are lost to the degenerative process. Therefore, current strategies that are under scientific investigation include gene therapy and cell-replacement therapy. Although gene therapy has

been valued in the treatment of various conditions, the drawback in this case once again lies in its use in late stage AMD. The data presented by Maguire *et al.* in 2009 shows that the best visual improvements after the use of adeno-associated viruses to restore the functionality of RPE65, a marker of cellular maturity in RPEs, has been seen only in children, whose photoreceptors have not already been lost to the degeneration process.[9] Furthermore, gene therapy is generally most useful in conditions where a loss-of-function mutation is observed or enzyme replacement is necessary, both of which are not the case for AMD and thus have limited therapeutic use for the prevention of retinal degeneration.

Current research endeavors regarding the treatment of AMD highlight the use of embryonic stem cells and induced pluripotent stem cells to replace retinal cells that have been lost to the degenerative process. Various groups focus on the inhibition of Wnt and BMP signaling pathways along with the activation of IGF signaling in order to derive retinal cells from pluripotent stem cells.[10-12] Additionally, differentiated retinal cells derived in such a manner have been shown to exhibit integration and survival after transplantation, even providing a rescue of sight in a *Crx*-deficient mouse model, albeit to a limited extent.[13-15] To date, non-mammalian vertebrates have been shown to have the remarkable ability to replace neurons that have been lost by damaging events.[16-18] However, this phenomenon has yet to be seen to any significant extent in humans. Therefore, despite advances in therapeutic treatments for AMD by cell-replacement strategies, mechanistic studies promise to be invaluable clinical assets in order to elucidate preventative strategies for the retinal degenerative process.

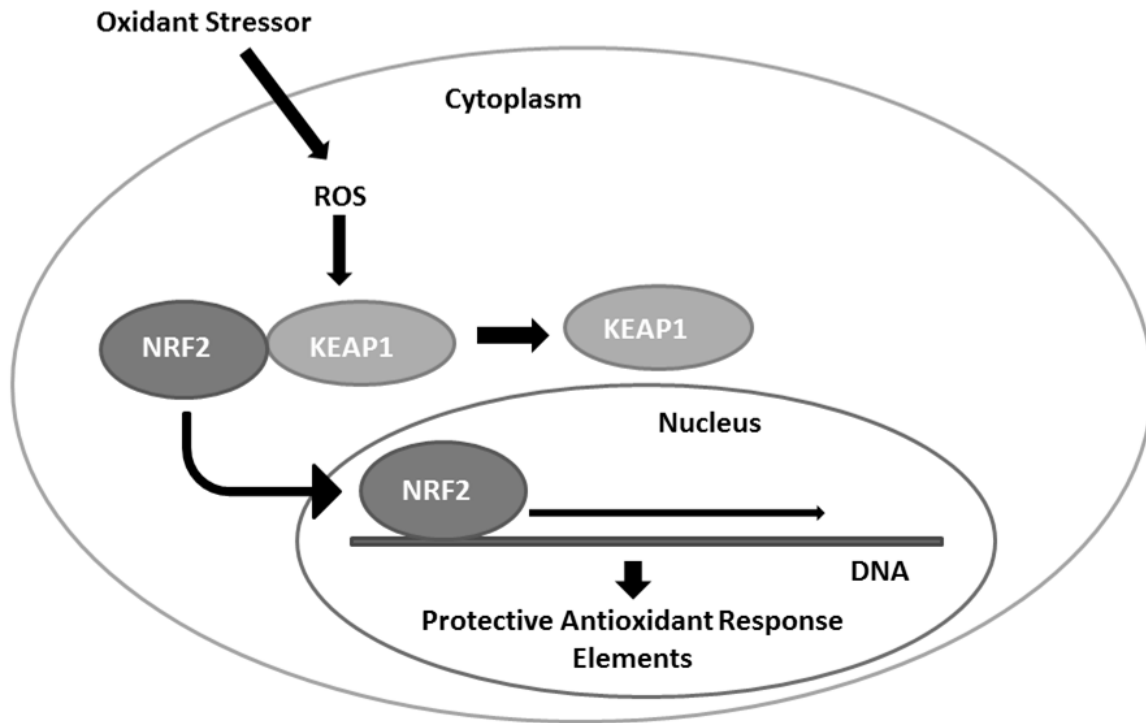
### Oxidative Stress as a Key Factor in the Progression of AMD

In light of the potential of understanding the mechanisms through which AMD progresses, many investigators focus on defining factors that contribute to the degenerative nature of AMD. From such information, strategies can be developed to help slow or prevent the degenerative process. Current research speculates the beneficial effects of omega-3 fatty acids and various antioxidant molecules in measures to prevent AMD, implicating the role of diet.[19] In one such study, omega-3 fatty acids were shown to have beneficial effects on AMD-like lesions in DKO<sup>rd8</sup> mice by anti-inflammatory mechanisms of docosahexaenoic acid and eicosapentaenoic acid (EPA).[20] Other environmental factors that have been implicated in the development of AMD include smoking and exposure to smoke, which have been shown to cause systemic oxidative stress,[21, 22] as well as genome instability and DNA damage caused by exposure to blue and ultraviolet (UV) light.[23] Genetic factors have also been proposed to play a role in the progression of AMD. It has been shown that mutation-induced dysfunctional expression of complement factor H is strongly associated with AMD development through the induction of chronic inflammation, leading to excessive cellular damage to retinal cells.[24, 25]

Because the underlying cause of AMD is complex and multifactorial, a single underlying factor that drives the degenerative process is considerably difficult to define. This problem is compounded due to the fact that most animal species lack a macula, therefore finding the appropriate animal model system for *in vivo* studies is quite difficult.[26] Recent studies have associated oxidative stress as a key element in the progression of AMD, presenting evidence of a decreased expression in glutathione S-transferase-1, increased lipid peroxidation, and a decreasing trend in catalase activity that continues with age.[27] As a result, there is an age-

related increase in autofluorescent lipofuscin between the RPE layer and Bruch's membrane. This substance contains a molecule known as A2E, a pyridinium bisretinoid byproduct of the visual phototransduction pathway. Importantly, A2E is known to be a potent generator of reactive oxygen species (ROS) in the RPE, the accumulation of which can lead to cell death.[28] Therefore, understanding how the RPE reacts, and what pro-survival mechanisms are affected in response to oxidative stress, has potential for further investigations into preventative measures against AMD. One way to investigate cellular response to stress is by focusing on the production of endogenous factors that can play a role in pro-survival mechanisms and how the expression of such factors are affected by cell stress.

One of the most notable signaling pathways that have been associated with oxidative stress response is the NRF2/KEAP1 pathway. This signaling pathway implements cellular responses to oxidative stress through the production of antioxidant response elements (AREs).[29, 30] Under basal conditions, KEAP1 is bound to NRF2, which then directs NRF2 into degradation in the cytosol. However, as visually outlined in Figure 4, the accumulation of ROS in the event of oxidative stress leads to the dissociation of KEAP1 from NRF2, stabilizing this factor. NRF2 is then translocated into the nucleus where it induces the expression of AREs such as NAD(P)H dehydrogenase quinone 1 (NQO1), heme oxygenase 1 (HMOX1), as well as the catalytic and regulatory subunits of glutamate-cysteine ligase (GCLC and GCLM respectively).

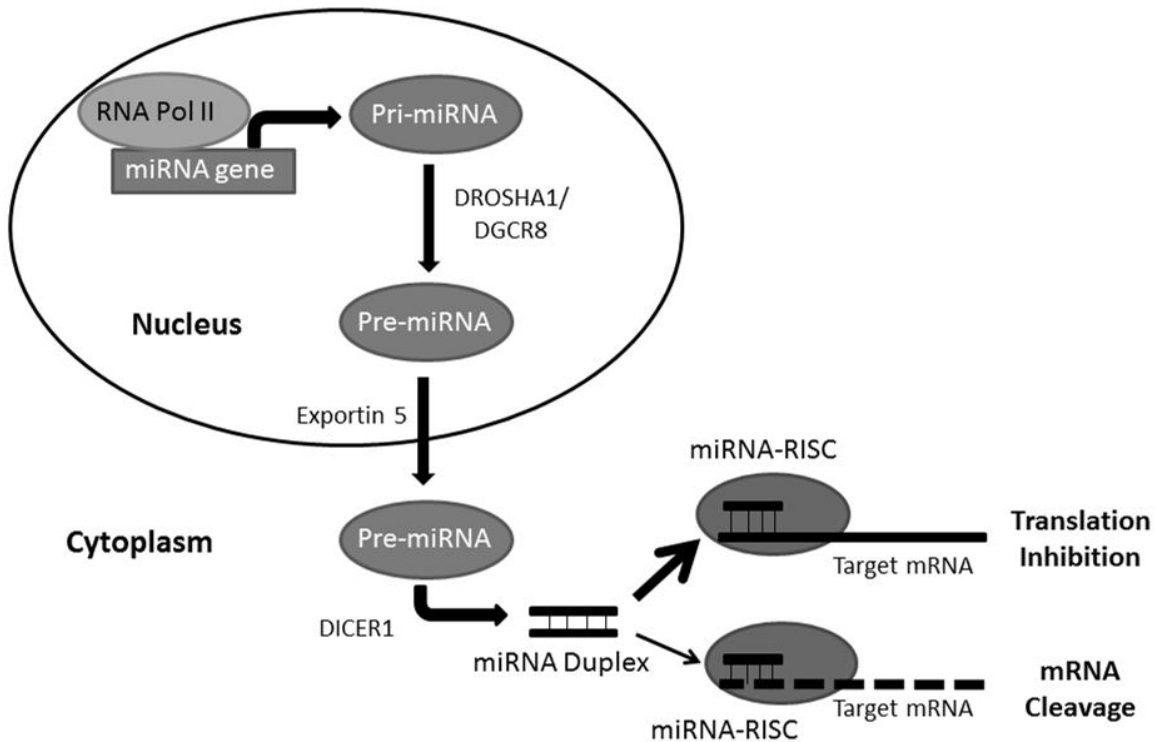


**Figure 4: NRF2/KEAP1 Signaling Pathway.**

#### Basic Biology of miRNAs

Various studies highlight the role of small, noncoding RNA molecules known as microRNAs (miRNAs) in the progression of various human disease conditions.[31] These 22-25 nucleotide small RNA molecules silence gene expression via the degradation or translational inhibition of target mRNA molecules. Such molecules had escaped the notice of many researchers until 1993, when Victor Ambros, Rosalind Lee, and Rhonda Feinbaum discovered that lin-4, a gene known to play a significant time-dependent role in *C. elegans* larval development, does not encode a protein. In fact, when expressed, the gene produces a pair of small RNA molecules that have been suggested by the group to inhibit lin-14 expression by RNA-RNA antisense interactions.[32]





**Figure 5: miRNA Biogenesis Pathway.** Complete processing of miRNA transcripts leads to the loading of the mature miRNA molecule onto the RNA-induced silencing complex, which targets specific messenger RNA sequences for gene silencing.

As outlined in Figure 5, the production of mature miRNAs follows a distinctly different pathway from that of functional messenger RNAs. miRNA genes are transcribed by RNA polymerase II to produce a primary miRNA (pri-miRNA) transcript, which is then processed by a microprocessor complex comprised of two factors, DROSHA1 and DGCR8, to produce the pre-miRNA transcript. This product is exported out of the nucleus by Exportin 5 and then processed by DICER1 to form a miRNA duplex composed of 5p and a 3p strands. This duplex dissociates and the mature 5p component is loaded onto the RNA-Induced silencing complex to form the miRNA-RISC, which then binds to the 3' untranslated region (3'UTR) of specific mRNA transcripts.

With regards to function, an individual miRNA molecule is as important to gene expression as an individual transcription factor, in this case to regulate the expression of multiple target genes at the post-transcriptional level.[33] After a mature miRNA transcript is loaded onto the RISC, the miRNA-RISC can target specific messenger RNA (mRNA) transcripts for gene silencing depending on the extent of complementary binding. Complete complementarity effectively leads to the degradation of the mRNA molecule by the RISC. Partial complementarity between an miRNA-RISC and an mRNA transcript doesn't degrade the mRNA molecule, however it does prevent its translation into a functional protein. Therefore, any binding of an miRNA-RISC to an mRNA molecule effectively leads to gene silencing at the post-transcriptional level. It is speculated that miRNAs may be involved in the regulation of all major cellular functions in the cell, including differentiation, replication, mobility, and apoptosis through the inhibition of key mRNA gene targets. Therefore, such molecules present much potential in the study of development, physiology, and disease in humans.

#### MicroRNAs in RPE Cells

With regards to the context of retinal cells, past studies highlight the expression and inhibition of miRNAs such as miR204, miR302,[34] miR184, miR187, and miR211[35] in the maintenance of RPEs. For example, it has been reported that the continuous expression of miR204 and the continuous inhibition of miR302 contributes to the expression of markers such as ZO-1, RPE65, MERTK, and MiTF through the specific targeting of CTNNBIP1, a reported inhibitor of Wnt signaling, and TGFBR2, a factor that aids in the direction of the mesodermal and endodermal cell fates, respectively.[34] Of particular note, it has been shown that miRNAs

play a role in RPE cell viability in the pathogenesis of AMD through the dysregulation of protective miRNAs[31] or the accumulation of ALU RNA elements. In both instances, the disease progression has been suggested to be driven by the loss of DICER1, a key protein involved in miRNA biogenesis.[36] Additionally, recent studies have observed shifts in miRNA expression profiles when comparing human ARPE-19 cells that have undergone H<sub>2</sub>O<sub>2</sub>-induced oxidative stress or treatment with the antioxidant curcumin.[37] Although this study involved only acute treatments of H<sub>2</sub>O<sub>2</sub> rather than chronic treatments, which would be a more appropriate experimental design to study AMD with, this indeed suggests the presence of miRNA-dependent pro-apoptotic and anti-apoptotic mechanisms in RPE cells that are activated in response to stress signaling.

#### Research Focus

As described previously, miRNAs are thought to play a role in how cells react to stress and disease conditions. Data from immortalized cell lines such as ARPE-19 may not accurately and comprehensively represent the disease due to the highly proliferative nature of the cell line's biology. Therefore, RPEs derived from human embryonic stem cells (hESCs) appear to be the ideal candidate for studies on the miRNA profiles of the AMD disease phenotype. Although there have been investigations into miRNA and gene profiling of short-term stress phenotypes, there has yet to be a comprehensive study looking into such profiling associated with long-term stress with a gradually increasing concentration of stressor, which would be a more appropriate model of AMD. This would be more adequately reflective of the accumulation of lipofuscin products under the RPE layer. Based on previously published methods of deriving retinal cell

types from hESCs, including the RPE cell fate[11] our lab sought to develop a model system displaying the chronically stressed phenotype associated with AMD through paraquat-induced chronic oxidative stress.[38] Once a model system has been developed, changes in miRNA expression can then be monitored by quantitative PCR analysis with modifications designed for the analysis of mature miRNA molecules.[39] This information can then be cross-referenced to the expression profile of protective antioxidant factors such as those found in the NRF2/KEAP1 pathway.

## **Materials and Methods**

### **Cell Culture and RPE Determination Conditions**

Undifferentiated embryonic stem cells or induced pluripotent stem cells derived from a patient with Leber's congenital amaurosis were maintained in Essential 8 basal medium (Gibco) supplemented with 1% Essential 8 supplement (Gibco) and 1% Pen/Strep Amphotericin B (Lonza) on culture vessels coated with Matrigel (BD Biosciences). The media of these cells were changed every day and grown in a 37° C incubator with 5% CO<sub>2</sub> and 5% O<sub>2</sub> before differentiation. Differentiated RPE cells were maintained in MEM/EBSS with 2.00 mM L-Glutamine and Earle's balanced salts (Hyclone) supplemented with Knockout<sup>tm</sup> serum replacement (Gibco), 1% for maintenance of mature RPEs and 5% for the proliferation of RPEs to confluency before maturation, 1% Pen/Strep Amphotericin B (Lonza), 1% Glutamax<sup>tm</sup> (Gibco), 125 mg taurine, 10 µg hydrocortisone, 6.5 ng triiodo-thyronin, and 0.1% plasmocin prophylactic (InvivoGen). The media of RPE cells was changed every other day, grown and maintained in a 37° C incubator with 5% CO<sub>2</sub>.

For the differentiation of embryonic stem cells into the RPE fate, undifferentiated cells were subjected to a 3 week of treatment with 10 ng/mL DKK1 (R&D Systems), noggin (R&D Systems), and IGF1 (R&D Systems) in DMEM/F-12 1:1 with 2.50 mM L-Glutamine and 15 mM HEPES (HyClone), 1% sodium pyruvate (Corning), 1% N-2 supplement (Gibco), 1% HEPES (HyClone), 1% Pen/Strep Amphotericin B (Lonza), and 0.1% plasmocin prophylactic (InvivoGen). Spontaneously appearing pigmented foci were picked and transferred to a separate culture vessel coated with Matrigel for expansion and complete formation of traditional cobblestone-like morphology.

#### Stress Treatment of RPEs

Once hESC-derived RPEs have grown to 100% confluency, cells were subjected to treatment with either paraquat (PQ), alternatively known as methyl viologen (Acros Organics), and hydroquinone (Acros Organics) in order to induce either a low stress or chronic high stress phenotype. To induce a low stress phenotype, RPE cells were treated with either stressor. The cells were treated with either 20  $\mu$ M of paraquat or 50  $\mu$ M of hydroquinone over 9 days and followed by 40  $\mu$ M of paraquat or 100  $\mu$ M of hydroquinone subsequently over 7-10 days.

To induce a high stress phenotype, RPE cells were treated in a similar manner. Paraquat treatment involved 40  $\mu$ M of paraquat over 9 days then 80  $\mu$ M over the following 6 days, and finally 160  $\mu$ M over 5 days. In a similar fashion, hydroquinone treatment involved 100  $\mu$ M over 9 days, 200  $\mu$ M over the next 7 days, and 400  $\mu$ M over 1 day. After each series of treatments, RNA was extracted for miRNA analysis by real-time quantitative PCR.

### RNA Extraction, Reverse Transcription, and PCR Analysis

Total RNA was extracted from RPE cells using the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's recommended instructions. RNA samples were then quantified by a NanoDrop 2000 apparatus (Thermo Scientific). cDNA was synthesized from 0.5-1 µg of total RNA template using the iScript cDNA Synthesis kit (Bio-Rad), the protocol of which was modified to accommodate 400 nM of a reverse transcription stem-loop primer. Up to 5 reverse transcription stem-loop primers were used per reaction. Reactions occurred in a T100 Thermal Cycler (Bio-Rad) according to the manufacturer's recommended instructions with the addition of stem-loop oligonucleotide primers for mature miRNA transcripts.

Real-Time Quantitative PCR analysis was performed using a Bio-Rad CFX Connect Real-Time PCR Detection System. Acquisition of data was then performed on a Bio-Rad CFX Manager software. Each 20 µL reaction was done in triplicate, each replicate comprising of a miRNA-specific forward primer, which binds to a miRNA of interest, and a universal reverse primer that binds to the stem-loop structure created by the reverse transcription stem-loop primer. Both primers were used at a final concentration of 400 nM with 1 µL cDNA diluted five-fold, 7.4 µL of DEPC-treated water, and 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad). Sample data values from quantitative PCR analysis were normalized to U6 small nuclear RNA-2 (RNU6-2).

Target miRNA	Mature Sequence	Forward Primer	Reverse Transcription Stem-loop Primer
200a	CAUCUUACCGGACAGU	GCGGCCATCTTACCGGACA	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAGTGCTGGA
27b	AGAGCUUAGCUGAUUG GUGAAC	GGCGCAGAGCTTAGCTGAT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGATGGTGAAC
21	UAGCUUAUCAGACUGA UGUUGA	GGCGCGTAGCTTATCAGACT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAGATGTTGA
29b1	GCUGGUUUCAUAUGGU GGUUUAGA	GGCGGCTGGTTTCATATGG	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAGGTTTGA
29a	UAGCACCAUCUGAAAUC GGUUA	GCGGGTAGCACCATCTGAA	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAATCGGTTA
302c	UAAGUGCUUCCAUGUU UCAGUGG	GGCGCTAAGTGCTTCCATGT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGATTCAGTGG
126	CAUUUUUACUUUUGGU ACGCG	GGCCGGCGCATTATTACTTT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAggtacg GGTACGCG
146a	UGAGAACUGAAUCCA UGGGUU	GGCGCGTGAGAACTGAATTC	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGACATGGGTT
144	GGAUAUCAUAUAC UGUAAG	GCGGGCGGATATCATCATAT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAACTGTAAG
23a	GGGGUUCUGGGGAUG GGAUUU	GCGGGGGTTCCTGGGGA	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGATGGGATT
149	UCUGGCUCCUGUCUU CACUCCC	GGCTCTGGCTCCGTGTCT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGATCACTCCC
27a	AGGGCUUAGCUGCUUG UGAGCA	GGCGAGGGCTTAGCTGCT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGATGTGAGCA
20a	UAAAGUGCUUAUAGUG CAGGUAG	GGCCGCTAAAGTGCTTATAG	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAGCAGGTAG
92a	AGGUUGGGAUCGGUUG CAAUGCU	GGCGAGGTTGGGATCGGTT	GCTACTTAGCGCGCTACTTACTGGACACTGGCAGTCGCGAGCAATGCT
Primer	Sequence		
Universal Reverse Primer	GCGCTACTTACTGGACACTG		

**Table 1: List of Primers Used for qPCR Analysis of mature miRNA transcripts.**

Gene Target	Forward Primer	Reverse Primer
RNU6-2	TGGAACGATACAGAGAAGATTAG	TGGAACGCTTCACGAATT
GCLC	AAGTGGATGTGGACACCAG	CTGTCATTAGTTCTCCAGATGC
GCLM	GTTGACATGGCCTGTTGAG	AACTCCATCTTCAATAGGAGGT
HMOX1	AACTCCCTGGAGATGACTC	CTCAAAGAGCTGGATGTTGAG
NQO1	ACATCACAGGTAACTGAAGG	ACATCACAGGTAACTGAAGG
P21	GACACCACTGGAGGGTGACT	CAGGTCCACATGGTCTTCCT

**Table 2: List of Primers Used for qPCR Analysis of Gene Targets.**

#### Dichlorofluorescein Assay for ROS generation

To screen for ROS generation at a given time, 2',7'-dichlorofluorescein 3',6'-diacetate (Acros Organics) diluted in DMSO to make a 10 mM stock solution was diluted to 5  $\mu$ M with 1x Hank's Balanced Salt Solution (Cellgro). RPE cells were then incubated in this solution for 30 minutes before images were taken under fluorescent microscopy.

#### Lentiviral Transduction of RPE cells for Manipulation of miRNA Expression

miRNA decoy expression plasmids used in this study were purchased from the Addgene plasmid depository, including miR27b decoy (Addgene plasmid# 46603) and miR29b decoy (Addgene plasmid# 46605)[40] expression plasmids. The miRNA expression plasmid used for the overexpression of miR144-5p was purchased from Genecopoeia (HmiR0275-MR03). Data obtained from the use of this plasmid was referenced to the use of a non-silencing, scrambled miRNA expression plasmid that was also purchased from Genecopoeia (CmiR0001-MR03-B).

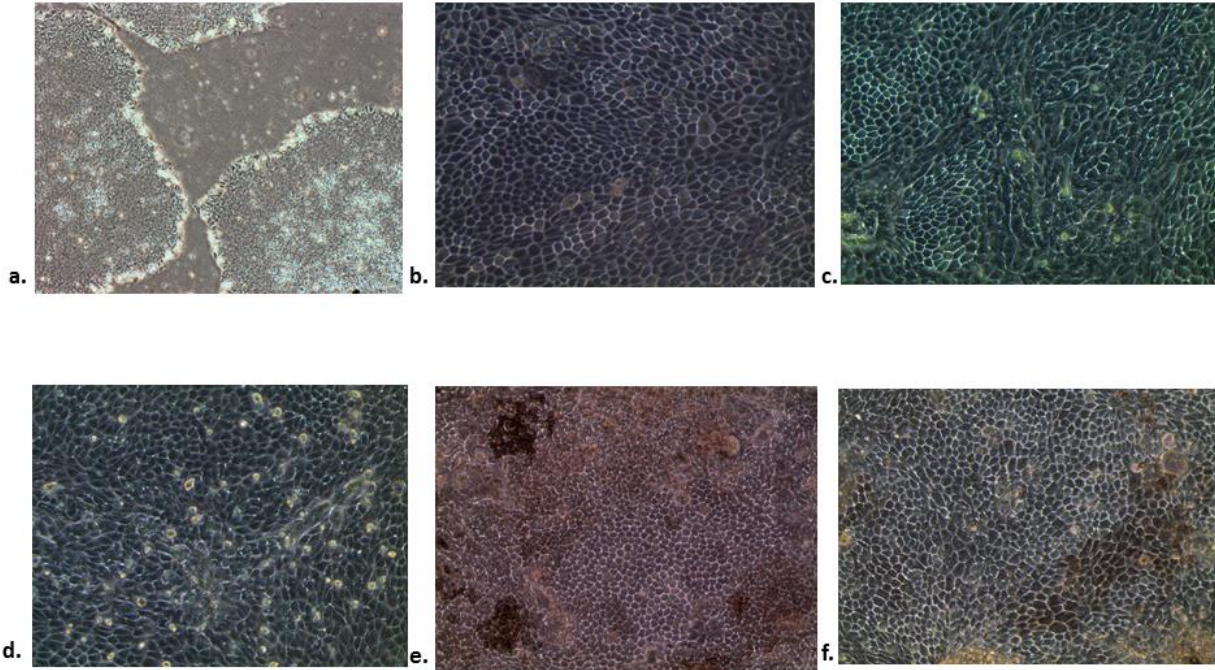


HEK293T cells were plated in 10-cm dishes and allowed to grow to ~70% confluency. 4 µg of miR decoy expression plasmid was incubated with 2 µg pMD2 VSVG and 2µg of psPAX2 lentiviral packaging plasmids in 750 µL of Cellgro Complete serum-free/low protein medium, 1X (Corning). 45 µL of *TransIT*-LT1 (Mirus Bio) also incubated with 750 µL of Celgro Complete medium. The two components incubated for 5 minutes at room temperature and then incubated together for 15 minutes. The mixture was then added directly onto the cells. The cells incubated at 37° C with 5% CO<sub>2</sub> for 16 hours at which point, the lentivirus-containing media was collected and replaced with Cellgro Complete medium with 1% FBS. Lentivirus-containing media was then collected every 12 hours for up to 48 hours post-transfection. Before transduction of RPE cells, lentivirus-media was filtered through a 0.45µm vacuum-driven filter.

Once filtered, virus-containing media supplemented with polybrene (at a final concentration of 4 µg/mL) was incubated with RPE cells over the course of 2 weeks, with media changes after every 2 days.

## Results and Discussion

### Establishment of Oxidative Stress Phenotype Reflective of AMD



**Figure 6: Establishment of the AMD phenotype by the induction of oxidative stress in hESC-derived RPE cells** **a.** Undifferentiated hESC colonies in feeder-free condition. **b.** hESC-derived RPE cells displaying typical cobblestone-like morphology without stressor treatment. **c.** hESC-derived RPE cells subjected to 1 week of treatment with hydroquinone. **d.** hESC-derived RPE cells subjected to an additional week of oxidant treatment, with 200 uM of hydroquinone. **e.** hESC-derived RPE cells subjected to 1 week of treatment with paraquat. **f.** hESC-derived RPE cells subjected to an additional week of paraquat treatment at a 160 uM concentration.

We began this study by the derivation of RPE cells from undifferentiated embryonic stem cells or iPSCs derived from patients affected with Leber's congenital amaurosis, as seen in Figure 6a. These cells were then subjected to treatment with DKK1, noggin, and IGF according to published protocols[11]. After 2-3 weeks of treatment with these factors, clusters of cells exhibiting a cobblestone-like epithelial morphology were manually extracted and transferred

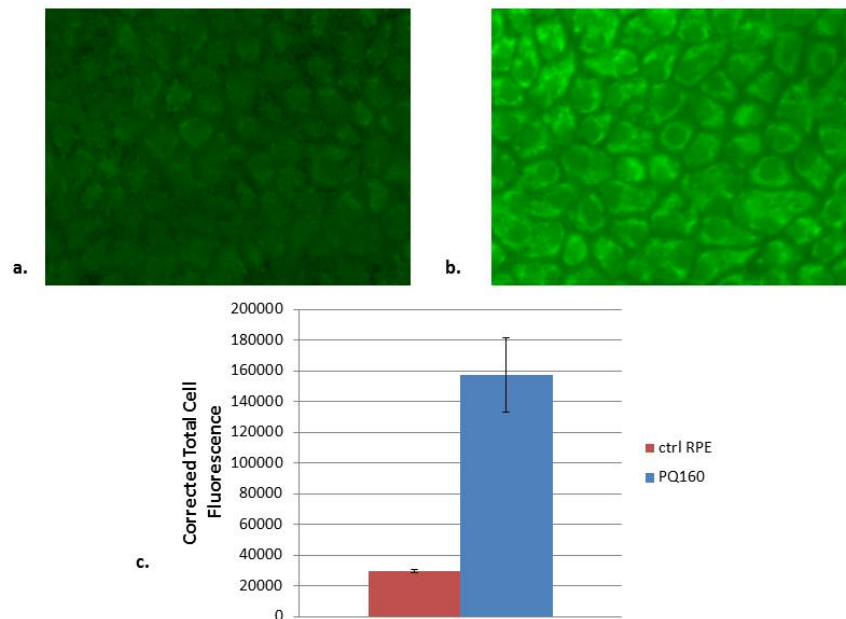
into separate culture vessels for proliferation and maturation, resulting in a monolayer of pigmented cells with a uniform morphology, as seen in Figure 6b.

We then conducted experiments to identify sublethal concentrations of hydroquinone and paraquat for the treatment of hESC-RPEs, which would allow us to induce an oxidatively stressed state with RPE cells without the activation of cellular pathways associated with programmed cell death. This will allow us to identify pro-survival molecular pathways involved in the cellular response to stress by PCR under high and low oxidative stress conditions. As noted previously, we also aimed to develop a protocol of oxidative stress induction based on the idea of a gradually accumulating stressor.

With the use of hydroquinone to induce a low stress phenotype, we found that the RPE cells were able to stay alive at 50  $\mu$ M of hydroquinone for 9 days, seen in Figure 6c at the 1-week time point, and then 100  $\mu$ M over the next 7 days. In order to induce a high, chronic stress phenotype, we found we were able to use 100  $\mu$ M of hydroquinone over 9 days, 200  $\mu$ M over the next 7 days, and then 400  $\mu$ M over the following day.

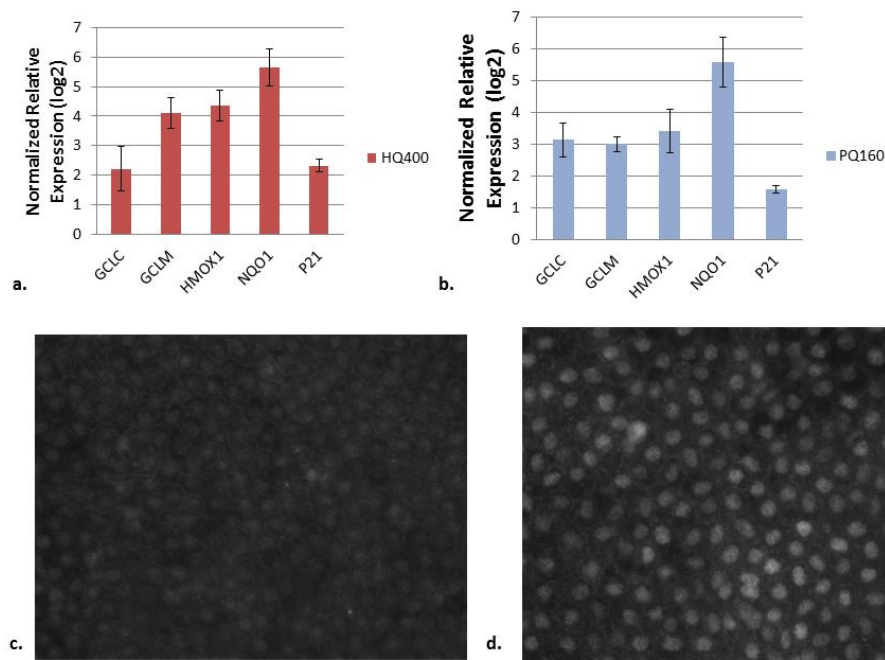
In terms of the using paraquat to induce a low stress phenotype, we found that RPE cells were able to stay alive at 20  $\mu$ M paraquat over 9 days, which is seen in Figure 6e at the 1-week time point, and then at 40  $\mu$ M over the following 10 days. To induce a high, chronic stress phenotype with paraquat, we were able to use 40  $\mu$ M of paraquat over 9 days, 80  $\mu$ M over the next 6 days, and then 160  $\mu$ M over the following 5 days. Upon further increase in concentration of either hydroquinone or paraquat or time of stressor treatment, RPE cells have often been seen to undergo apoptosis.

With the use of both paraquat and hydroquinone, we were able to observe a change in the traditional cobblestone-like morphology of RPE cells. Although this is less pronounced in the use of paraquat, we have noted that these cells take on a more elongated morphology reminiscent of fibroblasts, which we believe indicates an extent of an epithelial-to-mesenchymal transition which is reflective of the proliferative characteristics of RPE cells. We were able to then validate the generation of ROS associated with the incubation with oxidant stressor by DCF staining of intracellular ROS, as seen in Figure 7.



**Figure 7: Assessment of ROS Generation Associated with the use of Paraquat by DCF Assay.** **a.** Untreated hESC-RPE cells incubated with DCF, resulting in little to no fluorescence. **b.** hESC-RPE cells treated with paraquat at 160  $\mu$ M (PQ160) after increasing concentrations of stressor, resulting in higher fluorescence intensity as seen in the quantification in **c.** by Image J software.

## Correlation of Oxidative Stress Phenotype with NRF2/KEAP1 Activity and miRNA Analysis



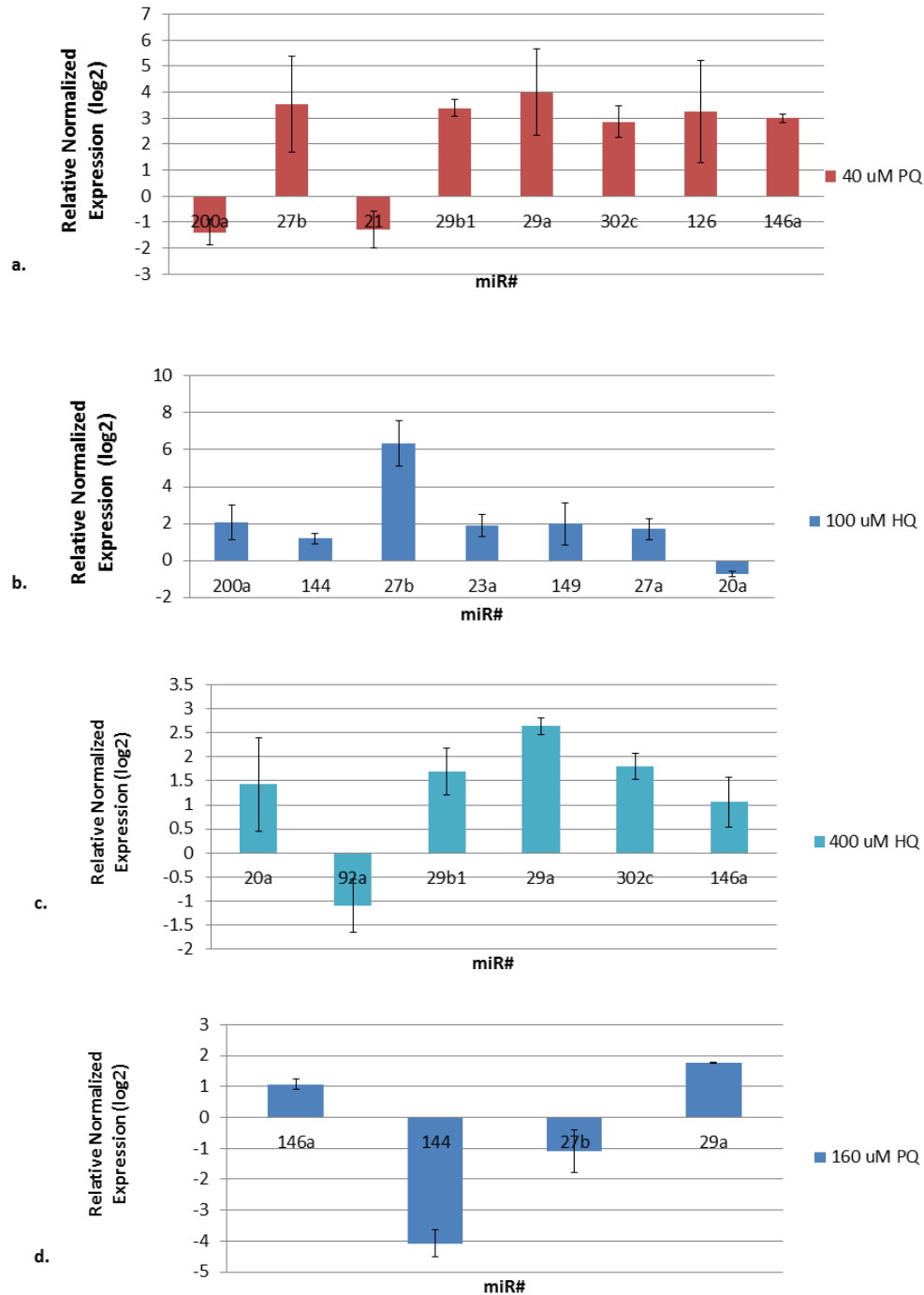
**Figure 8: Gene Analysis of Products of NRF2/KEAP1 Pathway Generated by Stress Signaling** Gene expression

analysis of RPE cells subjected to chronic oxidative stress by hydroquinone up to **a.** 400  $\mu$ M of hydroquinone (HQ400) and **b.** 160  $\mu$ M paraquat (PQ160). **c.** Unaffected RPEs compared to **d.** RPEs after 1 week treatment of 160  $\mu$ M paraquat, both stained for NRF2.

Once an observable phenotype was seen in cells treated with oxidative stressors, we sought a means to measure this phenotype through the analysis of pro-survival mechanisms. We elected to check for the activity of the NRF2/KEAP1 pathway, a pathway known to play a significant role in the production of endogenous antioxidant response elements (AREs) in lieu of stress signaling. As seen in Figure 8a and 8b, both paraquat and hydroquinone were able to induce the production of downstream products of the NRF2/KEAP1 pathway, including GCLC, GCLM, HMOX1, and NQO1 by at least a 4-fold increase compared to untreated RPE control cells. Interestingly, we also found that P21, a factor associated with cell cycle arrest and anti-

proliferative conditions[41] is also upregulated with stressor treatment. Additionally, p21 has been shown to stabilize NRF2 by competitive binding, preventing KEAP1 from binding to NRF2. This ultimately prevents the KEAP1-induced ubiquitination of NRF2 and therefore promotes a pro-survival oxidative stress response.[42] We have also been able to confirm the translocation of NRF2 into the nucleus and therefore the induction of endogenous antioxidant element expression by immunostaining, as seen Figure 8c and 8d.

Next, we looked for potential miRNA candidates that may exhibit significant change in expression due to oxidative stress signaling. Through various publications, we found a number of potential miRNA candidates that may play a role in oxidative stress signaling as well as cancer pathology. This list of miRNAs include miR200a[43], miR27b[44], miR23b[45], miR146a[46], miR29a[47], miR29b[48], miR143[49], miR144[50]. As seen in Figure 9a and 9b, various miRNAs such as miR27b, miR29b1, miR302c, miR126, and miR146a are upregulated by at least a 4-fold difference with a lower concentration of paraquat, and miR200a, miR27b, miR23a, miR144, miR149, and miR27a are upregulated by a similar amount. Both profiles suggest a negative regulatory effect on gene mRNA transcripts, which may in fact play a role in the survival of RPEs in light of an oxidant stressor. As seen in Figure 9c and 9d, various miRNAs such as miR20a, miR29b1, miR29a, miR302c, and miR146a are upregulated under chronic oxidative stress conditions with a higher concentration of hydroquinone. Interestingly, there are increases in the expression of miR146a and miR29a under chronic oxidative stress conditions with a higher concentration of paraquat, along with a rather striking decrease in miR144. This suggests a dramatic upregulation in the gene targets associated with miR144-target UTR binding.



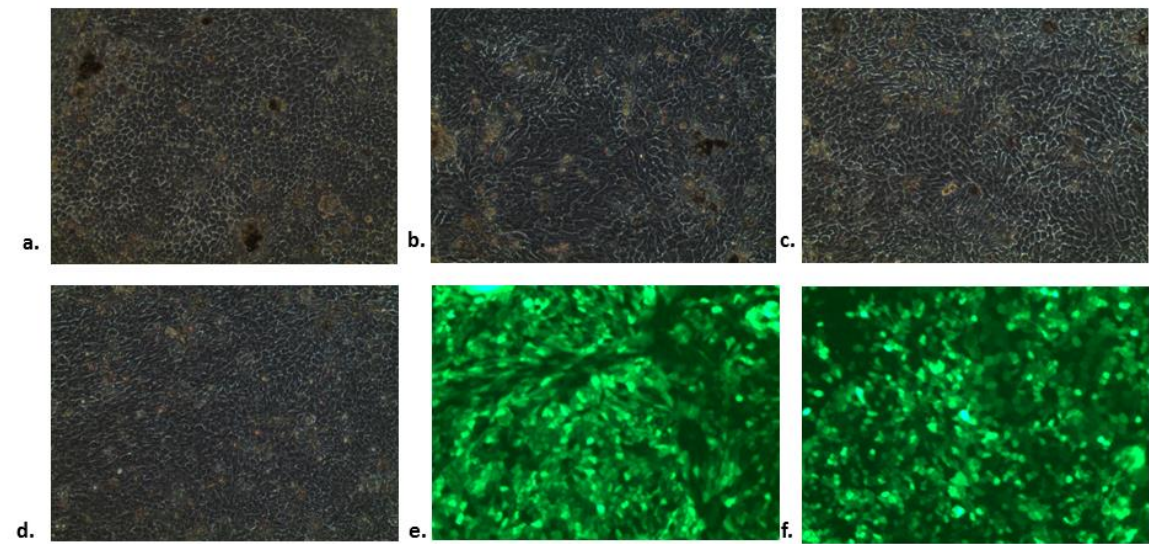
**Figure 9: Changes in miRNA expression profile associated with the oxidative stress phenotype** a. and b. miRNA expression profile of RPE cells subjected to low experimental oxidative stress with paraquat and hydroquinone. miRNA analysis was performed on RPEs treated with up to 40  $\mu$ M of paraquat (PQ40) and 100  $\mu$ M of hydroquinone (HQ100) with increasing concentrations of stressor as previously noted. c. and d. miRNA expression profile of RPE cells subjected to experimental chronic oxidative stress with paraquat and hydroquinone. miRNA analysis was

performed on RPEs treated with up to 400  $\mu$ M of hydroquinone (HQ400) and 160  $\mu$ M of paraquat (PQ160) with increasing concentrations of stressor as previously noted.

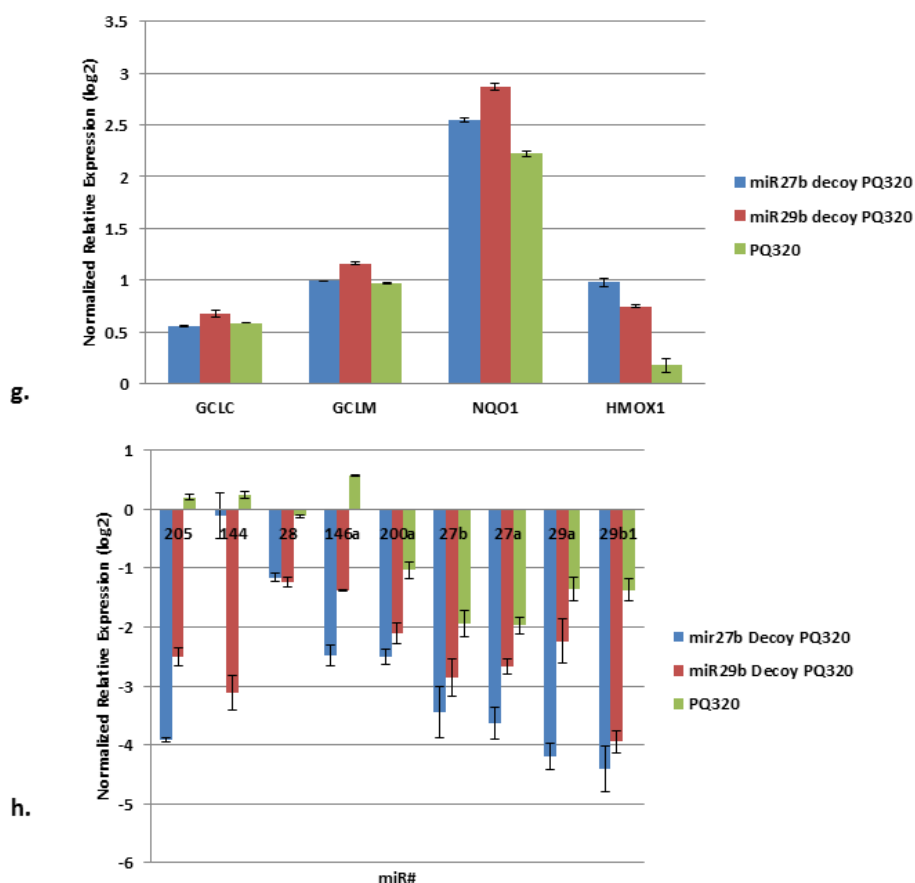
Low Stress	High Stress	Both
Up: miR126, miR149, miR23a, miR126	Down: miR144 Up: miR20a	Up: miR29b1, miR29a, miR302c, miR146a, miR27b, miR144, miR27b up in low stress, down in high stress

**Table 3: Changes in miRNA Expression Associated with High and/or Low Oxidative Stress conditions**

Modulation of miRNAs Associated with Oxidative Stress and its Effect on Stress Phenotype



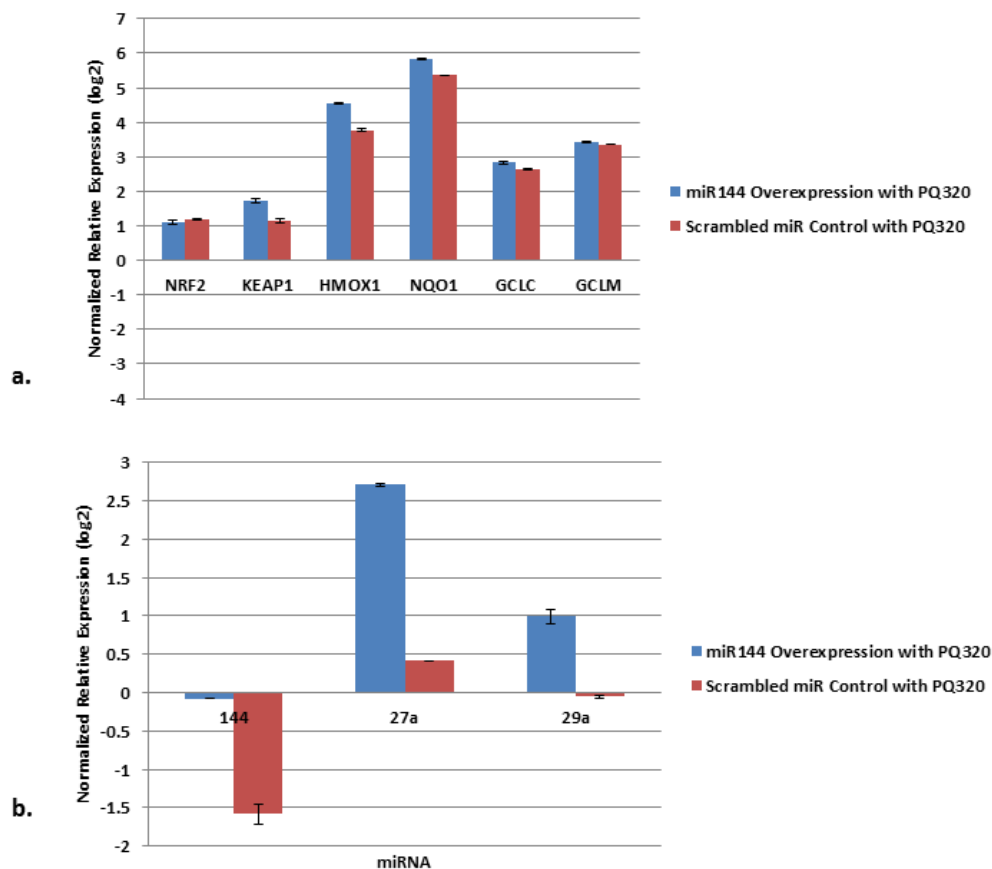




**Figure 10: Analysis of Oxidative Stress in Light of miRNA inhibition.** a. Untreated hESC-RPEs. b. RPE cells treated with 320  $\mu$ M of paraquat for 5 days. c. RPE cells expressing miR27b decoy treated with 320  $\mu$ M of paraquat for 5 days. d. RPE cells expressing miR29b decoy treated with 320  $\mu$ M of paraquat for 5 days. e. and f. miR27b and miR29b decoy RPEs (respectively) expressing GFP under fluorescent microscopy. g. Quantitative real-time PCR analysis of NRF2/KEAP1 pathway products expressed by miR27b and miR29b decoy-expressing RPEs after 5-day treatment with 320  $\mu$ M of paraquat. h. Quantitative real-time PCR analysis of pathology-associated miRNAs expressed by miR27b and miR29b decoy-expressing RPEs after 5-day treatment with 320  $\mu$ M of paraquat.

Based on the data described above, we next sought to directly assess the effects of the manipulation of miRNA expression on the induction of an oxidative stress phenotype and RPE survival. With regards to inhibiting miRNA expression, we specifically focused on two miRNAs, miR27b and miR29b using a lentivirus-mediated expression method to express miRNA decoy

constructs. As seen in Figure 10a-10f, despite robust expression of miR27b and miR29b decoys, there is no clear increase in RPE death over the course of a 5-day treatment with 320  $\mu$ M of paraquat. Although a mild disruption of the uniform, cobblestone-like morphology could be seen with paraquat treatment, the expression of the miRNA decoys didn't induce any morphology significantly different from that induced by paraquat treatment alone. Interestingly however, quantitative real-time PCR analysis showed an upregulation of HMOX1 by up to a 2-fold difference in RPEs expressing a miRNA decoy compared to unaffected RPEs treated with paraquat, as seen in Figure 10g. Even more intriguingly, it seems that the inhibition of miR29b and miR27b expression caused the inhibition of various other miRNAs such as miR205, miR28, miR146, miR200a, miR27a, and miR29a. This suggests either a global inhibition of the miRNA biogenesis pathway or potentially a cross-talk between the various oxidative stress response miRNAs and antioxidant response element genes. The decoys, through the activation of a negative regulator of other miRNAs, may have resulted in the inhibition of stress-associated miRNAs. Additionally, this suggests the striking upregulation of various other factors that may affect cell viability and functionality through the inhibition of miR27b or miR29b by miRNA decoy activity.



**Figure 11: Analysis of Oxidative Stress in Light of miR144 Overexpression.** a. Quantitative real-time PCR analysis of antioxidant response element expression after 1 week of treatment with 320  $\mu$ M paraquat (PQ320) under conditions with elevated expression of miR144, compared to treated hESC-RPEs expressing a non-silencing scrambled miRNA construct. b. miRNA analysis of the samples described in a.

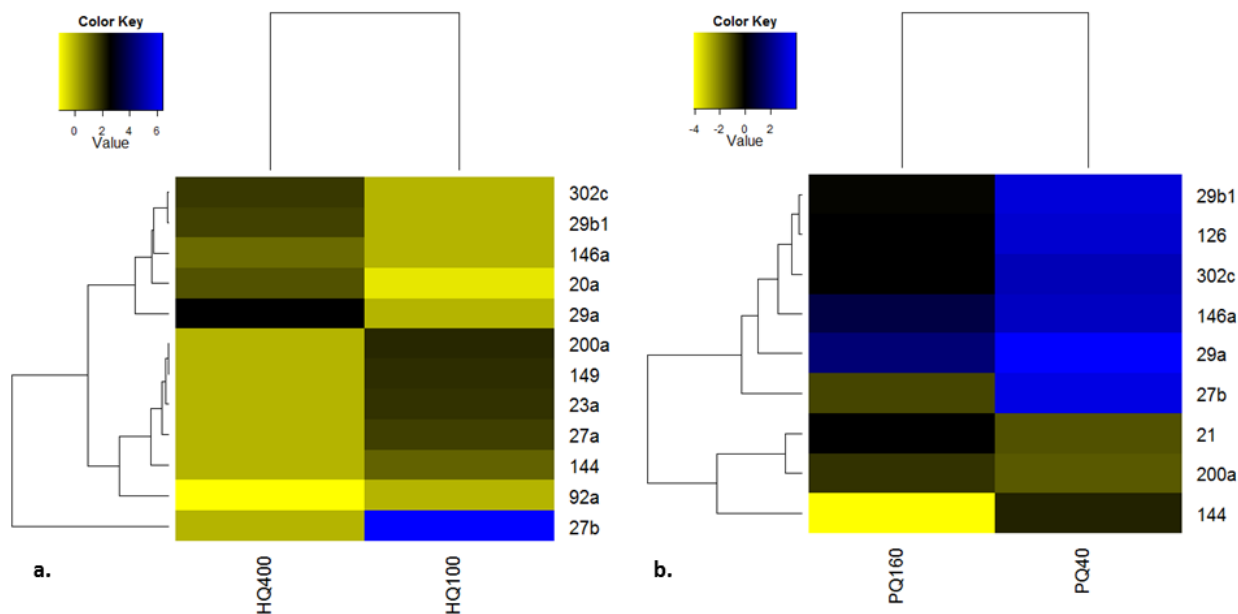
Because of the marked decrease in miR144 expression in high stress conditions, we also sought to assess the effects of miR144 overexpression on the oxidative stress response implemented by RPEs in high stress conditions. To address this question, we induced the elevated expression of miR144 as well as a scrambled, non-silencing miRNA construct in a separate culture, both via lentivirus-mediated expression in hESC-RPEs. As seen in Figure 11a, when compared to hESC-RPEs expressing a non-silencing miRNA construct, cells expressing

heightened levels of miR144 displayed slightly elevated expression of antioxidant response elements when treated with 320  $\mu$ M paraquat for 1 week, particularly of HMOX1 and NQO1 by approximately a 0.5-fold change. However, with regards to miRNA expression, the elevated expression of miR144 with a similar stress treatment has been seen to induce the elevation of miR27a and miR29a, which further points to the hypothesis that the expression of miRNAs involved in a similar cellular function are affected by a change in the expression of one of the miRNAs involved. In this case, a pro-survival change in miRNA expression induced the elevated expression of miRNA27a and miRNA29a both of which have been shown to play a role in stress conditions.

### **Conclusion**

The etiology of AMD is one with an exceptionally complex nature. It is one that researchers find difficult to define due to a lack of a good model system when the limitations presented by immortalized cell lines and animal models are taken into consideration. In this study, we have generated an *in vitro* disease model using human embryonic stem cell-derived RPE cells that we believe reflects the nature of age-related macular degeneration using a protocol that allows us to observe the oxidative stress phenotype using time-dependent increases in stressor concentration. Using this model, we have been able to observe changes in miRNA expression of miRNAs such as miR144, miR29b1, miR29a, miR27b, and miR23a, which have previously been implicated in cancerous conditions. Additionally, these microRNAs exhibit changes in expression between low, short-term stress and high chronic stress, suggesting miRNA-based mechanisms

for immediate and long-term stress response for the purpose of maintaining cellular homeostasis, as outlined in the heatmaps presented in Figure 12.



**Figure 12: Differential Expression Between Low, Short-Term Stress (HQ100 and PQ40) and High, Chronic Stress (HQ400 and PQ160) with the Use of Paraquat and Hydroquinone.**

We have also been able to correlate these findings with enhanced expression of products found in the NRF2/KEAP1 pathway. Interestingly, previously published publications indicate the anti-cancer role of NRF2/KEAP1 activity through cytoprotection against oxidant damage.[50] This, when taken into account the heightened expression of miRNAs such as miR29a and miR29b1, which have been speculated to have DNA-demethylating and anti-tumor capacities[48, 50], may indicate an anti-proliferative, antioxidant-associated mechanism used as a defense against cell death. However, further studies involving the expression of gene transcripts associated with cancer will have to be performed in order to further elucidate a

causal mechanism of action for such miRNAs which may play a role in pro-survival conditions in light of oxidative stress.

Of additional note, we have been able to make a preliminary observation of the effects of stress-related miRNA inhibition by the inhibition of miR27b and miR29b through the use of a decoy construct. We have also been able to note the effects of elevated stress-associated miRNA expression through the overexpression of miR144, which led to the increase in the expression of miR27a and miR29a, additional miRNAs implicated in RPE stress response. These observations suggest a mechanism of inter-miRNA communication as seen in current research,[50] where the inhibition of one miRNA affects the expression of various other related miRNAs which may play a role in biological mechanisms such as redox homeostasis. Further investigation will have to be performed in order to determine the role of factors that promote the repression of such miRNAs and then elucidate a mechanism portraying a regulatory network of stress-related miRNAs. However, such observations noted in this study suggest a promising potential in the role of miRNAs with regards to regulatory mechanisms that govern the response of RPE cells to chronic oxidative stress. This provides a new level of insight as well as potential target molecules of interest in studies involving the progression of AMD.

## References

1. Bharti, K., et al., *A regulatory loop involving PAX6, MITF, and WNT signaling controls retinal pigment epithelium development*. PLoS Genet, 2012. **8**(7): p. e1002757.
2. Kaarniranta, K., et al., *Autophagy and heterophagy dysregulation leads to retinal pigment epithelium dysfunction and development of age-related macular degeneration*. Autophagy, 2013. **9**(7): p. 973-84.
3. Rizzolo, L.J., *Barrier properties of cultured retinal pigment epithelium*. Exp Eye Res, 2014.
4. Wimmers, S., M.O. Karl, and O. Strauss, *Ion channels in the RPE*. Prog Retin Eye Res, 2007. **26**(3): p. 263-301.
5. Sparrow, J.R., D. Hicks, and C.P. Hamel, *The retinal pigment epithelium in health and disease*. Curr Mol Med, 2010. **10**(9): p. 802-23.
6. Lamba, D., M. Karl, and T. Reh, *Neural regeneration and cell replacement: a view from the eye*. Cell Stem Cell, 2008. **2**(6): p. 538-49.
7. Birch, D.G. and F.Q. Liang, *Age-related macular degeneration: a target for nanotechnology derived medicines*. Int J Nanomedicine, 2007. **2**(1): p. 65-77.
8. Frampton, J.E., *Ranibizumab: a review of its use in the treatment of neovascular age-related macular degeneration*. Drugs Aging, 2013. **30**(5): p. 331-58.
9. Maguire, A.M., et al., *Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial*. Lancet, 2009. **374**(9701): p. 1597-605.
10. Tucker, B.A., et al., *Use of a synthetic xeno-free culture substrate for induced pluripotent stem cell induction and retinal differentiation*. Stem Cells Transl Med, 2013. **2**(1): p. 16-24.
11. Lamba, D.A., et al., *Efficient generation of retinal progenitor cells from human embryonic stem cells*. Proc Natl Acad Sci U S A, 2006. **103**(34): p. 12769-74.
12. Mellough, C.B., et al., *Efficient stage-specific differentiation of human pluripotent stem cells toward retinal photoreceptor cells*. Stem Cells, 2012. **30**(4): p. 673-86.
13. Hambright, D., et al., *Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in un-immunosuppressed mouse retina*. Mol Vis, 2012. **18**: p. 920-36.
14. Lamba, D.A., J. Gust, and T.A. Reh, *Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice*. Cell Stem Cell, 2009. **4**(1): p. 73-9.
15. Warre-Cornish, K., et al., *Migration, integration and maturation of photoreceptor precursors following transplantation in the mouse retina*. Stem Cells Dev, 2014. **23**(9): p. 941-54.
16. Karl, M.O. and T.A. Reh, *Regenerative medicine for retinal diseases: activating endogenous repair mechanisms*. Trends Mol Med, 2010. **16**(4): p. 193-202.
17. Luz-Madrigal, A., et al., *Reprogramming of the chick retinal pigmented epithelium after retinal injury*. BMC Biol, 2014. **12**(1): p. 28.
18. Wang, S.Z., et al., *Generating retinal neurons by reprogramming retinal pigment epithelial cells*. Expert Opin Biol Ther, 2010. **10**(8): p. 1227-39.
19. Pinazo-Duran, M.D., et al., *Do Nutritional Supplements Have a Role in Age Macular Degeneration Prevention?* J Ophthalmol, 2014. **2014**: p. 901686.
20. Popp, N., et al., *Evaluating Potential Therapies in a Mouse Model of Focal Retinal Degeneration with Age-related Macular Degeneration (AMD)-Like Lesions*. J Clin Exp Ophthalmol, 2013. **4**(5): p. 1000296.
21. Cano, M., et al., *Cigarette smoking, oxidative stress, the anti-oxidant response through Nrf2 signaling, and Age-related Macular Degeneration*. Vision Res, 2010. **50**(7): p. 652-64.

22. Kunchithapautham, K., C. Atkinson, and B. Rohrer, *Smoke-exposure causes endoplasmic reticulum stress and lipid accumulation in retinal pigment epithelium through oxidative stress and complement activation*. J Biol Chem, 2014.
23. Szaflik, J.P., et al., *DNA damage and repair in age-related macular degeneration*. Mutat Res, 2009. **669**(1-2): p. 169-76.
24. Calippe, B., X. Guillonneau, and F. Sennlaub, *Complement factor H and related proteins in age-related macular degeneration*. C R Biol, 2014. **337**(3): p. 178-84.
25. Ding, J.D., et al., *The Role of Complement Dysregulation in AMD Mouse Models*. Adv Exp Med Biol, 2014. **801**: p. 213-9.
26. Pennesi, M.E., M. Neuringer, and R.J. Courtney, *Animal models of age related macular degeneration*. Mol Aspects Med, 2012. **33**(4): p. 487-509.
27. Jarrett, S.G. and M.E. Boulton, *Consequences of oxidative stress in age-related macular degeneration*. Mol Aspects Med, 2012. **33**(4): p. 399-417.
28. Finnemann, S.C., L.W. Leung, and E. Rodriguez-Boulan, *The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium*. Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3842-7.
29. Levonen, A.L., et al., *Redox regulation of antioxidants, autophagy, and the response to stress: Implications for electrophile therapeutics*. Free Radic Biol Med, 2014.
30. Kansanen, E., et al., *The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer*. Redox Biol, 2013. **1**(1): p. 45-49.
31. Mendell, J.T. and E.N. Olson, *MicroRNAs in stress signaling and human disease*. Cell, 2012. **148**(6): p. 1172-87.
32. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14*. Cell, 1993. **75**(5): p. 843-54.
33. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function*. Cell, 2004. **116**(2): p. 281-97.
34. Li, W.B., et al., *Development of retinal pigment epithelium from human parthenogenetic embryonic stem cells and microRNA signature*. Invest Ophthalmol Vis Sci, 2012. **53**(9): p. 5334-43.
35. Wang, F.E., et al., *MicroRNA-204/211 alters epithelial physiology*. FASEB J, 2010. **24**(5): p. 1552-71.
36. Kaneko, H., et al., *DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration*. Nature, 2011. **471**(7338): p. 325-30.
37. Howell, J.C., et al., *Global microRNA expression profiling: curcumin (diferuloylmethane) alters oxidative stress-responsive microRNAs in human ARPE-19 cells*. Mol Vis, 2013. **19**: p. 544-60.
38. Chang, X., et al., *Paraquat inhibits cell viability via enhanced oxidative stress and apoptosis in human neural progenitor cells*. Chem Biol Interact, 2013. **206**(2): p. 248-55.
39. Fiedler, S.D., M.Z. Carletti, and L.K. Christenson, *Quantitative RT-PCR methods for mature microRNA expression analysis*. Methods Mol Biol, 2010. **630**: p. 49-64.
40. Mullokandov, G., et al., *High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries*. Nat Methods, 2012. **9**(8): p. 840-6.
41. Gartel, A.L. and S.K. Radhakrishnan, *Lost in transcription: p21 repression, mechanisms, and consequences*. Cancer Res, 2005. **65**(10): p. 3980-5.
42. Chen, W., et al., *Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response*. Mol Cell, 2009. **34**(6): p. 663-73.
43. Eades, G., et al., *miR-200a regulates SIRT1 expression and epithelial to mesenchymal transition (EMT)-like transformation in mammary epithelial cells*. J Biol Chem, 2011. **286**(29): p. 25992-6002.



44. Lee, J.J., et al., *MiR-27b targets PPARgamma to inhibit growth, tumor progression and the inflammatory response in neuroblastoma cells*. *Oncogene*, 2012. **31**(33): p. 3818-25.
45. Jin, L., et al., *Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF-alpha in breast cancer*. *Cancer Res*, 2013. **73**(9): p. 2884-96.
46. Ji, G., et al., *MiR-146a regulates SOD2 expression in H2O2 stimulated PC12 cells*. *PLoS One*, 2013. **8**(7): p. e69351.
47. Zhong, S., et al., *MiR-222 and miR-29a contribute to the drug-resistance of breast cancer cells*. *Gene*, 2013. **531**(1): p. 8-14.
48. Amodio, N., et al., *DNA-demethylating and anti-tumor activity of synthetic miR-29b mimics in multiple myeloma*. *Oncotarget*, 2012. **3**(10): p. 1246-58.
49. Akagi, I., et al., *Relationship between altered expression levels of MIR21, MIR143, MIR145, and MIR205 and clinicopathologic features of esophageal squamous cell carcinoma*. *Dis Esophagus*, 2011. **24**(7): p. 523-30.
50. Narasimhan, M., et al., *Identification of novel microRNAs in post-transcriptional control of Nrf2 expression and redox homeostasis in neuronal, SH-SY5Y cells*. *PLoS One*, 2012. **7**(12): p. e51111.