Honors Project. Determining The Window Of Rhodopsin Degradation By RPE Cells

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Abstract: Macular Telangiectasia (MacTel) is a rare, late onset, degenerative eye disease whereby the photoreceptors (cones and rods) of the macula become diseased and slowly degenerate, thus reducing vision. These photoreceptors are subjected to harsh stresses, which cause them to shed the outer 10% of each cell on a nightly basis. This results in the build up of debris which another cell type, the retinal pigment epithelium (RPE) removes and recycles every night to reveal a fresh photoreceptor free of debris[4]. Excessive debris accumulated between the retina and RPE cells has been consistently observed in post mortem retinal samples from patients with MacTel .[3] We hypothesize that patients with MacTel may have RPE cells with reduced functionality, which ultimately reduces their efficacy of clearing debris and contributes to debris accumulation over time, leading to photoreceptor death and loss of vision.[2] To test this hypothesis, iPS cells will be collected from MacTel patients and differentiated into RPE cells. These cells will then be compared with iPS-derived RPE from normal (non-diseased), age-matched controls for the efficiency of rhodopsin degradation, a function of normal operating RPE cells. We will describe efforts to create and optimize a phagocytosis assay whereby photoreceptor outer segments are 'fed' to normal RPE and then the time-course for degradation of internalized rhodopsin is assayed using western blot analyses. This work will 1) provide a key functional assay that is optimized for future use with the more precious iPS-derived RPE, and 2) establish a baseline of normal RPE phagocytosis and debris processing.

Background

The eye is an incredible organ. Cornea cells undergo a crystallization process that allows for transparency and protection. They encapsulate the Iris, which ranges in colors from shades of blue to green, browns and onyx. The eyes are said to be the crown jewels of the body. In the brain, there are hundreds of millions of neurons devoted to visual processing and take up about 30 percent of the cortex[1]. When light enters the eye through the cornea, it travels to the lens where it is refracted and focused to the back of the eye on the retina, with the majority of light focused onto the central macula, and particularly the foveal center (Figure 1). Residing at this site of central vision are photoreceptors that enable high-resolution vision called Cones. In contrast, Rods become the prominent photoreceptor beginning at the outer regions of the macula and extending to the peripheral borders of the eye. Rods are photoreceptors necessary for high sensitivity, but

convey only low-resolution vision[7]. Together, the Rods and Cones comprise the cell types known as the Photoreceptors and they are responsible for our impressive ability to see vibrant colors and avoid end tables in the dark.

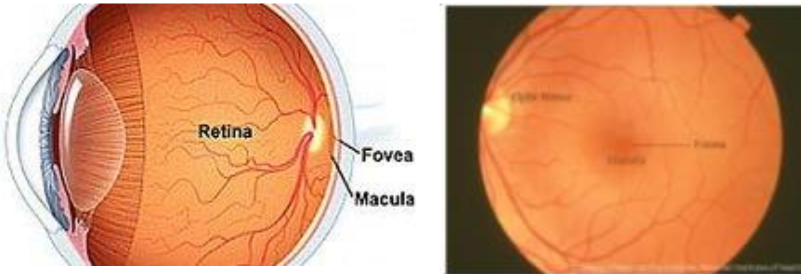

Figure 1: The retina absorbs light focused by the lens. The light from the central part of your vision is mainly focused onto the macula region, particularly centered at the fovea, where the highest density of Photoreceptors is located.

The stresses of light cause damage to these Photoreceptors and in order to maintain proper eyesight, the outermost 10% of the cones and rods known as Photoreceptor Outer Segments (POS), is shed every night[6]. This process helps ensure that the outer segment disks, the regions of the photoreceptors that absorb light and initiate the visual signals that are eventually processed by the brain, only live around ten days with new segment discs being made every night. The photoreceptors constantly make new outer segment discs that are placed at the proximal most ends of the POS. This pushes the older discs further distal until those that are about 10 days old make up the distal-most 10% of the POS and will be shed each night.[7] This process is energetically expensive for the retina, but is worth it to prevent build-up of oxidative radicals and other results of accumulated light-damage within the cells, which would ultimately cause the death of the photoreceptors. This nightly shedding process creates sub-retinal debris consisting of the shed photoreceptor segment discs, which must be dealt with in order to maintain retinal function. [3] Think about what would happen if the trash was not taken out and recycled from your own living area. Eventually the space would fill, and you would loose various functions a living area provides; this would be the result in the retina if the shed POS were not completely removed and processed. Retinal Pigment Epithelial cells (RPE) phagocytose and recycle this debris as one of their several functions (Figure 2).

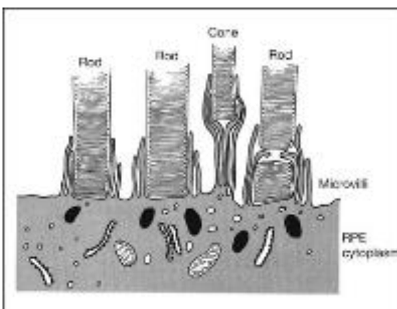

Figure 2: Photoreceptor outer segment discs mature with distal migration towards the RPE. New segments are replaced at the proximal edge of the photoreceptor, while the aged (damaged and over stressed) discs shed at the distal tip and are engulfed then recycled by RPE cells for degradation.
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Macular Telangiectasia (MacTel) is a rare, late onset, degenerative eye disease whereby the photoreceptors (cones and rods) of the macula become diseased and slowly degenerate (die), thus reducing vision. Symptoms begin in patients in their early 50's and are estimated to affect between ~1 in 10,000 – 100,000 people between the ages 40 – 80[2]. However, MacTel is likely to be under-diagnosed due to lack of good diagnosis procedures, stemming from poor insight about the macula region and the fact that most retinal doctors are not yet aware of this condition and thus misdiagnose it. For this project, we addressed the issue of why photoreceptors are dying in MacTel patients.[2] We hypothesize that MacTel may involve defective RPE cells resulting in inefficient phagocytosis and processing of shed photoreceptor outer segments. Overtime, accumulation of this debris causes death of photoreceptors. This hypothesis is supported by analysis of post mortem patients with MacTel, which showed an accumulation of debris between the photoreceptors and the RPE layer (Figure 3).

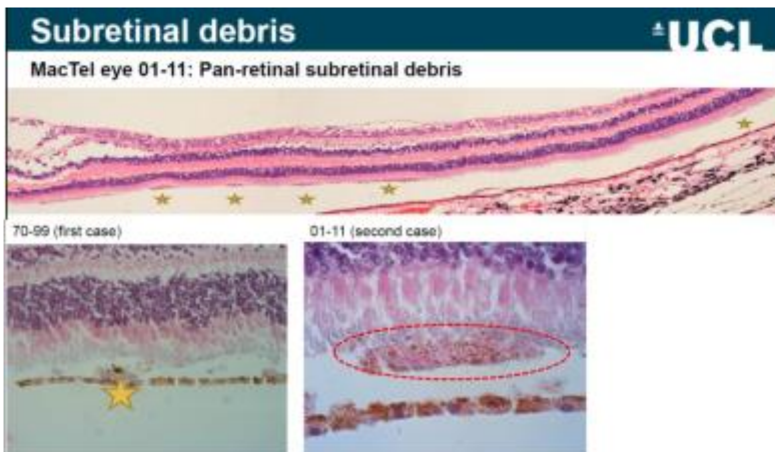

Figure 3: In post-mortem sections from patients with MacTel, subretinal debris accumulation is observed between the retina (top) and the RPE layer (bottom). This abnormal debris is depicted by stars and then one large debris is circled in the lower right image. (image courtesy of Dr. Mike Dorrell and Dr. Marcus Fruttiger; unpublished)

Since MacTel samples are not directly attainable from live patients, people are opposed to having tissue removed from their retina, for good reason. Thus, we must obtain RPE samples from MacTel patients in other ways. To evaluate our hypothesis of MacTel patients having less efficient RPE cells we set out to create induced pluripotent stem cells (iPSCs) from blood cells taken from MacTel patients and then differentiate them toward RPE cells. These induced RPE cells will be compared with induced RPE cells from healthy non-MacTel patients as a control. Assuming that MacTel has a genetic component, which is supported by family heritance patterns[5], the induced RPE cells from MacTel blood cells will retain the abnormal properties that relate to the disease once they are re-programmed into RPE cells. Our aim is to first generate a baseline of RPE maturation and RPE phagocytosis. First we need to determine how long it takes for a normal type RPE cell to mature. One of the main functions of the RPE is to form a tight monolayer that regulates passage of all materials, including liquids thus; RPE cells form a monolayer that is indicative of maturation[6]. Once a monolayer is formed via tight junctions, dye will no longer be able to travel across a trans-well, and will be contained outside the monolayer. Maturation recognition can also be accomplished

by determining at what time RPE cells release the survival protein PEDF, which is only expressed by mature RPE cells. [6] Thus, we will also analyze RPE maturation by determining when the cultured RPE cells begin to express PEDF.

Once we know when maturation takes place, cells can be used that have been cultured for an optimal amount of time to ensure maturity without waiting longer than necessary to test for RPE efficiency. One of the key features in this is how well RPE cells from both groups phagocytose the photoreceptor outer segment (POS). These studies are being performed at the Lowy Medical Research Institute. However, another aspect of RPE efficiency is based on the ability to degrade and recycle the POS components once they have been phagocytosed. This can be analyzed by determining how long it takes RPE cells to degrade rhodopsin, the light sensing protein within rods and thus one of the major proteins in a POS. One can imagine that inefficient processing of the POS segments would cause debris to build up within the RPE, thus slowing their ability to recycle key factors. Eventually, this debris within the RPE would reduce the RPE's health and result in the inability to perform the key function of POS debris removal. Since the functions of RPE cells and Photoreceptors follow a circadian rhythm, we hypothesize that healthy RPE cells can accomplish this in under 24 hours (although such a timeline has yet to be established), and MacTel RPE cells to be less efficient to some degree.

By determining a baseline for RPE maturation we can compare if MacTel RPE cell types are maturing at the same rate as normal (non-diseased) RPE cell types later on. Subsequently, determining a normal window of Rhodopsin degradation by normal RPE cell types can provide us with a standard with which to compare MacTel RPE cell types in order to determine their efficacy. This will allow us to evaluate if the disease might be caused by decreased efficiency of phagocytosis and processing of POS by MacTel RPE. This window of normal degradation may also be useful as a better diagnostic test for suspected MacTel patients, as well as to better monitor MacTel patients' status of debris degradation.

Methods and Results

RPE formation of monolayer

Transwell plates are designed to have 2 components, an upper well where cells are cultured and a lower well. To test for the presence of a monolayer, we cultured RPE cells onto Trans-well plates to get a qualitative assessment to narrow down the window maturation, which would be later confirmed by our qPCR results (Figure 4). A qualitative time frame was established by injecting dye into the transwell, and waiting for an hour to determine (visually), if any of the dye had transferred into the lower well. Through our tests, we established that RPE cells form a monolayer around 5-7 days.

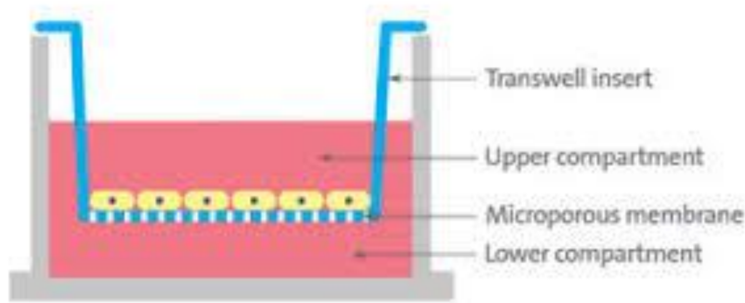

Figure 4. Transwell set up. RPE cells cultured in the Transwell insert, and tested for dye retention to indicate monolayer formation.

RPE maturation determination:
We analyzed the length of time that it takes cultured RPE cells to mature by analyzing levels of PEDF expression in RPE cells that have been cultured for differing amounts of time (younger vs. older). This will give us information about how long subsequent RPE cells derived from iPSCs will need to be cultured before full maturation allows us to analyze their phagocytosis functionality. It is desirable to always compare mature RPE, but using the shortest amount of time possible for model system efficiency and cost purposes. qPCR was used to analyze PEDF expression, a marker of mature RPE cells, in order to establish a baseline of RPE maturation.

Homogenization:
RPE cells were cultured then rinsed with ice cold PBS. The cells were lysed directly in a culture dish by adding 1 ml of TRIZOL Reagent per 3.5 cm diameter dish and scraping with rubber policeman scraper. The cell lysate was passed several times through a pipette and vortexed thoroughly. The amount of TRIZOL reagent added is based on the area of the culture dish (1 ml per 10 cm2).

Phase separation:
0.2 ml of chloroform was added per 1 ml of TRIZOL Reagent. Tubes were capped and vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. Samples were then centrifuge at 12,000 x g for 15 minutes at 2C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was transferred carefully without disturbing the interphase into a fresh tube.

RNA precipitation:
RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 mL of isopropyl alcohol per 1 mL of TRIZOL Reagent used for the initial homogenization. Samples were then incubated at 20C for 10 minutes and centrifuged at 12,000x g for 10 minutes at 2C. The RNA precipitate formed a gel-like pellet on the bottom of the tube.

RNA wash:
Supernatant was removed completely and the RNA pellet was washed once with 75% ethanol, adding 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. The samples were mixed by vortexing and centrifuging at 7,500 g for 5 minutes at 2C.

cDNA preperation:
In a PCR tube, 5 µg of RNA was added to 1 µL of the gene-specific primer PEDF, 1 µL of Annealing Buffer and 8 µL of RNase/DNase free water. The PCR tube was then incubated in a thermal cycler at 65°C for 5 minutes, and then immediately placed on ice for 1 minute. Then while on ice, 2X First-Strand Reaction Mix 10 µl SuperScript™ III/RNaseOUT™ Enzyme Mix 2 µl were added. The sample was then vortexed briefly to mix, collected by brief centrifugation and incubate as follows: 50 minutes at 50°C followed by termination of the reactions at 85°C for 5 minutes. The 5µL 0f cDNA from each test group was then added into a 96 well plate with 245µL of SYBR green indicator, which use 2 sets of primers that flank either side of the PEDF transcripts. The plate was read using a qPCR to indicate which test group of RPE cells was expressing PEDF.

Our results indicate that PEDF expression begins to significantly rise above the expression levels of GAPDH and Ribosomal proteins at day 5 and then peak around day 14 (Figure 5). This suggests that RPE cells begin to show signs of maturation as early as 5 days and have fully matured within 14 days.

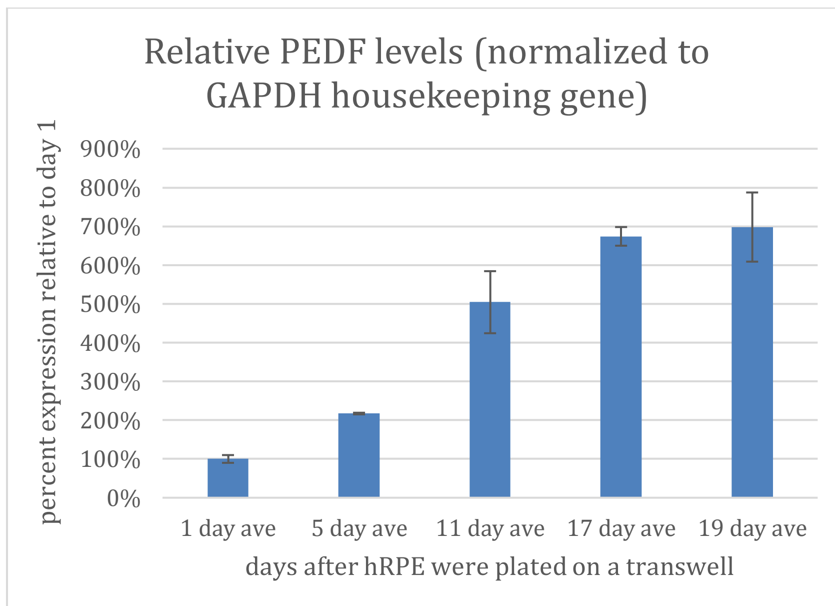

Figure 5. PEDF expression normalized to housekeeping genes- GAPDH and Ribosomal proteins. Compared to levels of PEDF expressed by immature RPE one day after plating (100%), PEDF expression dramatically increases around day 5 (200%) and plateaus around day 14, two weeks after plating (500% – 700%). This suggests that RPE cells are fully mature within at around 2 weeks after plating.

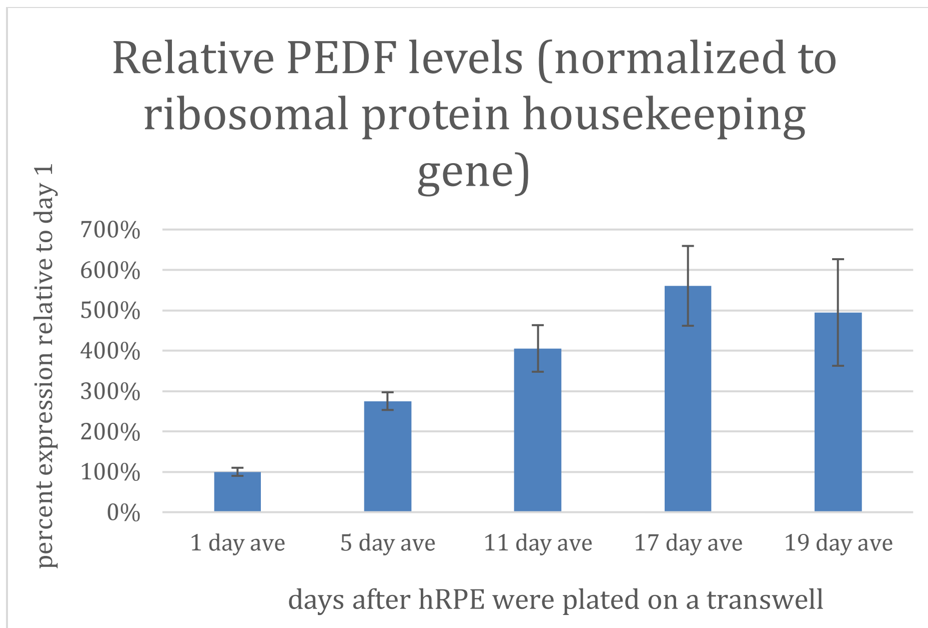

Figure 5b. Comparing PEDF expression with a second house-keeping gene for ribosomal protein production confirms that upregulation of PEDF expression begins around 5 days and plateaus around 2 weeks.

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Project #2: Western Blot to find window for Rhodopsin degradation:
Photoreceptor outer segments (POS) will be added to the RPE cells for 4 hours, allowing the RPE to bind and phagocytose the POS. The presence of rhodopsin within the RPE cells will then be analyzed at different times following POS exposure as an indication of whether or not the POS debris has been degraded. We should see Rhodopsin in wells where the cells have had less time to process the POS (thus these cells have some intact rhodopsin), but not in wells with RPE given adequate (longer) time to process and degrade the phagocytosed POS debris… thus establishing a window for normal POS degradation by healthy RPE.

There were several areas of optimization that were performed in order to derive the final assay. 1) Several concentrations of POS added to the RPE were tested. 2) Time of exposure of the RPE to the POS before washing any unbound POS and starting the "degradation time clock" was compared for 2 hours vs. 4 hours exposure, 3) Western blot conditions, including gels, buffers, antibodies, and antibody binding conditions, and 4) concentrations of RPE lysate loaded onto the western blot gels for analysis.

Plating Wells:
200,000 of the appropriate cells (RPE or control L-cells) were added into each well, with an adequate number of wellsfor 5 different time points with 3 controls. Cells were incubated and maintained for 14 days to allow for a monolayer to form and for the cells to become mature. The experiment was split in 5 test groups, each analyzed at varying time following the end of the 4-hour exposure to POS; 12 hours,

18 hours, 24 hours, 36 hours, and 48 hours. Negative controls: L-cells (cannot phagocytize) and hRPE that were never exposed to POS. Positive control for rhodopsin will be POS directly lysed in RIPA buffer; photoreceptor outer segments will have extensive amounts of rhodopsin. Additionally, a RPE group was exposed to POS for 4 hours then immediately lysed (0 hour), having time to uptake Rhodopsin, but not enough time to degrade it.

Adding POS:
Photoreceptor outer segments were added to each well at a concentration of 20 uL per well (optimization #1; differing amounts of POS were added during earlier permutations to determine the maximum level of POS addition that could be added without inducing RPE toxicity). POS were then incubated with hRPE for 4 hours with warm media (optimization #2 based on results of the timecourse for phagocytosis based on results from LMRI). The media was then removed and the RPE cells were gently washed 2x with PBS, then 1x with media (500 uL) for 5 mins each. The final wash was removed and 1 mL of fresh media was added and the cells were incubated for the duration of the time course for each group (12, 18, 24, 36, and 48 hours until time to isolate protein.

LYSATE ISOLATION.

Media was removed and the cells washed gently with ice-cold 1x PBS keeping cells on ice. PBS was then removed and cells were dried as much as possible (keeping plate of cells on ice). Ice-cold RIPA buffer was added (500 uL per well) and cells were incubated in this in lysis buffer (RIPA) for 15 – 20 minutes on ice. The bottom of the plate was scraped with a cell scraper to help remove cells and the solution was pipetted up and down to fully lyse cells. The cellular lysate (liquid) was removed from the wells of the plate and put into Eppendorf. All tubes were kept on ice then centrifuged @ 13,000 rpm in refrigerated centrifuge for 10 minutes (at 4 degrees Celsius). The supernatant was then collected from the pellet and added to labeled Eppendorf tubes. A bradford assay was performed to get see how much protein was in each Lysate well by comparing the wells to known concentrations from a standard protein that was serial diluted. Samples were made into aliquots, each sample into 40 ug / tube with each tube labeled clearly and the final concentration labeled. 5 uL of standard or unknown was added to the appropriate wells of a 96 well plate along with 250 uL of Bradford QuickStain to each well then measured at 595 nm on the microplate reader. A standard curve from known concentrations was then made. 20 ug of protein lysate was loaded onto the gel (~3 ug of photoreceptor outer segment positive control), RPE and L-cell lysate.

WESTERN BLOT ANALYSIS:

The gel is comprised of a Bolt 8% gel and Bis-Tris Plus running buffer. The gel was ran at 200 V for about 20 minutes. Then transferred using Mini gel tank: Transfer buffer = 25 mL 20x transfer buffer (we used NuPage Bis-Tris because we didn't have

any Bolt transfer buffer, even though the gel we used was Bolt) + 50 mL of methanol + 1 mL antioxidant + 425 mL HiQ water (bringing it all to 500 mL). Run for PVDF paper at 20V for 1 hr (nitrocellulose would be 10 V for 1 hr). Note: this did not seem to transfer all of the protein. Not sure if it was because it wasn't the correct buffer, or if it just needs longer in general. Because of issues with the gel transfer, we optimized the running and transfer buffers and altered the conditions for the electrophoresis (optimization #3).

Attempt 2:
Using Bolt 12% gels this time, with the correct Bolt running buffer, and loading as much protein lysate as possible onto the gels. We also used the more sensitive SuperSignal West Pico chemiluminescence. 55ug of Lysate, 6uL of 4X sample buffer, 2.5 uL of 10X reducing agent and water filled to make16.5 uL total. We used all of the RPE1 samples and the L-cell1 samples, with the exception of using RPE2; no POS since the RPE1; no POS sample is low with regards to its concentration. We also used 55 ug to maximize the amount of protein lysate that we will add and maintain about 25 uL total to load in the different wells.

We ran the transfer at 20V constant for 1 hr 45 minutes and the transfer of the molecular weight marker still wasn't complete. Changed to 200 mA constant for another 30 minutes. The pvdf membranes had some very strange debris all over them after transfer (RPE more than the L-cells, but the RPE was the only one that had the extra 30 minutes at 200 mA). The membranes almost look slightly transparent there. We blocked for 6 hours to try to get rid of debris and then added the primary antibody solution with a 1:1000 dilution of anti-rhodopsin antibody. The anti-Rhodopsin with indicator bound to the positive control wells of both the L cell negative control group and the RPE test group (Figure 6), but not in any other test well. We think this is because not enough protein was in the RPE Lysate wells. However, this indicates that our protocol is working because we got positive results in the wells with positive controls (L Cell wells, and POS well).

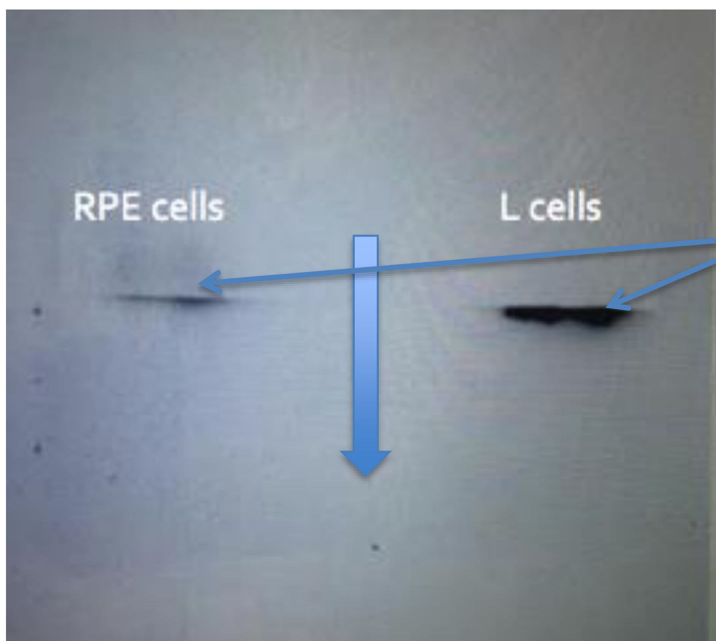

Figure 6. Western Blot image shows the anti-Rhodopsin antibody bound to Rhodopsin from the photoreceptor outer segment lysate. This specific signal in our positive control well indicates that the western blot conditions are working, but we just do not have adequate lysate from the lysed RPE cells.

Attempt 3

In order to generate higher concentrations of RPE lysate for gel loading, we performed the optimized phagocytosis assay again, with the intention of combining more lysate from each time point into concentrated samples. However, upon analysis of the protein lysate concentrations by Bradford assay, it was obvious that we had not obtained any protein lysates in our samples during this trial (Figure 7). Thus, we have successfully optimized the western blotting conditions, but need to load higher concentrations of RPE lysate in order to be able to visualize the remaining, unprocessed rhodopsin. It should be noted that, while rhodopsin is a major component of the POS, it is not naturally expressed by RPE. While this makes it a good candidate for analysis of POS processing by RPE, it also means that it's protein concentration will be a very small fraction of the whole RPE lysate that is loaded onto the wells of the western blot.

The attempt to produce a western blot image revealing a window of degradation was abandoned after the Bradford assay procedure. We suspect there was a problem with the RPE cells before the POS were added which resulted in the death of most if not all of the RPE cells (Figure 8).

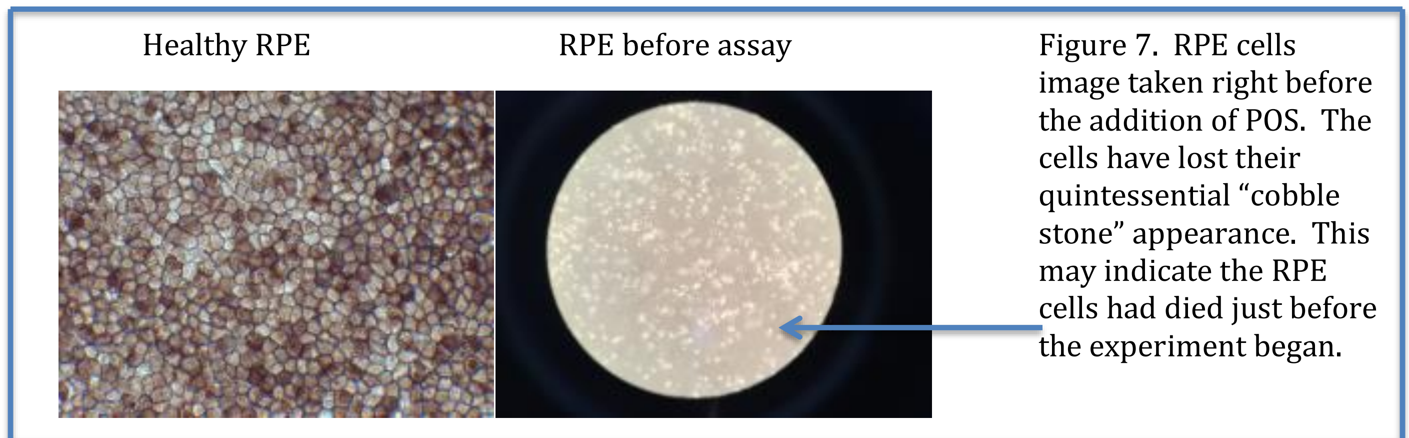

Figure 7. RPE cells image taken right before the addition of POS. The cells have lost their quintessential "cobble stone" appearance. This may indicate the RPE cells had died just before the experiment began.

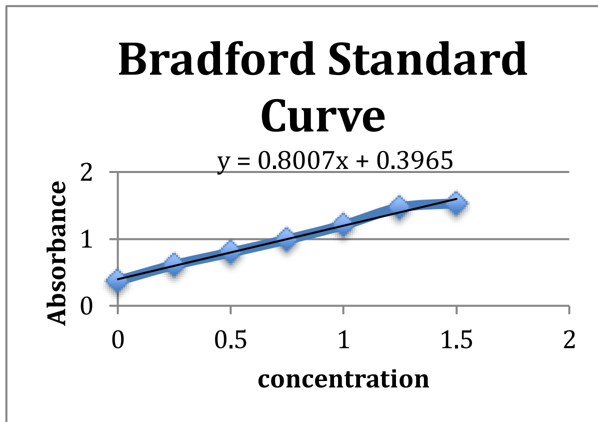

Figure 8. Analysis of RPE protein concentration did not rise above the absorbance levels of 0 mg/mL concentration from the known Bradford reagent.

Lysate Test Times	Absorbance at 595 nm	Calculated Protein Lysate Concentration
0H	0.308	-.11 mg/mL
12H	0.450	.067 mg/mL
18H	0.447	.063 mg/mL
24H	0.448	.064 mg/mL
36H	0.424	.034 mg/mL
48H	0.401	.0056 mg/mL
POS	0.334	.33 mg/mL
L cell	0.378	-.023 mg/mL

Conclusions:

Western Blot: The results from the lysates are inconclusive. After 3 attempts we have developed a functional assay that is optimized for future use with the more expensive iPS-derived RPE cells. If more time and POS were available the experimental assay would likely prove to be optimized, although the one problem with obtaining high enough concentrations of RPE lysate remains. We have proven the concept works as depicted by Rhodopsin being seen in the positive control well (Figure 6). This shows that our assay can identify the presence of Rhodopsin, but due to very small protein concentration within the well, a window of degradation could not be visualized in the experiment. Next, I would like to run the experiment again with more protein isolated from lysates to establish a window of degradation. Additionally, I would like to have more testing times to include more time points prior to 24 hours to better define the window of rhodopsin degradation. Then, the assay is ready for economically feasible testing of Rhodopsin degradation with MacTel iPS cells differentiated to RPE for comparison.

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