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An exploration of RNA and miRNA expression and their role in cell cycle regulation of human primary trabecular meshwork cells

Kyle Joseph Gonsalves
University of Iowa

An Exploration of RNA and miRNA Expression and Their Role in Cell Cycle Regulation of Human Primary Trabecular Meshwork Cells

by

Kyle Joseph Gonsalves

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Markus Kuehn, Associate Professor

SCHOLARLY ABSTRACT

Kyle Gonsalves,[1,3]; Tania, Cruz[1]; Lin Cheng[1,3]; Kuehn, Markus[1,3,4]

[1]Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, IA, USA [2]Department of Pediatrics, University of Iowa, Iowa City, IA, USA [3]VA Center for the Prevention and Treatment of Visual Loss, Iowa City, IA, USA [4] Wynn Institute for Vision Research

In the Kuehn lab, it has been shown that inducible pluripotent stems cells that have been induced to be trabecular meshwork cell-like (iPSC-TM) have a unique ability to regenerate dysfunctional trabecular meshwork (TM) cells by sharing specific unknown factors. In this thesis will discuss the novel means by which I isolate primary human Trabecular Meshwork (pTMs) and efficiently prepare cell cultures for experimentation, such as a sequencing experiment in which I studied expression changes that arose when the TM cell culture's cell cycle control is manipulated. Previous research has shown that pTM grow atypical when 100% confluent compared to other epithelial cells creating an interesting timeframe by which to observe their unique cell cycle control. Using newly isolated TM cell cultures I investigated expression of mRNA and miRNA to understand their roles in cell cycle control of these atypical cultures. With regards to the isolation of TM cell cultures were able to show that the "Crawling Out" methodology is an effective way to establish a pure TM cell line with both a low contamination rate and less passages/time. With these cultures we were able to establish 50 mRNAs and 19 miRNAs that were differential expressed in the TM cell cultures that were atypically grown. When reviewing the literature many of these expression changes were linked to carcinogenesis, and the progression/prognosis of various cancer types.

PUBLIC ABSTRACT

Kyle Gonsalves,[1,3]; Tania, Cruz[1]; Lin Cheng[1,3]; Kuehn, Markus[1,3,4]

[1]Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, IA, USA [2]Department of Pediatrics, University of Iowa, Iowa City, IA, USA [3]VA Center for the Prevention and Treatment of Visual Loss, Iowa City, IA, USA [4] Wynn Institute for Vision Research

Glaucoma is the second most prominent cause of blindness worldwide, affecting 60.5 million people. Primary open-angle glaucoma denotes a specific form of glaucoma in which an increase in pressure in the eye is caused by a dysfunction of the Trabecular Meshwork due to an unknown cause. In the Kuehn lab we have shown previously that we can regenerate dysfunctional TM cells in culture, as well as in mice when placed with induced to be trabecular meshwork cell-like (iPSC-TM) through some means of cell-cell transfer. In this thesis I will discuss the novel means by which I isolate primary human Trabecular Meshwork (pTMs) and efficiently prepare cell cultures for studies in gene expression changes that arose when the TM cell culture's ability to grow is manipulated. Previous research has shown that pTM grow atypical when their plate has been covered compared to other cell types creating an interesting timeframe by which to observe their unique growth patterns. After successful isolating several sets of TM lines from donor tissue, we were able to observe the differences in genes as well as microRNA, factors that regulate gene, when comparing normal cells to their atypical growth counterparts. With these cultures we were able to establish 50 genes and 19 miRNAs that were different many of which were related to the forming and progression of various cancer types.

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CHAPTER 1: INTRODUCTION TO TRABECULAR MESHWORK AND THE IMPORTANCE OF THEIR CELL CYCLE CONTROL MECHANISMS ON PRIMARY OPEN ANGULAR GLAUCOMA

Glaucoma is the second most prominent cause of blindness worldwide, affecting 60.5 million people(Quigley & Broman, 2006) and can be characterized by the progressive loss of Retinal Ganglion cells (RGCs) as well as the degradation of the optic nerve head(Stone et al., 1997). The most prominent form of this disease in the United States is primary open-angle glaucoma (POAG), which is often associated with an increase in intraocular pressure (IOP). Primary open-angle denotes specific characteristics of this form of glaucoma, where the iridocorneal angle is viably open during a gonioscopy(Kwon, 2008), and the word "primary" denotes that there are no identifiable factors upon inspection. POAG affects more than 2.5 million Americans over 40 years of age, with 130,000 suffering from functional blindness(Tamm, 2002). It also affects a large majority of patients suffering from juvenile-onset glaucoma(Stone et al., 1997).

An increase in IOP is caused by the disturbance in the balance between the outflow and production of the aqueous humor in the eye which is maintained by the Trabecular Meshwork (TM)(Chang, Folz, Laryea, & Overby, 2014). The TM is a layer of extracellular matrix found in the anterior segment of the eye in the iridocorneal angle that serves as a passageway and filtration system for the aqueous humor of the eye. TM cells are the major contributors in the maintenance of IOP and by extension, are of importance when diagnosing and researching POAG (Abu-Hassan, Acott, & Kelley, 2014). In POAG these cells are often the first to be disrupted and create a cascade of effects that lead to the deterioration of the RGCs and optic nerve head(Levin & Albert, 2010). The major issue with this is that TM cells, once degraded, do not proliferate naturally in the eye(Gonzalez, Hamm-Alvarez, & Tan, 2013). Although labs are attempting to regrow or replace these cells as a potential treatment and there has been much success in other model organisms such as mice, there are still factors in human TM regeneration that we are interested in investigating(W. Zhu et al., 2017). Consequently, this leads to investigating techniques that could be beneficial in the regeneration of the TM initial degradation in human patients as a means of combating the widespread effects of POAG(W. Zhu et al., 2016).

In previous studies done in the Kuehn lab, it has been shown that TM function can be regenerated using induced pluripotent stem cells (iPSC) (W. Zhu et al., 2016). These cells, once differentiated into TM-like cells (iPSC-TM) can be injected into the anterior chamber of the eye leading to a rescue of TM function, decreased IOP, and improved outflow facility in an *in vivo* mouse model of glaucoma(W. Zhu et al., 2017). Interestingly, transplantation of iPSC-TM results in an increase in cellular density within the TM. However, it appears that this is not due to implantation of iPSC-TM, but rather due to proliferation of existing endogenous TM cells.

This suggests that something is being shared between the iPSC-TMs and the primary TMs. In order to investigate this, our lab has done several siRNA knockdowns of Connexin 43's gene, GJA1, and seen significant decreases in proliferation in *in vitro* mouse cultures. When experimenting with co-cultured techniques the lab found interesting results. When TMs were placed in iPSC-TM cultured media, media that has been placed on cells for a certain number of days allowing the collection of important released factors(W. Zhu et al., 2016), as well when placed in a plate with a filter barrier between them and the iPSC-TMs, their rate of proliferation did not significantly increase. However, once they were directly co-cultured, placing the two cell types in the same well together, there was an increase in the TM cells compared to the controls. This suggests that something is being exchanged from the iPSC-TMs to the endogenous TM cells. An example of one of the possible means of cell-to-cell communication that could play a role in their increased proliferation is through hexametric assembled connexins, which form what is known as a gap junction. These gap junctions create a small window of exchange between the two cells but are limited with regards to the size of the products they can transport. With a width of approximately 35 A and only bale to pass particles with masses less than 1 kD, this limited size means that there are only a few probable molecules that can be exchanged between through these means (Calderón & Retamal, 2016). It is due to this that I am interested in investigating one such molecule, microRNA. MicroRNAs are a class of 19—25 nucleotide non-coding RNAs with epigenetic function in silencing and post translational regulation of gene expression(Lenkala et al., 2014) While gap junctions are a possible means of transport, they are far from the only methods in which growth factors can move, such as vesicles(Lazar, Götte, & Gallwitz, 1997) . Due to this it is also important to explore other possible interactions that can occur between these cell types. However, while investigating this specific phenomenon is important, taking a step back and focusing on means by which TM cells achieve proliferative and/or prevent apoptosis can give us contexts by which to examine our future and past findings. Also, by investigating both changes in mRNA and microRNA in cell cycle control in the TM we will be able to create a large dataset by which

to siphon out interesting regulatory patterns. With this project as a basis, we hope to achieve a means of manipulating the proliferation rates in endogenous TM to counteract the negative effects of POAG. Within this study I will discuss the novel means by which I isolate primary human Trabecular Meshwork and efficiently prepare cell cultures for experimentation, such as a sequencing experiment in which I studied expression changes that arose when the TM cell culture's cell cycle control is manipulated.

CHAPTER 2: ISOLATION OF HUMAN PRIMARY TRABECULAR MESHWORK CELLS FROM DONOR TISSUE USING NOVEL TECHNIQUE

The first component of any cell culture work is the ability to acquire a culture that allows the researcher to manipulate cells that are the closest representation of the original cells/tissue as possible. Once these are extracted, there are several techniques to modify these cells, such as genetic manipulation or media conditioning in order to achieve the experimental goal. However, a primary cell that retains as much of the same chemical and biological characteristics and processes as their *in-vivo* counterparts is ideal for creating re-usable stocks that can be manipulated in the future. As such it is with great care that when isolating stocks of human primary Trabecular Meshwork cells (hpTMs) we are using the best possible methods. The traditional procedures often call for breaking down the TM tissue using combinations of proteins, such as collagenase and human serum albumin, and mechanical disruption in order to create a cell culture that can be further isolated into a single cell type. In the Kuehn lab for hpTMs isolated before 2018, excluding line 17-062, we have applied the "traditional" methodology (Steely et al., 1992) and successful cell cultures have been created. However, due to the nature of the process, the original cultures have been inundated with contamination from other cell types which takes a couple of passages to eliminate. When removing the TM tissue from the donor eyes (Fig 1), it is nearly impossible to not collect some extra cell types (Fig 1 b.), namely Schlemm's canal or Fibroblast cells (Perkumas & Stamer, 2012). In particular fibroblast can overtake a culture due to their rapid expansion rate(Otten, Johnson, & Pastan, 1971). With an abundance of contamination from other cell types, these primary stocks can no longer be used for TM specific experiments and are considered unfit for our current experiments. This is the major reason why it is imperative that the cell cultures produced by every line of TM tissue has as minimal contamination as possible.

What we have found, interestingly enough, is that TM cells can overcome these contaminations if they are healthy enough to outgrow competing cells. This process normally takes three to four passages of the mixed cell culture during which we have observed a significant increase in TM cell morphology (Fig 3.) and a decrease in contaminating cell types. Which brings us to the important question: If it is necessary for the TM cells to out compete other contaminating cells in order to create pure TM cell culture, how can we improve their chances? Another factor that lends to the improvement of the primary cell culture is the fact that we are invested in both hastening the process by which we can obtain our viable Passage 0 (P0) stocks, as well as using less passaging to

obtain said stock. Passaging cells is the process by which a cell culture, is removed from the well via trypsin/EDTA and replanted into another well (Davis, 1994). This is often times used to divide one set of cells into multiple different stocks and can be used in order to plate a specific number of cells for an experiment(Steely et al., 1992), or to allow for an expansion of said stock. This technique allows for the expansion of overly confluent cells as well as the increase in number of cells at a staggering rate. To put this in perspective, we often passage one well of a 6-well plate which can hold up to 1 million hpTM cells into a separate 6 wells. There, our newly passage cells should grow approximately six-fold. Passaging is a necessary process in cell culture, yet it has its negative effects as well. With each passage and expansion of these cells, they become less inclined to proliferate and will eventually become apoptotic(Davis, 1994). Although it is not a hard and fast rule and there is a little leeway, in the Kuehn lab we tend to stop using hpTMs after their 6^{th} to 7^{th} passage. This prevents their natural inclination to decrease their rate of proliferation(Davis, 1994) at these passages, as to not impede our experimental analysis of the results obtained using these high passage lines. But this also means that we can only passage these cell lines a very limited number of times before we are forced to discontinue our use of them. This can be a disturbance to our lab's experiments if tissues are not isolated properly or if there is a decrease in the number of donors over a long period of time.

It is for these reasons that we have been have working to adopt and refine a newer method of creating our TM primary cultures, that has been used more prominently with hopes that it will result in better isolation and purification process. Our methodology involves the extraction of the TM unaided by proteins or mechanical disruption and is colloquially referred to as the "Crawling Out" method. This method involves placing the tissue under a glass coverslip inside a 12 well plate to allow the TM cells to adhere to the bottom of the well and start dividing outwards from the tissue. This method creates an interesting image of the cells seemingly crawling away from the tissue (Fig 1. d). Once the cells were isolated, we used a standard means of confirming that the cultures contained TM cells using the drug Dexamethasone as a means of triggering the increase of myocilin expression in our cells which we can quantify(Steely et al., 1992). Dexamethasone is a commonly used drug that has been shown to induce a robust increase in the expression of myocilin in TM cells and is often a basis by which TM relate labs confirm the purity of their lines. We also use immunofluorescent labeling to distinguish between TM and Schlemm's canal cells using VE Cadherin(Perkumas & Stamer, 2012) as well as Desmin(O'Brien, Metheney, & Polansky, 1999) to check for Fibroblast contamination.

With this process we hope to accomplish a structured and reproducible method for the isolation and cultivation of primary cells from the eye with an emphasis on low contamination by other cell types, high yield and the ability to create sustainable lines with fewer passages.

METHODS

PROCESSING OF ANTERIOR SEGMENT FOR CELL CULTURE

Donor eyes were provided through the Iowa Lions Eye Bank. These eyes were obtained from the hours of 6 am to 10 pm every day. The anterior segment is removed, inserted into a 50ml tubes of Biopsy Media (BM), which contains MEM-alpha, (Thermofisher), 10% FBS, penicillin and streptomycin, then placed in the -20 Celsius cold room for storage. Within six hours of processing, six hours being the maximum, the anterior segment are taken to a sterile cell culture hood to be processed for isolation. The BM is drained from the tube and the anterior segment is removed and placed on a 33mm sized petri dish. The segment is then dissected into 4 equal quadrants. With forceps the iris and ciliary body are removed exposing the small brown strip of TM tissue.

TRADITIONAL (PROTEIN AIDED) METHODS

The tissue is removed using a 1.5 mm Meyhoefer curette and the clump of tissue is digested in a solution of 0.8 mg/ml of Collagenase A and 0.8 mg/ml of Human serum albumin in PBS. The solution is placed the solution in a 37 Celcius water bath for 2 hours. After it is fully digested it is centrifuged at 1500 rpms for 5 mins and the supernatant is removed. 0.5 ml of BM is placed into the tubes allowing the pelleted TM to be resuspended. The solution is then plated in one well of a 12 well plate and allowed to grow until the culture is ready to be passaged.

"CRAWLING OUT" METHODS

The tissue is removed from the anterior segment using forceps as one complete strand. This tissue is then placed in a 12 well plate with 0.5 ml of BM with 20% FBS added. A glass cover slip is placed on top to prevent the tissue from floating off the bottom of the plate allowing the cells to migrate from the tissue. After approximately three to four weeks the TM cells are then harvested and plated into a 6 well plate for expansion.

EXPANSION AND CULTIVATION OF P1 STOCKS

The culture is maintained in a 6 well plate. Fresh BM is added every 2 days until the culture reaches 100% confluency. The cells are then passaged from 1 well into a new 6 wells of a 12 well plate using 0.25% trypsin EDTA

(Thermofisher). The resulting culture is referred to as P0s. These are observed for contamination using microscopy and allowed to expand, generally taking 4 weeks to reach morphological homogeneity for the "Crawling Out" Method and up to 4-8 weeks for the traditional protein aided method. Throughout these weeks passaging can occur when confluency is reached, normally occurring four to five times before a stock is created with the "traditional" method and one to two times for the "Crawling Out" Method. Once homogenous they are passaged into four wells of a 6-well plate and denoted as P1. They are allowed to expand, and three stocks are frozen in liquid nitrogen using 1 ml of 10% DMSO in BM in cryotubes.

DEXAMETHASONE TREATMENT/ MYOCILIN RT-PCR

After three successful passages stocks from each line are plated for 24 hours into a control well and a treated well. For the control wells the cells were treated with standard cell culturing procedures, 2 ml of BM every two days for ten days. The dexamethasone treated cells were given an additional 500 nM of dexamethasone for ten days. Next the treated and untreated cells were extracted using the RNAeasy Kit (Quiagen) using the instructions necessary for total RNA and eluted into 50 ul of heated H20. The concentration of RNA was checked using a nanodrop and 500 nM is converted to cDNA using reverse transcriptase. A qPCR using the SYBR qPCR assay (Thermofisher) with 10 nm of treated and untreated cDNA, in triplicate is done using human myocilin primers. The real-time PCR was run at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 1 min at 55°C and myocilin expression was analyzed using the using 2-ΔΔCt method.

IMMUNOCYTOCHEMISTRY ASSAY

We stained for VE Cadherin, a Schlemm's canal cells marker, Desmin, a specific marker for fibroblast cells, and Vimentin, a non-specific marker as a control. After 3-4 days of growth in a 6 well plate, the cells are passaged to chambered cover slides and allowed to settle for 24 hrs. The cells were then washed briefly in PBS and fixed in 4% formaldehyde for 5 min. Cells were then permeabilized for 5 min with 0.5% Triton X-100 in PBS and washed 3 times in PBS with 5 min between washes. Polyclonal anti-VE Cadherin/Desmin/or Vimentin was then added at a 1/400, 1/400, and 1/800 dilution, respectively, in PBS with 10 % skim milk. Coverslips were placed "cells down" for 1 hr onto a droplet of the primary antibody solution placed on parafilm and covered with an airtight plastic cover. After 1 hr, the coverslips were gently put back into small plastic dishes, washed 3 times with PBS and treated with a secondary antibody solution. The secondary antibody solution was prepared with 10% skim milk in PBS, plus 1/200 dilution of goat anti-rabbit FITC-conjugated, affinity purified IgG (Biosource, Camarillo, CA). In

all experiments, 1/1000 dilutions of diamidino phenylindole (DAPI; Sigma) were added to observe DNA simultaneously. Coverslips were then placed on droplets of secondary antibody solution on Parafilm for 1 hr as before. After staining with secondary antibodies, the coverslips were washed as above in PBS, then placed on glass slides in a glycerol-based antifade solution (n-propyl gallate in pH8.0 Na borate). Slides were evaluated using 10X magnification using a Nikon Eclipse 80i fluorescent microscopy (Nikon).

RESULTS

ISOLATION TIME FRAME

Lines 17-031 the traditionally isolated TM culture took 6 weeks in order to properly grow to confluency from the degraded tissue to be passaged into a stock line. As for the TM cultures isolated from the "Crawling Out" method 18-052, 18-062, and 18-063 they each took 5 weeks to grow to an appropriate amount to be passaged. However, line 17-062 took 3 weeks to be isolated and 18-019 was isolated after 8 weeks.

DEXAMETHASONE TREATMENT

After 10 days of dexamethasone treatment all lines displayed at least a 2-fold increase in myocilin production over their control counterparts. The "traditional" line 17-031 showed a 2.13-fold increase, whereas the "Crawling Out" lines showed an average 3.5-fold increase with line 17-062 having an extreme difference at 5.65-fold increase in myocilin expression.

IMMUNOHISTOCHEMISTRY ASSAY

We ran a control, unspecific marker over all samples and the fibroblast using Vimentin, an unspecific marker and were able to successfully show expression in all lines. When testing for Fibroblast contamination we were able to confirm through Desmin that neither methods yielded any contamination for any of the lines. However, while using VE Cadherin to check for Schlemm's canal cell contamination we saw much more frequent staining in the "traditionally" isolated lines (17-031) compared to the "Crawling Out" lines.

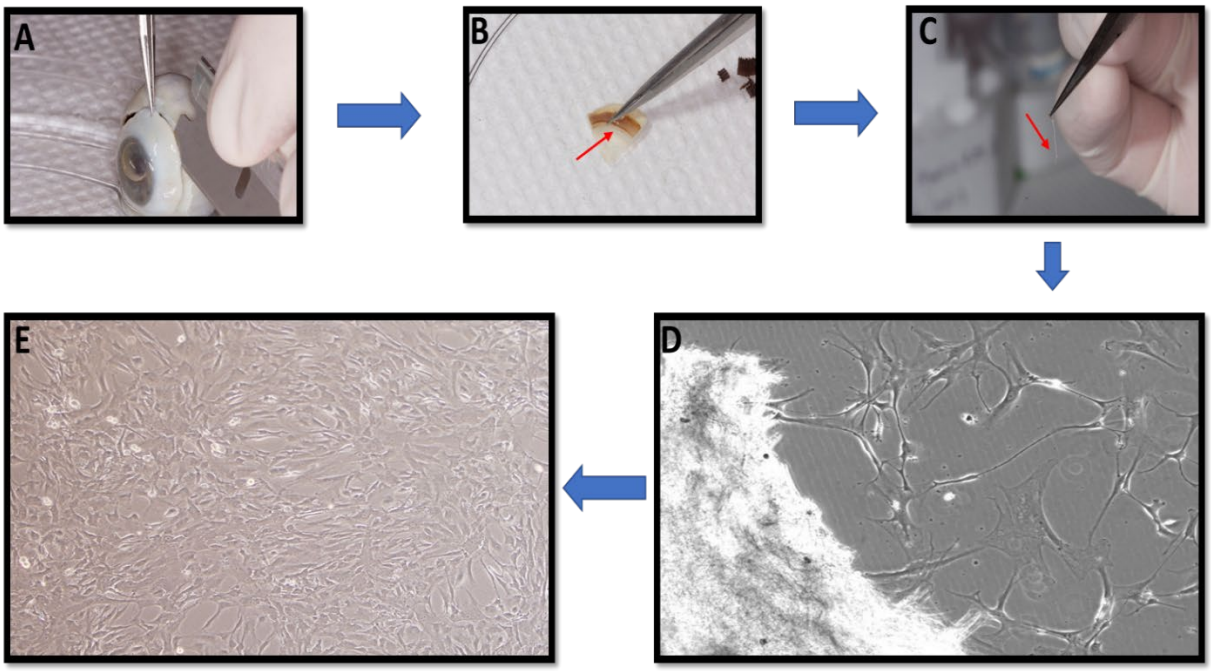

FIGURE 1: WORKFLOW OF TRABECULAR MESHWORK CELL LINE ISOLATION USING " CRAWLING OUT" METHOD**:** (A) Dissection of the anterior segment of the donor eye (B) Removal of the iris and ciliary body and dissection of the TM tissue, red arrow point towards darkened region of the TM (C) An isolated fully intact segment of trabecular meshwork tissue from one quadrant, red arrow pointing to TM (D) Microscopy image of TM cells migrating away from the tissue via the "Crawling Out" method (E) Fully isolated Trabecular Meshwork culture, line 18-019, P0 stock (Images A,B, and C of TM isolation provided by Scott Whitmore)

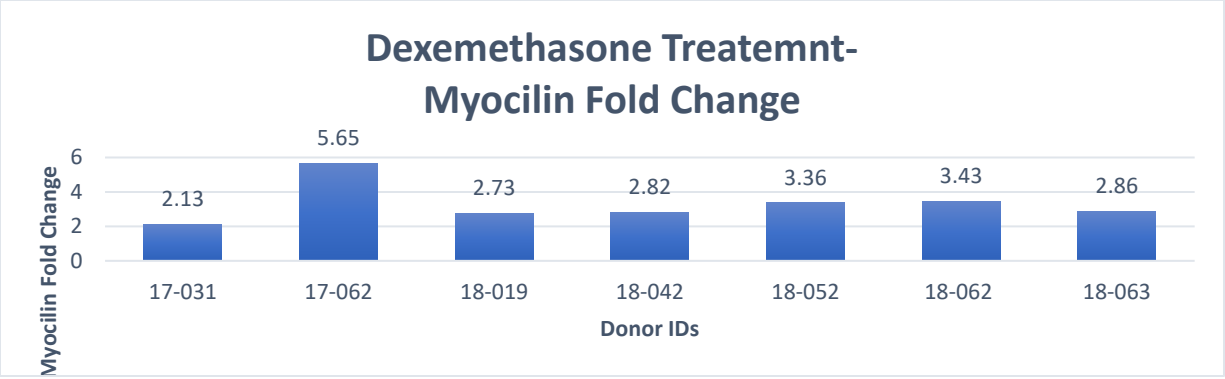

FIGURE 2: MYOCILIN FOLD CHANGES IN ISOLATED HPTMS: Fold change compared to untreated stock cells 17-031 used traditional isolation methods; whereas all other cell lines were extracted using the "Crawling out" methodology.

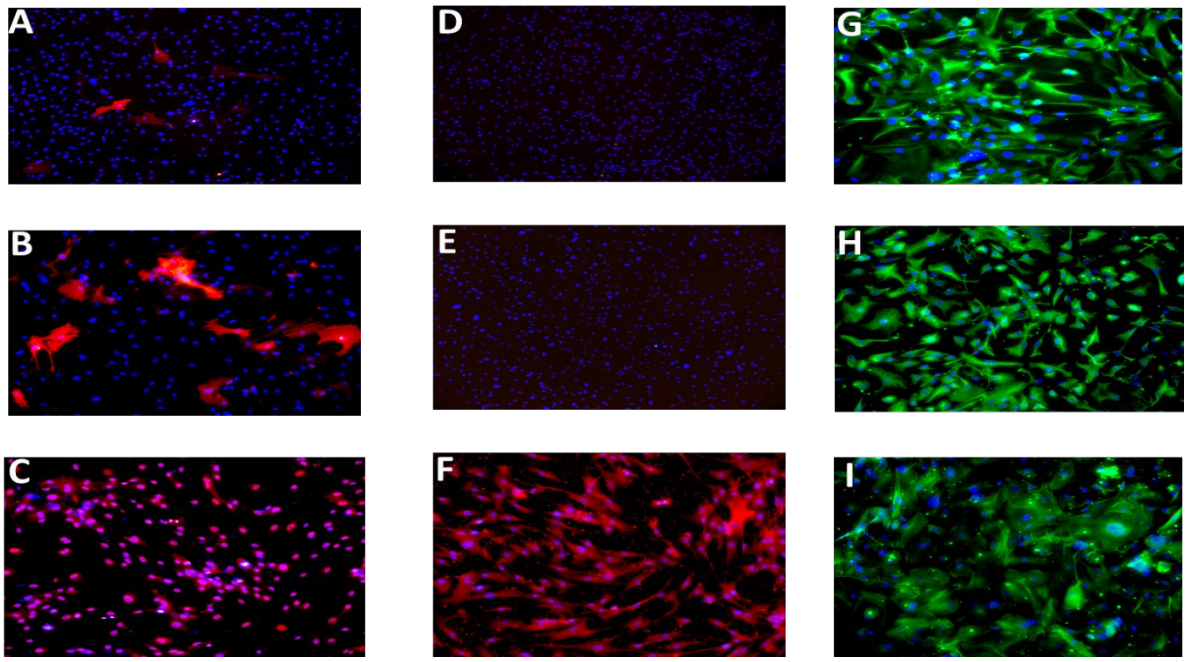

FIGURE 3: IMMUNOFLUORESCENCE STAINING CONFIRMATION OF TRABECULAR MESHWORK CELLS (A, D, G) Line 17-062, a line isolated using "Crawling Out" procedure immunofluorescent stained for, right to left, VE Cadhrin, Desim and Vimentin, **;** (B, E, H) Line17-031, a traditionally isolated cell culture line, immunofluorescent staining for, right to left, VE Cadherin, Desmin and Vimentin, (C,F,I) Fibroblast P5, unaltered line, immunofluorescent stained for, right to left, VE Cadherin, Desim and Vimentin. (Images provided by Lin Cheng)

CONCLUSION

The data shows that the "Crawling Out" Method is an effective means to establish pure TM cell cultures with both low contamination rates while also taking fewer passage to reach a useable state of homogeneity. With the dexamethasone treatment, those lines that were established with the "Crawling Out" methodology displayed an increase in myocilin expression greater than 17-031, which used the traditional method. We observed that line 17-062 was an extremely robust line, whose myocilin expression after dexamethasone treatment was 1.9 times the average of the other tested lines. When investigating further we noticed that the donor of this line was 37 years old, which was young compared to our typical donors, averaging at approximately 73 years of age. This distinction was even further confirmed as the average time frame for the culture to form a solid layer in the 12 well plate that can be transferred for expansion was 6 weeks, while the tissues extracted from line 17-062 were passaged in under 3 weeks, about half the average of the other lines. This most likely points to the fact that isolations work better with younger donors' eyes. There have several studies that show that over time the TM naturally degrades, even in non-glaucomatous patients (Alvarado, Murphy, Polansky, & Juster, 1981). This natural degradation could be important when isolating these cells since proliferation rates are the determining factor for the amount of time needed for the "crawling out" phase of isolation. The opposite trend was observed with donor 18-019 which was able to successfully create a viable TM cell culture line. Yet it was necessary to culture the tissue for 8 weeks, the longest timeframe, wherein other lines averaged around 4-6 with the "Crawling Out". This line was isolated from a donor who was 88 years old which is by far the oldest donor that we received. This suggests that these younger more robust donor eyes will be much more viable to create larger lines at a much faster rate. However, that also means that these lines will have less natural degradation which could be a key factor in testing the ability of certain drugs, genetic manipulations or cell-cell interactions to induce proliferation in declining cells similar to those afflicted with POAG. With older donors we could make a modification to our method by adding bFGF (250 ng/ml) to the BM placed with the tissue to enhance cell growth in older donor cultures(Abu-Hassan et al., 2014). This could allow for a more equal isolation when comparing older and younger donors as to make less differences when analyzing downstream data. However, this would be manipulation that would need to be factored in later experiments. This balance between creating multiple lines quickly through younger donors and retaining some POAG characteristics in older donor cultures is something that warrants further investigation.

As far as the “Crawling Out” method, when these lines were immunofluorescence labeled for fibroblast and Schlemm’s canal contamination we found almost no fibroblast contamination in either methods. However, in the traditionally isolated cultures in passage 0 we found larger amounts of Ve Cadherin expression suggesting that Schlemm’s canal contamination maybe be a consequence of this isolation.

Another factor that was novel in the “Crawling Out” method that was not previously observed in traditional method was that there seemed to be “stem cell”-like TM cells found in three lines [17-062, 18-052, and 18-062] which gave rise to large clusters of TM cells. These clusters can no longer be seen after two to three passages, but it is apparent that they cause a rapid increase in TM cell number for a brief period of time. While most likely not a contamination from other cell types, these cells may exhibit slightly different behaviors than normal TM culture and it would be interesting to use stem cell markers in order to confirm that this may be the case. It could be that investigating methods that can prevent these cells from forming or allowing them to differentiate quickly so that homogeneity can be achieved with fewer passages as well as understanding why these clusters form and how they can play a role in TM cell proliferation.

CHAPTER 3: RNA TRANSCRIPTIONAL LEVEL CHANGES RELATED TO TM CELL CYCLE REGULATION

Having created a sustainable means by which to isolate and culture our TM cells, we were interested in exploring possible experiments that could us these cultures to further our lab's research goals. With TM cell proliferation as the forefront of our research, I was interested in looking at both the transcription of RNA and miRNA in our different lines. However, we needed a specific methodology in which to investigate this without knowledge of which drugs or genetic manipulations would simulate proliferation and/or an apoptotic response, that would also be unique to this specific cell type. Yet we did see an interesting pattern of growth in our cell lines that was fairly consistent.

Over the years we observed that when TM cultures grew on plates, they would form a single monolayer at the bottom and once that monolayer was completed, there would be a decrease in the division of the cells. This is termed post-confluent inhibition in which cultures, often times epithelial cells, that have reached full confluency will decrease their mitotic rate and remain stationary despite renewal of nutrients (Middleton, 1972). With regards to other cells, such as fibroblast (Otten et al., 1971), it is common to see the cells grow on top of each other once the plate has reached 100% confluency. This was interesting enough to warrant further investigation but there was also something else atypical about the 100% confluent TM cells: there was little to no cellular debris even after several days. When observing other cell types, ie. TM5 an immortalized line of TM cells, there is usually a cycle in which the plate reaches 100% confluency and older cells die and float into the media. In other cases, the cells will form a monolayer and begin to peel off the plate as one large fragment after a few days. These two responses are common, yet primary TM cells do not behave in this manner. Once they have reached 100% confluency it seems as though most cellular division stops. Yet the cells remain as an intact monolayer for approximately a week after the fact, which is much longer than most cultures. Eventually they do start to die off, but it occurs very rapidly taking only a single day for all of the cells to be found floating in the dish.

This observation was crucial because it allows us to observe a specific time frame in which the TM cells seem to neither proliferate or apotose. The time frame between 100% confluency and when the line expires is a snap shot for understanding the mechanisms by which cellular division in TM cells is unique from other cell types. With this in mind, we set out to investigate if this phenomenon could be examined with regards to proliferation or cell

cycle regulation. We decided to allow our cultures to grow until they reached this point and investigate the change in gene transcription. We are interested in finding transcriptional differences that could account for this stagnant phase in the TM cell culture. With this we hope to find certain genes that can be manipulated in our cultures to prevent their degradation and understand the interactions as well as the downstream effects that these genes may have on human TM cells.

METHODS

TM CELL CULTURE AND TOTAL RNA EXTRACTION

The cells were plated from stocks all Passage 3. We used 6 lines that were processed from TM tissue as described in the previous chapter (17-062, 18-019,18-042,18-052,18-053). Cells were plated into 4 l wells which contained 50,000 cells each, 2 wells are designated Dividing (50 %) in which they are grown and extracted before they reach high confluency, and the second 2 are designated Confluent (100%) in which they are grown and are extracted 3 days post 100% confluency. All cultures were all grown in 2 ml of Biopsy Media (BM) which is changed every two days. The cells labeled "Dividing" were observed and removed for RNA extraction after 4-5 days once they reached approximately 50% confluency. The cells labeled "Confluent" were allowed to culture till they reached 100% confluency. However, once they reached 100%, they were extracted two days afterwards as to make sure that they had reached the point at which division was sparse. We also checked the media for an excess of cellular debris and/or if a line was peeling. Those that had this were removed and a new line of P3 cells were used.

Once they reached the desired confluency, they were extracted using the microRNAeasy Kit (Quiagen) using the instructions necessary for total RNA, both RNA and miRNA, and eluted into 50 ul of heated H20. The concentration of RNA was confirmed and 200 ngs of RNA was sent to the DNA core at the University of Iowa for quality analysis using Agilent Model 2100 Bioanalyzer. Only RNA with an RNA integrity Number (RIN score) of 7.0 were accepted and 4 ug of RNA was sent to the DNA core for RNA-Sequencing.

RNA SEQUENCING FOR MRNA

Using a Poly-A enriched RNA to make cDNA libraries we sequenced (150-bp paired end) on the Illumina HiSeq 4000 Genome Sequencer. The fastq reads were then processed on a high-performance compute cluster with the bcbio-nextgen fastrna-seq pipeline, with human hg19 as the reference genome, and Salmon used for quantification of read counts. Read counts were imported into R using tximport and gene annotation was obtained

from Ensembl using the biomaRt package. The raw counts were normalized and transformed using rlog while a principal component analysis (PCA) was performed to visualize sample clusters. For statistical analysis of the data, the read counts were imported into R/DESeq2. We created a model incorporating the donor identifier and the conditions ("Dividing" and "Confluent") using DESeq2. Pairwise comparison between conditions, while controlling for patient effect, was performed using a Wald test to determine significance. A False Discovery Rate (FDR) less than 10% was considered to be statistically significant. Results from the statistical analysis were exported to Microsoft Excel format using the writexl package and were visualized in R with customized volcano plots generated using the ggplot2 graphic package. The data was also uploaded to Ingenuity Pathway Analysis (IPA) in order to assess conical pathways and upstream analysis.

RESULTS

With the mRNA-Seq were able to create a dataset containing 50 differentially expression genes with a significant up/down-regulation in the combined 5 "Confluent" lines compared to their "Dividing" counterparts. With 40 of these genes being upregulated and 10 of which were significantly down regulated. Of the 50 genes there was a range of fold-changes ranging from 23.57-fold increase to a .0.64-fold decrease in expression. Once the data was placed into IPA two immune system related pathways of interest were significant shown with high upregulation, the Classic Complement System and the the MHC class I-dependent pathway

TABLE 1: MRNA GENE EXPRESSION DATA: padj= p-value adjusted for multiple testing; this is also considered to be a false discovery rate (FDR), 0.1 or 10% a commonly used threshold for FDR. Blue represents upregulation in the 100% lines vs their 50% counterparts Green represents downregulation in the 100% lines vs their 50% counterparts. Total of 50 genes with differential gene expression: 40 upregulated, 10 downregulated.

baseMean	log2FoldChange	foldChange	pvalue	padj	gene_name
263.64	1.56	2.96	2.98E-14	3.96E-10	BCYRN1
32.17	4.56	23.57	3.61E-10	5.93E-06	KRT17
15741.60	0.65	1.57	5.74E-08	0.0001	HLA-B
284.37	1.89	3.69	2.89E-07	0.0008	CHRDL2
152.40	-1.75	0.30	3.73E-07	0.0008	CAMK2A
1392.07	1.54	2.92	6.13E-08	0.0014	NDUFA4L2
2226.74	0.94	1.92	1.12E-06	0.0020	C1QTNF1
374.74	-0.97	0.51	2.88E-06	0.0028	SYNE2
81.12	1.91	3.76	8.43E-07	0.0040	KCNJ15
4436.82	1.08	2.11	7.80E-06	0.0045	SCG2
173.99	1.05	2.07	5.36E-06	0.0065	ADGRD1
17780.18	0.66	1.57	1.48E-05	0.0081	C1S
550.92	1.03	2.05	6.55E-07	0.0081	TYMP
1823.10	1.47	2.78	2.50E-06	0.0087	A2M
4033.08	0.51	1.42	2.60E-05	0.0114	LRIG1
17440.26	0.69	1.61	3.13E-05	0.0133	C1R
3084.11	0.88	1.84	2.12E-05	0.0147	TNC
397.03	-1.00	0.50	3.19E-05	0.0147	ADAMTS9
574.88	0.60	1.51	2.58E-06	0.0200	NPAS2
485.28	0.57	1.49	4.49E-05	0.0200	HLA-H
304.54	-1.15	0.45	3.16E-05	0.0200	LMNB1
40.08	2.37	5.17	6.05E-06	0.0200	PCDH1
7547.19	1.07	2.10	9.25E-05	0.0225	IFITM1
3845.14	0.95	1.93	0.0001	0.0271	ICAM1
17138.91	0.54	1.46	0.0001	0.0307	HLA-A
4306.11	0.87	1.82	0.0001	0.0307	CFB
605.78	-1.24	0.42	5.99E-05	0.0307	KIF20A
352.97	1.12	2.18	8.79E-05	0.0307	SOD3
79.26	1.08	2.12	6.44E-05	0.0307	FNDC1
13074.55	-0.85	0.55	0.0002	0.0361	GPRC5A
341.26	0.91	1.88	8.18E-05	0.0361	EIF3CL
31076.03	-0.99	0.50	0.0002	0.0413	NQO1

TABLE 1-CONTINUE

5946.08	-0.71	0.61	0.0002	0.0413	GREM1
16285.74	1.13	2.19	0.0003	0.0622	MGP
6822.16	0.81	1.75	0.0004	0.0626	RGS5
6583.50	0.38	1.31	0.0004	0.0626	VEGFA
2096.73	0.57	1.48	0.0001	0.0646	CCDC9B
11504.81	-0.75	0.59	0.0005	0.0657	G6PD
117.58	1.88	3.68	0.0001	0.0657	C11orf96
338.63	1.56	2.95	0.0004	0.0753	AGT
32400.77	0.53	1.45	0.0006	0.0857	IGFBP3
12901.33	0.47	1.39	0.0007	0.0857	HLA-C
6612.35	0.43	1.35	0.0007	0.0857	SLC39A14
2346.13	0.89	1.85	0.0002	0.0857	COL16A1
501.74	-0.64	0.64	0.0004	0.0857	SELPLG
326.92	0.61	1.53	0.0004	0.0857	HAUS7
482.02	0.77	1.70	0.0005	0.0886	ZC3H12A
120.96	0.88	1.85	0.0002	0.0886	ARHGEF37
3527.83	0.84	1.79	0.0003	0.0897	ITGA7
570.89	0.78	1.72	0.0006	0.0919	TLE2

CONCLUSION

We were able to create a gene expression data set that contained 50 distinct genes that were either upregulated [40] or downregulated [10] in the "Confluent" category vs their "Dividing" counterparts (Table 1). The first gene that struck our interest was a highly upregulated and significant product: Brain Cytoplasmic RNA 1 (BCYRN1) which was upregulated 2.96-fold. What was particularly interesting with regards to BCYRN1 is the fact that several papers have shown that when BCYRN1 is upregulated or genetically overexpressed in non-neural cells, there is an increase in proliferation and a decrease in apoptosis(Booy, McRae, Koul, Lin, & McKenna, 2017). BCYRN1 or as it can also be referred to as BC200, is a long non-coding RNA (lncRNA) that has been associated mainly with the brain and neurons (Hu & Lu, 2015). Its altered expression in brain tissue seems to be linked to several neurodegerative diseases as well as several studies of the natural aging process(Gu, Lu, Zhou, & Liu, 2018). It is rarely significantly expressed in other tissues, barring the testes. However there have been several studies that have linked it to human tumors(Booy et al., 2017; Gu et al., 2018; Hu & Lu, 2015; Iacoangeli et al., 2004). Many of these studies have described it as having a regulatory function in breast cancer(Iacoangeli et al., 2004), yet it has been found to be differentially expressed in other cancers such as in the lungs, skin, stomach, ovaries and cervix(Hu

& Lu, 2015). With these findings it seems that it is related to proliferation, however there are two concerns. The first was found in Tiedge et al. in which they assert that it is in fact not a proliferation marker but a marker for the ability of, in this case, breast cancer cells to become "potentially invasive malignancies". This overexpression is linked to the carcinoma's ability to spread to surrounding tissues(Iacoangeli et al., 2004). The second issue is that the ***upregulation*** of BCYN1 is associated with an ***increase*** in proliferation in cancer cells. Yet the TM data shows an upregulation of BCYN1 in the 100% confluent cells vs the 50% cells. This would indicate that there is an increase in BCYN1 expression in TM cells that we concluded have a decreased rate of proliferation and/or increased apoptosis. This means that with regards to BCYRN1 the literature has been directly opposed to the outcome of our mRNA-Seq. However, I believe there are two possibilities that can explain why there is a difference in our findings. The first would be that the TM's BCYRN1 regulation is different from that of cancer cells. While there has been much evidence to support BCYRN1 role in proliferation in cancer cells, this lncRNA is still vastly understudied in other cell types. It could be possible that this long non-coding RNA has a distinctive role in the TM that is not directly linked to its role in cancer cells. Another possibility is that it is upregulated in response to the post-confluent inhibition of the TM culture. Wherein the cells responded to the negative conditions of over confluence by both decreasing proliferation similar to other cell types but also upregulating anti-apoptotic responses, a distinction that could set the TM cells apart. While many papers have associated BCYRN1 to cancer cells, there has been little study into whether its regulator role serves to increase proliferation or to decrease apoptosis (Hu & Lu, 2015). Due to the nature of the TM cells there is, as stated before, little to no cellular debris when inspecting the confluent cells. This means that while proliferation has decreased, as there are no new cells forming, there is also a large window in which the cells are not dying. BCYN1 could be a response to overgrowth in which the culture decreases its apoptotic response as a means of creating a monolayer homeostasis.

With further investigation it would be interesting to see if regulation of BCYRN1 follows similar trends if increased proliferation or decreasing in the TM cell's apoptotic response are achieved through manipulation. What is also interesting is that a similar trend also occurs with several other genes such as Kertin17 which had an astonishing 23.57-fold increase in expression. KRT17 is the gene that encodes for Kertin, type 1 a cytoskeletal protein whose overexpression has been implicated in the formation of Psoriasis, an immunological skin disease that results in epidermal hyperproliferation(J. Liu, Liu, Cao, & Wen, 2018). It is also a molecular marker for carcinogenesis, progression, and prognosis of various cancer types(Kim et al., 2015). With studies that investigate genetically

induced KRT17 knock downs in mice, there is a resulting decreases in proliferation, and migration(Kim et al., 2015; J. Liu et al., 2018). This points towards an interesting connection to cancer.

While less is known about many of the other gene's link to cancer compared to the top two results of our mRNA-Seq, others are often are used as basic cancer cell markers, such as CHRDL2(Sun et al., 2017), and NDUFA4L2(L. Liu et al., 2016; Lv et al., 2017). This is interesting because the 100% cells are expressing more cancer related genes. This could suggest a shift in gene expression in order to sustain the cells in the post-confluent conditions that differ from basic post-confluent inhibition. It would be interesting to investigate if these 100% cells or genetically manipulated versions of these cultures would have consequences to TM proliferation in a human eye perfusion experiment and whether that would result in a cancer-like TM cell type similar to TM5s.

Once analyzed through IPA, two systems with a large variety of genes that were differentially expressed in the "Confluent" cells seem to be the Classical Compliment System and the MHC class I-dependent pathway which includes several of the HLA complex genes. The Classical Compliment System is interesting because it is implicated in some of the most basic functions of the TM(Ren & Danias, 2010). While direct links to proliferation in cell culture were not found when reviewing the literature, both of these processes are related to immune responses to cancer(Afshar-Kharghan, 2017; Kochan, Escors, Breckpot, & Guerrero-Setas, 2013; Rutkowski, Sughrue, Kane, Mills, & Parsa, 2010). This further justifies our interest in these cells and their relation to cancer. However, the first question that should be asked is whether or not this is an artifact of the culturing of cells. Before any further studies can be done, ie. genetic manipulations of TM cell cultures or even *in vitro* manipulation in a model organism, it is imperative that we investigate this phenomenon in the human tissue. This could be done using profusion of donor eyes before isolation, with the manipulation of the tissue through growth factors. With this technique we could run a parallel mRNA-Seq study in order to ascertain if similar results to our cell culture experiments can be found.

CHAPTER 4: AN INVESTIGATION INTO THE ROLE OF MIRNA IN THE CELL CYCLE IN HUMAN PRIMARY TRABECULAR MESHWORK CELL CULTURE

While investigating the differential expression using mRNA-Seq was an attempt to find some of the basic genes that may control the cell cycle in the TM, it would only be half of the picture we were seeking. With our knowledge from previous research(W. Zhu et al., 2016; W. Zhu et al., 2017) our lab concluded that most likely that there would be some factor that was being passed from the iPSC-TMs to our pTMs that allows them to proliferate irregularly. We were interested in investigating this. However, while that project was being worked on, we felt it better to investigate possible targets that could be linked to our co-culture experiment once completed. miRNA having become a relatively newer topic made for an excellent candidate, both due to its prominence in tumorigenesis and tumor suppression (Lenkala et al., 2014; W. Liu & Wang, 2019; J. Y. Zhu et al., 2009) as well as its ability to move between cells via gap junctions(Calderón & Retamal, 2016). With this we set out to use the same methods previously used to create our gene expression data set to investigate possible miRNA that be involved in the regulation of either proliferation or prevention of an apoptotic response. However, another advantage that we had in investigating miRNA was our ability to manipulate them in cell culture with much more ease than we would with normal genetic manipulation. With the use of miRIDIAN microRNA Hairpin/Mimics we could easily simulate up/down-regulation of specific miRNA with our cultures and investigate their effects on proliferation. With this knowledge we are hoping to find key miRNA, and their targets, so that we have a clearer picture of the interactions between iPSC-TMs and TM, to create a foundation on which to build our current research.

METHODS

TM CELL CULTURE AND TOTAL RNA EXTRACTION

The cells were plated from stocks all Passage 3. We used 6 lines that were processed from TM tissue as described in the previous chapter (17-062, 18-019,18-042,18-052,18-053). Cells were plated into 4 l wells which contained 50,000 cells each, 2 wells are designated Dividing (50 %) in which they are grown and extracted before they reach high confluency, and the second 2 are designated Confluent (100%) in which they are grown and are extracted 3 days post 100% confluency. All cultures were all grown in 2 ml of Biopsy Media (BM) which is changed every two days. The cells labeled “Dividing” were observed and removed for RNA extraction after 4-5 days once they reached approximately 50% confluency. The cells labeled “Confluent” were allowed to culture till

they reached 100% confluency. However, once they reached 100%, they were extracted two days afterwards as to make sure that they had reached the point at which division was sparse. We also checked the media for an excess of cellular debris and/or if a line was peeling. Those that had this were removed and a new line of P3 cells were used.

Once they reached the desired confluency, they were extracted using the microRNAeasy Kit (Quiagen) using the instructions necessary for total RNA, both RNA and miRNA, and eluted into 50 ul of heated H20. The concentration of RNA was confirmed and 200 ngs of RNA was sent to the DNA core at the University of Iowa for quality analysis using Agilent Model 2100 Bioanalyzer. Only RNA with a RNA integrity Number (RIN score) of 7.0 were accepted and 4 ug of RNA was sent to the DNA core for RNA-Sequencing.

RNA SEQUENCING FOR MICRORNAS

A small RNA workflow was used to generate cDNA libraries that were sequenced (150-bp paired end) on the Illumina Mi Sequencer. The fastq reads were then processed on a high-performance compute cluster with the bcbio-nextgen smallRNA-seq pipeline. In this pipeline, Atropos was used for adaptor trimming and STAR was used for alignments, with human hg19 as the reference genome. The program Seqcluster was then used to collapse mapped reads and quantify read counts. The microRNAs counts were imported into R and the raw counts were normalized and transformed using rlog. A principal component analysis (PCA) was performed to visualize sample clusters. For statistical analysis of the data, a DESeq2 object was created from the imported counts data by using the isoDE command. We created a model incorporating the donor identifier and the conditions ("Dividing" and "Confluent") using DESeq2. Pairwise comparison between conditions, while controlling for patient effect, was performed using a Wald test to determine significance. A False Discovery Rate (FDR) less than 10% was considered to be statistically significant. Results from the statistical analysis were exported to Microsoft Excel format using the writexl package and were visualized in R with customized volcano plots generated using the ggplot2 graphic package.

MIRNA HAIRPIN/MIMIC MANIPULATION

Passage 3 cells from two stocks [18-019 and 18-063] stocks were plated in 6 well plate to grow for 2-3 days. Eleven miRIDIAN microRNA Hairpin Inhibitor 2 nmol [hsa-miR-122-5p, hsa-miR-7-5p,hsa-miR-625-3p,hsa-miR-4483,hsa-miR-222-3p,hsa-miR-130b-5phsa-miR-584-5p,hsa-miR-138-1-3p,hsa-miR-4435,hsa-miR-1304-3p, and hsa-miR-3180] and seven miRIDIAN microRNA Mimic 2nmol [hsa-miR-1307-5p ,hsa-miR-184 ,hsa-miR-

210-3p ,hsa-miR-27b-3p ,hsa-miR-99a-5p ,hsa-miR-7974, andhsa-miR-181c-5p] were purchased from Dharmon and resuspended in H20 to 100 uM concentrations. The cells were removed using 0.25% trypsin EDTA (Thermofisher) and counted using at Hemocytometer. The cells were seeded in 96-well plates at a density of 10 X 10^3 cells/well. Cells were set up in triplicates with each of the 18 batches, including one batch with all 18 mimic/hairpins, and 17 which have 17 mimics/hairpins with one removed, as well as an unaltered negative control with hpTMs and a base line control containing only media. Cells are left for 24 hrs in order to attach to the bottom. After 24 hrs they are washed with ice-cold PBS. They are then transfected with Lipofectamin RNAiMaxTransfect Reagent guidelines (Thermofisher). Diluting the Lipofectamin and mimics/hairpin in each in 25 ul of Opti-MEM Medium, mixing the dilutions at a 1:1 ratio and incubating for 5 mins at room temperature. Each 100 ul mimic/hairpin-lipid complex is then placed into its respective wells, excluding the unaltered controls. The cells were to be incubated at 37 degrees Celsius and analyzed using at MTT assay cell proliferation assay(abcam) at 550 nm with a TECAN GENios microplate reader as instructed at 24/48/72/96 hr. There procedures were used in a similar fashion for the second attempt, however only 5 mimics/hairpins were used [hsa-miR-222-5p, has-miR-1307-5p, hsa-miR-122-5p, hsa-miR-7-5p and hsa-miR-184]. A standard curve was created using these two stocks grown to a fix number of cells (10,000, 20,000, 50,000, 100,000) with the MTT assay.

RESULTS

MIRNA EXPRESSION ANALYSIS

With the small-RNA-Seq were able to create a dataset containing 18 differentially expression miRNAs with a significant up/down-regulation in the combined 5 “Confluent” lines compared to their “Dividing” counterparts. With 7 of these genes being upregulated and 11 of which were significantly down regulated. Of the 18 miRNA there was a range of fold-changes ranging from 2.64-fold increase to a 0.72-fold decrease in expression

TABLE 2: MIRNA GENE EXPRESSION DATA: padj= p-value adjusted for multiple testing; this is also considered to be a false discovery rate (FDR), 0.1 or 10% a commonly used threshold for FDR. Blue represents upregulation in the 100% lines vs their 50% counterparts Green represents downregulation in the 100% lines vs their 50% counterparts. Total of miRNAs 7 of which were upregulated and 11 downregulated.

baseMean	log2FoldChange	foldChange	pvalue	padj	gene
782.49	-1.21	0.43	7.8E-09	3.5E-06	hsa-miR-222-5p
1338.78	1.00	2.00	3.0E-05	0.0066	hsa-miR-1307-5p
80.20	-1.68	0.31	0.0001	0.0083	hsa-miR-122-5p
119.56	-1.19	0.44	0.0001	0.0083	hsa-miR-7-5p
54.99	1.40	2.64	0.0002	0.0166	hsa-miR-184
136.35	-0.86	0.55	0.0004	0.0263	hsa-miR-625-3p
1799.11	0.65	1.56	0.0005	0.0284	hsa-miR-210-3p
99.90	-1.04	0.49	0.0005	0.0284	hsa-miR-4483
177266.32	-0.77	0.59	0.0006	0.0298	hsa-miR-222-3p
652.95	-0.58	0.67	0.0013	0.0593	hsa-miR-130b-5p
749.49	-0.54	0.69	0.0015	0.0620	hsa-miR-584-5p
788.70	-0.49	0.71	0.0025	0.0880	hsa-miR-138-1-3p
299315.71	0.49	1.41	0.0030	0.0880	hsa-miR-27b-3p
77.04	-0.73	0.60	0.0027	0.0880	hsa-miR-4435
3100.66	0.48	1.39	0.0029	0.0880	hsa-miR-99a-5p
535.56	-0.48	0.72	0.0036	0.0968	hsa-miR-1304-3p
110.59	-1.86	0.27	0.0037	0.0968	hsa-miR-7974
1794.02	0.44	1.35	0.0042	0.0979	hsa-miR-181c-5p
62.35	-0.85	0.56	0.0042	0.0979	hsa-miR-3180

MIRNA HAIRPIN/MIMIC MANIPULATION

In our first experiment after 24 and 48 hrs, wells that were transfected with miRNA containing batches of 17 mimics/hairpins were found to contain total cell death with absorbance measuring at the same levels as the empty controls. With the second attempt , made using only five of the miRNA candidates [hsa-miR-222-5p, hsa-miR-1307-5p, hsa-miR-122-5p, hsa-miR-7-5p, andhsa-miR-184], the same procedure was attempted again with the minimum require amount of ssRNA to transfect the cells. However, after 24 and 48 hrs of incubation there was still total cell death in the transfected cells with absorbance at the same levels as the empty controls.

CONCLUSION

With our parallel confluency experiment we were able to determine a set of 19 miRNA that were either upregulated [7] or downregulated [11] in the 100% cultures over the 6 lines in comparison to their 50% counterparts (table 2). With so few significant mircoRNA in our list we were able to individually look at each in-depth. However once looking at the relevant literature regarding these microRNAs many were relegated to either large miRNA

screens of certain cell lines or very broad gene expression datasets with regards to cancer(W. Liu & Wang, 2019; J. Y. Zhu et al., 2009). The literature often points to expression dataset naming said miRNA as one of many being differentially expressed in certain cancers, with little to no reference as to how their ability to regulate gene expression of certain genes would affect their increased/decreased prevalence in cells. This means that while it is interesting that we once again found data similar to the mRNA-Seq gene expression that ties our miRNAs to cancer cell regulation, it is difficult to pinpoint information regarding how these miRNAs specifically interact with certain genes.

Although the literature was unable to give a more nuanced look into these 19 miRNA's roles in cell cycle control, I was interested in understanding more about the specific targets of these miRNAs and if any of them were related to the mRNA data that we collected. In order to investigate these possibilities, we used an online database for predicting mircoRNA targets termed miRDB(Wang, 2016; Wong & Wang, 2014). This database allows for the systematic analysis of high-throughput experimental data to identify novel features that are important to target downregulation, allowing for a simple means by which to investigate probable targets for microRNAs. With this tool, I was able to see the most likely targets of each microRNA, in which a set of approximately 1000 genes would be listed with each microRNA. This list, however, would contain a target prediction score between 50-100 for each target gene using a target prediction algorithm. As suggested by the creator of this tool, the confidence that this gene is a genuine target dwindles as the target score gets closer to 50 and it is recommended that only those above 80 are most likely "real". What is interesting about this is that I would often find gene targets that corresponded with my previous mRNA dataset. Yet these genes would never have a target score above a 66. What was also interesting is that when looking at specific targets and linking them to microRNA, even if it was a low score, none of the highly significant cancer related gene showed, ie. BCYRN1, KRT17 CAMK2A. This is possibly due to the fact that these are all upregulated in the 100% but when investigated, some of the downregulated genes such as CAMK2A, SYNE2, or ADAMTS9, did not correspond with any of the microRNA even on a lower prediction score. However, there were some interesting connections that could be found. Although the direct gene targets were not linked to the previous dataset, often times there would be a target that was in the same family or complex as one differentially expressed in the previous data set. For example, the microRNA hsa-miR-184 which is upregulated has a predicted target with a score of 80 with CAMK2B. This is the beta subunit of CAMK2A which is downregulated in my previous data set. These two are important isoforms and subunits of Calcium/calmodulin-dependent protein kinase II

that are known to play a role in neurodevelopment(Akita et al., 2018). While not directly linked to cell cycle control, they seem to be related more to the regulation of the neuronal excitatory response. It is interesting to see that this linkage can be found between the datasets. While these two datasets seem to be pointing towards a link to cancer related genes, more investigation is needed with a clearer targeting system and more refined investigation into the mechanisms that link these microRNA to the cancer genes.

In order to investigate the effects of these significant miRNAs in our TM cells we attempted to manipulate the levels of them in culture. With miRIDIAN microRNA Hairpin Inhibitors, which bind to their corresponding miRNA preventing them from binding to their target genes, and Mimics, which act as a synthesized miRNA binding and inhibiting their target, we wanted to investigate whether or not they would inhibit proliferation in our healthy TM lines. For this we transfected two lines with batches of the hairpins/mimics in order to make sure that interactions between these regulating miRNAs were not a factor. We removed one inhibitor or mimic form each batch and used the MTT proliferation assay to observe if there was a decrease in proliferation in certain batches over others. While the idea was to eliminate certain microRNA that were not contributing to proliferation, we were unable to successfully sustain a line in which the hairpins/mimics were placed. However, with both attempts using batches of 17 and batches in which the 5 most significant miRNA were manipulated, we were meet with total cell death. With this knowledge, it would most likely be a better idea for future experiments to test the effects on cell cycle control with a single hairpin/mimic or smaller set of two to three batches in order to investigate if interactions between the manipulations are the cause of the changes in cell cycle control.

REFERENCES

Abu-Hassan, D. W., Acott, T. S., & Kelley, M. J. (2014). The Trabecular Meshwork: A Basic Review of Form and Function. *Journal of ocular biology, 2*(1), http://fulltextarticles.avensonline.org/JOCB-2334-2838-2302-0017.html. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25356439

https://www.ncbi.nlm.nih.gov/pmc/PMC4209746/.

Afshar-Kharghan, V. (2017). The role of the complement system in cancer. *The Journal of clinical investigation, 127*(3), 780-789.

Akita, T., Aoto, K., Kato, M., Shiina, M., Mutoh, H., Nakashima, M., . . . Shiihara, T. (2018). De novo variants in CAMK 2A and CAMK 2B cause neurodevelopmental disorders. *Annals of clinical and translational neurology, 5*(3), 280-296.

Alvarado, J., Murphy, C., Polansky, J., & Juster, R. (1981). Age-related changes in trabecular meshwork cellularity. *Investigative ophthalmology & visual science, 21*(5), 714-727.

Booy, E. P., McRae, E. K., Koul, A., Lin, F., & McKenna, S. A. (2017). The long non-coding RNA BC200 (BCYRN1) is critical for cancer cell survival and proliferation. *Molecular cancer, 16*(1), 109.

Calderón, J. F., & Retamal, M. A. (2016). Regulation of connexins expression levels by microRNAs, an update. *Frontiers in physiology, 7*, 558.

Chang, J. Y., Folz, S. J., Laryea, S. N., & Overby, D. R. (2014). Multi-scale analysis of segmental outflow patterns in human trabecular meshwork with changing intraocular pressure. *Journal of Ocular Pharmacology and Therapeutics, 30*(2-3), 213-223.

Davis, J. M. (1994). *Basic cell culture: a practical approach*.

Gonzalez, J. M., Hamm-Alvarez, S., & Tan, J. C. (2013). Analyzing live cellularity in the human trabecular meshwork. *Investigative ophthalmology & visual science, 54*(2), 1039-1047.

Gu, L., Lu, L., Zhou, D., & Liu, Z. (2018). Long noncoding RNA BCYRN1 promotes the proliferation of colorectal cancer cells via Up-regulating NPR3 expression. *Cellular Physiology and Biochemistry, 48*(6), 2337-2349.

Hu, T., & Lu, Y.-R. (2015). BCYRN1, a c-MYC-activated long non-coding RNA, regulates cell metastasis of non-small-cell lung cancer. *Cancer cell international, 15*(1), 36.

Iacoangeli, A., Lin, Y., Morley, E. J., Muslimov, I. A., Bianchi, R., Reilly, J., . . . Tiedge, H. (2004). BC200 RNA in invasive and preinvasive breast cancer. *Carcinogenesis, 25*(11), 2125-2133.

Kim, H.-S., Lee, J.-J., Do, S.-I., Kim, K., Do, I.-G., Kim, D.-H., . . . Sohn, J. H. (2015). Overexpression of cytokeratin 17 is associated with the development of papillary thyroid carcinoma and the presence of lymph node metastasis. *International journal of clinical and experimental pathology, 8*(5), 5695.

Kochan, G., Escors, D., Breckpot, K., & Guerrero-Setas, D. (2013). Role of non-classical MHC class I molecules in cancer immunosuppression. *Oncoimmunology, 2*(11), e26491.

Kwon, Y. H. (2008). *A Patient's Guide to Glaucoma*: FEP International.

Lazar, T., Götte, M., & Gallwitz, D. (1997). Vesicular transport: how many Ypt/Rab-GTPases make a eukaryotic cell? *Trends in biochemical sciences, 22*(12), 468-472.

Lenkala, D., LaCroix, B., Gamazon, E. R., Geeleher, P., Im, H. K., & Huang, R. S. (2014). The impact of microRNA expression on cellular proliferation. *Human genetics, 133*(7), 931-938.

Levin, L. A., & Albert, D. M. (2010). *Ocular Disease: Mechanisms and Management: Expert Consult-Online and Print*: Elsevier Health Sciences.

Liu, J., Liu, L., Cao, L., & Wen, Q. (2018). Keratin 17 promotes lung adenocarcinoma progression by enhancing cell proliferation and invasion. *Medical science monitor: international medical journal of experimental and clinical research, 24*, 4782.

Liu, L., Lan, G., Peng, L., Xie, X., Peng, F., Yu, S., . . . Tang, X. (2016). NDUFA4L2 expression predicts poor prognosis in clear cell renal cell carcinoma patients. *Renal failure, 38*(8), 1199-1205.

Liu, W., & Wang, X. (2019). Prediction of functional microRNA targets by integrative modeling of microRNA binding and target expression data. *Genome biology, 20*(1), 18.

Lv, Y., Nie, S. L., Zhou, J. M., Liu, F., Hu, Y. B., Jiang, J. R., . . . Liu, J. S. (2017). Overexpression of NDUFA4L2 is associated with poor prognosis in patients with colorectal cancer. *ANZ journal of surgery, 87*(12), E251-E255.

Middleton, C. (1972). Contact inhibition of locomotion in cultures of pigmented retina epithelium. *Experimental cell research, 70*(1), 91-96.

O'Brien, E. T., Metheney, C. D., & Polansky, J. R. (1999). Immunofluorescence method for quantifying the trabecular meshwork glucocorticoid response (TIGR) protein in trabecular meshwork and Schlemm's canal cells. *Current eye research, 19*(6), 517-524.

Otten, J., Johnson, G. S., & Pastan, I. (1971). Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. *Biochemical and biophysical research communications, 44*(5), 1192-1198.

Perkumas, K., & Stamer, W. (2012). Protein markers and differentiation in culture for Schlemm's canal endothelial cells. *Experimental eye research, 96*(1), 82-87.

Quigley, H. A., & Broman, A. T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. *British journal of ophthalmology, 90*(3), 262-267.

Ren, L., & Danias, J. (2010). *A Role for Complement in Glaucoma?*, New York, NY.

Rutkowski, M. J., Sughrue, M. E., Kane, A. J., Mills, S. A., & Parsa, A. T. (2010). Cancer and the complement cascade. *Molecular Cancer Research, 8*(11), 1453-1465.

Steely, H. T., Browder, S. L., Julian, M. B., Miggans, S. T., Wilson, K. L., & Clark, A. F. (1992). The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. *Investigative ophthalmology & visual science, 33*(7), 2242-2250.

Stone, E. M., Fingert, J. H., Alward, W. L., Nguyen, T. D., Polansky, J. R., Sunden, S. L., . . . Nichols, B. E. (1997). Identification of a gene that causes primary open angle glaucoma. *Science, 275*(5300), 668-670.

Sun, J., Liu, X., Hong Gao, L. Z., Ji, Q., Wang, Z., Zhou, L., . . . Li, Q. (2017). Overexpression of colorectal cancer oncogene CHRDL2 predicts a poor prognosis. *Oncotarget, 8*(7), 11489.

Tamm, E. R. (2002). Myocilin and glaucoma: facts and ideas. *Progress in retinal and eye research, 21*(4), 395-428.

Wang, X. (2016). Improving microRNA target prediction by modeling with unambiguously identified microRNA-target pairs from CLIP-ligation studies. *Bioinformatics, 32*(9), 1316-1322.

Wong, N., & Wang, X. (2014). miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic acids research, 43*(D1), D146-D152.

Zhu, J. Y., Pfuhl, T., Motsch, N., Barth, S., Nicholls, J., Grässer, F., & Meister, G. (2009). Identification of novel Epstein-Barr virus microRNA genes from nasopharyngeal carcinomas. *Journal of virology, 83*(7), 3333-3341.

Zhu, W., Gramlich, O. W., Laboissonniere, L., Jain, A., Sheffield, V. C., Trimarchi, J. M., . . . Kuehn, M. H. (2016). Transplantation of iPSC-derived TM cells rescues glaucoma phenotypes in vivo. *Proceedings of the National Academy of Sciences, 113*(25), E3492-E3500.

Zhu, W., Jain, A., Gramlich, O. W., Tucker, B. A., Sheffield, V. C., & Kuehn, M. H. (2017). Restoration of aqueous humor outflow following transplantation of iPSC-derived trabecular meshwork cells in a transgenic mouse model of glaucoma. *Investigative ophthalmology & visual science, 58*(4), 2054-2062.