EXPRESSION OF AUTOPHAGY TRANSCRIPTS AND PROTEINS IN THE OCULAR LENS SUGGESTS A ROLE FOR AUTOPHAGY IN LENS CELL AND CELLULAR DIFFERENTIATION

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Marc Kantorow, Department of Biomedical Science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Author: Lyndzie Mattucci

Title: Expression of Autophagy Transcripts and Proteins in the Ocular Lens Suggests a Role for Autophagy in Lens Cell and Cellular Differentiation

Institution: Florida Atlantic University

Thesis Advisor: Dr. Marc Kantorow

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The lens is an avascular organ that focuses light onto the retina where neural signals are transmitted to the brain and translated into images. Lens transparency is vital for maintaining function. The lens is formed through a transition from organelle-rich epithelial cells to organelle-free fiber cells. Lens cell differentiation, leading to the lack of organelles, provides an environment optimal for minimizing light scatter and maximizing the ability to focus light onto the retina. The process responsible for orchestrating lens cell differentiation has yet to be elucidated. In recent years, data has emerged that led our lab to hypothesize that autophagy is likely involved in lens cell maintenance, cell differentiation, and maintenance of lens transparency. As a first step towards testing this hypothesis, we used RT-PCR, western blot analysis, immunohistochemistry, confocal microscopy, and next generation RNA-Sequencing (RNA-Seq) to examine autophagy genes expressed by the lens to begin mapping their lens function.

DEDICATION

This manuscript is dedicated to my daughter, who has been with me every step of the way. Completing this segment of my life represents a new beginning for the two of us. I also dedicate this work to my parents, without whom, pursuing this degree would have never been possible. Your support, love, and encouragement made this possible, and I can never thank you enough.

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TABLES

FIGURES

INTRODUCTION

The lens is an avascular epithelial tissue whose primary function is to focus light onto the retina, where neural signals can be translated into images in the brain. The lens is composed of anterior cuboidal epithelial cells that overlie posterior fiber cells that exist as a continuum of differentiated states (Brennan et al 2012). The lens can be broken down into and evaluated as four separate functional zones: central epithelium (EC), equatorial epithelium (EQ), peripheral fibers (FP), and central fibers (FC) (Leonard et al 2008). Epithelial cells form a monolayer on the anterior portion of the lens. As they are found in the equatorial region and finally, in the center of the lens, the epithelial cells elongate and begin the process of losing their organelles, including nuclei (Bassnett and Mataic, 1997), mitochondria, and endoplasmic reticulum (Bassnett and Beebe 1992). At the posterior region of the lens equator, the epithelial cells are capable of mitosis; however, this is where differentiation commences, which is a continuous process throughout life that slows with age (Delamere NA 2006). Fiber cells are long, narrow and tightly packed together to form a honeycomb-like array (Sugiyama et al. 2011). Intracellular dimensions of fiber cells are approximately 20nm, which is small when compared to the distance of the visible light wavelength. Degrading organelles also provides a cytoplasmic environment with a refractive index, of approximately 1.37, that is conducive to minimizing light scatter (Johnsen and Widder, 1999). This, together with the tightly packed formation of cells and their contents, minimizes light scatter and promotes transparency (Delamere 2006).

Since lens fiber cells are not renewed once differentiation has commenced, lens cell transparency and its maintenance become essential to the viability and function of the lens. Lens cells are exposed to a host of exogenous environmental and endogenous oxidative stress factors that can compromise this transparency throughout life, making for an ideal model for studying how long-lived cells in the body manage stress as

well as for studying cell differentiation (Brennan et al. 2012, Wang et al. 2010). Cataracts are a major disease of the eye, where lens transparency has deteriorated and visual clarity is lost (Delamere NA 2006). As the lens ages, the lens structure loses elasticity (Burd et al. 2011) membranes and lipid stores becomedamaged (Costello et al. 2012), and core lens proteins become damaged and potentially aggregate, all of which likely contribute to loss of lens transparency and lead to age-related cataracts (Hooi et al. 2013). The only treatment currently available for those affected with cataracts is surgical intervention in which the lens is removed and replaced with an intraocular lens to restore vision (Delamere 2006). With an aging population, age-related cataracts and other age-related eye diseases not only create a more difficult and dangerous situation for older adults by limiting vision, they create a burden to the economic and healthcare systems (Ghodes et al. 2005). Although, age-related cataracts are an issue with the elderly population, they are not the only type of cataract. Congenital cataracts cause approximately one-third of infant blindness cases, and are a major cause of vision loss in children, worldwide. Congenital cataracts can occur as a single condition or as a result of a systemic condition affecting many tissues. Congenital cataracts can be inherited as autosomal dominant, autosomal recessive, or X-linked. To date, more than 40 loci have been identified and linked with congenital cataracts, with more than 26 of them associated with mutations of specific genes (Chen et al 2011). By understanding those mutations that cause congenital cataract formation, we can better understand those functions that are essential for lens maintenance of transparency and differentiation, especially combined with an understanding of those mechanisms occurring in the lens wild type condition.

Chen et al. (2011) assessed autosomal recessive congenital cataracts (arCC) in 12 consanguineous Pakistani families. In this study, nine mutations were found in the gene encoding FYVE and coiled-coil domain containing 1 (FYCO1) in the 12 Pakistani families and 1 Arab Israeli family. All but one mutation resulted in nonsense mutations, making the resulted transcripts potential targets for nonsense-mediated mRNA decay. One mutation, Leu1376Pro, was found to be a missense mutation, resulting in a disruption in the conserved uncharacterized golgi dynamics (GOLD) domain, suggesting a necessity for FYCO1 function. Because FYCO1 has been shown to be an important protein for autophagy (Pankiv et al 2010) these data imply that autophagy is important for lens differentiation and function, and its disruption could be a causative factor in cataract formation.

Autophagy is a complex mechanism, with various proteins interacting in multiple pathways to maintain cell integrity by removing and recycling varying cellular components such as protein, lipids, and sugars (Brennan et al 2012, Kusama et al 2009). Three types of autophagy have been classified to date: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy, herein referred to as autophagy, is a well-characterized derivative of autophagy that utilizes sequestration of cellular materials by double membrane structures, called autophagosomes, that are trafficked and fuse with endosomes and eventually lysosomes for degradation of their components and subsequent recycling (Fan and Zong 2013, Wang and Qin 2013). As mentioned above, one such protein shown to have a critical role in maintaining the seamless flow of events for autophagy is FYCO1. FYCO1 plays a critical role in mediating plus-ended migration of autophagosomes along microtubules via interaction with kinesin by its coiled-coil domain. FYCO1 interacts with other autophagy components such as LC3 (and related homologues), Ptinds3P, and Rab7, to accomplish this task (Pankiv et al 2010). Autophagy has been shown to degrade and recycle organelles and cellular components by recruiting proteins and other degradation mechanisms to break down compromised organelles or to degrade organelles in response to intra- or extra-cellular signals (Fan and Zong 2013, Wang and Qin 2013). For example, when mitochondria become dysfunctional or are no longer needed, a similar process, called mitophagy, degrades the mitochondria (Karbowski et al 2011). Because of its role in organelle degradation and cell maintenance, it is interesting to speculate that autophagy may have a large role in these processes in the lens.

To date, autophagy has not been extensively studied in the lens. Autophagosomes have been demonstrated in both lens epithelium and fiber; however, not explicitly explained as such (Menko et al, 1984). Matsui et al. (2005) originally suggested that autophagy was not responsible for organelle degradation based on their finding that the lens cells continued to differentiate in Atg5 -/- transgenic mice.

However, Nishida et al. (2009) showed that autophagolysosomes did form in Atg5 -/- and Atg7-/- transgenic mice, which were once believed to be required for initiating autophagy, suggesting that an alternative pathway must exist. Morishita et al. (2013) demonstrated “lens-specific” knockout mice for Atg5 and Vps34 still form an organelle-free zone however, develop congenital cataract at 6 months for Atg5 and birth for Vps34 respectively. However, there are some concerns with analysis of this data. In addition to ATG5-independent forms of autophagy, there is also Beclin-1-independent (Grishchuk et al.

2011) and Pik3c3-independent autophagy (Zhou et al. 2010), suggesting that many pathways of autophagosome formation exist (Park et al. 2013) calling into question the reliability that "autophagy" was deleted in either genotype. Careful examination of the Pik3c3 knockout mice by the data presented even demonstrates organelle retention and altered distribution and lysosomal accumulation, demonstrating that Pik3c3-mediated autophagy is very important in this process, although this observation was not called by the authors and is our own interpretation of the data presented. In the previously mentioned studies, the roles of the newly discovered process of mitophagy (degradation of mitochondria via a process similar to macroautophagy) (Ding et al. 2012, Ding et al. 2012) and microautophagy (sequestration of cellular material via the lysosome) (Li et al. 2012) were not explored. The conclusions are fully merited, and further investigation into the role of autophagy in the lens is warranted.

We hypothesized that autophagy is an important mechanism that maintains lens cell integrity, thus, maintaining lens transparency and is potentially important for lens cell maintenance, differentiation, and maturation. As a first step in testing this hypothesis, we began by examining those spatial expression patterns of autophagy components in the lens wild type condition and whether or not autophagy could be induced by serum starvation in cultured human lens cells. Our data provide evidence that autophagy components are expressed throughout the lens, in both lens epithelium and fiber cell sub compartments, and that autophagy can be induced in human lens cells. These data, in conjunction with human cataract studies suggest that autophagy is critical for lens maintenance of transparency and is likely to be important for lens cell differentiation.

MATERIALS AND METHODS

Gene expression analysis of specific autophagy transcripts from pooled microdissected human lens epithelium and fibers

The levels of autophagy transcripts were analyzed from Affymetrix (U133A) microarray (Affymetrix, Santa Clara, CA) gene signature intensities detected upon hybridization with reverse transcribed and fragmented total lens RNA isolated from pooled microdissected human lens epithelium (7–9 mm central) and fibers (rest of lens; average age 57.8, age range 47–69). These data were previously reported in Hawse et al (2005). Raw affymetrix chip data were normalized between lens epithelium and fiber cell populations using the housekeeping genes *GAPDH* (glyceraldehyde-3-phophate dehydrogenase), *PGK* (phosphoglycerate kinase), and *TRP* (trieosphate isomerase) as standards. Selected autophagy transcripts were further evaluated by semi-quantitative real-time PCR (RT–PCR) using the SuperScript® III one-step RT–PCR system with Platinum Taq polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and *GAPDH* as control. We assayed 50–100 ng of total RNA from human microdissected lens tissue. RNA was isolated from microdissected human lens epithelium and fiber cells as previously described (Goswami et al 2003) using the Total RNA kit (Ambion, Woodland, Tx) according to the manufacturer's instructions. A summary of primers used is provided in Table 1. PCR cycle numbers were chosen to be linear at the indicated amounts of RNA and cycle numbers (Table 1).

The corresponding levels of autophagy proteins were further analyzed by western analysis. Protein samples were mixed with 2× Laemmli sample buffer (0.5 M Tris-HCl, pH 6.8, Glycerol 10% [w/v], SDS 0.1% [w/v], 0.0025% Bromophenol Blue, and 5% 2-Mercaptoethanol) at a 1:1 volume ratio and heated at 100 °C for 5 min. Samples were separated by electrophoresis on 8%, 10%, and 15% sodium dodecyl sulfate-polyacrylamide gels where appropriate at room temperature using a Bio-Rad mini Protean® vertical

electrophoresis system (Bio-Rad, Hercules, CA). Proteins were transferred onto Hybond™ ECL™ nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using a Bio-Rad mini Trans Blot® electrophoresis system (Bio-Rad) for 1.5 h at 100 V. Following transfer immunoblots were rinsed in phosphate buffered saline (PBS) pH 7.2 for 2 min. Immunoblots were then blocked in 5% milk in Tris Buffered Saline with Tween (TBST; 5% fat-free dry milk, 0.1% Tween-20, 150 mM NaCl, and 50 mM Tris at pH 7.5) for 1 h before incubation with the appropriate primary antibody diluted in 5% milk TBST (anti-LC3B antibody [Abcam, Cambridge, MA] 1:1,000, anti-RB1CC1/FIP200 [Bethyl Labs, Montgomery, TX] 1:1,000, anti-FYCO1 [Bethyl laboratories] 1:1,000 and anti-BNIP3L/NIX [Enzo Life Sciences, Plymouth Meeting, PA] 1:2,000). Blots were washed in TBST and incubated for 1 h with 1:5,000 DyLight goat anti rabbit 800 conjugated secondary antibody (Thermo Scientific, Rockford, IL) followed by rinsing in PBS pH 7.2 for 2 min. Immunoblots were imaged for 2 min on the Odyssey Imaging System (LI-COR Biosciences, Lincoln, Nebraska).

Spatial localization of LC3B and FYCO1 protein in mouse lens

Animal husbandry and experiments were conducted in accordance with the approved protocol of Animal Institute Committee (Albert Einstein College of Medicine, NY) and the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Noon of the day that the vaginal plug was observed was considered as E0.5 of embryogenesis. Pregnant female mice were euthanized by CO2 and sacrificed following standard procedure. Mouse embryos were dissected and then fixed in 10% neutral buffered paraformaldehyde overnight at 4 °C before paraffin embedding. Serial sections were cut in 5 μm thick sections through the mid-section of the lens. Immunohistological staining was performed following standard procedures described below. Antigen retrieval was performed to unmask the paraffin embedded tissues before antibody incubation.

Whole mouse head sections were processed from a postnatal day 1 (P1) mouse, and LC3B and FYCO1 proteins were visualized by immunohistochemistry using the ImmPRESS Reagent kit according to the manufacturer's instructions (Cat no. MP-7401; Vector Laboratories, Burlingame, CA). Briefly, tissues were deparafinized and hydrated using xylene and ethanol gradients and then rinsed in tap water for 5 min.

The sections were blocked with 2.5% horse serum for 1 h. Primary FYCO1 (Cat no. A302–796A; rabbit polyclonal; Bethyl Labs) and LC3B antibodies (rabbit polyclonal; Sigma-Aldrich, St Louis, MO) were both diluted in 2.5% horse serum at 1:250, added to the sections and incubated overnight at 4 °C. The sections were washed in phosphate buffered saline contiatween 20 (PBS-T) for 5 min and incubated with the ImmPRESS reagent (Cat no. MP-7401; anti-rabbit immunoglobulin peroxidase, Vector Laboratories) at room temperature for 30 min according to the manufacturer's instructions. The sections were washed again in PBS-T and incubated with ImmPACT DAB Peroxidase Substrate (Cat no. SK-4105; Vector Laboratories) for 4 min at room temperature. For the sections that were counterstained, Vector's Hematoxylin QS (Cat No H-3404) was used according to the manufacturer's instructions. Tissue sections were incubated with hematoxylin counterstain for 30 s at room temperature and dipped in tap water for 10 s to remove excess stain. Sections were cleared and mounted with VectaMount Permanent Mounting Medium (Cat no. H-5000; Vector Laboratories). Identical procedures were performed using only rabbit secondary antibody as a control. Sections were visualized using an Olympus Provis AX70 (Olympus, Center Valley, PA) fluorescent microscope and images captured using Magnafire software (Optronics, Goleta, CA).

Human lens cell culture

A human lens epithelial cell line (HLEB3) (Andley et al 1996) (a gift from Dr. Majorie Lou, University of Nebraska-Lincoln, Lincoln, NB) was grown and cultured in Dulbecco Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Invitrogen), gentamicin (50 units/ml; Invitrogen), penicillin-streptomycin antibiotic mix (50 units/ml; Invitrogen), and amphotericin B (1.25 µg/ml; Invitrogen) at 37 °C in the presence of 5% CO2. For induction of autophagy by serum starvation HLEB3 lens cells were plated in 24 well plates at a density of 50,000 cells per well overnight. For serum starvation, HLEB3 cells were transferred to serum-free media with or without addition of 50 µM chloroquine, an autophagy inhibitor that prevents autophagosome fusion with lysosomes (Mizushima et al 2010), and assessed for autophagy at 24 h post treatment by staining with an LC3B specific antibody and fluorescent confocal microscopy as described below.

LC3B accumulation assays

HLEB3 lens cells were plated onto coverslips and treated as described above for induction of autophagy using serum starvation. Immunofluoresence staining was conducted by fixing cells with 3.7% formaldehyde in PBS, blocking with 1% BSA and permeabilizing with 0.25% TritonX-100 in PBS. Following permeabilization, a rabbit polyclonal anti-LC3B (Sigma-Aldrich) at 1:1,000 was incubated overnight at 4 °C. Cells were washed three times with PBS, and subsequently incubated with Alexa Fluor 488 goat anti-rabbit secondary (Invitrogen) for 1 h at room temperature at a 1:2,000 dilution. HLEB3 cells were washed three times with PBS and the nucleus counterstained using 300 nM DAPI (Invitrogen) for 2 min. Cells were washed three times with PBS and mounted onto glass slides using ProLong Gold antiFade reagent (Invitrogen). Immunofluoresence staining was visualized with a Zeiss LSM 700 Confocal microscope (Zeiss, Thronwood, NY). LC3B puncta were quantified in at least 50 cells per treatment using the AxioVision 4 software (Zeiss) by manual visual selection of "events" as described below and the mean and standard deviation calculated. Fully rounded intense green staining of LC3B was counted as a single puncta or "event" representing an autophagosome; diffuse staining is believed to be cytoplasmic LC3 I and was not counted as puncta. Data presented is representative of 3 independent experiments. Differences between treatments and controls were determined using Tukey's test following one-way ANOVA. A p-value less than 0.001 was considered statistically significant.

Table 1. List of primers used for semi-quantitative RT-PCR of human autophagy transcripts

Gene	Forward Primer	Reverse Primer	NCBI #	Cycles	Ng RNA	Annealing temp
beclin 1	CGGGAAGTCGCTGAAGACAG	CCATCCTGGCGAGGAGTTTC	NM 003766.3	30	100	55
atg14	GAGCGGCGATTTCGTCTACT	CTGAAGACACATCTGCGGGG	NM 014924.4	35	50	55
mtor	TTCTGGTGCGACACCGAATC	CATCGGGTTGTAGGCCTGTG	NM 004958.3	30	100	55
rb1cc1/ fip200	GGAGCTTGTGCACCTGAACT	GAAGCACCCTCACCTGGTTTG	NM 014781.4	35	50	55
ralb	GCTCGTCGTGGGAAACAAGT	TGACAAAGCAGCCCTTCCAC	NM 002881.2	35	50	55
atg4a	GAGTAAGGGCACCTCTGCCTA	GTTCATTCGCTGTGGGGACT	NM 052936.3	35	50	55
atg12	GAGGTCTGTAGTCGCGGAGA	TGGATGGTTCGTGTTCGCTC	NM 004707.3	30	100	55
map1lc3b/ atg8	AAGTGGCTATCGCCAGAGTCG	CTGAGATTGGTGTGGAGACGC	NM 022818.4	25	100	55
rab7	GACACAGCAGGACAGGAACG	TTGTACAGCTCCACCTCCGT	NM 004637.5	25	100	55
fyco1	GAAGCTGAAGGCCACCCAAG	GGGCATCTGACTTCTGCCAG	NM 024513.3	35	50	55
bnip3l/ nix	ACTCGGCTTGTTGTGTTGCT	TCCCTGCTGGTGTGCATTC	NM 004331.2	25	100	58
pink1	TCTGCAGTCCTCTGCTCACA	GCTCATCCGTCACTTTCGCT	NM 032409.2	35	50	55
p62	CTCACCGTGAAGGCCTACCT	TAGCGGGTTCCTACCACAGG	NM 003900.4	35	50	55
gapdh	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC	NM 003900.4	35	50	60

RESULTS

Preliminary Result-Gene expression analysis of autophagy mRNA transcripts

Microarray analysis revealed 42 autophagy-associated genes, which were grouped into functions; induction (represented by 14 genes), expansion/closure (represented by 8 genes), fusion/degradation (represented by 6 genes), mitophagy (4 genes), chaperone mediated autophagy (CMA, represented by 4 genes), adaptor proteins (2 genes), and autophagy inhibitors (4 genes) (Figure 1). Each gene investigated was found to be present in both the epithelial cells and fiber cells, even though gene expression was not necessarily found to be uniform between both the two types of cells.

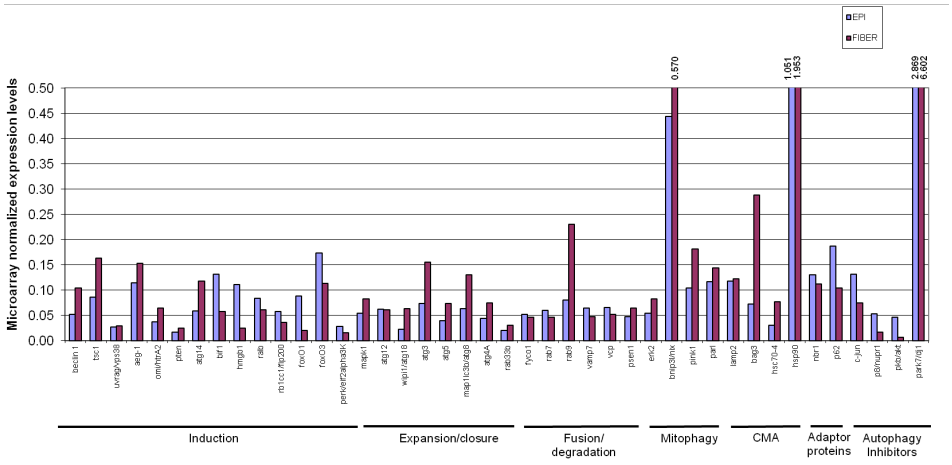

Figure 1. Affymetrix gene expression data of autophagy transcripts

My role for this data was to help identify and correlate absolute transcript levels with their autophagic functions based on intensities of raw chip data.

Primary Results

Beginning with the raw data from the microarray, 13 autophagy mRNA transcripts were chosen for further analysis. Using the induction genes *beclin 1, atg14, rb1cc1/fip200, ralb, and atg4a*; the

expansion genes *atg12* and *map1lc3b/atg8*; the fusion genes *fyco1* and *rab7*; the mitophagy genes *bpnip3l/nix* and *pink1*; the adaptor protein *p62*; and the inhibitor *mtor*, a separately prepared sample of human epithelial and fiber lens cells was used to investigate expression using RT-PCR (Figure 2). The RT-PCR data corroborate the results concluded from the microarray data; autophagy mRNA transcripts were present in both lens epithelial and fiber cells, further suggesting that autophagy may play a role in lens development and differentiation.

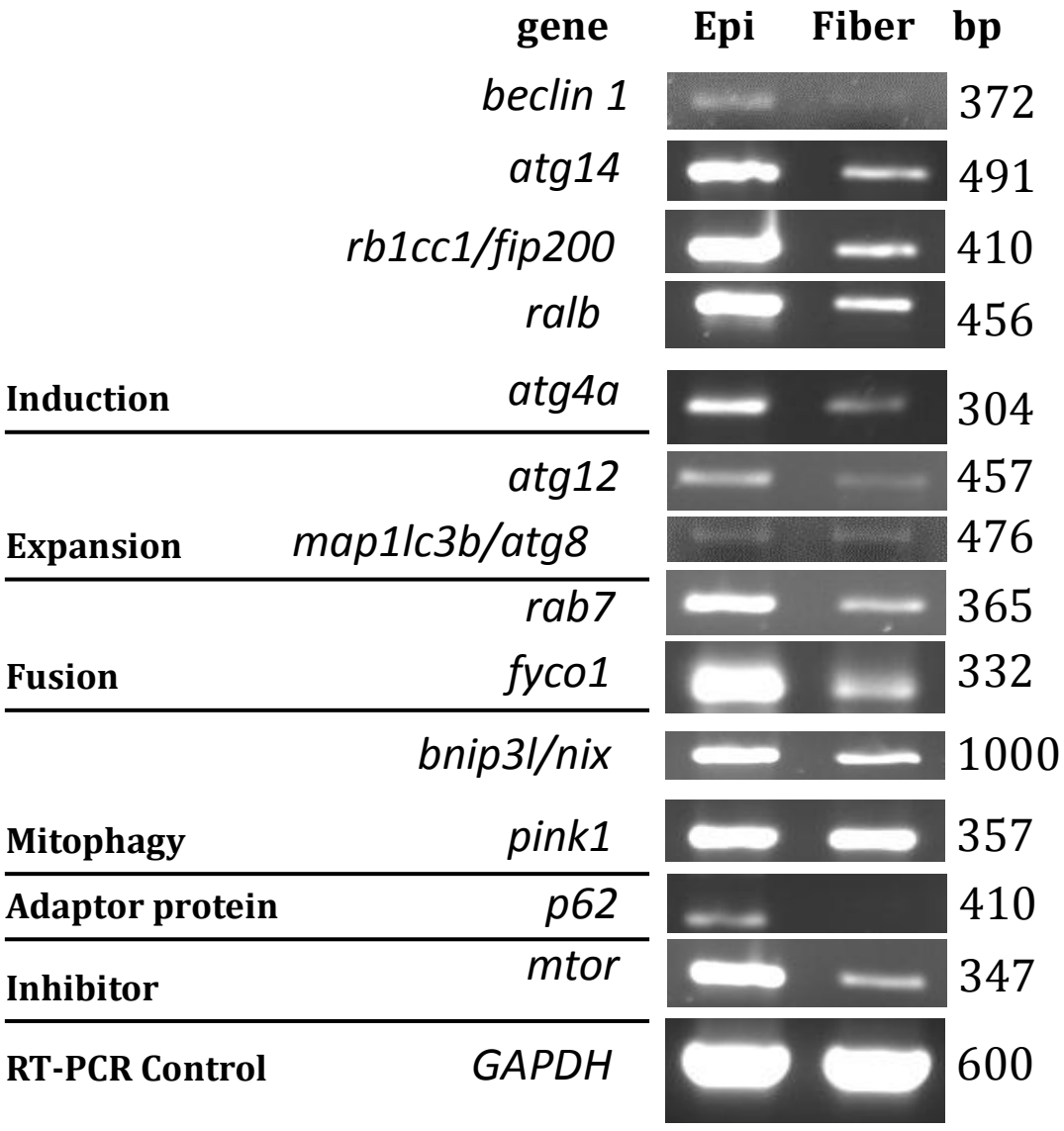

Figure 2. Semi-quantitative RT-PCR confirmation of select Autophagy Transcripts

My role for this set of data was to help design the primers and image.

Four of the mRNA transcripts investigated in the RT-PCR experiment were chosen and further analyzed for protein expression using western blot analysis. An induction protein, RB1CC1/FIP200; an expansion protein, LC3B; a fusion protein, FYCO1; and a mitophagy protein BNIP3L/NIX were isolated from a third pool of human lens epithelial and fiber cells and then, examined for expression. LC3B showed 3 distinct bands interestingly, with the 18kDa band consistent with the cytosolic form LC3I and the 16kDa band consistent with membrane-bound LC3II. RB1CC1, or FIP200; FYCO1 and BNIP3L, or NIX, all exhibited bands where we expected them to be, 200 kDA, 200 kDA, and 40 kDA, respectively.

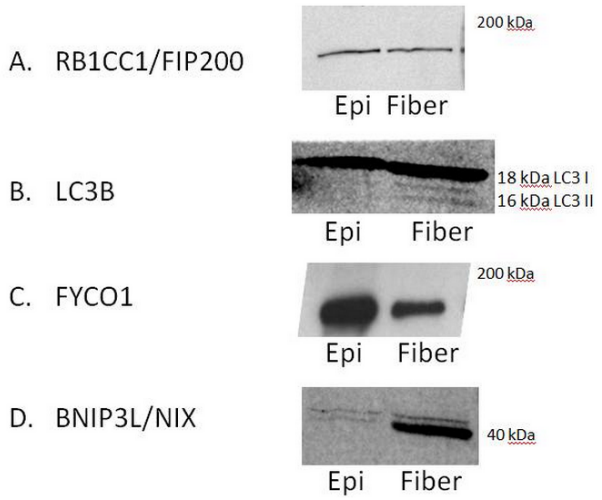

Figure 3. Western blot analysis of select autophagy protein expression in the lens

My role was to aid in data analysis.

Using LC3B-specific and FYCO1-specific antibodies, localization of the corresponding proteins could be specifically assessed. Figure 4 shows how the autophagosomal marker LC3B was found to be present within both lens epithelial and lens fiber cells, but more specifically, highest levels were found in nuclear fibers, indicating that autophagy may have had a role in lens cell differentiation. FYCO1 immunostaining showed a similar staining pattern as that of LC3B, once again, suggesting a role for autophagy in the lens.

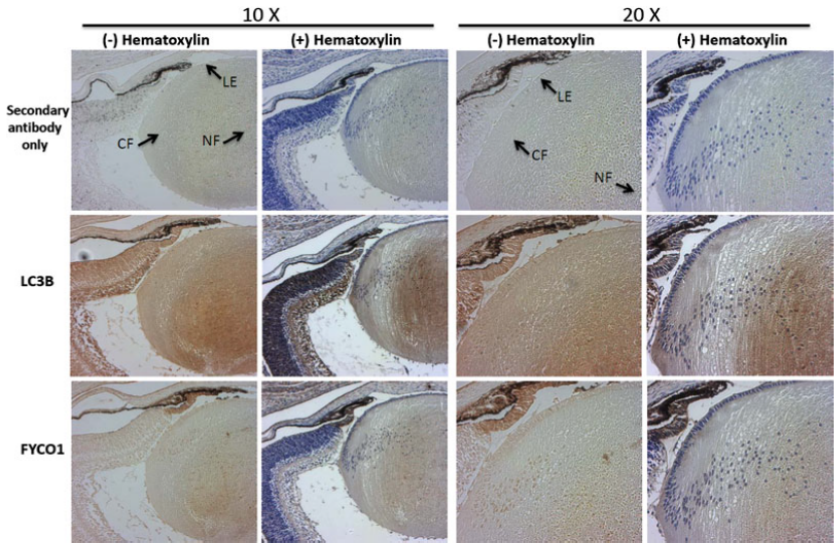

Figure 4. Immunohistochemisty staining of LC3B and FYCO1 expression in the lens

Sections stained by WL Kantorow. Analysis by Brennan et al. (2012). My role was to help WL Kantorow and Brennan image, analyze, and interpret this data.

To examine whether or not autophagy could be induced in the lens, human epithelial lens cells were exposed to serum-free media and chloroquine and then measured for the presence of the autophagosomal marker, LC3B. Chloroquine inhibits autophagosome/lysosome fusion, exposing any LC3BII present on the surface of the autophagosome. Upon analysis of LC3B-positive puncta present on the surface of the autophagosomes, the serum starved epithelial cells demonstrated a fourfold increase as opposed to the complete media (Figure 5).

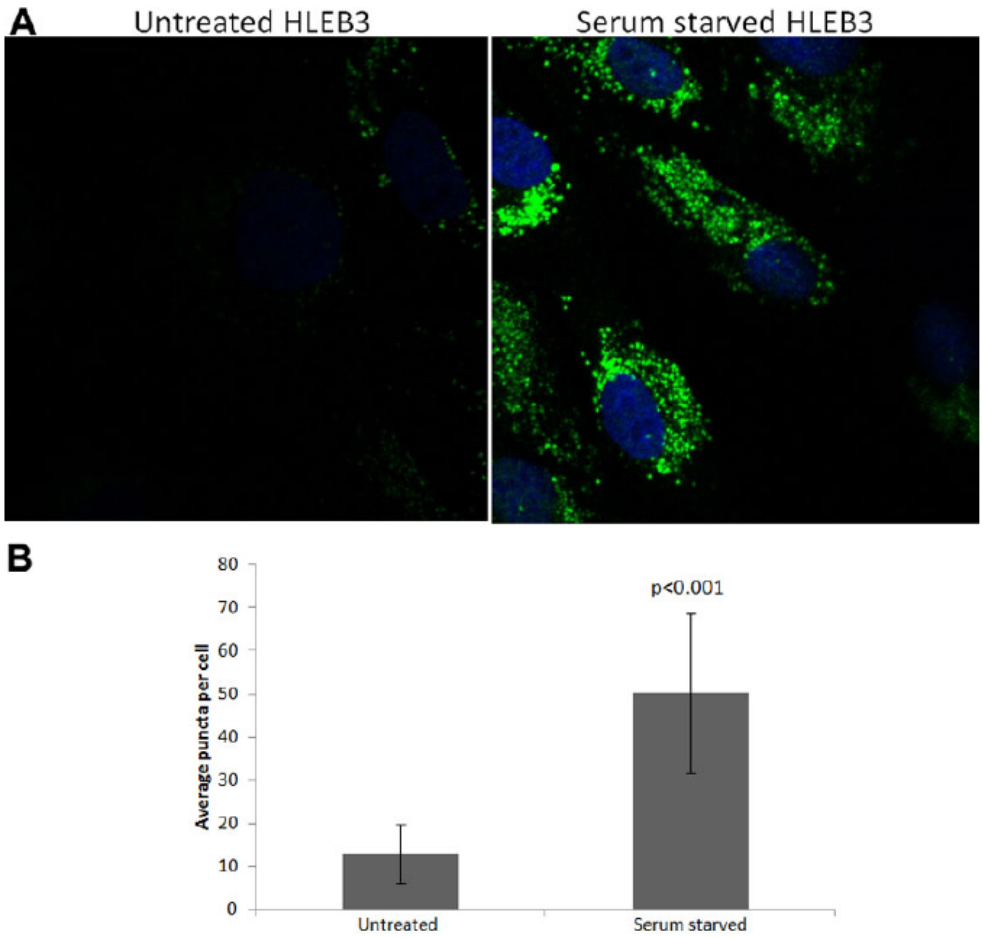

Figure 5. Induction of autophagy through serum starvation and analysis of LC3B-positive puncta

My role was data analysis.

DISCUSSION

Lens differentiation is a process first described in the late 19th century. Since this time, much has been learned about lens cell differentiation. Throughout the years, many researchers have discovered key players found to be involved in this rather obscure process; however, assembling a comprehensive picture has yet to be fully elucidated. Autophagy is a ubiquitous recycling process necessary for maintaining cellular health (Wang and Qin. 2013). Investigating the presence of mRNA transcripts across the different phases of lens differentiation reveals expression patterns and provides insight into how the different autophagy genes may be utilized throughout lens cell differentiation. The purpose of this study looked at spatial expression of mRNA transcripts known to be associated with autophagy in human and mouse lens cells.

Beginning with the raw data from Hawse et al. (2005) (Figure 1), our laboratory further investigated mRNA transcript expression and corresponding protein expression, spatial expression patterns, and the possibility of inducing autophagy in the lens. We narrowed the list down from 42 genes in the microarray data to 14 genes in the RT-PCR data. Our data, shown in Figure 2, confirmed the microarray analysis, that the mRNA transcripts examined were present in both lens epithelial and fiber cells. Since mRNA expression doesn't necessarily correlate to protein expression, our laboratory performed protein analysis via western blot (Figure 3) for the autophagy proteins LC3B, a marker for autophagy (Kabeya et al. 2000) and FYCO1, whose disruption has been shown to cause cataracts (Chen et al. 2011). Once again, the expression further confirmed the microarray data, and now, the RT-PCR data; LC3B and FYCO1 are present in both lens epithelial and fiber cells.

The RT-PCR and western blot analysis only revealed whether the mRNA or protein was present in the lens epithelial cells or lens fiber cells. Although it is common to refer to lens cells as existing in two states, epithelial cells and fiber cells, closer examination shows a dynamic continuum in which all stages of

differentiation are continuously displayed; thus, simply stating lens cells are categorized as either epithelial or fiber grossly underrepresents the intricately orchestrated event of differentiation found in the lens (Menko et al 1984). In order to get a more detailed picture of expression, spatial expression patterns for two proteins, LC3B and FYCO1, were investigated using immunohistochemistry staining techniques for microdissected sections of the mouse eye lens (Figure 4). Using LC3B- and FYCO1-specific antibodies, we were able to see a magnified view of LC3B and FYCO1 protein expression. While both proteins were found in the lens epithelium and fiber cells, as expected, we were able to see that both exhibited higher expression in the nuclear fibers, indicating that autophagy may have played a role in differentiation.

Since autophagy is a ubiquitous process found in different tissues, and it appeared that the autophagy machinery was available in the lens, our next step was to investigate whether or not we could induce autophagy in the lens. Exposing lens cells to serum starvation to induce autophagy and exposing them to chloroquine to prevent fusion of autophagosomes and lysosomes would supposedly allow us to see an increase in LC3, suggesting an increase in autophagosomes. Upon analysis, we clearly saw a four-fold increase in LC3-positive puncta in the serum-starved cells versus the cells treated in complete media (Figure 5), indicating that autophagy can be induced in the lens with serum starvation.

Our lab has investigated many autophagy genes. Through our own conclusions and through gathering information present in the literature, we created a beginning list of some autophagy genes present in the lens and their roles in autophagy (Table 2), which seems to be proving the case for the hypothesis that autophagy has a role in lens cell differentiation. In the literature, some alternative autophagy pathways have been unveiled. Nashida et al. (2009) described an Atg5 -/- independent pathway, induced by etoposide, staurosporine, and starvation, and an Atg7 -/- independent pathway, induced under etoposide, exposure, in mouse embryonic fibroblasts (MEFs). Both pathways produced autophagosomes without the presence of LC3-II, a protein that has been characterized to have a crucial role in autophagosome elongation. Zhou et al. (2010) described a Pik3c3-independent pathway in neurons. While Pik3c3 is a member of the PI3K complex III, they showed that autophagy was not disrupted with deletion of the Pik3c3/Vps34 gene. While Grishchuk et al. (2011) described a beclin-1 independent autophagy pathway that appeared to promote apoptosis in cortical neurons. The discovery of these "alternative autophagy" pathways proves the importance of conserving this process. With implications in various tissues

throughout the body, it makes sense that there would be alternative methods in place to ensure cellular homeostasis through autophagy will be maintained, including in the lens.

Table 2. Identified autophagy genes and their functions, compiled by myself, Chauss, and Brennan

Induction	Role in Autophagy	Reference
Beclin 1	Member of PtdIns 3-kinase complex, involved in activation of macroautophagy	53
TSC1	Acts as a GTPase-activating protein for Rheb, thus inhibiting TOR	25
UVRAG	Member of PtdIns 3-kinase complex, regulates macroautophagy	52
AEG1	Gene encodes oncogenic protein that induces macroautophagy independent of Beclin-1 and PtdIns 3-kinase	7
Omi/HtrA2	Degrades the Bcl-2 family-related protein Ha x -1 to allow macroautophagy induction	49
Pten	Dephosphorylates PtdIns (3,4,5) P3 inhibiting PDK1 and PKB/Akt activity	2,77
Atg14	Component of PtdIns 3-kinase complex, targets this complex toward autophagic machinery	61
Bif-1	Interacts with Beclin 1 via UVRAG and is required for macroautophagy	74
HMGB1	Binds Beclin-1 to displace BCL-2 inhibiting apoptosis and promoting macroautophagy	75
RalB	Activation of phagophore assembly through ULK1-Beclin1-Vps34 complex assembly and Exo84 interaction	9
RB1CC1/ FIP200	Component of ULK1 complex, required for phagophore formation, phosphorylation of ULK1/2	30
FoxO1	Regulates macroautophagy independent of transcriptional control	84
Fox O3	Stimulates macroautophagy through transcriptional control of autophagy genes	83
PERK/ eif2α3K	Phosphorylated due to ER stress which induces LC3 conversion and macroautophagy	44,3
Expansion/ Closure		
MAPK1	MAPK/ERK regulates the maturation of autophagosomes	15
Atg12	Ubiquitin-like protein, conjugates Atg5, member of Atg12-5-16 complex, essential for Map1LC3B/Atg8 activation, involved in mitochondrial homeostasis	57,67
WIPI1/Atg18	Binds PI3P by WD40 β-propeller domain, involved in retrograde movement of Atg9	66,65
Atg3	E2 ubiquitin ligase, conjugates PE to Map1Lc3B after Atg7 processing of C-terminus of cleaved Map1LC3B/Atg8, can conjugated to Atg12	67,35
Atg5	Contains ubiquitin-folds, member of Atg12-5-16 complex	57
Map1LC3B/ Atg8	Atg8 homolog, involved in autophagosome biogenesis and cargo recruitment to autophagosomes, marker of autophagosomes	39,81,40
Atg4a	Cysteine protease of yeast Atg8 homologs, required for Map1LC3B/Atg8 activation, able to deconjugate PE of processed Map1LC3B	40,50
Rab33B	Binds Atg16L1, involved in autophagosome maturation by regulation of autophagosome to lysosome fusion, OATL binding partner	33,34
Fusion/ Degradation		
FYCO1	Rab7 effector, binds Map1LC3B and phosphatidylinositol-3-phosphate, coordinates plus-end directed autophagosome transport	63,64
Rab7	Transport of early to late endosomes, docking protein for amphisome to lysosome fusion	63,72,28,36
Rb9	Involved in trafficking from late endosomes to the trans-golgi, believed to be a key component of the ATG5/7 alternative macroautophagy pathway	72,69
VAMP7	SNARE protein, required for autophagosome formation, autophagosome maturation via facilitation of autophagosome to lysosome fusion	58,22
VCP	AAA+ ATPase, required for autophagosome maturation, mutations to vcp results in accumulation of ubiquitin-containing autophagosomes	38,76
PSEN1	Protease, part of the γ-secretase complex, involved in lysosomal degradation	47
Mitophagy		
ERK2	Localizes to the mitochondria, regulates mitophagy	18
BNIP3L/NIX	Bcl2 related, necessary for selective mitochondrial clearance	71
Pink1	Decreased MMP causes altered Pink1 processing, results in spanning of Pink1 across the outer mitochondrial membrane, recruiting Parkin for mitophagy	78
PARL	Mitochondrial protease that regulates PINK1 localization and stability	70
Chaperone Mediated Autophagy		
Lamp2	Lysosomal membrane receptor for chaperon-mediated autophagy allowing translocation of substrates across the lysosomal membrane	17
BAG3	Directs Hsp70 misfolded protein substrates to dynein targeting them to aggresomes for selective degradation	24
Hsc70-4	Aids in targeting of cytosolic proteins to the lysosome for degradation	14
hsp90	Assists in LAMP-2A stabilization during its lateral mobility in the lysosomal membrane	4
Adaptor Protein		
NBR1	Binds ubiquinated proteins allowing degradation by macroautophagy	42,46
p62	Interacts with Atg8 via its LIR domain, adaptor for degradation of ubiquitin-labeled molecules	46,8
Autophagy Inhibitors		
mTOR	Serine/threonine kinase that controls cell growth and metabolism in response to nutrients, growth factors, cellular energy and stress	1,29
c-Jun	Transcription factor, inhibits mammalian macroautophagy induced by starvation	82
p8/Nupr1	Inhibits macroautophagy by repressing the transcriptional activity of FoxO3	43
PKB/Akt	Upstream regulator of mTOR	2
Park7/DJ1	Overexpression suppresses macroautophagy through the JNK pathway	68

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VITA

The following manuscripts and abstracts were published during my graduate studies

Brennan LA, Kantorow WL, Chauss DC, McGreal R, He S, Mattucci L, Wei J, Riazuddin SA, Cvekl A, Hejtmancik JF, Kantorow M. 2012. Autophagy gene expression in the eye lens and its induction in lens cells. *Molecular Vision* (June): 1773-1786.

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