Relative expression of apoptotic genes in the retina of Mus musculus

By

James Joseph Bruce

April, 2012



Allegheny College

Department of Biology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Christy Donmoyer

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Dr. Ron Mumme

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Abstract

Apoptosis, or programmed cell death, is a process which is vital for survival and maintenance of certain tissues, but can be destructive when it occurs in other tissues, such as neural tissue. Interphotoreceptor retinoid-binding protein (IRBP) is a large glycoprotein responsible for photoreceptor development and maintenance, as well as the transport of retinoids. IRBP-deficient mice experience photoreceptor loss via apoptosis and abnormal photoreceptor development and organization. The purpose of this study was to investigate the role of four genes involved in retinal apoptosis, two of which are pro-apoptotic (BAX and Bnip31) and two that are anti-apoptotic (AATF and Bcl-2). To investigate the roles of these genes, their expression in the retinas of P25 IRBP$^{-/-}$ (KO) mice was compared to that of age-matched C57BL/6 (wild-type; WT) mice retinas. My hypothesis was that expression of the two pro-apoptotic genes would be higher in the retinas of the KO mice than in those of the WT mice and that expression of the anti-apoptotic genes would be higher in the WT mice retinas than in those of the KO mice. The results of this study showed that expression of all target genes did not differ between the strains. The most likely explanation for a lack of difference in expression between the two strains is that these four genes are not involved in the retinal apoptosis observed in IRBP$^{-/-}$.

Introduction

Apoptosis, defined as programmed cell death, is essential for normal development. Integral in mediating cell death are caspases, which can induce apoptosis in one of two pathways (Fig. 1). The extrinsic pathway involves cytochrome c binding to cell surface receptors and activating caspases and the TNF pathway. The intrinsic pathway involves other caspases binding to receptors on the outer mitochondrial membrane and inducing intracellular cell death pathways (Budihardjo et al. 1999).

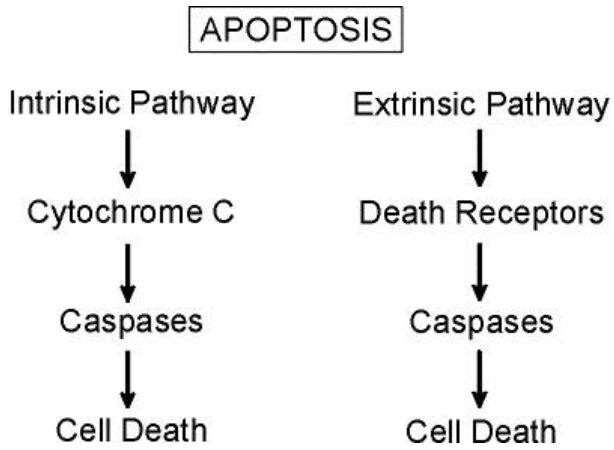

Figure 1: Intrinsic and extrinsic apoptotic pathways. In each pathway, caspases play an integral role by either being released from receptors or binding to receptors. In both cases, cell death is the end result (University of Toronto at Mississagua).

If too much or too little apoptosis occurs, tissue damage can occur (Nakagawa et al. 1999). In some neural cells, for example, apoptosis occurs as part of differentiation. However, in other cells such as photoreceptors, apoptosis is

uncommon and results in an overall degeneration of the tissue and loss of function (Péquignot et al. 2003). When apoptosis occurs in cells leading to a developmental abnormality, a genetic mutation is likely to be the cause (Oppenheim 1991).

In the visual cycle, vitamin A (all-*trans* retinol) is released from rods following light exposure (photic bleaching), carried through the interphotoreceptor matrix (IPM) to the retinal pigment epithelium (RPE) where it is converted into 11-*cis* retinal, and finally carried back to the rod outer segments where it binds to opsin for the resynthesis of rhodopsin (Ripps et al. 2000). A key step in this process is the passage through the IPM from the rods to the RPE. Retinoids are insoluble in the aqueous media, so transport proteins are generally required to escort these proteins across the IPM (Qtaishat et al. 2005).

Interphotoreceptor retinoid-binding protein (IRBP) is a large and abundant glycoprotein found in the interphotoreceptor matrix (IPM) and is considered to be important in the transport of retinoids across the IPM from the photoreceptors to the apical region of RPE cells (Qtaishat et al. 2005). The compound is also thought to be responsible for other functions, including the promotion of the release of 11-*cis* retinal from retinal pigment epithelial (RPE) cells and all-*trans* retinol from photoreceptors following light exposure (bleaching), as well as binding fatty acids required for structural integrity (Jin et al. 2009). When IRBP is deficient, such as in an $IRBP^{-/-}$ mouse, normal photoreceptor development does not

occur (Liou et. al. 1998). Furthermore, in the absence of IRBP, structural abnormalities in the photoreceptor outer segments and RPE are present by 2 months of age. These structural abnormalities include a reduced number of photoreceptors and abnormally short outer segments (Ripps et al. 2000).

Given that IRBP is involved in the transport of retinoids and the release of all-*trans* retinol from rods, it would be expected that a lack of IRBP would result in a defective visual cycle. However, while photoreceptor structure is affected by the absence of IRBP, the visual cycle occurs at a normal rate (Placzewski et al. 1999). In addition, electroretinography assays have shown that retinas of IRBP-deficient mice produce response amplitudes comparable to those of wild-type mice during the early stages of life (Ripps et al. 200). However, the light response amplitudes gradually decrease as the animal ages (Peachey and Ball 2003). The reason for this reduction in visual performance is not due to defective retinal cells, but the death of the cells. A study conducted by Wisard et al. (2011) showed that rod segments in $IRBP^{-/-}$ mice were 28% shorter than those of C57BL/6 wild-type mice.

It has been determined that retinal apoptosis in $IRBP^{-/-}$ mice spikes at age postnatal day 25 (P25), but the mechanisms by which IRBP promotes apoptosis are not completely understood (Wisard et al. 2011). Apoptosis pathways are complex and involve a variety of inhibitory and promoting genes (Péquignot et al. 2003). In

mammals, the mechanism by which apoptosis occurs can either be extrinsic or intrinsic (Péquignot et al. 2003). The extrinsic pathway involves tumor necrosis factor (TNF-α) signaling, leading to the caspase cascade, whereas the intrinsic pathway can involve any number of cellular stresses inducing caspase activation. My aim in this study was to investigate which genes act in conjunction with IRBP to induce the abnormal retinal apoptosis observed in IRBP-deficient mice.

The focus of my study was four genes, two pro-apoptotic and two anti-apoptotic. The two pro-apoptotic genes studied Bnip31 and Bcl-2 associated X protein (BAX). Bnip31 is a protein that interacts with the Bcl-2 family of proteins, which prevent apoptosis. However, overexpression of Bnip31 overcomes the mitigating effects of Bcl-2 (Reed et al. 1998). The BAX protein is a mitochondrial membrane protein which induces cell death at the mitochondrial level, promoting the release of cytochrome c and caspase from the mitochondria, which intracellularly signal the cell to undergo apoptosis (Wolter et al. 1997). Previous studies have shown that inhibition of BAX results in a reduction of apoptosis in neural cells (Péquignot et al. 2003).

The two anti-apoptotic proteins studied were Apoptosis antagonizing transcription factor (AATF) and Bcl-2. AATF, as its name suggests, is a ubiquitous protein which interacts with DNA to inhibit apoptosis. Specifically,

AATF defends against endoplasmic reticulum (ER) stress-mediated apoptosis by promoting transcription of the protein ATK1, which aids in cell survival (Ishigaki et al. 2010). In addition, AATF phosphorylates Dlk, which induces apoptosis by a mechanism which is not yet understood (Page et al. 1999). Bcl-2 inhibits apoptosis by acting on a wider range of organelles, including the mitochondria, endoplasmic reticulum, and nuclear membrane (Hockenbery et al. 1993). The main site of Bcl-2 regulation is the mitochondrial membrane, where it inhibits the release of cytochrome c, thus preventing the activation of caspase and the associated apoptotic pathway (Kluck et al. 1997).

The purpose of my study was to determine the relative expression of these pro- and anti-apoptotic genes in C57BL/6 (wild-type; WT) mice in comparison with $IRBP^{-/-}$ mice. I hypothesize that, since apoptosis is occurring in the retinas of the IRBP-knockout mice, there will be higher expression of the two pro-apoptotic genes in the knockout group versus the wild-type group. Conversely, I expect to see lower expression of the anti-apoptotic genes in the knockout mice versus their wild-type counterparts. Also, based on the specific nature of each of the genes and how they are involved in apoptosis, I intend to gather information about which apoptotic pathway is responsible for the degeneration observed in the $IRBP^{-/-}$ mice.

Materials and Methods

Subjects

To provide a model for retinal degeneration, $IRBP^{-/-}$ mice pups were chosen as the experimental group and C57BL/6 (WT) pups as the control group. Four litters of each strain were reared to age postnatal day 25 (P25). All animals were kept at room temperature, fed regular mouse chow and water, and kept on a 12-hour light/dark cycle.

Tissue Collection

Once the pups were raised to postnatal day 25, they were sacrificed individually via 5 minutes of CO_2 inhalation followed by cervical dislocation. The eyes were immediately enucleated from the sacrificed pups and a microdissection was performed to extract the retinas. To obtain enough genetic material, the retinas of at least four mice (8 eyes) were pooled for each sample. The retinas were placed in 1mL of TRIzol (Invitrogen) and flash frozen to -70℃.

RNA Isolation

Retina samples were thawed, transferred to a 4.5 mL Falcon tube, and an additional 1.5 mL of TRIzol solution was added to each. Each sample was homogenized using a Brinkmann Polytron PT 3000 homogenizer at 11,000 rpm for

30 seconds. The total RNA was then isolated from the homogenate using the Direct-zol RNA MiniPrep kit (Zymo Research, Protocol Appendix B). The three-step thermocycling procedure required for this kit was performed using a Techgene PCR Thermocycler (Techne). Upon completion of total RNA isolation, RNA yield was determined using a NanoDrop spectrometer. Only samples with RNA concentrations greater than 100 ng/µL and $A_{260/280}$ ratios greater than 1.8 were kept.

cDNA Synthesis

cDNA was synthesized using the isolated RNA as a template and the iScript cDNA Synthesis Kit (Bio-Rad, Protocol in Appendix C). This step requires 1 µg of RNA for each reaction. The correct amount of RNA from each sample was determined by analyzing the results of NanoDrop spectrometry. Resulting cDNA was stored at -20℃.

Primer Design

Two oligonucleotide primer sequences were designed for each of the following genes: Bnip31 (gene ID 12177), BAX (gene ID 12028), AATF (gene ID 56321), and Bcl-2 (gene ID 12043) using the Nucleotide Database from the NCBI website (www.ncbi.nlm.nih.gov, Table 1). Using this database, corresponding mRNA sequences in mice were determined for each gene of interest. Next, a

"BLAST" was performed on each sequence using the NCBI website. Each sequence was analyzed to identify intron and exon sites, and the primer sequences were designed using the primer design tool from Operon (operon.com/primerdesign). This tool provided multiple forward and reverse sequences, which are the sequences of each gene in the 3'-5' direction as well as the 5'-3' direction. One of each was chosen, and lyophilized oligo primers were ordered from Eurofins MWG/Operon Biotechnologies. These dry primers were then resuspended to create a final stock concentration of 100 µM. Since the primers were separated into forward and reverse portions, a 10 µM primer solution containing both forward and reverse primers was created in RNase-free water.

Table 1: Primer sequences for target genes and the reference gene, β-actin. *Denotes primers designed by Mary Ogechi Nwokedi (Allegheny College 2011).

Gene	Sequence	Base Length	% GC Content	T_M (℃)
Bnip31* Forward	TGGAGCACGTTCCTTCCTCGTC	22	59.1	58.6
Bnip31* Reverse	GCTGTCACAGTGCGAACTGCC	21	61.9	58.9
BAX Forward	CACGTCCACGATCAGTCAC	19	57.9	62.3
BAX Reverse	TGATCTGTTCAGAGCTGGTG	20	50	60.4
Bcl-2 Forward	GACTTCTGCAAATGCTGGAC	20	50	60.4
Bcl-2 Reverse	TGAGATCAAAGCCCAGACTC	20	50	60.4

AATF Forward	ACAGTTGCTGAATCCGAGAC	20	50	60.4
AATF Reverse	TTCCGCTCTATGAGTTCTCG	20	50	60.4
B-actin* Forward	GGCATTGCTGACAGGATGCAGAAG	24	59	58.7
B-actin* Reverse	TGAGGCCAGGATGGAGCCAC	20	60	58.9

Quantitative Real-Time PCR(qRT-PCR)

To quantify the relative amount of cDNA present in each sample, qRT-PCR was performed using StepOne Software v2.0, the StepOne Thermocycler. Each sample contained 10 µL of RNase-free water, 0.5 µL of cDNA, 0.5 µL of the desired primer, and 9 µL ofSYBR Green PCR Master Mix (Applied Biosystems), which contains *Taq* polymerase for cDNA amplification. This fluorescent dye is used as a marker by the thermocycler to quantify DNA sequences. Once the amount of target DNA sequences is replicated to a specific quantity (the threshold value), the instrument records the amount of replication cycles the sample has undergone . B-actin was used as a reference gene against which all other genes were compared. Threshold values and melt curves were recorded for each gene/cDNA sample combination.

Data Analyses

To compare relative gene expression between the strains, ΔC_T, or the difference of each gene/cDNA sample's C_T from that of the reference gene, was first determined. Then, ΔC_T values for each gene were compared with their opposite-strain counterpart of the same age. For statistical analysis, a T-test was performed on all data.

Results

RNA Isolation

RNA isolation success was evaluated by NanoDrop spectroscopy. Spectroscopy results showed that RNA isolation was completed successfully in all samples (Table 2). Success was defined as RNA concentrations greater than 125 ng/µl and A_{260}/A_{280} ratios greater than 1.75.

Table 2. RNA concentrations and purity ratios determined by NanoDrop spectroscopy. RNA was successfully isolated from each sample and success was defined as RNA concentrations greater than 125 ng/µl and A_{260}/A_{280} ratios greater than 1.75.

Sample	RNA Concentration (ng/µl)	A_{260}/A_{280} ratio
WT 1	129.7	1.83
WT 2	161.8	1.85
WT 3	250.9	1.89

WT 4	225.2	1.95
KO 1	284.1	1.94
KO 2	209.1	2.01
KO 3	203.9	1.87
KO 4	143.6	1.96

qRT-PCR

For this study, four genes were chosen based on the pro- or anti-apoptotic properties of the proteins for which they code. The expression of these four genes (BAX, Bnip 31, AATF, and Bcl-2) was measured in the retinas P25 C57BL/6 (wild-type) and IRBP knockout mice by quantitative real-time PCR using SYBR Green (Applied Biosystems) analysis. For each qRT-PCR reaction, a melt curve was generated to indicate the presence of a single PCR product for the targeted genes. β-actin was used as a reference gene to normalize gene expression quantities. Each gene-strain combination was run in triplicate and ΔC_t and RQ values were calculated.

For the pro-apoptotic genes studied in this experiment (BAX and Bnip31), expression was slightly higher in the IRBP knockout retinas (average BAX ΔC_T = 14.75, average Bnip31 ΔC_T = 10.66) than in the wild-type retinas (average BAX

ΔC_T = 14.05, average Bnip31 ΔC_T = 10.66). However, the difference in expression between the two genes was statistically insignificant (p = 0.15209 for BAX ΔC_t, p=0.88457 for Bnip31 ΔC_t; Table 1). These results, although statistically insignificant, are inconsistent with my hypothesis that pro-apoptotic genes are more highly expressed in the IRBP knockout retinas as compared with wild-type retinas. Results obtained by qRT-PCR on anti-apoptotic genes were also inconsistent with my hypothesis. I hypothesized that expression of the two anti-apoptotic genes (AATF and Bcl-2) would be higher in the wild-type retinas than in knockout retinas. However, the expression of these genes was not found to be statistically different between the strains (p=0.753533 for AATF ΔC_t and p=0.473618 for Bcl-2 ΔC_t; Table 1). Overall, the results of all qRT-PCR experiments yielded no significant results.

Table 3. ΔC_t values from qRT-PCR experiments with $IRBP^{-/-}$ and C57BL/6 retina cDNA and four genes. Each gene-strain combination was run in triplicate and n=4. β-actin was used as the reference gene.

Strain	BAX (ΔC_T)	Bnip31 (ΔC_T)	AATF (ΔC_T)	Bcl-2 (ΔC_T)
C57BL/6 (Wild-Type)	13.74	11.70	18.06	14.33
	15.09	9.82	11.12	15.81
	14.47	9.05	9.66	13.57
	12.89	8.84	12.31	15.70
Average	14.05	9.85	12.79	14.85
Std. Deviation	0.95	1.30	3.68	1.09
$IRBP^{-/-}$	15.91	11.25	15.05	19.76
	14.66	11.61	10.34	13.96
	16.19	11.63	9.85	12.68
	12.26	8.17	13.01	14.25
Average	14.75	10.66	12.06	15.16
Std. Deviation	1.79	1.67	2.43	2.72
p-value	0.51209	0.473618	0.753533	0.858409

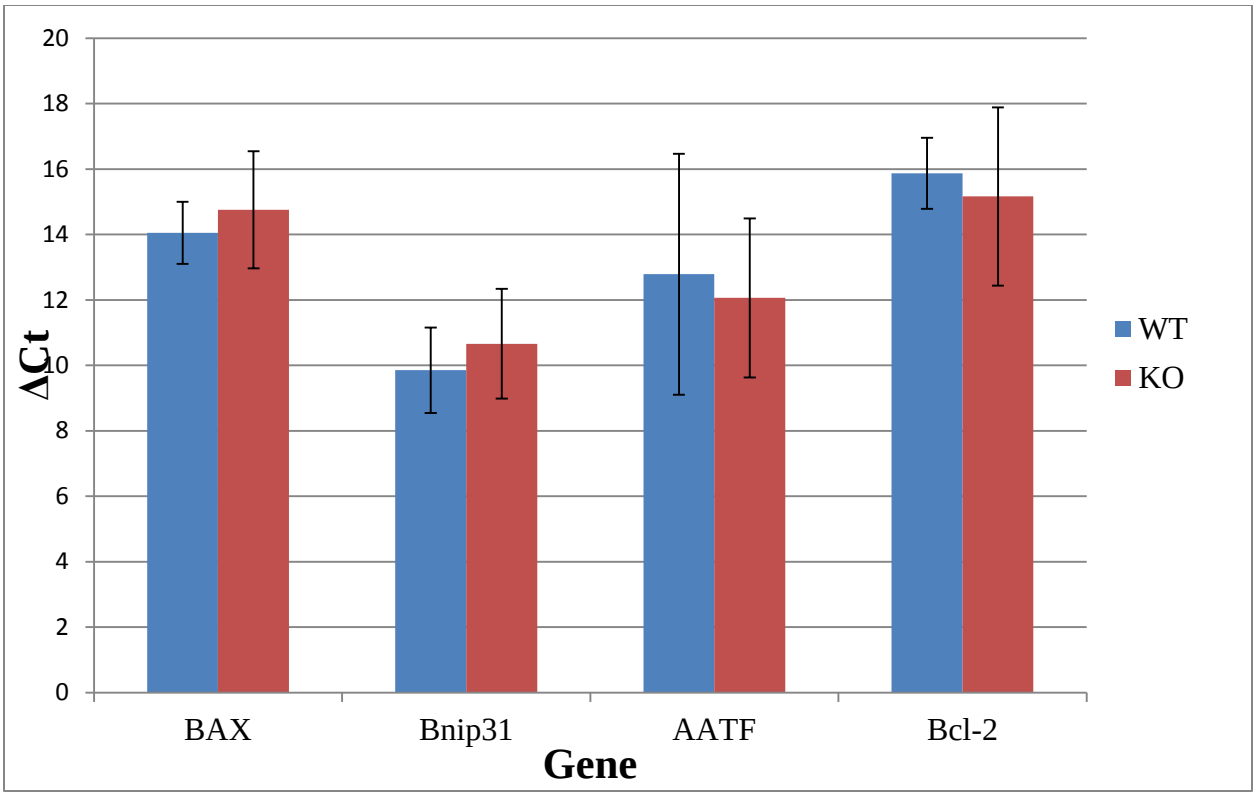

Figure 2: Average ΔC_t values obtained from qRT-PCR trials of four genes in C57BL/6 (WT) and IRBP$^{-/-}$ (KO) strains of mice. Reactions were performed in triplicate and n=4. β-actin was used as the reference gene and target gene ΔC_t values were calculated by subtracting average C_T from the average β-actin C_T for each sample. All results were insignificant ($p>0.05$).

Discussion

The IRBP$^{-/-}$ strain of *Mus musculus* is a viable model for studies on retinal gene expression because, aside from visual impairment, the phenotype is identical to that of the wild-type C57BL/6 strain (Liou et al. 1998). Despite the observation that IRBP$^{-/-}$ mice have reduced ERG response amplitudes (Peachey and Ball 2003), previous studies have confirmed that the visual cycle occurs at a normal rate in the retinas of these mice (Placzewski et al. 1999). In addition, a study conducted by Wisard et al. (2011) showed that the rods of IRBP$^{-/-}$ mice are significantly shorter than those of wild-type mice. Overall, previous studies have shown that photoreceptor development and function in IRBP$^{-/-}$ mice are irregular as compared with wild-type mice.

Wisard et al. (2011) showed that apoptosis is most prevalent at age P25 in IRBP deficient (-/-) *Mus musculus*. However, the genetic cause of this abnormal apoptosis is not completely understood. The goal of this study was to investigate the relative expression of four genes involved in retinal apoptosis, specifically two pro- and two anti-apoptotic genes in the retinas of P25 IRBP deficient mice as compared with the retinas of age-matched C57BL/6 wild-type mice. My hypothesis was that the two pro-apoptotic genes (BAX and Bnip31) would be more highly expressed in the IRBP$^{-/-}$ mice retinas than in the wild-type retinas and that

the two anti-apoptotic genes (AATF and Bcl-2) would more highly expressed in the wild-type mice retinas than in the knockout retinas.

Contrary to my hypothesis, the results of this study showed that there was not a significant difference in the expression of any of the studied genes. Furthermore, this study showed that the two pro-apoptotic genes were slightly more highly expressed in the wild-type group than in the knockout group and the two anti-apoptotic genes were more highly expressed in the knockout retinas, though these differences were insignificant. Therefore, the results suggest that the four genes, BAX, Bnip31, AATF, and Bcl-2, do not play an important role in the apoptosis observed in $IRBP^{-/-}$ mice retinas.

A similar study was conducted by an Allegheny College student, Mary Ogechi Nwokedi, in 2011. In this study, Bnip31 expression in the retinas of P10 $IRBP^{-/-}$ retinas was compared with that of C57BL/6 mice retinas. The study showed that expression did not differ significantly between the two strains. However, since the subjects in this study were P10, it is possible that pro-apoptotic genes were not yet expressed since apoptosis in $IRBP^{-/-}$ mice retinas peaks at age P25 (Wisard et al. 2011). The results of my study confirm that Bnip31 does not play a significant role in the retinal apoptosis observed in $IRBP^{-/-}$ mice.

There are a few explanations as to why none of the studied genes were expressed differently in the retinas of the two strains of mice. Unsuccessful tissue extraction and RNA isolation could have been a possible explanation, but NanoDrop spectroscopy results showed that RNA was successfully extracted and isolated (Table 2). The first possible explanation is that apoptotic pathways are complex and involve many inhibitory and promoting proteins (Péquignot et al. 2003). Although the genes studied in this experiment have been shown to play a role in retinal apoptosis, it is possible that the apoptosis pathway occurring in the IRBP deficient retinas involves other genes. There are hundreds of genes involved in retinal apoptosis (Mouse Genome Informatics), so identifying the culprit gene or genes is going to be a long and ongoing process. A second possible reason for a lack of disparity in gene expression between the two strains is that there are other genes that mediate different aspects of the apoptotic pathways in which the pro-apoptotic genes are involved. It is possible that BAX and/or Bnip31 are expressed in both strains, but the wild-type mice are able to express genes which mitigate the effects of these pathways. If this was true, the difference in the expression of these genes between the two strains would be minimal. Furthermore, another possible reason for a lack of difference in expression between the two strains is that gene expression often follows circadian rhythms (Granda et al. 2004). However, tissue

extraction was performed at random times in this study since there is no previous research on circadian cyclical expression in retinal tissue.

The final proposed reason for which there was no difference in expression is that the IRBP$^{-/-}$ mice are not true knockouts. It is certainly possible, though hopefully not true, that breeding between the two strains occurred accidentally. Interbreeding between the knockout and wild-type strains would result in little or no differences between the expression of all genes, including the target genes of this study.

In the future, I propose that this experiment be repeated at a larger scale. Since retinal tissue is so small and therefore contains a relatively small amount of RNA, more samples should be analyzed before it can definitively said that there is no difference in the expression of these genes between the two strains. Furthermore, I propose that other genes which play a role in promoting or inhibiting apoptosis be studied. Apoptosis is not completely understood and, while there is some evidence that certain genes may play a role in abnormal retinal apoptosis, there is no evidence as to which specific genes are in play in the IRBP$^{-/-}$ mouse. Finally, I propose that all mice used in genetic expression research at Allegheny College be genotyped. In hindsight, this should have been done before I began my experiment, but my discovery that there may have been interbreeding will hopefully help future projects.

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Appendix A: qRT-PCR C_T values for IRBP$^{-/-}$ and C57BL/6 (Wild-Type) mice. The reference gene was run in duplicate for each sample and each target gene was run in triplicate. *Denotes that qRT-PCR was unable to determine a cycle threshold value due to an unexpected error.

C_T Values for IRBP$^{-/-}$ Mice					
Gene	B-actin	BAX	Bnip31	AATF	Bcl-2
Sample 1	17.93	30.93	29.42	31.67	37.41
		30.24	Undetermined*	33.68	37
	17.96	30.35	28.97	Undetermined*	37.75
Sample 2	21.63	33.19	Undetermined*	28.8	Undetermined*
		31.9	28.25	29.19	Undetermined*
	21.92	32.71	28.85	29.6	32.81
Sample 3	19.67	32.86	30.01	31.25	32.75
		33.57	29.96	27.93	32.15
	19.7	32.95	39.86	29.45	32.19
Sample 4	21.27	32.86	29.75	34.19	34.59
		33.57	29.26	Undetermined*	Undetermined*
	21.75	32.67	30.05	34.84	36.93

C_T Values for C57BL/6 (Wild-Type) Mice					
Gene	B-actin	BAX	Bnip31	AATF	Bcl-2
Sample 1	18.44	31.62	30.28	32.77	31.32
		30.63	29.54	35.97	30.77
	18.24	31.16	30.31	Undetermined*	29.84
Sample 2	19.24	33.42	28.7	29.91	34.48
		33.44	27.94	31.86	34.29
	18.92	Undetermined*	28.29	29.03	34.1
Sample 3	18.46	32.73	28.5	30.36	35.93
		32.24	27.66	Undetermined*	33.34
	18.51	33.9	27.61	Undetermined*	33.9

Sample 4	18.54	31.85	28.36	29.99	32.37
		32.09	29.45	28.65	32.93
	18.49	Undetermined*	28.9	Undetermined*	32.85

Appendix B

Direct-zol™ RNA Mini Prep (Zymo Research) Protocol

1. Add 1 volume ethanol (95-100%) to each volume of sample homogenate in TriZol Reagent. Mix well by vortexing.
2. Load the mixture into a Zymo-Spin™ IIC Column and centrifuge at ~12,000 x g for 1 minute. Transfer the column into a new Collection Tube and discard the Collection Tube containing the flow-through.
3. Add 400 µl Direct-zol™ RNA PreWash to the column and centrifuge at ~12,000 x g for 1 minute. Discard the flow-through. Repeat this step.
4. Add 700 µl RNA Wash Buffer to the column and centrifuge at ~12,000 x g for 1 minute. Transfer the column carefully from the Collection Tube into an RNase-free tube.
5. Add 25 µl of DNase/RNase-Free Water directly to the column matrix and centrifuge at ~12,000 x g for 30 seconds. The eluted RNA can be used immediately or stored at -70℃.

Appendix C

iScript™ cDNA Synthesis Kit Protocol

Components	Volume per Reaction
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Nuclease-free water	x µl
RNA template (1 µg total RNA)	x µl

Incubate complete reaction mix:

5 minutes at 25℃
30 minutes at 42℃
5 minutes at 85℃
Hold at 4℃ (optional)

Store cDNA at -20℃.