REGULATION OF CASPASE-3 ACTIVATION BY PHOSPHORYALTED Ab-CRYSTALLIN AND ITS ROLE IN DIFFERENTIATION

by

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ABSTRACT

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The lens is responsible for focusing light into the retina. It accomplishes this through its maturation from an epithelial cell into a fiber cell. A large amount of research has been done on cellular differentiation. Nevertheless, we still lack knowledge on many different aspects of differentiation, including a complete theory on the mechanism behind differentiation. Due to the lens' unique structure and cell types, this is an ideal model for studying differentiation. Our research has shown that αB crystallin, a small heat shock protein, is able to modulate cytochrome C levels and protect the mitochondria under oxidative stress. Also, cytochrome C release is often followed by caspase 3 activation. In addition, research has shown that low levels of caspase 3 activation is essential in driving

differentiation. My work examined if αB crystallin could modulate cytochrome C to lower caspase 3 levels to allow for differentiation rather than apoptosis.

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INTRODUCTION

The lens is an arcuate, transparent structure behind the iris of the eye. It has no blood supply, making it somewhat isolated, and it obtains its nutrients and gets rid of its waste using the surrounding fluid, primarily the aqueous humor. The primary function of the lens is to focus light onto the retina to allow organisms to see. While lenses may vary in thickness, shape, the way that they focus light and even in the addition of certain structures, the overall function of a lens remains the same across all organisms (11). The lens's ability to focus light is accomplished by the lack of organelles in cells at the center of the lens and the densely packed lens crystallins (9). Crystallins are the most prevalent proteins in the lens and comprise of two families, the α- and βγ-crystallins (9). Combined, they make up to 90% of water-soluble proteins in the mammalian lens and are thought to be essential in allowing for lens transparency (9). In addition to crystallin proteins, there are also membrane proteins (like N-Cadherin and E-Cadherin), gap-junction proteins (like Gja3 and Gja8), and cytoskeletal proteins (Filensin and CP49) which make up the remaining 10% of proteins found in the mammalian lens (9). Failure to fully complete the development process in the lens may result in a wide range of lens structural abnormalities and cataracts (8). Lens development first starts with the formation of the neural plate (8). Then the partitioning of the neural plate border followed by lens placode formation and the start of lens differentiation program (8). Once the placode is formed it is then invaginated and the lens vesicle is formed and is made up of undifferentiated

precursor lens cells (8). Then through the use of multiple pathways the lens cells are able to exit the cell cycle so that proliferation can stop and lens cell differentiation occurs (8). Differentiation is the process of epithelial cells in the lens transitioning to become fully mature fiber cells. In the lens there are four regions that represent different stages of differentiation, as shown in Figure 1.

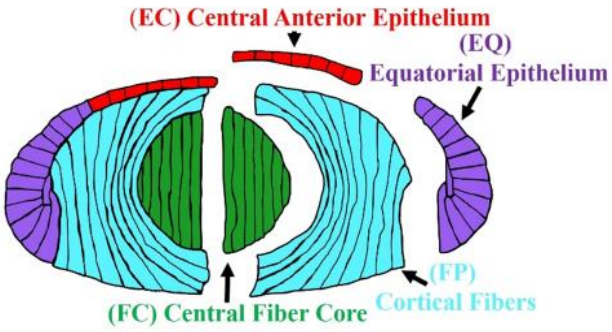

Figure 1. Illustration of the four progressive stages of lens differentiation. While the do serve as stages of differentiation they are also specific regions in the lens.

The EC, also known as the central anterior epithelium, is where epithelial cells are being produced. At this stage the epithelial cells are quiescent until they reach equatorial epithelium, also known as EQ. Cells begin to prepare for differentiation and this is also referred to as the transition zone of the lens. This is then followed by the cortical fiber region of the lens where differentiation has begun. A hallmark feature of differentiation is the degradation of organelles. This is essential for the final stage of differentiation, the central fiber region, also known as FC. It is at this stage that fiber cells are fully developed to form the organelle free zone, also known as the OFZ, which is essential for

lens' transparency. The OFZ is not full formed until approximately day 15 in chicks (18). A transparent organelle free zone is dependent on the epithelial cells' ability to complete differentiate. While the mechanism is not fully elucidated, research has shown that many different pathways play major roles in differentiation such as the wnt signaling pathway, SHH pathway, RA, and FGF pathways. They are all responsible for a variety of different cell types and play roles in cell survival, proliferation, and determining cell fate (17). The pathway I am most interested in was found specifically in EQ region and involves a low level of caspase 3 activation. It is thought that this low level of caspase 3 is essential for the initiation of differentiation (6,7). This is based on research done on IGF-1R. It was shown in previous research that IGF-1R/NFκB survival signal can regulate caspase 3 levels. Results revealed that when IGF-1R and NFκB survival signal is blocked this causes an elevation in caspase 3 levels and this prevents differentiation and induces apoptosis. On the other hand, when the IGF-1R and NFκB survival signal is active, survival proteins from Bcl-2 and IAP families are expressed, caspase 3 levels are lowered, and cells proceed towards differentiation (6,7). Furthermore, our lab has shown that αB-crystallin, a small heat shock protein, is able to translocate to the mitochondria and protect it from oxidative stress by interacting with cytochrome to control cytochrome c release thus lowering caspase 3 activation (13). In addition, we also discovered that phosphorylation at serine 59 is essential to αB-crystallin's ability to translocate to the mitochondria to protect it under oxidative stress (13) I hypothesize that controlled release of cytochrome c by αB-crystallin could regulate low levels of caspase 3 that initiate cellular differentiation without inducing apoptosis, as seen in Figure 2**.**

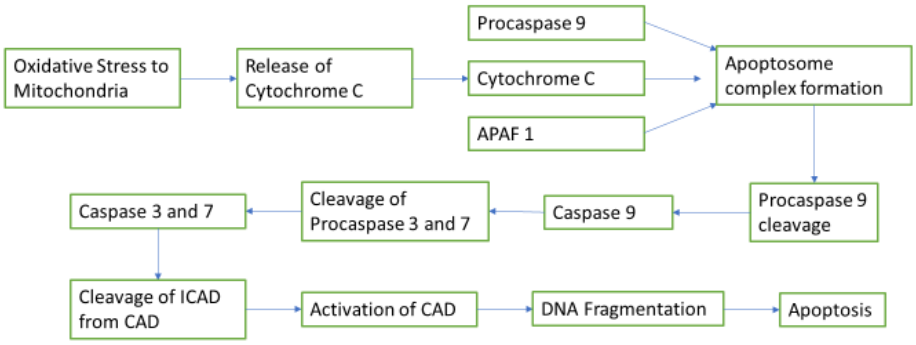

Figure 2. This is a general diagram of the apoptotic pathway that we believe may be responsible for driving cells into differentiation

My work is to test this hypothesis by measuring caspase 3 levels in whole cultured lenses. Previous research in our lab has established the requirement for key phosphorylation of αB-crystallin and upstream kinase pathways, that coordinate this important property of αB-crystallin. By using inhibitors of the kinases related to the phosphorylation of αB crystallin, we can hope to show an association between αB crystallin phosphorylation and differentiation. The use of apoptotic pathways or at least parts of it to achieving cell survival is not an uncommon theory. Previous research has shown that pro-survival pathways can use pro-apoptotic molecules such as Bax, Bcl-xs, cytochrome c release, and coincidentally activated caspase 3 as molecular switches for differentiation without driving cells into apoptosis (16). Identifying the role and requirements for αB-crystallin control of caspase 3 levels could provide a novel requirement for lens cell differentiation

that would advance our understanding of lens formation and provide insight into the role of αB-crystallin in the formation and disease states of more complex tissues.

αB-crystallin is a 20kD, 175 amino acid, multimeric, small heat-shock chaperone protein (sHSP) required for homeostasis of multiple tissues including the eye lens, retina, heart and brain. α-crystallin expression makes up 40% of lens in humans. While αA-crystallin, is found almost exclusively in the lens, αB-crystallin is found ubiquitously throughout the body (10). Correspondingly, mutation or altered levels of αB-crystallin are associated with multiple degenerative diseases including cataract, retinal degeneration, cardiomyopathy, and Lewy body disease (10). Based on its wide-ranging importance understanding the protective and homeostatic properties of αB-crystallin is critical for understanding degenerative diseases and could lead to the development of therapies to treat these diseases. αB-crystallin is localized to the mitochondria suggesting direct effect on mitochondrial function. Research has shown that αB-crystallin has three known serine sites that are phosphorylated post-translationally and that those sites are affect its ability to translocate to the mitochondria, site serine 59 being the most essential of the three (2,14). In addition, serine 59 has been shown to be phosphorylated at that site through the P38 MAPK/MAPKAPK 2 pathway (2,14). It follows that P38 MAP Kinase phosphorylates MAPKAP Kinase 2 which in turn phosphorylates αB-crystallin at serine 59. By preventing the phosphorylation of αB-crystallin, I aim to first observe the effects of αB-crystallin on levels of caspase 3 activation. I believe that when αB crystallin is not properly phosphorylated, this will cause an increase in levels of caspase 3 activation, suggesting the induction of apoptosis. I also aim to observe how this might affect differentiation. I hypothesize that if αB crystallin is not phosphorylated it will cause cells

to not properly differentiate, affecting proteins that are essential for differentiation. To test these hypotheses, we intend on using previously unpublished data that was made by Dr. Brennan, midsagittal sections that I have made from lenses treated with P38 MAPK inhibitor and MAPKAP Kinase 2 inhibitor, and western blots on protein expression of lenses that I treated with either P38 MAPK inhibitor or MAPKAP Kinase 2 inhibitor.

METHODS

SDS/ Western Blotting

To measure for protein expression, western blots were performed. Samples were diluted 1:1 with 2x Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH approx. 6.8) (Sigma-Alrich, St. Louis, MO) and heated for 5 minutes at 100°C. Samples were run at room temperature through 5% stacking gel (0.5 M Tris base pH 6.8, 10% SDS, 10% APS, and 1% TEMED) then 10% acrylamide resolving gel (1.5M Tris base pH 8.8, 10% SDS, 10% APS, and 1% TEMED) using BioRad Mini-PROTEAN® Tetra Handcast System (Hercules, CA). Wet transfer was used to transfer proteins onto Immobilon-P PVDF Transfer Membrane (Millipore Sigma, Burlington, MA), both using Bio-Rad Mini-PROTEAN® vertical electrophoresis system (Hercules, CA). Bradford protein assay was used to determine protein concentration

Immunostaining for lens sections

Whole eyes isolated from day 10 embryonic chicks were fixed in 3.7% paraformaldehyde solution for 24 hours at 4˚C and then incubated in 30% sucrose solution prior to cryofreezing and cryosectioning. 40µm sections were cut serially in the anterior to posterior direction. Sections were then permeabilized with 0.25% Triton-X 100 buffer, blocked for 1 hour in blocking buffer at 37 ˚C, and incubated sequentially in

primary antibody at 4˚C overnight. Cryosections were then incubated in secondary antibody at 37 ˚C for 1 hour. F-actin was labeled with Alexa 488 phalloidin (Invitrogen), Alexa fluor555 (Invitrogen) to serve as a secondary stain, Nuclei were stained with DAPI (Invitrogen) .

Examining phosphorylation distribution of αB crystallin, P38 MAPK, and MAPKAPK2

To examine the phosphorylation distribution of αB crystallin, P38 MAPK, and MAPKAPK2 western blots were performed by Dr. Lisa Brennan. The lenses from day 13 embryonic chick lenses were removed and then micro-dissected so that western blots could be performed on each of the regions of the lens. The western blots were probing for αB crystallin, phosphorylated serine 59 αB crystallin, P38 MAPK, phosphorylated (Thr 180/Tyr 182) P38 MAPK, MAPKAPK2, phosphorylated threonine 222 MAPKAPK2, and phosphorylated threonine 334 MAPKAPK2. The western blots were then imaged, and the densitometries of the blots were quantified.

In addition, sections were made from lenses that were untreated, treated with P38 MAPK inhibitor (SB203580), or treated with MAPKAPK2 inhibitor (MK25) and they were then incubated at 37˚C for 24 hours in an incubator. These sections were then stained with phosphorylated threonine 222 MAPKAPK2, MAPKAPK2, and P38 MAPK rabbit antibodies. Furthermore, secondary antibodies were placed on these sections. Alexa 488 phalloidin (Life Technologies) for F-actin, Alexa Fluor 555 (Life Technologies) to serve as the secondary for the primary antibodies, and DAPI (Invitrogen) for the nuclei These sections have yet to be analyzed and observed under a confocal microscope.

Examining the effect of P38 MAPK and MAPKAPK2 on αB crystallin

To examine the effect of P38 MAPK and MAPKAPK2 on the phosphorylation of αB crystallin, whole lenses treated in P38 MAPK inhibitor (SB203580), MAPKAPK2 inhibitor (MK25), and lenses left untreated were incubated for 24 hours at 37˚C. Protein extractions were performed on the lenses and western blots were performed by Dr. Lisa Brennan. The western blots probed for αB crystallin and phosphorylated serine 59 αB crystallin. The western blots were then imaged, and densitometries of blots were recorded. In addition, sections were made and stained for MAPKAPK2 and pThr222 MAPKAPK2 using a specific antibody (Cell Signaling) for both

Examining the effect of αB crystallin on caspase 3 levels and cytochrome C release

To examine the effects of αB crystallin on levels of activated caspase 3 and cytochrome C release, whole lenses were treated with P38 MAPK inhibitor (SB203580), MAPKAPK2 inhibitor (MK25), or left untreated. These lenses were then incubated for 24 hours at 37˚C and protein extractions and western blots were performed by Dr. Lisa Brennan. The western blots probed for cleaved caspase 3 and cytochrome C. The western blots were imaged, and densitometries were recorded.

In addition, sections were made from lenses that were untreated, treated with P38 MAPK inhibitor (SB203580), or treated with MAPKAPK2 inhibitor (MK25) and they were then incubated at 37˚C for 24 hours in an incubator. These sections were then stained with cleaved caspase 3 primary antibody (Cell signaling). Furthermore, secondary antibodies were placed on these sections. Alexa 488 phalloidin (Invitrogen) for F-actin, Alexa Fluor 555 (Invitrogen) to serve as the secondary for the primary antibodies (Cell Signaling), and DAPI (Invitrogen) for the nuclei.

Examining the effect of αB crystallin on lens differentiation

To examine the effects of αB crystallin on lens differentiation, whole lenses were treated with P38 MAPK inhibitor (SB203580), MAPKAPK2 inhibitor (MK25), or left untreated. These lenses were then incubated for 24 hours at 37˚C and protein extractions and western blots were performed by Dr. Lisa Brennan. The western blots were probed for three specific differentiation markers: CP49, Filensin, and P27. The western blots were imaged, and densitometries were recorded.

RESULTS

αB crystallin expression in the stages of differentiation

Figure 3 suggests that as lens cells progress through differentiation, the band intensity for phosphorylated Serine 59 αB crystallin increases in Figure 3a. In Figure 3b when compared to total αB crystallin there is a significant increase in phosphorylated αB crystallin. Figure 3c, 3d, and 3e all show the phosphorylation of the kinases necessary for phosphorylating αB crystallin occurring mainly in the EQ and FP regions.

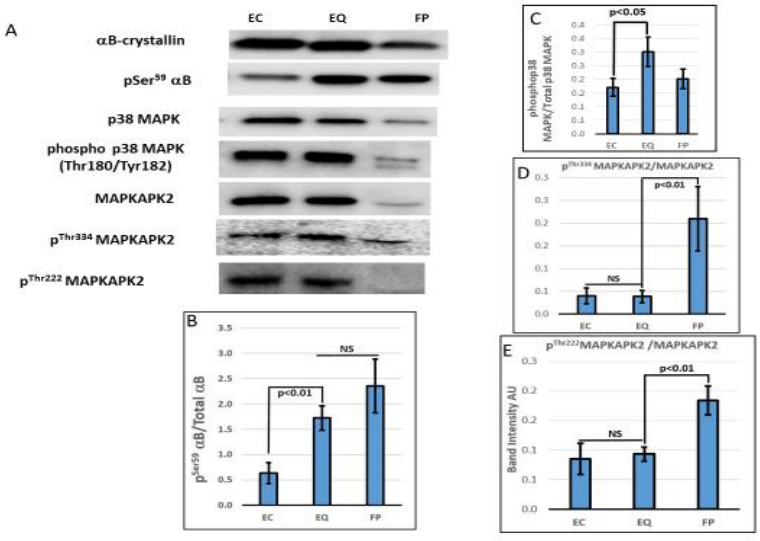

Figure 3. Whole lenses probing for αB crystallin, phosphorylated αB crystallin, the kinases phosphorylating αB crystallin, and the phosphorylated kinases at progressive stages of differentiation. Western blot performed by Dr. Lisa Brennan

αB crystallin and phosphorylated αB crystallin in whole lenses treated with MK25 and SB203580

Figure 4 indicates that when treated with p38 MAPK inhibitor or MK25 there is a significant decrease in band intensity for pSer59 αB crystallin compared to the control asseen in Figure 4a and 4c. Furthermore when compared to total αB crystallin there is a significant decrease in band intensity as seen in Figure 4d.

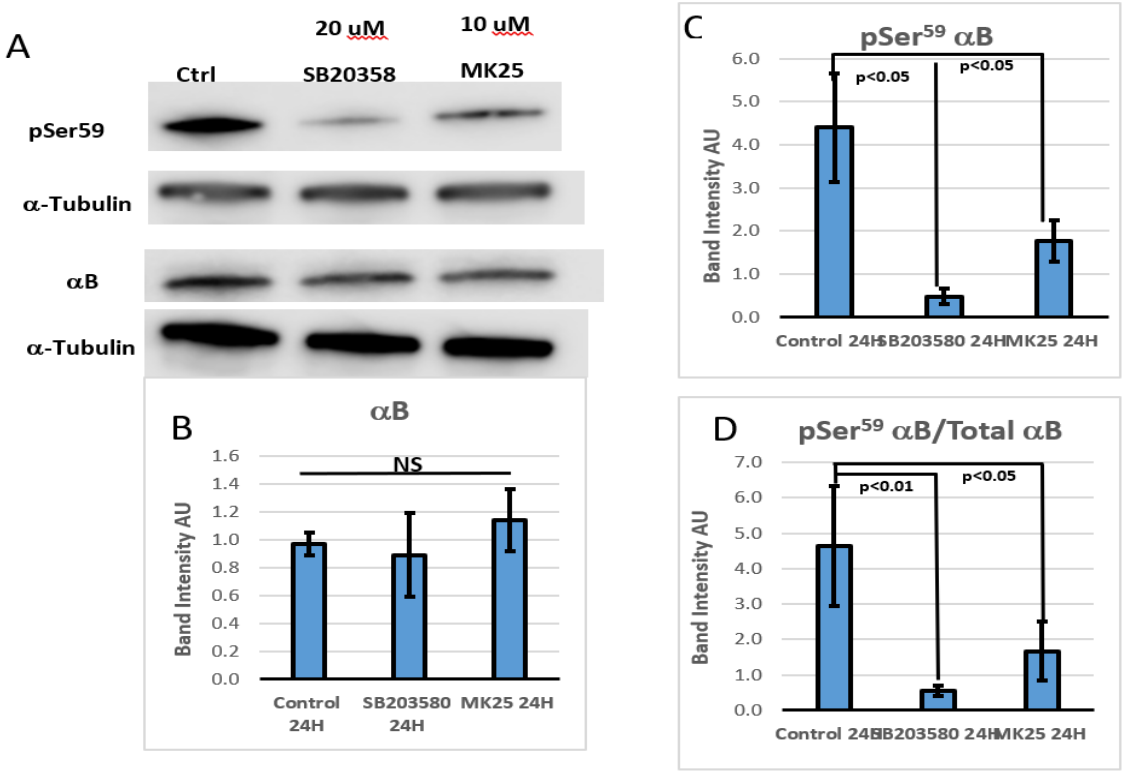

Figure 4. Western blots to measure the level of αB crystallin and phosphorylated Serine 59 αB crystallin in control whole lenses, lenses treated with p38 MAPK inhibitor (SB203580), and lenses treated with MAPKAP2 inhibitor (MK25). Western Blot performed by Dr. Lisa Brennan

In Figure 5, the sections show that when whole lenses are treated with MK25, there is a slight decrease in pSer59 αB crystallin compared to the control. It is also important to

note that the difference between the control group and the SB203580 treatment, there does not appear to be a difference. The concentration of pSer59 αB crystallin seems to be the most at the EC region of the lens.

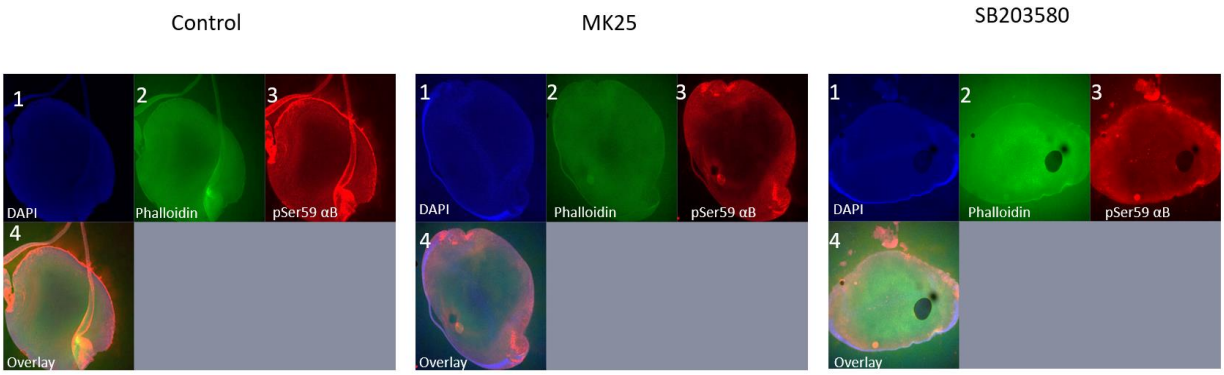

Figure 5. Stains of three midsagittal Day 10 whole lens sections viewed under a confocal microscope. One is untreated, the other is treated with MAPKAPK2 inhibitor, and the last is treated with p38 MAPKAPK2

MAPKAPK2 and phosphorylated MAPKAPK2 in whole lenses treated with MK25 and SB203580

Figure 6 indicates that when whole lenses are treated with MK25 there appears to have the largest decrease in MAPKAPK2, followed by the SB203580 treatment. Then the control group seems to have the most MAPKAPK2. The differences do not appear prominent and MAPKAPK2 is mostly found in the epithelial region of the lens sections. Overall it doesn't seem that there is much of a significant difference in MAPKAPK2 concentration in the lens.

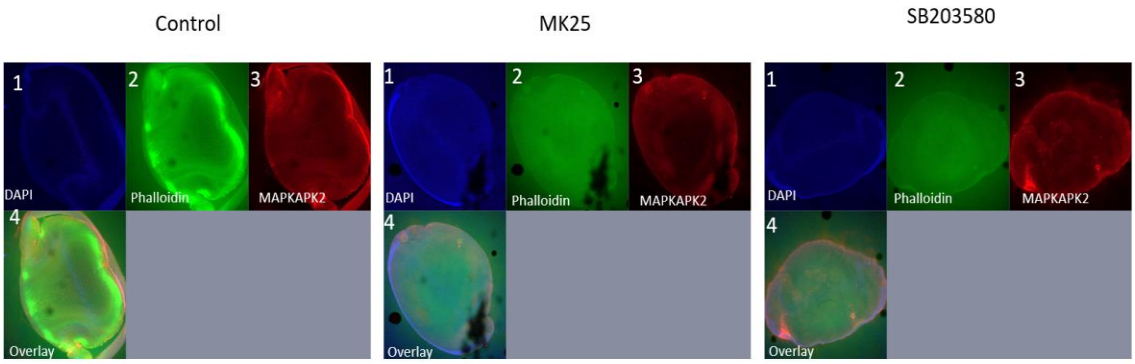

Figure 6. Stains of three midsagittal Day 10 whole lens sections viewed under a confocal microscope. One is untreated, the other is treated with MAPKAPK2 inhibitor, and the last p38 MAPKAPK inhibitor.

Additionally, Figure 7 shows that there is an increase in phosphorylated MAPKAPK2 when treated with either MK25 or SB203580.

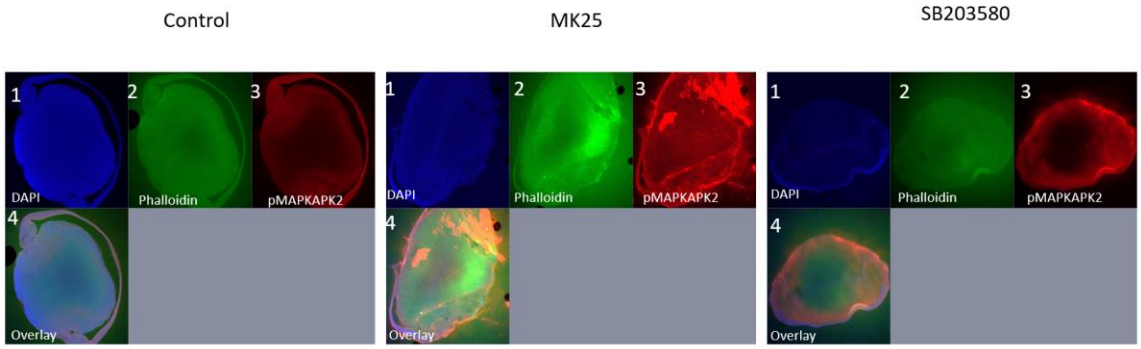

Figure 7. Stains of three midsagittal Day 10 whole lens sections viewed under a confocal microscope. One is untreated, the other is treated with MAPKAPK2 inhibitor, and the last is treated with P38 MAPKAPK2 inhibitor. We probed for Thr222 MAPKAPK concentrations

Cleaved Caspase 3 and Cytochrome C release when treated with MK25 and SB203580

Figure 8 shows that when treated with either SB203580 or MK25, there is an increase in cytochrome C as seen in Figure 8b and cleaved caspase 3 shown in Figure 8d. It is important to note that this increase is not significant, and this is likely due to the small sample size (N=2). More samples will be run on this and we would expect to see a

much more significant increase in cleaved caspase 3 and cytochrome C when treated with either P38 MAPK inhibitor (SB203580) or MAPKAPK2 inhibitor (MK25).

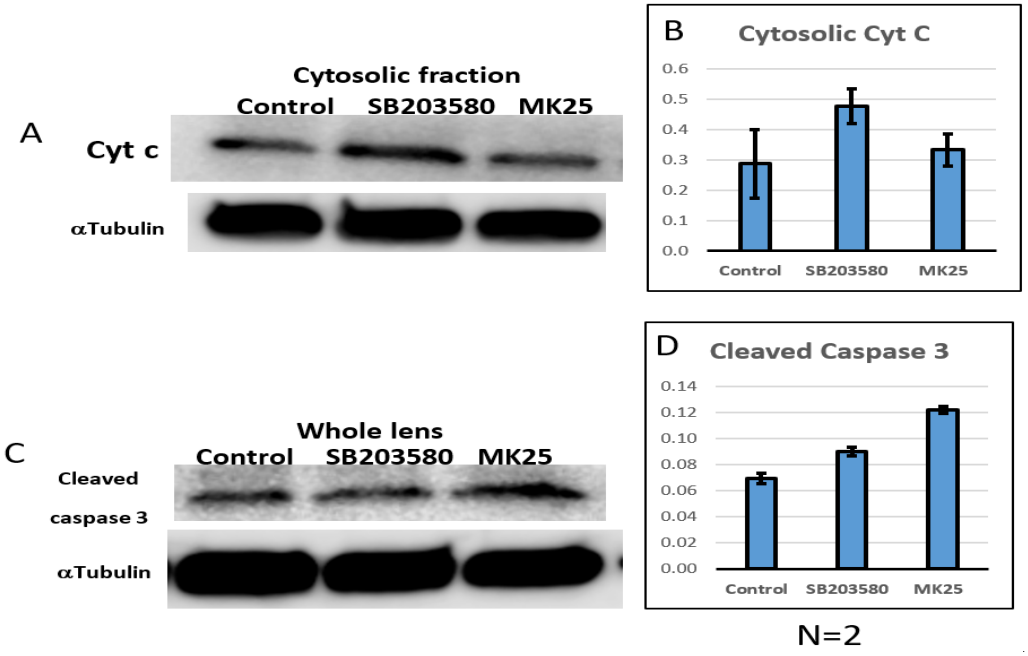

Figure 8. Western blots to determine how αB crystallin affects levels of cytochrome C release and caspase 3 activation. We measure the levels of cytochrome C in the cytosol and cleaved caspase in whole lenses that were inhibited by both a P38 MAPK inhibitor (SB203580) or a MAPKAPK2 inhibitor (MK25) through western blot (N=2). Western blot performed by Dr. Lisa Brennan.

Furthermore, in Figure 9, there seems to be no real difference between the MK25 treatment and the untreated lens section but there is an increase in cleaved caspase in the SB203580 treatment.

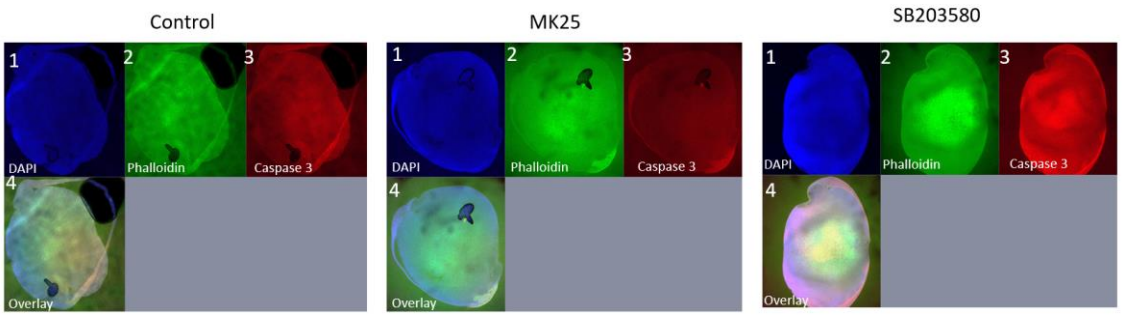

Figure 9. Stains of three midsagittal Day 10 whole lens sections viewed under a confocal microscope for caspase 3 activation. One is untreated, the other is treated with MAPKAPK2 inhibitor, and the last is treated with P38 MAPKAPK2 inhibitor.

Differentiation markers in whole lenses treated with MK25 or SB203580

Figure 10b shows that when treated with MK25 CP49 experiences a significant decrease compared to the control. In addition, in Figure 10d when treated with either SB203580 or MK25 P27 showed significant decrease compared to the control. On the other hand, Filensin showed no significant changes in any of the treatments in Figure 10c.

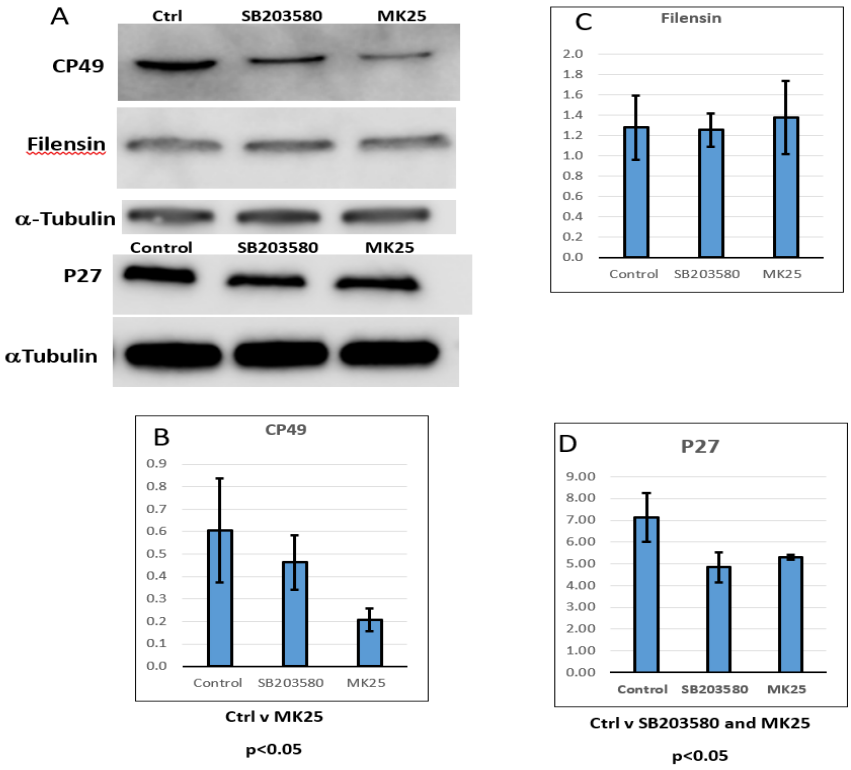

Figure 10. Western blots of 3 markers of differentiation in whole lenses that were untreated, treated with P38 MAPK inhibitor (SB203580), or treated with MAPKAPK2 inhibitor (MK25). CP49 and Filensin are both structural proteins and P27 is a cell cycle regulatory protein. Western blot performed by Dr. Lisa Brennan

DISCUSSION

Figure 3 suggests an important role for phosphorylated αB-crystallin in differentiation. We notice a significant increase in phosphorylated αB-crystallin compared to non-phosphorylated αB-crystallin at both EQ and FP, the region where differentiation occurs and where differentiation has ended respectively. This suggests that αB-crystallin is being phosphorylated to be in the initiation of differentiation in some capacity. Furthermore, we notice a significant increase in phosphorylated P38 MAPK at EQ, and a significant increase in phosphorylated threonine 334 and threonine 222 for MAPKAPK2 at FP. This suggests the importance of both P38 MAPK and MAPKAPK2 in the process of differentiation and potentially in the phosphorylation of αB-crystallin.

Figure 4 shows that P38 MAPK and MAPKAPK2 play a role in the phosphorylation of αB-crystallin. When whole lenses are treated with either P38 MAPK or MAPKAPK2 inhibitor we notice a significant decrease in phosphorylated αB-crystallin compared to the control. While this does not eliminate the possibility that these kinases are not directly responsible for the phosphorylation of αB-crystallin it does at least show that this pathway is related to the phosphorylating αB-crystallin.

Figure 8 is meant to show the relation between phosphorylated αB-crystallin and the release of cytochrome C and the levels of caspase 3 activation. While we do not have

enough samples to be able to make any conclusion, we can predict what can be expected based on what we know from previous findings. We would expect to see an increase in cleaved caspase and cytosolic cytochrome C with the inhibition of P38MAPK or MAPKAPK2, given what we believe. Since αB-crystallin is no longer able to be phosphorylated by the P38 MAPK pathway, αB-crystallin is no longer able to translocate to the mitochondria and regulate cytochrome C release to help reduce the activation of caspase 3. This is what we believe will drive a cell to differentiation rather than apoptosis.

Figure 10 attempts to analyze the effects of αB-crystallin on the expression of differentiation markers. When whole lenses are treated with P38 MAPK inhibitor or MAPKAPK2 inhibitor there is a significant decrease in P27 and when treated with MAPKAPK2 inhibitor there is a significant decrease in CP49 compared to the control. Filensin shows no significant changes when treated with either SB203580 or MK25. Perhaps this is due to Filensin being regulated by a completely different mechanism than CP49 and P27. This data suggests that αB-crystallin can affect more than one differentiation marker. What this means for differentiation is that it is potentially able to affect it globally by regulating a few of these markers of differentiation. In mice it has been shown that CP49 and P27 both play important roles in the prevention of cataracts and when mutated or loss it causes the lens to become opaque (1,12). Downregulating either of these genes may potentially cause similar effects.

Regarding the results from the stained whole lens sections, it was mostly inconclusive and contradicted many of the results obtained in the western blots. Figure 6 showed that MAPKAPK2 levels were increased in the control group compared to MK25

and SB203580. This is especially evident when comparing control to MK25. There should be no real significant difference in MAPKAPK2 levels across the three treatments. MK25 and SB203580 are only really meant to inhibit the phosphorylation of MAPKAPK2 and P38 MAPK. Therefore, this should not affect the level of MAPKAPK2.

Figure 7 shows that when treated with MK25 or SB203580, there are higher levels of pThr222 than when compared to the control. Knowing the role that both MK25 and SB203580, this is the opposite of what we would expect. The control should have the highest levels of phosphorylated MAPKAPK2, while the MK25 treatment and the SB203580 treatment would both have significantly lower levels.

Figure 5 is meant to show the level of phosphorylated serine 59 αB-crystallin in three different whole lenses. It seems that the control and SB203580 treatment has similar levels while the MK 25 has slightly less. This goes against what we believe and what the western blots from Figure 3. P38 MAPK and MAPKAPK2 have been shown to play a role in the phosphorylation of αB-crystallin at serine 59. Therefore, it is expected that inhibition of either of these two kinases would cause a decrease in phosphorylation compared to the control. Furthermore, pSer59 αB-crystallin seems to be the most abundant in the EQ region which goes against what was shown in Figure 3.

Figure 9 shows the levels of cleaved caspase 3 in the three different treatments. It showed that SB203580 had the highest level of caspase 3 activation. While MK25 and control had similar levels. I would expect to see similar levels between MK25 and SB203580, while control had a significantly lower level of cleaved caspase. This

contradicts Figure 8. While, the results aren't significant, they do show that MK25 and SB203580 have higher levels of cleaved caspase and this is a clear marker for apoptosis.

CONCLUSION

In conclusion, there may possibly be a link between phosphorylated pSer59 αB-crystallin, cleaved caspase 3 levels, and differentiation. Unfortunately, the data we collected is too inconclusive to make a definitive interpretation. We were not able to obtain enough samples to make western blots for cleaved caspase 3 and cytochrome C to show any significant results. In terms of sections, none of the sections that we collected showed any data that we can make any real conclusions from. I believe that there was an issue with the antibodies. I believe that they were binding non-specifically. That would explain why there was such a significant amount of pSer59 found in the EQ region of the lens in Figure 3. Furthermore, it would have been nice to have been able to make sections and stains for P38 MAPK, phosphorylated P38 MAPK, cytochrome C, and a few of the differentiation markers that we outlined here. It would have paired well with the western blots that were performed on these proteins.

As far as future research, I believe that using αB-crystallin knockout mice that are then transfected with different forms of αB-crystallin may be a worthwhile project. The main issue in this project was the fact that P38 MAPK and MAPKAPK2 are responsible for the phosphorylation of so many different genes. This means we cannot definitively say that preventing the phosphorylation of αB-crystallin was what would prevent cells from not completing differentiation. If the project came back with definitive results, at

best we would have simply been making an association. I believe using knockouts would have made for a much more direct conclusion that points more towards αB-crystallin in the role of differentiation. In addition, I believe that it might be worthwhile exploring what happens beyond low caspase 3 activation when driving towards differentiation. We do not really know what happens once caspase 3 activation is lowered beyond the fact that the cells are moving away from apoptosis. Other research has shown the link between gene expression and differentiation, more specifically the accessibility of chromatin playing a role in gene expression that then drives each stage of differentiation. It may be worth looking into a possible correlation or association between caspase 3 activation and the accessibility of chromatin.

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