Mechanical Insult Uncouples the Protective Function of PEA15 on Extracellular Matrix Remodeling and Apoptosis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Glaucoma is an optic neuropathy in which the eye’s intraocular pressure can become elevated and exert mechanical insult on the optic nerve head (ONH). This causes dramatic ONH remodeling, astrocyte activation, and retinal ganglion cell death, causing vision loss. Proteomic analysis of human ONH astrocytes exposed to pathologically relevant mechanical insult revealed increased phospho-protein enriched in astrocytes (PEA15). PEA15 plays roles in extracellular matrix (ECM) remodeling and apoptosis, however this has yet to be explored in a glaucomatous context. PEA15 demonstrated protection by decreasing levels of matrix metalloproteinase 2 (MMP2) and apoptosis. However, mechanical insult uncoupled this protection by PEA15 leading to increases in MMP2 and apoptosis. Phosphorylation of serine 104 on PEA15 was shown to be critical in uncoupling this protection as mutation of this residue rescued anti-apoptotic activity and suppression of MMP2. Overall glaucomatous mechanical insult appears to abrogate protective functions of PEA15 in apoptosis and ONH remodeling.
Acknowledgments

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Finally I would like to thank my family and friends for the support given when completing my masters. It must be difficult to listen to me distress about my research problems when you barely even understand what glaucoma is.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Ca(^{2+})/Calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>COUP-TFII</td>
<td>chicken ovalbumin upstream promoter transcription factor II</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
</tr>
<tr>
<td>DMBA</td>
<td>dimethylbenzanthracene</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated signal kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FLICE</td>
<td>Fadd-like interleukin-1 beta-converting enzyme</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>hepatic nuclear factor 4 alpha</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>membrane type 1 matrix metalloproteinase</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence</td>
</tr>
<tr>
<td>NT</td>
<td>non-targeting</td>
</tr>
<tr>
<td>ONH</td>
<td>optic nerve head</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEA15</td>
<td>phosphoprotein enriched in astrocytes</td>
</tr>
</tbody>
</table>
PFA – paraformaldehyde
PKB – protein kinase B
PKC – protein kinase C
POAG – primary open angle glaucoma
PRRA – primary rat retinal astrocytes
PVDF – polyvinylidene fluoride
qRT-PCR – quantitative reverse transcriptase polymerase chain reaction
RCF – relative centrifugal force
RGC – retinal ganglion cell
RIPA – radioimmunoprecipitation assay
ROS – reactive oxygen species
RSK2 - p90 ribosomal s6 kinase 2
SDS – sodium dodecyl sulfate
TIMP – Tissue inhibitor of metalloproteinase
TM – trabecular meshwork
TNF-α – tumour necrosis factor α
TRAIL – TNF-related apoptosis inducing ligand
TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling
WDR36 - WD repeat containing protein 36
XIAP – X inhibitor of apoptosis
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Chapter 1
Introduction
1 Introduction

Glaucoma is a group of optic neuropathies that are the second leading cause of blindness globally. It is commonly associated with increased intraocular pressure that exerts mechanical strain on the retinal ganglion cell axons and astrocytes present in the optic nerve head of the eye (Weinreb and Khaw 2004). This contributes to extensive remodeling of the ONH (Downs et al. 2009), astrocyte activation, and retinal ganglion cell (RGC) death for which there is currently no known mechanism or cure (Fiory et al. 2009). During astrocyte activation, normal functions such as extracellular matrix remodeling, glutamate recycling, and ROS scavenging are altered in ways that are both harmful and beneficial to other astrocytes and neurons (Pekney and Nilsson 2005, Guo et al. 2014). These alterations are characterized by increases in glial fibrillary acidic protein (GFAP), as well as altered expression of many proteins such as those involved in apoptosis and extracellular matrix remodeling (Pekney and Nilson 2005, Yan et al. 2000, Rogers et al. 2012). Once initiated, the progression of these pathological processes greatly resembles those of neurodegenerative diseases, giving glaucoma the alias of the “neurodegenerative disease of the visual system” (Gupta and Yucel 2007).

We have previously conducted proteomic analyses of human optic nerve head astrocytes subjected to biomechanical insult in order to identify proteins of importance that may contribute to tissue remodeling, astrocyte activation, and the loss of RGCs. The level of mechanical strain – defined as the change in length divided by the total original length of the cell - was applied at a pathologically relevant level to human optic nerve head astrocytes in the range of 2 – 12% strain, as predicted from previous modeling of glaucomatous eyes (Rogers et al. 2012, Sigal et al. 2007). This strain altered the expression of many proteins but one of specific interest identified from the gene ontology analysis with significantly increased expression was phosphoprotein
enriched in astrocytes (PEA15, Rogers et al. 2007).

This ubiquitously expressed protein acts a molecular adaptor binding to and altering the function of other proteins involved in cell proliferation, apoptosis, and glucose metabolism. Two phosphorylation sites are present on PEA15 that are very important to its function as they dictate which targets and pathways PEA15 can bind and alter. When PEA15 is unphosphorylated at serine 104, it is able to bind ERK (extracellular signal-regulated kinase) and increase the activation of ERK in two independent ways as well as prevent ERK-dependent transcription (Perfetti et al. 2007, Renault et al. 2003, Vaidyanathan and Ramos 2003, Formstecher et al. 2001). When phosphorylated at serine116, PEA15 is known to have anti-apoptotic actions by binding and blocking the formation of the DISC (death inducing signaling complex) in the extrinsic apoptotic pathway (Fiory et al. 2009, Renault et al. 2003, Condorelli et al. 1999). PEA-15 has also been implicated in regulating ECM remodeling although this has been largely unexplored in astrocytes. Transfections of PEA-15 into several cancer cell lines were shown to decrease the levels of MMP2 and MMP9 (Glading et al. 2007), however the mechanism by which this occurs has remained elusive.

Although the role of PEA15 has yet to be characterized in the context of glaucoma and mechanical insult, based on previous proteomic studies and research from other diseases it seems likely that PEA15 is involved in the glaucoma pathogenesis. PEA15 plays a prominent role in neurodegenerative diseases with mechanisms similar to that of glaucoma (Thomason et al. 2013, Zabel et al. 2006). Furthermore PEA15 was predicted from two proteomic analyses utilizing models of glaucomatous injury (Rogers et al. 2012, Tezel et al. 2012). PEA15 has also shown protective roles in apoptosis and suppression of MMPs, both of which have pathological implications in the ONH during glaucoma. Finally, PEA15 is enriched in astrocytes; a key cell
type that is essential to the health of the RGCs in the ONH. It is therefore important to investigate the protective role of PEA15 in apoptosis and ECM remodeling induced by glaucomatous mechanical insult in ONH astrocytes.

1.1 Hypothesis
Increased levels of astrocytic PEA15 are accompanied by changes in its phosphorylation and activity that play an important role in the mechanism directing apoptosis and ECM remodeling associated with the pathology of glaucoma.

1.1.1 Specific Aims
1. To determine the level and phosphorylation state of PEA15 in astrocytes undergoing mechanical strain.

2. To determine how PEA15 affects changes in the level of MMPs in astrocytes undergoing mechanical strain.

3. To determine how PEA15 affects apoptosis in astrocytes undergoing mechanical strain.
Chapter 2
Literature Review
2 Literature Review

2.1 Glaucoma

Glaucoma is a group of optic neuropathies that are the primary cause of irreversible blindness globally (Weinreb et al. 2014). There are two main types of glaucoma; open-angle glaucoma and angle-closure glaucoma, both of which result in optic nerve defects and visual field loss (Quigley 2011). Angle-closure glaucoma is an acute condition that results when the angle between iris and the cornea closes leading to a dramatic elevation in eye pressure (Quigley 2011). Primary open-angle glaucoma (POAG) is the most common form of glaucoma. It is a chronic condition that develops over months to years and progresses similar to that of a neurodegenerative disease (Leske et al. 2008, Gupta and Yucel 2007). It commonly results from blockage of aqueous humour drainage leading to gradual increases in intraocular pressure (IOP), however development also occurs under normotensive (normal pressure) conditions. Interestingly both hypertensive and normotensive POAG are characterized by many of the same detrimental mechanisms such as astrocyte activation, leading to RGC death. Throughout this next section I will discuss the risk factors associated with the development of POAG, the methods for diagnosing and treating POAG, and the mechanisms through which the disease progresses.

2.1.1 The Outflow System and Optic Nerve Head

The outflow system of the eye is a balancing act of aqueous humour production and drainage to maintain a normal pressure in the range of 12-22 mmHg in the eye (Weinreb and Khaw 2004). Aqueous humour is produced in the ciliary body (Figure 1), flows through the pupil into the anterior chamber, and exits through a porous tissue called the trabecular meshwork (TM)
Figure 1. Diagram of aqueous humour production and outflow pathways (Ito and Walter 2013). Aqueous humour is produced in the ciliary body and flows through the pupil and mainly out through the porous trabecular meshwork. A small portion also flows out through the uveoscleral pathway.

Figure 2. Diagram of the eye and optic nerve head (Clark et al. 2001).
into Schlemm’s canal which then drains into the bloodstream (Weinreb and Khaw 2004). During the aging process there is an increase in TM stiffness. If it becomes too stiff this can lead to a decrease in the rate of aqueous humour outflow and a gradual increase in intraocular pressure of the eye (Paulaviciute-Baikstiene et al. 2013). Once IOP rises above 22 mmHg, the patient is classified as ocular hypertensive - a significant risk factor for glaucoma (Coleman and Miglior 2008).

The increase in IOP exerts mechanical stress and strain on the delicate structures at the posterior of the eye. Cell bodies of the retinal ganglion cells are present in the retina and their axons converge to form the optic nerve, which travels back to the lateral geniculate nucleus (Weinreb and Khaw 2004). The area where the axons converge is termed the optic nerve head (ONH) and it consists solely of RGC axons, blood vessels, and glia (Figure 2, Weinreb and Khaw 2004). The lamina cribrosa of the ONH is a porous area adjacent to the sclera that undergoes significant deformation as a result of increased IOP, and it is likely in this area that damage to the RGC axons is initiated (Morgan 2000). This deformation occurs as a result of increased mechanical insult in combination with pathological ECM remodeling and is termed “optic cupping”. Astrocytes in the glaucomatous ONH are seen to secrete higher levels of MMPs, which weaken the lamina cribrosa making it more susceptible to pressure induced damage. This will be discussed further in section 2.2.

2.1.2 Risk Factors

The largest risk factors for susceptibility to POAG are age, race, elevated IOP, myopia, and family history (Coleman and Miglior 2008, Douglas 1998). Individuals above the age of 60 and those of African American descent are at a significantly higher risk of developing glaucoma (Leske et al. 2008). Those with increased IOP also have a dramatically increased risk, as elevated
IOP is tightly correlated to death of the retinal ganglion cells (RGC) leading to vision loss (Weinreb et al. 2014, Quigley 2011, Leske et al. 2008, Coleman and Miglier 2008). Because myopic (near sighted) eyes have slightly higher IOP's than emmetropic (correct refractive vision) or hyperopic (far sighted) eyes, myopia is also strongly correlated with increased risk of POAG across all races (Chen et al. 2012). Those with a family history of glaucoma have a 22% risk of developing glaucoma at some point in their lives, compared to 2-3% risk for those without relatives (Gementezi et al. 2012). In spite of this statistic, glaucoma is a very complex genetic disease for which the genes responsible are unknown. Glaucoma is linked to twenty genetic loci, however only three genes known to cause congenital forms of POAG have been identified, those being myocilin, optineurin, and WDR36 (Koga et al. 2010).

Myocilin is associated with early onset glaucoma (<35 years) and POAG, and is expressed in both the trabecular meshwork (TM) and the optic nerve head - two key tissues related to the pathology of glaucoma (Clark et al. 2001). Mutations in myocilin lead to excess protein accumulation in the ECM of TM leading to decreased drainage of aqueous humour and an increase in IOP (Clark et al. 2013). Implications of extracellular myocilin in the ONH are still being explored, however myocilin has been show to reduce neurite outgrowth and regeneration (Koga et al. 2010). Optineurin is associated with normal tension glaucoma and mutations have been seen to directly induce apoptosis in RGCs (Koga et al. 2010, Chalasani et al. 2007). WDR36 (WD repeat containing protein 36) protein depletion has been seen to induce apoptosis in the trabecular meshwork through activation of the stress protein p53 (Skarie and Link 2008, Blanco-Marchite et al. 2011). Although mutations in the aforementioned proteins are responsible for some cases of glaucoma, this represents less than 5% of all cases (Blanco-Marchite et al. 2011). The pathology POAG is genetically complex and characterized by the presence of many risk mutations that are observed at a higher incidence in POAG patients (Fingert 2011). These
genetic mutations combined with a myriad of environmental factors contribute to the development and progression of glaucoma, similar to neurodegenerative diseases (Fingert 2011). Currently there are no treatments to address this neurodegenerative aspect of the disease as will be further discussed in the following section.

2.1.3 Diagnosis and Treatment

Glaucoma is undiagnosed in 50% of people in developed countries (Quigley 2011). Because vision loss is irreversible, there is great need for improved diagnostic methods. Currently the best method of diagnosis is through imaging of the posterior of the eye to observe for cupping of the optic disc and thinning of the neuroretinal rim – the area between the edge of the scleral and the optic cup (Quigley 2011, Leske et al. 2008, Weinreb and Khaw 2004, Morgan 2000). The periphery of the optic cup can be visualized by kinks in the blood vessels as they advance into the brain (Morgan 2000). Tonometry is also used to measure IOP, however this method is unreliable because of the large variability between patients (Morgan 2000). Furthermore, 50% of all POAG cases do not have pressures in the ocular hypertensive range (>22mmHg) after a single screening (Weinreb and Khaw 2004), making this method ineffective. Because damage to the visual system could be present anywhere from the retina to the lateral geniculate, examining the optic nerve head represents a very small part of this large disease (Gupta and Yucel 2007). Therefore, better targets to earlier detect the glaucoma pathology are markedly needed.

Currently the only treatment available for glaucoma is aimed at reducing IOP (Weinreb and Khaw 2004, Gupta and Yucel 2007). Available drugs either decrease the inflow of aqueous humour or increase the outflow (Weinreb and Khaw 2004). Other options include performing surgery (trabeculectomy) or laser treatment (laser trabeculoplasty) on the TM to increase
aqueous humour drainage (Quigley 2011). These methods are effective at reducing visual field loss in patients with elevated IOP, however they are accompanied by a large caveat: many patients of POAG are normotensive, and conversely many people with elevated IOP do not develop POAG (Morgan 2000). Furthermore these treatments completely ignore other mechanisms mediating the pathology of POAG outside of the TM such as astrocyte activation, apoptosis and ECM remodeling in the ONH, which are common to all forms of POAG. Since elevated IOP is not the sole causative factor of visual field loss it is important that we treat POAG as a neurodegenerative disease and explore other mechanisms as targets for effective treatments.

2.1.4 Pathology of Glaucoma

The pathology of glaucoma is thought to be initiated by mechanical insult from elevated IOP, however once this insult has begun, the disease progresses using many of the same mechanisms that are common to neurodegenerative diseases (Gupta and Yucel 2007). Initially, increased IOP causes mechanical strain (further discussed in section 2.3) leading to disruption of axonal transport (Nickells 2012, and Rastogi et al. 2009), decreased blood flow to the ONH (Douglas 1998), and glial activation (Johnson and Morrison 2009, Hernandez 2000, and Sofroniew and Vinters 2010). These initial adverse processes soon spiral into a myriad of other problems that characterize the pathology of POAG (Quigley 2011, Weinreb and Khaw 2004, Douglas 1998).

Previously observed in human, primate, and rodent models of glaucoma, disruption of axonal transport of the RGCs from the brain reduces the level of neurotropic factors in the optic nerve head that are essential to the survival of RGCs (Morgan 2000). Since neurotropic factors such as BDNF suppress the intrinsic apoptotic pathway, this leads directly to apoptosis of the
RGCs and visual field loss (Putcha et al. 2003). Blood flow in the retina and ONH is also disturbed causing ischemia/reperfusion injury as a result of increased pressure on the blood vessels (Grunwald 1999). Subsequent generation of ROS can harm astrocytes causing glial activation, and directly induce RGC death via the intrinsic apoptotic pathway (Gupta and Yucel 2007, Hernandez 2000). Glial activation also causes a myriad of problems within the optic nerve head such as reduction in ROS scavenging, glutamate excitotoxicity, secretion of apoptotic cytokines, and pathological ECM remodeling (as discussed in depth in the following section) by the astrocytes (Sonofrew and Vinters 2010, Hernandez et al. 2008). Thus glaucoma is initiated by mechanical strain on the ONH caused by elevated IOP that sends several homeostatic mechanisms out of balance leading to astrocyte activation, ischemia/hypoxia, disrupted axonal transport, and ultimately RGC death.

2.2 Pathological Extracellular Matrix Remodeling

ECM remodeling is a continual process of ECM degradation while ECM deposition occurs simultaneously (Agapova et al. 2003, Hernandez 2000, Prasanna et al. 2011). Pathological ECM remodeling occurs when these two processes are no longer homeostatic. During glaucoma, significant levels of pathological ECM remodeling occur in both the TM and the ONH (Agapova et al. 2003). Throughout this next section the role of MMPs in glaucoma and their possibility as a future therapeutic target will be presented.

MMPs are a family of endopeptidases responsible for the degradation of the ECM during tissue remodeling (Johnson et al. 1998). Common processes such as fetal development, wound healing, angiogenesis, and inflammatory cell migration require the breakdown of the ECM by MMPs (Johnson et al. 1998). Four classes of the more than 20 MMPs exist: collagenases,
gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs, Johnson et al. 1998, Pan and Hung 2002). MMPs are very tightly regulated through transcription, proenzyme activation, and inhibition by TIMPs (tissue inhibitors of metalloproteinase, Nagase and Woessner 1999). Increased expression can be effected from many different stimuli such as growth factors and cytokines, and are very specific to the MMP (Nagase and Woessner 1999).

ECM remodeling is a balance between the level of MMPs and their corresponding inhibitors. The normal ratio of MMPs to TIMPS is 1:1 (Groed et al. 2013), and the disruption of this balance can lead to a variety of diseases such as those of the cardiovascular system, arthritis and cancer (Groef et al. 2013). POAG is characterized by an increase in the levels of TIMPS in the TM, causing decreased activation of the MMPs present (Groef et al. 2013). This leads to the accumulation of ECM components in the TM clogging the aqueous outflow pathway, and raising the IOP (Schlotzer-Schrehardt et al. 2003). Altered levels of MMP2 have received considerable attention as deregulation has been observed in humans (Maatta et al. 2005), dogs (Weinstein et al. 2007), and mice (Zhou et al. 2005). Decreased activity of MMP9 has also been reported to contribute to elevated IOP in both humans (Guo et al. 2012), and mice (Robertson 2013).

During glaucoma, extensive cupping of the optic nerve head is observed. As mentioned previously, this deformation is a combination of elevated IOP and tissue remodeling (Yan et al. 2000, Downs et al. 2010, Taylor 2012). The lamina cribrosa in the optic nerve head is primarily made up of collagens type I, III, IV, VI (Svoboda et al. 1998) along with laminin and α-elastin (Fukuchi et al. 1992). The content of elastin increases as we age to as much as 3.5 times when comparing the young to the elderly (Albon et al. 2000), contributing to increased stiffness in the ONH, and increased susceptibility to mechanical insult. Furthermore, this biomechanical insult
can induce pathological increases in MMP expression (Blain et al. 2007), which continues the cycle of dysregulated ECM remodeling.

Agapova et al. (2003) examined the levels of MMPs in monkey ONHs with experimental glaucoma, revealing elevated levels of MMPs. Immunohistochemical analysis of glaucomatous human as well as rat ONHs with induced IOP elevations, also revealed dramatic increases in MMPs (Yan et al. 2000, Guo et al. 2005). Upregulation and increased disorder of ECM components has also been reported after exposure of ONHs to elevated IOP. Changes in the make up of the ECM can lead to alterations in compliance and resiliency of the ONH, which could further exacerbate damage to RGCs caused by increases in IOP (Pena et al. 1998, Kirwan et al. 2005). From these observations it can be proposed that elevated levels of MMPs are a pathological process of glaucoma, which could serve as a potential target to decrease optic cupping of the ONH and prevent damage to the RGCs.

MMP2 and MMP9 are of specific interest to the remodeling of the ONH because they are known to be present in the lamina cribrosa at increased levels during glaucoma in humans (Yan et al. 2000, Sivak 2002). Both degrade collagen type IV (Yan et al. 2000) (one of the key components of the ONH) and are under transcriptional control of ERK when it phosphorylates the nuclear transcription factors Elk-1 for MMP9 (Hsieh et al. 2008) and Sp1 and Sp3 for MMP2. Most MMPs are secreted as inactive zymogens that are activated in the ECM. For example, the pro-MMP9 zymogen is cleaved by MMP2 and MMP3, and activation can be inhibited by TIMP1 (tissue inhibitor of metalloproteinase, Marta et al. 2003, Ramos-DeSimone et al. 1999). Unlike most other MMPs, pro-MMP2 is activated on the cell surface. It is cleaved when complexed with MT1-MMP and TIMP2, by a neighboring unbound MT1-MMP (Figure 3, Nagase and Woessner 1999, Bernardo and Fridman 2003). Once MMP2 is activated it
dissociates from the cell membrane (Bernardo and Fridman 2003). Although TIMP2 is required for MMP2 activation, high concentrations of TIMP2 can also inhibit MMP2 activation (Lu et al. 2004).

Increased IOP in glaucoma can be induced by MMP deregulation in the TM and can induce MMP deregulation in the ONH. Therefore the activity of MMPs in the eye can act as future targets for glaucoma therapy in two separate tissues; both the TM and ONH. Although it is known that biomechanical insult induces the expression of MMPs (Kirwan et al. 2004), the mechanism through which this works is not fully understood. Future research is needed to understand this mechanism, to facilitate proper targeting and inhibition of pathological ECM remodeling in the ONH.

2.3 Mechanical Insult

High intraocular pressure exerts mechanical strain on the RGCs and astrocytes of the lamina cribrosa causing activation of a variety of pathological mechanisms ultimately leading to RGC death and blindness (Weinreb et al. 2014, Sigal et al. 2004, Sigal et al. 2007, Sigal 2009). Because the optic nerve head is a discontinuation of the stiff corneo-scleral shell of the eye, it is vulnerable to higher levels of strain (Downs et al. 2010), and these strains can increase with age as the composition of the ONH changes (Albon et al. 2000). Mechanical strains previously modeled in the eye, showed the highest level of strain in the lamina cribrosa of the optic nerve head (Sigal et al. 2004, Sigal et al. 2007). This mechanical strain is defined as the change in length of a tissue during increased pressure, divided by the initial length of the tissue at rest (Sigal et al. 2007). Previous models of human eyes exposed to elevated levels of IOP have found compressive, shearing, and extensive strains exerted on the lamina cribrosa (Sigal 2006, Sigal et
Figure 3. Activation of MMP2 (Grzela et al. 2011). MMP2 is activated when bound to both MT1-MMP and TIMP2 in a complex. An adjacent MT1-MMP then cleaves the pro-MMP2 zymogen so the active form can dissociate from the membrane to degrade collagen type IV.

Figure 4. Extensive strains in the ONH during glaucoma (Sigal et al. 2007). Sections through a human ONH exposed to increased IOP show that levels of extensive mechanical strain can reach up to 15% as a result of elevated IOP during glaucoma. Strain is defined as the change in tissue length under pressure over the initial tissue length. N = nasal, T = temporal, S = superior, I = inferior.
Both extensive and compressive strains (also known as first and third principal strains respectively) are seen in the range of 12-25% in ONH with elevated IOP (Figure 4, Sigal et al. 2007, Sigal et al. 2014).

Mechanical insult from high intraocular pressure has biological consequences in addition to physical ones. This becomes evident when studying other diseases characterized by elevated biomechanical strains, such as osteo-arthritis, and many cardiovascular diseases such as atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction and aortic aneurysm (Messerli 2004). As introduced previously, mechanical strain in the eye leads to pressure on the RGC axons, ONH astrocytes, and optic nerve blood vessels. This causes disruption of axonal transport, glial activation and ischemia/reperfusion injury that characterize the pathology of glaucoma (Hernandez 2000). Furthermore, mechanical insult has been seen to induce the expression of MMPs that contribute to pathological ECM remodeling (Kirwan et al. 2004). Glaucoma research has only begun to determine the effect of mechanical insult on the various processes in the ONH, and further research is needed to identify appropriate targets to abrogate the pathological mechanisms initiated by mechanical insult.

2.4 Astrocytes

Astrocytes are the most abundant glial cell type and central support structure for RGCs in the ONH (Sofroniew and Vinters 2010, Hernandez et al. 2008). Astrocytes surround the RGCs and perform functions such as removing excess neurotransmitters and ions from the extracellular space, maintaining pH, providing energy substrates, growth factors and neurotropic factors to the RGC axons, scavenging excess reactive oxygen species, and maintaining the ECM (Gupta and Yucel 2007, Morgan 2008, Johnson and Morrison 2009, Sofroniew and Vinters 2010, Prasanna al. 2014).
et al. 2011, Hernandez et al. 2008). Proper function of astrocytes is especially important in the ONH because the formation of myelin around the axons of the RGCs is prevented past the lamina cribrosa into the retina (Perry and Lund 1990), making the astrocytes the main cell type surrounding the RGCs (Nguyen et al. 2011).

During the progression of POAG, astrocytes take on an altered function, phenotype, and distribution in the ONH in a process termed glial activation or reactive gliosis. This transformation is most easily visualized by enlargement of the astrocyte cell body and increases of glial fibrillary acidic protein (GFAP, Johnson and Morrison 2009). Secretion of various cytokines, growth factors, and ECM remodeling also begins once the astrocytes become reactive (Hernandez et al. 2008). Furthermore, astrocytes have been seen to migrate from the periphery of the lamina cribrosa into the nerve bundles (Hernandez et al. 2008, Miao et al. 2010).

It has been highly controversial whether glial activation is beneficial or damaging to the RGCs in the optic nerve head, with the truth likely being a mix of both helpful and harmful elements. A good example of this is seen when astrocytes react to ischemia/reperfusion injury in the ONH during glaucoma. Hypoxia in the ONH results in the production of harmful reactive oxygen species (ROS) that are damaging to all cell types (Guo et al. 2010). Astrocytes are the cell type with the highest concentration of antioxidants and are able scavenge these ROS, as well as provide neurons with substrates for antioxidants to reduce this damage (Pekney and Nilsson 2005). Conversely, astrocytes have also been seen to promote axon loss indirectly by increased production of nitric oxide – a free radical that promotes vasodilation (Hernandez et al. 2008). Nitric oxide can react with other ROS free radicals to produce the harmful product, peroxynitrite (Johnson and Morrison 2009). Inhibition of the enzyme that produces nitric oxide in astrocytes, iNOS (inducible nitric oxide synthase), has proven to be neuroprotective in a rat model of
glaucoma (Neufeld et al. 2002). Although astrocytes are reacting to reduce ROS and increase oxygen in the ONH through production of nitric oxide, the end result is a mix of helpful and harmful effects.

Other adverse outcomes of reactive astrocytes during POAG are the secretion of harmful cytokines such as tumour necrosis factor-α (TNF-α, Tezel et al. 2001). In severe glaucoma, RGCs increase expression of TNF-α receptors, leading to direct RGC apoptosis (Agarwal and Agarwal 2012). Glutamate excitotoxicity is also thought to lead to RGC death through two mechanisms present in activated ONH astrocytes; loss of glutamate recycling function (Prasanna et al. 2011, Hernandez 2008), in addition to pathological secretion of glutamate through reversal of glutamate transporters (Parpura et al. 2004). Astrocytes also direct changes in ECM remodeling in the ONH that occur during glaucoma. Increased pathological secretion of MMPs as discussed in the previous section can lead to increased vulnerability of the ONH to mechanical insult (Johnson and Morrison 2009). Furthermore astrocytes deposit new ECM components in areas that RGCs previously occupied, forming what is called a glial scar (Hernandez et al. 2008). Although this scar is meant to be protective, the deposition of new matrix has been reported to be unorganized and further aggravates the remaining RGC axons to mechanical insult (Johnson and Morrison 2009, Sofroniew and Vinters 2010). Thus astrocyte activation during glaucoma, although likely intended helpful, produces many harmful outcomes.

2.5 Apoptosis

Cells that have undergone damage beyond repair can induce their own programmed cell death or apoptosis (Keuhn et al. 2005). This is a controlled induction of cell death that uses energy to degrade the cell and does so without eliciting any inflammatory responses (Rastogi et
There are two primary pathways of apoptosis that are well described, known as the intrinsic and extrinsic apoptotic pathway (Rastogi et al. 2009).

### 2.5.1 Intrinsic Apoptosis Pathway

This apoptotic response is induced by damage to DNA through a variety of mechanisms such as UV or ionizing radiation, oxidative stress from ROS, errors during DNA replication, or DNA damage from genotoxins (Rastogi et al. 2009). In the context of glaucoma, RGC death is triggered by both ROS damage to DNA, as well as a neurotropic factor deprivation from loss of axonal transport (Putcha et al. 2003). Resulting damage induces changes in the levels of proteins in the Bcl2 gene family, which consists of three groups of proteins; apoptosis-promoting proteins (such as BAX or BAK), anti-apoptotic proteins (BCL-X and BCL2) or BH3-only domain-containing proteins that regulate the interaction between the previous two members listed (Nickells 2012). DNA damage can induce activation of p53, a key tumour suppressor gene that activates BH3-only proteins, which facilitate activation of pro-apoptotic proteins, and inhibition of anti-apoptotic proteins (Putcha et al. 2003). Pro-apoptotic proteins aggregate on the outer mitochondrial membrane to induce destabilization and pore formation resulting in the leakage of aggressive pro-apoptotic factors such as cytochrome C and Omi/htra2 (Putcha et al. 2003, Keuhn et al. 2005, Rastogi et al. 2009). Cytochrome C binds to another factor (Apaf-1) to form a complex termed the apoptosome, which then activates pro-caspase 9 to induce the caspase cascade (Rastogi et al. 2009). Omi/htra2 also induces the caspase cascade by binding and degrading IAPs (inhibitors of apoptosis) bound to several caspases, thereby activating them (Figure 5, Rastogi et al. 2009).

### 2.5.2 Extrinsic Apoptotic Pathway
Death inducing ligands and cell surface death receptors initiate the extrinsic apoptotic pathway. Some of the common death receptors/ligands are TNF receptor/TNF-α and Fas/Fas ligand (Rastogi et al. 2009). After exposure to stress, certain types of cells such as immune cells and astrocytes will release death ligands (Putcha et al. 2003, Thomason et al. 2013). In the context of glaucoma, astrocytes become activated after experiencing mechanical strain from elevations in IOP and release TNF-α which induces apoptosis in proximal RGCs expressing the TNF receptor (Tezel et al. 2001, Agarwal and Agarwal 2012).

![Diagram of the extrinsic and intrinsic apoptotic pathways](image)

**Figure 5. The extrinsic and intrinsic apoptotic pathways (Panayi et al. 2013).** The extrinsic apoptotic pathway begins by binding of the death ligand to the death receptor (top left) whereas the intrinsic apoptotic pathway is initiated by DNA damage from a variety of stresses (top right). Both converge to activate caspases resulting in apoptosis.
After the death ligand has bound to the death receptor, formation of the DISC (death inducing signaling complex) is formed. The DISC consists of the death receptors, an adaptor molecule termed FADD (Fas-associated death domain) and the initiator caspase 8 (Putcha et al. 2003, Rastogi et al. 2009). Both FADD and caspase 8 contain a death effector domain (DED) allowing them to interact closely leading to activation of caspase 8. Caspase 8 then triggers the caspase cascade by directly cleaving effector caspases or triggering the intrinsic apoptotic pathway by cleaving the BH-3 only protein, Bid (Figure 5, Rastogi et al. 2009).

Apoptosis through both the extrinsic and intrinsic apoptotic pathways is seen in astrocytes and RGCs during glaucoma and so a thorough understanding of both is needed to study this disease. This allows for effective targeting of apoptosis for glaucoma prevention and treatment.

2.6 PEA15

2.6.1 Relation to Glaucoma

PEA15 was identified from our previous proteomic analysis in which human ONH astrocytes underwent cyclical mechanical stretch for 2, 12 and 24 hours (Rogers et al. 2012). PEA15 was seen to increase 1.5 fold after 2 hours of 12% mechanical strain and was predicted from the gene ontology analysis. This analysis pinpoints proteins in pathways relevant to glaucoma including apoptosis, glial activation, neurodegeneration, DNA damage/repair, cellular remodeling, and stress responses (Rogers et al. 2012). PEA15s ability to elicit transcriptional changes has made it of specific interest and led us to pursue its role in mechanical insult during the pathology of glaucoma.
2.6.2 PEA15

This ubiquitously expressed protein was discovered in 1993 in striatal astrocytes (Araujo et al. 1993). It is a molecular adaptor that can bind and alter the function of other proteins involved in cell proliferation, apoptosis, and glucose metabolism (Greig and Nixon 2014, Fiory et al. 2009). It is, as the name suggests, highly enriched in astrocytes, however it is also present in neurons (Sharif et al. 2004). PEA15 is highly conserved among mammals with 96% similarity between mice and humans (Thomason et al. 2013). Two phosphorylation sites are present on PEA15 at serine 104 and serine 116 that are phosphorylated by protein kinase C (PKC) and CAMKII/PKB (Ca\(^{2+}\)/Calmodulin-dependent protein kinase II/PKB) respectively (Figure 6, Fiory et al. 2009). This phosphorylation occurs in a hierarchical manner in that PKC is able to phosphorylate ser104 at an enhanced rate when ser116 is phosphorylated (Renault et al. 2003). Another effect of phosphorylation at serine 116 is an increased lifespan of PEA15 by preventing ubiquitinylation and proteasomal degradation of PEA15 (Figure 7, Perfetti et al. 2007). PEA15 has a death effector domain (DED) present near its N-terminus allowing it to interact and alter the function of other proteins containing a DED (Fiory et al. 2009, Ramos et al. 2000). PEA15 also contains a nuclear export sequence (NES) that localizes PEA15 and anything it may bind to the cytoplasm (Formstecher et al. 2001).

2.6.3 Anti-apoptotic Role

PEA15 has several mechanisms through which it is anti-apoptotic. PEA15 is able to interact with the apoptotic mediators, FADD (Fas-associated death domain), and caspase 8 to inhibit formation of the DISC (death-inducing signaling complex) during the extrinsic apoptotic pathway. This has been shown to prevent FasL and TNF-\(\alpha\) induced apoptosis (Condorelli et al. 1999, Kitsberg et al. 1999, Condorelli et al. 2002), as well as apoptosis induced by the anti-
neoplastic agent TRAIL (TNF-related apoptosis-inducing ligand) which similarly induces apoptosis by binding the TNF death receptor (Hao et al. 2001). When phosphorylated at serine116, PEA15 is able to bind FADD and caspase 8 at an enhanced rate (Bock et al. 2010). A second anti-apoptotic mechanism of PEA15 occurs when it competes with IAPs (initiators of apoptosis) to delay the onset of apoptosis (Trencia et al. 2004). Normally the apoptotic activator, Omi/Htra2 is released from the mitochondria as a result of cell stress and cleaves IAPs bound to inactive caspases causing activation (Rastogi et al. 2009). PEA15 competes with XIAP bound to caspase 3, 7, and 9 for degradation by Omi/Hta2 (Trencia et al. 2004), ultimately delaying apoptosis mediated through the intrinsic apoptotic pathway. Thirdly, in transgenic mice overexpressing PEA15, activation of JNK and p38 has been observed to be reduced, leading to reduced levels of apoptosis (Figure 8, Formisano et al. 2005, Condorelli et al. 2002).

2.6.4 Interaction with ERK1/2

When unphosphorylated at serine 104, PEA15 is able to bind ERK1/2 (extracellular signal regulated kinase1/2) and sequester it in the cytoplasm preventing ERK dependent transcription, and cell cycle progression (Renault et al. 2003, Perfetti et al. 2007, Formstetcher et al. 2001, Vaidyanathan and Ramos 2003). This results in decreased phosphorylation of ERK1/2 nuclear transcription factor targets such as Elk-1 (Vaidyanathan and Ramos 2003), Sp1, and Sp3 (Boros et al. 2009). PEA15 can bind ERK1/2 regardless of ERKs state of phosphorylation, and ERK1/2 is still able to undergo phosphorylation while PEA15 is bound to it (Mace et al. 2013). This leads to the accumulation of inactive, phosphorylated ERK in the cytosol when PEA15 is bound to it. Once PEA15 is phosphorylated by PKC, it can no longer inhibit ERK through binding and ERK1/2 is released fully active to phosphorylate both cytoplasmic and nuclear targets (Figure 9, Mace et al. 2013).
Figure 6. Structure of PEA15 (Fiory et al. 2009). PEA15 has two phosphorylation sites, and a death effector domain (DED) that allow it to interact with other proteins.

Figure 7 (right). Regulation of PEA15 levels (Fiory et al. 2009). PEA15 is regulated through ubiquitinylation and proteosomal degradation. Phosphorylation of PEA15 on ser116 prolongs the lifespan of PEA15 by preventing this degradation. The transcription of PEA15 is promoted by the transcription factor COUP-TFII, and inhibited by HNF-4α.

Figure 8 (left). PEA15 inhibits apoptosis through the extrinsic apoptotic pathway (Fiory et al. 2009). PEA15 can bind both FADD and caspase 8 (also known as FLICE) to prevent formation of the DISC. Furthermore, PEA15 has been seen to reduce phosphorylation of the stress kinases JNK1/2 and p38.
Figure 9. ERK2 can still be phosphorylated when bound to PEA15 (Mace et al. 2013). Unphosphorylated PEA15 can bind ERK, however when PEA15 is phosphorylated by Akt/PKC it releases ERK. ERK can be phosphorylated when PEA15 is bound to it leading to sequestered ERK in the cytoplasm until it is released.

Figure 10. PEA15 induces insulin sensitivity (Fiory et al. 2009). PEA15 blocks translocation of the glucose transporter to the cell membrane through it's interaction with PLD1 resulting in insulin insensitivity.
Through another independent mechanism, PEA15 activates ERK by increasing the GTP-loading of Ras upstream (Ramos et al. 2000). Activation of Ras triggers a cascade for MEK1 (mitogen-activated protein kinase kinase) activation, which phosphorylates ERK1/2 (Mace et al. 2013). PEA15 also binds a substrate of ERK, RSK2 (p90 ribosomal s6 kinase) and prevents its nuclear translocation as well as 50% of its activity (Vaidyanathan and Ramos 2003).

2.6.5 Disease

Because of PEA15's prominent role in a wide range of pathologies, it has been termed a nodal point protein (Zabel et al. 2006). Since its recent discovery it has already been implicated in a diverse set of diseases including type 2 diabetes, cancer, and other neurodegenerative diseases.

2.6.5.1 Type 2 Diabetes

PEA15 is overexpressed in individuals with type 2 diabetes and contributes to its pathogenesis (Vigliotta et al. 2004, Condorelli et al. 1998). PEA15 overexpression in transgenic mice leads to both impairments in insulin secretion as well as insulin action (Vigliotta et al. 2004). PEA15 blocks translocation of the GLUT4 to the cell membrane inhibiting glucose transport into cultured muscle and adipose cells (Vigliotta et al. 2004). Furthermore PEA15 overexpression also leads to increased activation and lifespan of phospholipase D1 (PLD1, Zhang et al. 2000). PLD1 catalyzes the breakdown of glycerophospholipids into a variety of products including DAG (diacylglycerol), which activates PKC isoforms. Therefore, the interaction between PLD1 and PEA15 leads to increased activation of PKC-α, blocking activation of PKC-ζ/λ by insulin (Viparelli et al. 2008). Consequently, PEA15 expression is detrimental to insulin sensitivity and activation of glucose transport machinery contributing to diabetes (Figure 10, Fiory et al. 2009).
2.6.5.2 Cancer

PEA15s anti-apoptotic function has led to thorough investigation of its implication in cancer, although this function appears to vary considerably between different types of cancer. Furthermore, the stage of the cancer seems to also play a large role. I report here a brief summary of the protective and destructive roles of PEA15 in cancer research to date, although clearly there is much more to be discovered.

PEA15 has been observed to be harmful to several types of cancer. Levels of PEA15 are elevated in several human tumour cell lines (Dong et al. 2001), and PEA15 has been show to confer resistance to the neoplastic agent, TRAIL in glioma cells through inhibition of the extrinsic apoptotic pathway (Hao et al. 2001). Furthermore, PEA15 overexpressing mice showed early development of skin carcinogenesis induced by the tumour initiator DMBA, and a four-fold increase in the number of skin papillomas that developed (Formisano et al. 2005).

However, not all the roles of PEA15 in cancer have been negative. PEA15 has also been seen to promote autophagy through potent activation of JNK (c-Jun N-terminal kinase) in glioma cells, when biphosphorylated (Bock et al. 2010). Furthermore, PEA15 has been found protective in ovarian cancer as women with high PEA15-expressing tumours survived longer than those with low PEA15-expressing tumours (Bartholomeusz et al. 2008). This was due to PEA15s interaction with ERK. Ovarian tumours displayed increased levels of ERK activation and as a result, decreased cell proliferation and higher levels of autophagy (Bartholomeusz et al. 2008, Cagnol and Chambard 2010). Furthermore, an immunohistochemical analysis of PEA15 in breast cancer showed that PEA15 was inversely correlated with invasive behavior of breast cancer (Glading et al. 2007). It appears that depending on the type and stage of cancer, the role of PEA15 may be beneficial or detrimental.
2.6.5.3 Neurodegenerative Diseases

Although limited research is available on the role of PEA15 in neurodegenerative diseases, the results clearly implicate PEA15 for a role in this group of pathologies. In a proteomics study evaluating changes in protein expression levels in several neurodegenerative diseases, levels of PEA15 were found to be dysregulated in three of the four neurodegenerative diseases evaluated; Huntington’s disease, prion disease Scrapie, and in a model of impaired synaptic function (Zabel et al. 2006). Furthermore, PEA15 expression is also observed both increased and decreased in reactive astrocytes of two mouse models of Alzheimer’s (Thomason et al. 2013, Takano et al. 2012). Although the role of PEA15 in neurodegenerative diseases is a fairly new area of research, it could potentially be very interesting because PEA15 has been found deregulated in several pathologies that possess overlapping mechanisms.

2.6.5.4 Possible Role in Glaucoma

Glaucoma has been called the “neurodegenerative disease of the visual system” because of the many similarities between the pathogenesis of glaucoma and neurodegenerative diseases (Gupta and Yucel 2007). Since PEA15 has been implicated in several neurodegenerative diseases as discussed above, it seems likely that PEA15 may also be implicated in the glaucoma pathology. To my knowledge, there are no studies that have examined the role of PEA15 during glaucomatous mechanical insult. However, PEA15 has popped up in two proteomics studies using models of glaucomatous mechanical strain in vitro and in vivo (Rogers et al. 2012, Tezel et al. 2012). Both of these studies have shown increased levels of PEA15 after exposure to increased mechanical strain, which begs the question; what is this protein’s function in mechanically insulted cells?
PEA15 seems likely to be involved in apoptosis and ECM remodeling, two common pathological processes in glaucoma. Although the role of PEA15 has never before been examined in these pathological mechanisms during glaucomatous mechanical strain, there is some basis as to why PEA15 may be implicated. PEA15 is known to be anti-apoptotic via the extrinsic apoptotic pathway and has shown to be neuroprotective during ischemic brain injury (Koh et al. 2011). As mentioned in section 2.1.4, ischemia is a common trait of glaucomatous eyes since increases in IOP restrict blood vessels. Furthermore, PEA15 transfections into two breast cancer cell lines have shown to repress levels of several MMPs, namely MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 (Glading et al. 2007). During glaucoma MMPs are observed to be pathologically elevated which degrades and weakens the ONH exacerbating any injury caused by increased IOP.

Finally as the name of PEA15 suggests, this protein is highly enriched in astrocytes. Since astrocytes are one of the main cell types that occupy the ONH and provide extensive support to the RGCs, this seems an appropriate cell type in which to study these glaucomatous mechanisms.

PEA15 is highly enriched in astrocytes, becomes increased after exposure to glaucomatous mechanical insult, and has a basis for roles in apoptosis and ECM remodeling; two key pathological mechanisms in glaucoma. Furthermore PEA15 has been implicated in neurodegenerative diseases, which have comparable mechanisms to those seen in glaucoma. Because of these reasons, it seems fitting to examine the role of PEA15 in apoptosis and ECM remodeling in astrocytes during glaucomatous mechanical insult.
Chapter 3
Methods
3 Methods

3.1 Cell Culture

3.1.1 A7 Cell Culture

The A7 cell line is an immortalized neonatal rat ONH astrocyte cell line (Okoye et al. 1995). These cells were cultured in 100mm polystyrene cell culture dishes (Greiner bio-one, Ref#664160) in Dublecco media H21 with antibiotics (Gibco, Ref#12800) supplemented with 10% fetal bovine serum (Multicell, Ref#080105). Cells were kept in a humidified incubator at 37°C and 5% CO₂. Media was changed completely every other day. Cells were passaged to a new culture dish once a week, or if confluent, using a brief rinse with TrypLE (Gibco, Ref#12604-013) to detach cells followed by resuspension in complete media to inactivate trypsin. Cells were then pelleted by centrifugation at 200 rcf. Media was suctioned off of the cell pellet and cells were resuspended in fresh complete media free of trypsin. A7 cells were quantified using a hemocytometer and plated for the appropriate experiment.

3.1.2 Primary Rat Retinal Astrocyte Isolation and Culture

My lab has previously published this protocol to isolate and culture primary rat retinal astrocytes (Nahirnyj et al. 2013). Astrocytes remained quiescent in culture until a stress was applied at which point phenotypic changes occurred indicative of astrocyte activation (Nahirnyj et al. 2013). Twenty-one day old wistar rats were sacrificed by CO₂ and confirmed dead by cervical dislocation. Eyes were removed and placed in ice-cold MEM H17 media (Gibco, Ref#61100) supplemented with 5% horse serum (Invitrogen, Ref#16050-122) and 5% fetal bovine serum (Multicell, Ref#080105). Using a dissection microscope, retinas were dissected out of eyes and washed in serum-free media before being trypsinized with TrypLE (Gibco,
Ref#12604-013) and shaken for 40 minutes to break apart cells. Cells were then washed several times in serum free media and plated in T-75 flasks pre-coated with astrocyte growth media (Lonza, Ref#CC-4123). Media was changed the following day to remove aggregates and other cell types, and then twice weekly for 2 weeks replacing only half of the media at each change. When flasks appeared confluent, cells were shaken for 5h at 100rpm inside an incubator to detach microglia, which were discarded with media. Two days after shaking, cells were plated on 6-well, collagen IV coated, bioflex stretch plates (Flexcell International Corp, Ref#BF-3001C-IV) that were additionally coated with collagen I (Corning, Ref#354236).

3.1.3 HEK 293 Cells

The human embryonic kidney 293 (HEK293) cell line was used for immunoprecipitation experiments with mutant PEA15 plasmids since this cell line minimally expresses PEA15 (Doti et al. 2010). These cells were cultured in 100mm polystyrene cell culture dishes (Greiner bio-one, Ref#664160) in Dulbecco’s Modified Eagles Medium (Sigma, Ref#D5796) with antibiotics (Gibco, Ref#12800) supplemented with 10% fetal bovine serum (Multicell, Ref#080105). Cells were kept in a humidified incubator at 37°C and 5% CO₂. Media was changed completely every other day. Cells were passaged to a new culture dish once a week or if confluent using TrypLE (Gibco, Ref#12604-013) to detach cells.

3.2 Induction of Mechanical Insult

Mechanical insult was induced using the Flexercell® Tension Plus FX-4000T system using a vacuum pump and a custom baseplate able to hold up to 4, 6-well bioflex plates as previously reported (Rogers et al. 2012). Equiaxial strain was applied in a humidified incubator at 37°C and 5% CO₂ with 12% strain and 1Hz for the indicated times. Control cells were also
plated on bioflex plates and placed in a humidified incubator with the same settings. All cells were serum starved for 1 hour prior to mechanical insult.

3.3 Collection and Analysis of Pig Optic Nerve Heads

Previous to my arrival at the lab, other lab members exposed pig eyes to elevated IOP and collected the entire eye for analysis. Seventeen adult Yorkshire pigs were anesthetized with 2.5% isoflurane. Each eye was cannulated with two 27-gauge needles inserted into the anterior chamber, one used to manipulate IOP, the other connected to a pressure transducer used to measure IOP. Pressure was altered with a saline reservoir attached to a stand of adjustable height. Pressure was increased in increments of 20mmHg up to 60mmHg and back down to baseline, four times over a 1 hour period. Eye pressure measured with the transducer was confirmed using a tonometer (Mentor Tonopen XL). Control eyes were also cannulated with the IOP remaining unaltered. At completion of the 1 hour experiment, eyes were removed and the retina, optic nerve head, aqueous humour, and vitreous humour were collected and snap frozen using liquid nitrogen before being stored at -80°C. Pig optic nerve heads were analyzed for gene expression alterations using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

3.4 qRT-PCR

Total RNA was isolated from pig ONH or cells by homogenizing and extracting RNA using trizol (Ambion, Ref#15596-026). RNA was then digested using RQ1 RNase-Free DNase (Promega, Ref#M6101), and cDNA reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Ref#18080-093). PCR was carried out using 200nM of primers, RNase-free water and Power SYBR Green PCR master mix (Applied Biosystems,
Ref#4367659), with 1µL of cDNA for a total reaction volume of 10µL. Real-time PCR was performed using the Mastercycler Eppendorf Realplex for 40 cycles followed by a melting curve to confirm that a sole product was formed. Gene expression was quantified using the comparative Ct method in which Ct values determine relative levels of mRNA by normalizing to a housekeeping gene, in this case TBP (TA-TA binding protein). Levels of TBP were confirmed to have similar levels in treatment and control groups.

### 3.5 Western Blots

#### 3.5.1 Protein Collection and Quantification

Cells to be analyzed by western blot were detached using TrypLE, resuspended in complete media, and transferred to a 1.5mL centrifuge tube. Cells were pelleted, washed with PBS, and resuspended in RIPA buffer (Cell Signaling, Ref#9806). After sitting at room temperature for 10 minutes, samples were centrifuged at 10 000 rcf for 10 minutes to separate protein in the supernatant which was then collected and frozen at -80°C until used.

Protein lysates were quantified using the Lowry Protein Assay kit (Biorad, Ref#500-0116). Briefly, this method involves the production of Cu⁺ ions from the oxidation of peptide bonds under alkaline conditions. These Cu⁺ ions then reduce a Folin reagent to induce a colour change that is measured by a spectrophotometer (Lowry et al. 1951). A standard concentration of BSA is used against protein samples to determine levels of concentration.

Some experiments required separation of cytoplasmic and nuclear lysates. This was achieved using the NE-PER Nuclear Protein Extraction kit (Thermo Scientific, #78833), allowing the protein lysates for the nucleus and cytoplasm to be analyzed separately.
3.5.2  Running Gel and Transfer

Protein samples to be analyzed were diluted in RIPA to obtain equal protein concentrations, and mixed with equal volumes of 4x Laemmeli Sample buffer (Biorad, Ref#161-0747) containing 10% β-mercaptoethanol. Samples were boiled for 10 minutes before being loaded into a 15% SDS-polyacrylamide gel. The gel was submerged in Tris-Glycine-SDS buffer (Bioshop, Ref#TGS222.1) and run at 60V for 30 minutes, followed by 100V for another 1.5 hours. Protein transfer was performed onto a PVDF membrane (Millipore, Ref#IPFL00010) using the Pierce Fast Semi-dry Transfer System (Thermo Scientific, PI-88217). This system allows transfer to occur in 15 minutes at 15V. Membranes were then immediately blocked for 1 hour with 5% BSA (Fischer Scientific, Ref#CAS9048-46-8) in TBST-T (tris-buffered saline with 0.1% tween20), followed by incubation with the primary antibody of choice overnight at 4°C with gentle agitation. Antibodies used are seen in Table 1. After incubation with primary antibodies, membranes were washed 3 times for 10 minutes with TBS-T before incubation with the appropriate secondary antibody for 1 hour at room temperature. Reading of western blots was performed using the LI-COR Odyssey Infrared Quantitative Imaging System in which secondary IRDye antibodies fluoresce and at either 700nm or 800nm by an infrared laser. Densitometry was performed using the LI-COR Odyssey Software or ImageJ.

Table 1. List of antibodies and concentrations used for western blots.

<table>
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<tr>
<th>Antibody</th>
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<tr>
<td>Phospho-ERK1/2</td>
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<td>Cell Signaling, Ref#4370s</td>
</tr>
<tr>
<td>Total ERK1/2</td>
<td>1 in 1000</td>
<td>Santa Cruz, Ref#sc-153</td>
</tr>
<tr>
<td>Cleaved Caspase 8</td>
<td>1 in 1000</td>
<td>Abcam, Ref#ab25901</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1 in 10 000</td>
<td>Calbiochem, Ref#CB1001</td>
</tr>
<tr>
<td>PEA15</td>
<td>1 in 200</td>
<td>Santa Cruz, Ref#sc-28255</td>
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<tr>
<td>Phospho-serine</td>
<td>1 in 500</td>
<td>Abcam, Ref#ab9332</td>
</tr>
<tr>
<td>Donkey Anti-Rabbit IgG IRDye</td>
<td>1 in 10 000</td>
<td>Mandel Scientific, Ref#926-68023</td>
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<td>Mandel Scientific, Ref#926-32212</td>
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3.6 PEA15 Misexpression

3.6.1 PEA15 siRNA Knockdown

siRNAs are short double stranded RNAs that target complementary mRNAs for degradation to effectively silence the expression of a specific gene. siRNAs targeted to PEA15 were transcribed into cells to silence the expression of PEA15 to determine its effect on apoptosis during mechanical insult. Cells were plated on 6-well bioflex plates coated in collagen IV and manually pre-coated with collagen I (Corning, Ref#354236). The following morning, cells were transfected with ON-TARGET plus smartpool rat PEA15a siRNA (Thermo Scientific, Ref#L-083152-02). siRNAs were resuspended in siRNA buffer (Thermo Scientific, Ref#B-00200-UB-100) at 5µM and transfected into cells using DHARMAfect 1 Transfection Reagent (Dharmacon, Ref#T2001-02) using antibiotic free media following the protocol provided by thermo scientific. Cell media was changed 24 hours after transfection.

siRNA transfection efficiency was determined using siGLO Green Tranfection Indicator (Dharmacon, Ref#D001630-01) with flow cytometry (Appendix 1A). Levels of PEA15 mRNA after siRNA knockdown were determined using qRT-PCR (Appendix 1B), and levels of PEA15 protein were evaluated using western blotting (Appendix 1C).

3.6.2 PEA15 Overexpression

3.6.2.1 Growing and Isolating Plasmids

Plasmids containing PEA15 were purchased from the SPARC BioCenter at the Hospital for Sick Children (Toronto, Ontario) with the plasmid containing the full PEA15 sequence tagged to either fluorescent GFP (pcDNA-DEST47) or a V5 protein tag (pcDNA3.2-DEST).
plasmids were transformed into Subcloning Efficiency DH5α Chemically Competent E. coli (Invitrogen, Ref#18265-017). After the plasmid was added, the bacteria were heatshocked for 40s at 42°C, and left to grow suspended in 1mL of LB Broth in an orbital shaker at 37°C for 1 hour. Bacteria were added to agar plates containing ampicillin and allowed to grow overnight. Colonies were picked the following day and grown up in LB broth containing ampicillin. One mL of broth containing bacteria was set aside while DNA was isolated in the remaining bacteria using a Qiagen Spin Miniprep Kit (Qiagen, Ref#27106). DNA underwent Sanger sequencing at the Center of Applied Genomics (Toronto, Ontario). Once the correct sequence was confirmed, E.coli containing the plasmids was grown in 250mL of LB Broth overnight and DNA was isolated using the Endofree Plasmid Maxi Kit (Qiagen, Ref#12362). These plasmids were then transfected into cells to achieve PEA15 overexpression.

3.6.3  PEA15 Mutant Plasmids

In order to develop mutant phospho-plasmids of PEA15, primers were designed to mutate the phosphorylatable serine 104 and serine 116 residues to the unphosphorylatable residue, glycine, as has been previously reported (Trencia et al. 2003). Primer sequences can be seen in Table 2. PCR was performed using Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Scientific, Ref#F-549S) to mutate each of the two serine sites. After PCR, the product was transformed into competent E. coli and grown/isolated as described in the previous section. Mutant plasmids were sequenced at the Center for Applied Genomics (Toronto, Ontario) to confirm the presence of the desired mutation. Once serine 116 was mutated, this plasmid served as the template for the PCR reaction to mutate serine 104 to produce a doubly mutated plasmid. Mutations were introduced into both the GFP-tagged and V5-tagged PEA15 plasmids.
Table 2. Primers for PEA15 mutant phospho-plasmids. Mutated amino acids are highlighted in grey while the mutated base pairs appear in red.

<table>
<thead>
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<th>Site</th>
<th>Sequence</th>
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</tr>
<tr>
<td>Glycine 104 - Reverse</td>
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</tr>
<tr>
<td>Glycine 116 - Forward</td>
<td>CAGCGCGGTGAGGAAGAGATCATCAAAATTGGCTC</td>
</tr>
<tr>
<td>Glycine 116 - Reverse</td>
<td>GATCTCTTCTCTCACCGGGCTGCCGGATAATGCTTTG</td>
</tr>
</tbody>
</table>

3.6.3.1 Plasmid Transfections

Media was changed 30 minutes before transfection was performed using Polyjet In Vitro DNA Transfection Reagent according to the manufacturer’s instructions (SignaGen, SL10068). Briefly, 1μg of DNA and 3μL of polyjet reagent was used per well of a 6-well plate. DNA and polyjet were diluted in serum-free media with antibiotics and mixed immediately after dilution. Incubation of DNA with polyjet was allowed for 15 minutes at room temperature before being added to a 6-well plate containing cells at 80 – 90% confluence. Media was changed once again 8 hours after transfection. Levels of transfected PEA15 were evaluated by western blot for levels of V5-PEA15, and imaging fluorescent cells containing GFP-tagged PEA15 (Appendix 2A, B respectively).

3.7 Determining Levels of Apoptosis

3.7.1 Flow Cytometry

Cells were detached from wells by incubation with TrypLE and resuspended in 1.5mL centrifuge tubes using complete medium. Cells were pelleted by centrifugation at 0.3 rcf, washed in sterile PBS, and repelleted. PBS was suctioned off the cell pellet and cells were resuspended at a concentration of 1 million cells/mL in annexin binding buffer (Life Technologies,
Ref#V13246) containing propidium iodide (PI) at 10ug/mL. Cells were filtered through a cell strainer cap into a flow cytometry tube and incubated at room temperature with Annexin-V, Alexa Fluor®488 conjugate (Life Technologies, A13201) for 15 minutes. Cells underwent flow cytometry using the BD FACS Calibur Flow Cytometry machine that sorts’ cells based on fluorescence signal. The 488nm argon laser excited all cells undergoing early apoptosis targeted by the annexin V conjugate and the emitted light was collected in the FL1 channel in the range of 510 – 545nm. Propidium iodide identified the fluorescence of all dead cells in the FL3 channel at wavelengths of greater than 650nm. BD Cellquest Pro Software was used to gate the FL1 and FL3 channels to separate fluorescent cells from non-fluorescent to determine levels of apoptosis and cell death.

3.7.2 TUNEL Staining

After mechanical insult, cells were fixed in the bioflex plates using 4% PFA for 20 minutes, and stored in 1% PFA. Cells were stained using the DeadEnd Fluorometric TUNEL System (Promega, Ref#G3250) according to the manufacturer’s instructions. Briefly, cells were permeabilized with a 0.1% triton-X solution for 15 minutes followed by washing with PBS. A mix of reagents containing rTdt (recombinant terminal deoxynucleotidyl transferase) enzyme and fluorescent dUTP were incubated on slides at 37°C for 1 hour. This facilitated the incorporation of fluorescent dUTP bases onto 3’OH DNA ends indicative of apoptosis. Following the enzymatic reaction, sections of the silicone bioflex plates were washed and mounted on glass slides, and Vectashield Hardset Mounting Medium with Dapi (Vector Laboratories, Ref#H-1500) was applied before sealing with a coverslip and nail polish. Sections were then imaged and the number of TUNEL positive cells was quantified using cell-counting software. The number of
cells was also counted using the DAPI signal that stains cell nuclei allowing for the identification of the percentage of TUNEL apoptotic cells.

3.8 Zymography

Zymography is a technique used to analyze the level of MMPs present in cell culture media by adding MMP substrate into an SDS-polyacrylamide gel. The respective MMP is then reactivated and digests through the gel, which is stained and imaged.

More thoroughly, an 8% SDS-polyacrylamide gel was prepared as usual, however 0.1g/mL of gelatin was added into the stacking gel as MMP2 and MMP9 substrate. Media combined with sample buffer in non-reducing conditions was loaded into the gel along with an MMP2 standard (Calbiochem, Ref#PF037) and run for 30 minutes at 60V, followed by 100V for 1.5 hours. The gel was incubated in homemade renaturation buffer (2.5% Triton-X) to remove SDS and allow proteins to refold, followed by incubation for 24 hours with a development buffer (50mM Tris, 10mM CaCl$_2$, pH 7.6) at which point MMP2 and MMP9 digested the gel. Staining of the gel with Coomassie Blue solution (0.5% Coomassie blue R250, 30% isopropanol, 10% acetic acid) revealed clear areas of digestion by MMP2 and MMP9. These were imaged and quantified using densitometry with the ImageJ software. For easier interpretation of the levels of MMPs, the images of the zymograms were changed to black and white, and the colours inverted to look similar to a western blot.

3.9 Immunoprecipitation

Protein was collected using RIPA and a small volume of protein (enough for 1 western, approx. 50µL) was set aside as a control. Protein lysates were pre-cleared by incubation with
normal rabbit IgG (Santa Cruz, Ref#SC-2027) and Protein A/G PLUS-Agarose Immunoprecipitation beads (Santa Cruz, Ref#SC-2003) for 30 minutes at 4°C. Normal rabbit IgG was selected to pre-clear lysates based on the type of primary antibody used for the immunoprecipitation, which was PEA15 rabbit polyclonal IgG. After incubation, samples were centrifuged to pellet beads, which were then discarded. Lysates were incubated with the primary PEA15 antibody (Santa Cruz, Ref#sc-28255) and fresh agarose beads overnight at 4°C with gentle agitation. Beads were pelleted and washed with PBS 3 times, then resuspended in sample buffer containing β-mercaptoethanol. Samples were boiled for 10 minutes, beads were pelleted, and the supernatant loaded onto a 15% SDS-polyacrylamide gel and run as a normal western blot. Relative levels of PEA15 and phospho-PEA15 were determined by blotting for PEA15 and phospho-serine respectively.

3.10 Statistical Analysis

All statistics were performed using graphpad’s Prism software, version 5.0c. Statistical tests used were the ANOVA (analysis of variance) and Students T-test (both paired and unpaired) where appropriate. Significance was determined by a P value of less than 0.05, and denoted by asterisks. A P value of 0.01 – 0.05 was denoted by one asterisk (*), a P value between 0.001 and 0.01 was denoted by two asterisks (**) while a p value of < 0.001 was denoted by three asterisks (***)

The number of Ns reported in each experiment represents the number of times each experiment was performed (biological replicates), with each experiment having a minimum of 3 technical replicates.
Chapter 4
Results
4 Results

4.1 Aim 1: Mechanical Insult increases PEA15 protein

4.1.1 Primary Rat Retinal Astrocytes

In order to confirm that levels of PEA15 protein increase after mechanical insult as found previously (Rogers et al. 2012), primary rat retinal astrocytes (PRRA) were stretched for 1, 6, 12, and 24 hours using the Flexercell® Tension Plus FX-4000T system. A western blot of protein lysates revealed that increases in the level of PEA15 occurred after 6 hours and peaked at 12 hours (Figure 11A, C). To further characterize the levels and effect of mechanical insult on PEA15 interactors, blotting for the active forms of ERK1/2 and caspase 8 was performed. Both phospho-ERK1/2 and cleaved caspase 8 levels were observed to peak at 6 hours of mechanical insult (Figure 11A). Therefore mechanical insult induces increases in the level of PEA15, as well as activation of ERK1/2 and caspase 8.

4.1.2 A7 cells

The same experiment was performed in A7 cells (rat ONH astrocyte cell line) where mechanical insult was applied for 1, 6, 12 and 24 hours. Again levels of PEA15 protein were elevated up to 12 hours of mechanical insult (Figure 11B). Similar trends were also found in the activation of phospho-ERK and cleaved caspase 8 (Figure 11B). Phospho-ERK was seen to peak at 6 hours, whereas caspase 8 was elevated for 1 and 6 hours as seen in the PRRAs. Thus A7 cells behave similarly to PRRA when undergoing mechanical insult. Increases in PEA15 protein, and activation of ERK1/2 and caspase 8 was seen during mechanical insult in both cell types making A7 cells suitable for future experiments.
4.1.3 Phosphorylation of PEA15 Increases with Mechanical Insult

Due to a lack of working, commercially available antibodies for the phosphorylated forms of PEA15, the relative levels of PEA15 phosphorylation were determined by immunoprecipitation of total PEA15 followed by western blotting for phospho-serine. Since PEA15 levels were observed to increase at 6 and 12 hours, mechanical insult was induced in A7 cells for 6 hours, and PEA15 phosphorylation was determined. This revealed that mechanical insult induced significant increases in the level of PEA15 phosphorylation over 2 fold compared to control (Figure 12).

4.1.4 PEA15 Expression Increases in Pig ONH with Increased IOP

Pig ONH samples obtained previously were measured to determine levels of PEA15 expression after exposure to mechanical insult by artificially increasing the IOP for 1 hour. Pig eyes are especially suited for in vivo study of glaucoma as they are the most similar animal model of the human eye in both size and function (Guduric-Fuchs et al. 2009). After IOP was elevated for 1 hour the eye was harvested, the ONH was dissected out, mRNA was extracted, and qRT-PCR was performed. This revealed that even after only 1 hour of mechanical insult in vivo, expression of PEA15 was significantly increased 5-fold (Figure 13).
Figure 11. Mechanical insult induces increases in PEA15. Primary rat retinal astrocytes underwent 12% mechanical insult for the times indicated. Western blot analysis revealed increased levels of PEA-15 after 6 hours as well as activation of ERK2 and caspase 8 compared to 24h control (A, n=3). Mechanical insult in the A7 rat ONH astrocyte cell line similarly showed increases of PEA-15, and activation of ERK and caspase 8 (B, n=1). PEA15 was normalized to GAPDH and densitometry determined the fold change of PEA15 (C, n = 3).

Figure 12. Mechanical insult induces phosphorylation of PEA15. A7 cells underwent mechanical insult for 6 hours and protein lysates were collected. Subsequent PEA15 immunoprecipitation and blotting for phospho-serine showed increases in PEA15 phosphorylation (A). Densitometry revealed significant differences between control and mechanically insulted cells (B, n=3, p=0.01).

Figure 13. Raised IOP induces PEA15 expression in vivo. Seven pig eyes with IOP artificially elevated to 60mmHg for 1 hour showed increases in PEA15 expression in their ONH measured by qRT-PCR compared to 10 control eyes (n=17, p=0.006).
4.2 Aim 2: Role of PEA15 in Apoptosis during Mechanical Insult

4.2.1 PEA15 is Protective During Non-Stretch Conditions

To determine if PEA15 plays a role in apoptosis in astrocytes, misexpression of PEA15 was induced in A7 cells. siRNAs were used to bind and knockdown PEA15, while a V5-tagged PEA15 plasmid was transfected into A7’s to overexpress PEA15.

Optimization of PEA15 knockdown and overexpression were performed (Appendix 1 and 2). Efficacy of PEA15 siRNA was confirmed in three different ways; siRNA transfection efficiency was evaluated using siGLO transfection indicator and flow cytometry (Appendix 1A), PEA15 knockdown of mRNA was confirmed by RT-PCR (Appendix 1B), and knockdown of PEA15 protein was confirmed by western blot (Appendix 1C). PEA15 overexpression was confirmed by western blot of epitope tagged PEA15 protein (Appendix 2A) and imaging of transfected fluorescent GFP-PEA15 (Appendix 2B, C).

Forty-eight hours after PEA15 knockdown or overexpression had been induced, the level of apoptosis was measured using FITC-annexin-V labeling, a fluorescent marker of early apoptosis, and flow cytometry. Annexin-V works by binding phosphatidylserine, which is normally located on the cytoplasmic side of the plasma membrane. During apoptosis phosphatidylserine flips to the extracellular surface of the membrane allowing binding by annexin-V. In non-insulted control cells PEA15 was found to be significantly protective. Accordingly, PEA15 plasmid transfections halved levels of death (Figure 14A) whereas PEA15 siRNA knockdown doubled levels of apoptosis (Figure 14B).
**Figure 14.** Flow cytometry reveals PEA15 is protective in resting ONH astrocytes. The level of apoptosis was measured by annexin-V and flow cytometry 48 hours after either V5-tagged PEA15 plasmid transfection (A) or PEA15 siRNA transfection (B). PEA15 was found to be significantly protective in both cases (n=6, p<0.0001). NT = non-targeting siRNA.

**Figure 15.** TUNEL staining reveals PEA15 is protective in resting ONH astrocytes. The level of apoptosis was measured by TUNEL staining 30 hours after either V5-tagged PEA15 plasmid transfection (A, n=12, p=0.048), or PEA15 siRNA transfection (B, n=12). A trend of PEA15 protection was found in both cases. NT = non-targeting siRNA.
These experiments were repeated for evaluation of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining – a marker of late stage apoptosis. During imaging of cells, the signals for TUNEL and nucleus staining (DAPI, 4',6-diamidino-2-phenylindole) were quantified to determine the percentage of cells that were TUNEL positive and therefore apoptotic. Trends of PEA15 protection were observed for both PEA15 overexpression and siRNA knockdown, with a significantly decreased percentage of TUNEL positive cells in PEA15 overexpressing cells compared to control (Figure 15A). The level of apoptosis determined from TUNEL staining is consistent with the level detected by flow cytometry indicating that PEA15 is protective in resting conditions.

4.2.2 Mechanical Insult Uncouples the Anti-apoptotic Function of PEA15

To determine the role of PEA15 in apoptosis induced by mechanical insult, 24 hours after PEA15 misexpression was induced A7 cells underwent 24 hours of mechanical insult and the level of apoptosis was evaluated. This revealed a complete loss of the protective function of PEA15. Levels of apoptosis in cells with PEA15 overexpression or knockdown were virtually identical to cells transfected with a control plasmid or non-targeting (NT) siRNA, as detected by both flow cytometry (Figure 16) and TUNEL staining (Figure 17). Therefore PEA15s protective function appears to be uncoupled by mechanical insult.

4.2.3 Serine 104 is Important to the Anti-Apoptotic Function of PEA15

To determine if changes in PEA15 phosphorylation were responsible for the loss of anti-apoptotic function during mechanical insult, non-phosphorylatable mutant PEA15 plasmids were created. Using standard molecular biology techniques, serine 104 and serine 116 were mutated to non-phosphorylatable glycine residues, as has been done previously by another group (Trencia et
Figure 16. Mechanical insult uncouples the anti-apoptotic function of PEA15 as measured by flow cytometry. Twenty-four hours after transfection with V5-PEA15 plasmid or PEA15 siRNA, A7 cells underwent 24 hours of mechanical insult. The level of apoptosis was measured by annexin-V and flow cytometry with PEA15 overexpression (A, n=6) or PEA15 knockdown (B, n=6). NT = non-targeting siRNA.

Figure 17. TUNEL staining reveals that mechanical insult uncouples the anti-apoptotic function of PEA15. Twenty-four hours after transfection with V5-PEA15 plasmid or PEA15 siRNA, A7 cells underwent 24 hours of mechanical insult. The level of apoptosis was measured by quantification of TUNEL positive cells with PEA15 overexpression (A, n=12) or PEA15 knockdown (B, n=12). NT = non-targeting siRNA.
al. 2003). The PEA15 plasmid with mutated serine 104, serine 116, or both serine sites mutated will be denoted as PEA15-S104G, PEA15-S116G, and PEA15-S104/116G moving forward. Verification of mutations in these plasmids was accomplished by sequencing as well as transfections of these plasmids into the HEK293 cell line followed by immunoprecipitation of PEA15 and blotting for phospho-serine (Figure 18). Since HEK293 cells minimally express PEA15 and are highly transfectable (Doti et al. 2010), the bands within the western blot almost entirely reflect the recombinant protein from the transfected PEA15 mutant plasmids.

To first determine the level of PEA15 protection in resting cells, PEA15 mutant plasmids were transfected into A7 cells and level of apoptosis was determined by annexin-V labeling measured by flow cytometry. In non-mechanically insulted cells, three of the mutant PEA15 plasmids significantly decreased levels of apoptosis compared to the positive control empty vector: the wild type (WT) PEA15 plasmid, PEA15-S104G, and PEA15-S104/116G (Figure 19).

The same experiment was then performed with A7 cells exposed to 24 hours of mechanical insult. The only PEA15 plasmid that continued to confer significant protection after exposure to mechanical insult was PEA15-S104G (Figure 20A). To simplify the interpretation of these results, a chart denoting the level of protection under control and stretch conditions was created (Figure 20B). As can be observed, only PEA15 plasmids with no phosphorylation at position 104 conferred protection during stretch. Therefore no phosphorylation at serine104 appears integral to the anti-apoptotic function of PEA15 during mechanical insult.
**Figure 18. Verification of mutant PEA15 phospho-plasmids.** Transfection of PEA15 mutant plasmids into HEK293 cells was followed by immunoprecipitation and western blotting for phospho-serine. Wildtype (WT) PEA15 shows a lower band of doubly phosphorylated PEA15, PEA15-S104G and PEA15-S116G show a single band of monophosphorylation, while PEA15-S104/116G contains displays no phosphoserine.

**Figure 19. Mutant plasmids confer protection against apoptosis in resting ONH astrocytes.** PEA15 mutant plasmids were transfected into A7 cells and the level of apoptosis was determined after 48 hours by annexin-V fluorescent labelling measured by flow cytometry (n=3, p<0.0001). Negative control (-ve C) = untransfected cells, positive control (+ve C) = transfection with an empty vector.
Figure 20. Only PEA15-S104G mutant plasmid rescues protection after mechanical insult. PEA15 mutant plasmids were transfected into A7 cells and the level of apoptosis was determined after 24 hours of mechanical insult by annexin-V fluorescent labeling measured by flow cytometry (A, n=3, p=0.012). Negative control (-ve C) = untransfected cells, positive control (+ve C) = transfection with an empty vector. A chart documenting protection during control and mechanical insult helps to simplify the observed effect. Plasmids expressing recombinant proteins that are not phosphorylated at serine104 show a greater trend of protection (B).
4.3 Aim 3: PEA15 Regulates ECM Remodeling during Mechanical Insult

4.3.1 Mechanical Insult Induces Increases in MMP2

4.3.1.1 *In vitro*

To resolve the effect of mechanical insult on levels of MMP2, mechanical insult was applied to A7 cells for 1, 6, 12, and 24 hours and the media was collected and analyzed by zymography. This revealed increases in the level of MMP2 at 6 hours of mechanical insult (Figure 21). Thus mechanical insult leads to increases in MMP2 in the culture media.

4.3.1.2 *In vivo* Pig ONH

The pig ONH samples previously used to detect levels of PEA15 were also evaluated for expression of MMP2. Mechanical insult was applied to pig eyes by increasing the IOP to 60mmHg for 1 hour. RT-PCR analysis of the level of MMP2 expression in the pig ONH revealed significant increases of more than 3-fold in the ONHs with elevated IOP (Figure 22). This *in vivo* analysis of mechanical insult agrees with the results *in vitro*: mechanical insult induces increases in MMP2.

4.3.2 PEA15 Decreases Levels of MMP2

To identify the effect of PEA15 on levels of MMP2 in the media, PEA15 knockdown and overexpression was induced in A7 cells. Fresh media was incubated with these cells for 6 hours and analyzed by zymography. The results agreed with previous publications results (Glading et al. 2007) that PEA15 overexpression significantly decreases levels of MMP2, while PEA15 knockdown had the opposite effect by increasing levels of MMP2 (Figure 23). Thus PEA15 suppresses levels of MMP2, which can be viewed as protective in the context of glaucoma.
**Figure 21.** Mechanical insult induces increases in the level of MMP2 peaking at 6 hours. A7 cells underwent mechanical insult for the indicated times. Media was collected and analyzed by zymography (n=1).

**Figure 22.** Mechanical insult induces expression of MMP2 in pig eyes *in vivo*. Seven pig eyes were exposed to artificially elevated IOP for 1 hour and ONH were collected, along with 10 control eyes. qRT-PCR of MMP2 was performed. (n=17, p=0.04).

**Figure 23.** PEA15 suppresses the level of MMP2 in A7 cells. Media was collected 30 hours after A7 cells were transfected with PEA15-V5 plasmid (A, n=3, p=0.031) or PEA15 siRNA (B, n=3). Zymography was performed to determine the level of MMP2.
4.3.3  Mechanical Strain Inverts the Effect of PEA15 on MMP2 Levels

To determine the effect of mechanical insult on PEA15s regulation of MMP2, PEA15 knockdown and overexpression were induced in cells exposed to 6 hours of mechanical insult. Interestingly the opposite result was observed as in resting cells. PEA15 overexpression induced increases in MMP2 expression, whereas PEA15 knockdown had no effect (Figure 24). Therefore mechanical insult uncouples PEA15s suppression of MMP2, changing its function from protective to harmful.

4.3.4  ERK Mediates the Regulation of MMP2 by PEA15

When unphosphorylated at serine 104, PEA15 can bind ERK and sequester it in the cytoplasm to prevent ERK-dependent transcription. However, upon phosphorylation by PKC PEA15 can no longer bind to ERK. Since MMP2 is under transcriptional control of ERK when it translocates into the nucleus to phosphorylate downstream transcription factor targets, it is reasonable to hypothesize that PEA15 regulates the level of MMPs by altering ERK’s activation and cellular localization.

4.3.4.1  Mechanical Insult Induces Increases in Nuclear Phospho-ERK

To test this hypothesis, A7 cells were stretched for 6 hours, protein lysates were collected, and the nuclear and cytoplasmic fractions were separated. A western blot was run to detect nuclear fractions of phospho-ERK during both stretch and control conditions. As predicted, mechanical insult increased the fraction of nuclear phospho-ERK by approximately 2-fold (Figure 25). Therefore it appears that increased phosphorylation of PEA15 during mechanical insult releases ERK1/2 facilitating translocation of ERK to the nucleus.
**Figure 24. PEA15 overexpression combined with mechanical insult changes the way PEA15 regulates levels of MMP2.** A7 cells transfected with PEA15 plasmid (A, n=3 p=0.087) or PEA15 siRNA (B, n=3) underwent 6 hours of mechanical insult. Media was collected and analyzed by zymography for levels of MMP2.

**Figure 25. Mechanical insult increases the level of nuclear ERK.** After 6 hours of mechanical insult, A7 cell protein lysates were separated into nuclear and cytoplasmic fractions. Western blotting of the nuclear lysates was performed (A) and densitometry of these blots revealed a significant increase in the nuclear fraction of phospho-ERK after mechanical insult (B, n=3, p=0.048).

**Figure 26. ERK1/2 mediates the regulation of MMP2 by PEA15.** A7 cells were pretreated with ERK inhibitor (U0126) at the indicated concentration for 1 hour and mechanically insulted for 6 hours. Media was collected for zymography analysis of MMP2, while protein lysates were blotted for ERK activation (A). Densitometry revealed significant decreases in the level of MMP2 at 1µM (n=3, p=0.005) and 10µM of U0126 (B, n=3, p=0.003).
4.3.4.2 ERK Inhibition Blocks Increases in MMPs during Mechanical Insult

To confirm that changes in the level of MMP2 were mediated by PEA15's interaction with ERK, an inhibitor was used that blocks MEK (the kinase that phosphorylates ERK), termed U0126. The inhibitor was applied to A7 cells at concentrations of 0.1µM, 1µM, and 10µM, for 1 hour prior to and during mechanical insult. The cells underwent mechanical insult for 6 hours. Protein lysates and media were collected for western blot and zymography analysis. This revealed that as the concentration of U0126 increased, ERK activation decreased concurrent with decreases in MMP2 levels (Figure 26A). Levels of MMP2 were significantly decreased at 1µM and 10µM of U0126 (Figure 26B). This experiment confirms that PEA15 regulates levels of MMP2 through its interaction with ERK1/2.

4.3.5 Mutant PEA15-S104G Rescues Protective Suppression of MMP2

To further confirm that the interaction of PEA15 with ERK was responsible for the regulation of MMP2, a plasmid with mutated serine104 (the phospho-site required for the PEA15-ERK interaction) was used, termed PEA15-S104G. A7 cells were transfected with wild type PEA15, and PEA15-S104G. Twenty-four hours later, cells were stretched for 6 hours and media was collected. Zymography revealed significant decreases in the level of MMP2 in the PEA15-S104G transfected cells compared to the wild type PEA15 transfected cells (Figure 27). Thus no phosphorylation of serine 104 on PEA15 is essential to suppression of MMP2 as it allows for PEA15 bind and sequester ERK in the cytoplasm. This is evident since mutation of serine 104 rescued this protective function during mechanical insult.
Figure 27. Mutation at serine104 on PEA15 rescues protective function of MMP2 suppression. A7 cells transfected with wild type PEA15 and PEA15-S104G underwent 6 hours of mechanical insult. Media was collected for zymography analysis (A) and densitometry revealed significant decreases in the level of MMP2 present in the PEA15-S104G media (B, n=3, p=0.028).
Chapter 5
Discussion
5 Discussion

Glaucoma is a disease characterized by high levels of IOP exerting mechanical insult on the astrocytes and RGCs of the ONH. This contributes to increases in cell apoptosis and pathological ECM remodeling that ultimately result in RGC death and blindness. This project looked at the protective functions of the molecular adapter, PEA15 in the context of glaucoma for the first time. A protective role for PEA15 was identified in resting ONH astrocytes through a reduction in the levels of apoptosis, and the levels of MMP2 that contribute to pathological ECM remodeling in glaucoma. Glaucomatous mechanical insult was administered to cells at levels previously predicted in vivo and was seen to increase levels of PEA15 protein and phosphorylation. Mechanical insult also had the unique effect of uncoupling the protective functions of PEA15 from both apoptosis and ECM remodeling. In addition, PEA15 overexpression in the presence of mechanical insult was seen to further increase the levels of secreted MMP2. The phospho-site, serine 104 on PEA15 was identified as specifically important to inhibiting both the anti-apoptotic function, and the suppression of MMP2 during mechanical insult. Mutation of serine 104 to non-phosphorylatable glycine rescued the anti-apoptotic function and the suppression of MMP2 levels by PEA15.

5.1 Mechanical Insult Induces PEA15 Expression and Phosphorylation

During mechanical insult, PEA15 expression was observed elevated in vivo in pigs after 1 hour of raised IOP, while protein levels were elevated in vitro after 6 and 12 hours. This is consistent with previous proteomic findings in astrocytes of both rats and humans. Tezel et al. (2012) identified PEA15 elevated 1.8 fold in rats with artificially elevated IOP after 2 weeks,
while in Rogers et al. (2012), our laboratories found levels of PEA15 increased 1.5 fold after 2 hours of mechanical insult in human primary ONH astrocytes using the same stretch system.

Although levels of PEA15 have been previously identified to increase in the context of mechanical insult, this is the first time that PEA15 phosphorylation status has been evaluated during stretch, revealing increased total phosphorylation. Phosphorylation at serine 116 is known to increase stability and prevent ubiquitinylation, and proteasomal degradation of PEA15 (Fiory et al. 2009), likely contributing to elevated levels of the protein. As introduced previously, Akt and PKC phosphorylate the two serine sites on PEA15. Changes in mechanical stress exerted on cells is detected by several surface receptors, which activate a variety of signaling molecules including ERK1/2, PKC, Akt, and small GTPases such as Rho and Rac (Ingber 2003). Therefore it aligns that mechanical insult would induce increases in phosphorylation on PEA15 via the action of PKC and Akt.

Another item to consider is how the transcription of PEA15 is activated. The regulation of PEA15 expression is dependent on two steroid transcription factors; HNF-4α (hepatic nuclear factor 4α) and COUP-TFII (chicken ovalbumin upstream promoter transcription factor II, Ungaro et al. 2008). Repression is achieved by binding of HNF-4α to a hormone response element on the PEA15 promoter while competitive binding by COUP-TFII facilitates activation of PEA15 expression (Ungaro et al. 2008). Previously in two breast cancer cell lines, it was shown that the MAPK pathway including ERK1/2 activation induces expression of COUP-TFII (More et al. 2003). If this also occurs in astrocytes, it is possible that PEA15 is regulated via a feedback loop of ERK1/2 and PEA15. Under normal resting conditions, the relationship between PEA15 and ERK would theoretically act as a negative feedback loop. This is because PEA15 is able to bind and inhibit ERK in the cytoplasm preventing transcription of COUP-TFII (Mace et
al. 2013). However during mechanical insult, this loop would switch to positive feedback as PEA15 becomes phosphorylated at serine104 and is no longer able to bind to ERK. PEA15 can however, still indirectly activate ERK1/2 upstream through Ras (Ramos et al. 2000) ultimately leading to increases in transcription of COUP-TFII and PEA15. This hypothesis would be interesting to examine as it explains transcriptionally how the levels of PEA15 may increase. Functionally this also makes sense since PEA15 is anti-apoptotic and phosphorylation of PEA15 increases its anti-apoptotic activity in the extrinsic apoptotic pathway.

5.2 Mechanical Insult Uncouples the Anti-apoptotic Activity of PEA15

It is well established that PEA15 is able to inhibit apoptosis via the extrinsic apoptotic pathway by binding and blocking signaling between FADD and caspase 8 required to form the DISC (Kitsberg et al. 1999, Condorelli et al. 1999, Condorelli et al. 2002, Song et al. 2006, Renganathan et al. 2005, Renault et al. 2003, Trencia et al. 2003). This was shown in this project when PEA15 exhibited a protective effect in resting cells with PEA15 misexpression. As expected from previous literature, mutation to serine116 on PEA15 abrogated the anti-apoptotic function in control conditions (Kitsberg et al. 1999, Renganathan et al. 2005, Trencia et al. 2003). This is because phosphorylation of serine 116 is required for PEA15s interference in the DISC and confirms PEA15s anti-apoptotic role in the extrinsic apoptotic pathway (Renganathan et al. 2005). Therefore, PEA15 is anti-apoptotic in resting conditions as expected. This is the first time that this anti-apoptotic role of PEA15 has been explored in the context of glaucoma and biomechanical strain. Interestingly, mechanical insult uncoupled the protective function of PEA15 in ONH astrocytes, the reason for which will be explored in the following section.
5.2.1 Phosphorylation of serine104 abrogates the Anti-apoptotic Activity of PEA15

Since PEA15’s anti-apoptotic function in the extrinsic apoptotic pathway is only present when PEA15 is phosphorylated at serine 116 (Renganathan et al. 2005), we originally hypothesized that mechanical insult interfered with phosphorylation at this site. Experiments involving overexpression of mutant phospho-PEA15 plasmids combined with mechanical insult were performed, and levels of apoptosis evaluated. When mechanical insult was applied however, only overexpression of PEA15 with mutated serine104 (PEA15-S104G) rescued the anti-apoptotic function of PEA15. Therefore it appears that phosphorylation of serine116 coupled with no phosphorylation at serine104 confers protection against mechanical insult.

Based on these results we can speculate the mechanisms responsible for protection. Since the PEA15-S104G plasmid displayed protection and can be phosphorylated at serine116, it seems plausible that at least part of this anti-apoptotic activity is facilitated by interference of PEA15 in the extrinsic apoptotic pathway. Consistent with this mechanism is the fact that all plasmids with mutation to serine116 did not display anti-apoptotic activity. Furthermore, since no phosphorylation on serine104 was observed essential to protection from mechanical insult, binding partners of PEA15 in this phosphorylation state can be explored for anti-apoptotic activity. As introduced earlier, phosphorylation at serine104 blocks PEA15’s ability to bind ERK. Thus the PEA15-S104G plasmid is permanently able to bind ERK. ERK can induce cell death through a variety of mechanisms (Cagnol and Chambard 2010) henceforth inhibition of ERK action via PEA15 binding emerges as a potential secondary form of anti-apoptotic action of the PEA15-S104G plasmid during mechanical insult. This is not the first time that singly phosphorylated PEA15 has been reported protective. PEA15 present in human gliomas singly
phosphorylated at serine 116 was also observed to display anti-apoptotic action (Eckert et al. 2008).

Unfortunately little is known about the phosphorylation status of PEA15 required for binding with other proteins that could potentially be involved in preventing apoptosis, and moreover new binding partners of PEA15 are continually being discovered. Identification of new and known interactors that are restricted to binding while PEA15 is unphosphorylated at serine104 could prove useful not only in the context of glaucoma but several other diseases that implicate PEA15 as well. For example, PEA15 is able to bind and improve the stability and half-life of PLD1 (phospholipase D1) and PLD1 is able to suppress the level of DNA-damage induced apoptosis (Zhang et al. 2000, Hui et al. 2004). Currently there are no studies of which I am aware examining the interaction between PLD1 and PEA15 that take into account the phosphorylation status of PEA15, however this knowledge could help to further decipher the protective role of PEA15.

Another puzzling notion to consider is why the levels of PEA15 protein increase while there is a loss of anti-apoptotic function during mechanical insult. The reason why PEA15 was unable to decrease levels of apoptosis may be because mechanical insult is inducing apoptosis through a pathway other than the extrinsic apoptotic pathway. Apoptosis induced via mechanical insult may be mediated by ERK. This could explain why mutation at serine104 on PEA15 prevents apoptosis – because it allows PEA15 to bind and inhibit ERK, thereby blocking induction of apoptosis. Since mechanical insult induces phosphorylation of PEA15 and PEA15 can only interfere with the extrinsic apoptotic pathway while phosphorylated, it would be unable to prevent apoptosis induced through other mechanisms such as ERK1/2.
Future *in vivo* studies are underway that will confirm the protective function of PEA15 mutated at serine104 in a rat model of raised IOP, mimicking the mechanical damage induced in glaucoma. Viral transfection of PEA15-S104G and PEA15 siRNAs into the ONH of the eye will hopefully confirm the protective role of PEA15 with mutation to serine104. Identification of binding partners of PEA15 *in vitro* and in this *in vivo* model will help to elucidate the anti-apoptotic mechanisms at work.

### 5.3 PEA15 Regulates Levels of MMPs

Elevated secretions of MMPs are present in ONH of humans (Yan et al. 2000), monkeys (Agapova et al. 2003), and rodent (Guo et al. 2005) models of glaucoma. This pathological secretion of MMPs contributes to the cupping of the ONH that leads to damage of the RGC axons. Previously it has been shown that biomechanical insult as a result of elevated IOP can induce the secretion of MMPs (Kirwan et al. 2004), however the mechanism through which this occurs has remained elusive. Here we present a novel pathway in which the interaction between PEA15 and ERK normally suppress levels of MMP2. However, when glaucomatous biomechanical insult is introduced, this interaction is uncoupled leading to increases in MMP2 levels, *in vivo* and *in vitro*. I have shown that under resting conditions, PEA15 has low levels of phosphorylation and is able to bind ERK to decrease the expression of MMP2, which is under downstream nuclear transcriptional regulation of ERK (Pan and Hung 2002). During mechanical insult, increased phosphorylation of PEA15 prevents its interaction with ERK, allowing ERK to translocate into the nucleus, and activate transcription factors that initiate the transcription of MMP2, ultimately leading to increases in the media. This was confirmed when increased levels of nuclear ERK were detected and the inhibition of ERK suppressed MMP2 levels in the presence of biomechanical insult. Furthermore, transfection of the PEA15-S104G plasmid that
blocks the PEA15-ERK interaction, also suppressed levels of MMP2 during mechanical insult confirming that ERK mediates PEA15s regulation of MMP2.

It was interesting to note that PEA15 overexpression during mechanical insult lead to significant increases in the levels of MMP2 compared to a control plasmid. This can appear unaccountable since PEA15 and ERK are unable to interact during mechanical insult, due to the increased phosphorylation of PEA15. However, PEA15 is able to indirectly activate ERK by increasing the GTP loading of Ras in the ERK cascade (Ramos et al. 2000). This would ultimately lead to increases in MMP2 transcription and levels seen in the media.

These overall findings agree with several studies that have explored the role of PEA15 in cancer tumour cell invasion in cell lines. The expression of MMP-7 was observed decreased in human ovarian cancer cell lines overexpressing PEA15 (Lee et al. 2012). Furthermore PEA15 transfection was also seen to decrease levels of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 in several breast cancer cell lines (Glading et al. 2007). This agrees with the decreased levels of MMP2 observed in A7 cells shown in this study. Although PEA15 suppresses the level of MMPs present in resting cells, it would be interesting to identify if this trend is the same in vivo in tumors since mechanical strains are present. Breast cancer tumours were found to experience up to 13 times the mechanical load compared to normal breast tissue (Samani et al. 2007). Therefore, we would expect PEA15 to become phosphorylated and contribute to increasing the level of MMPs present, as found during this project. Coincidentally ovarian cancers display drastically increased levels of MMP2 (Schmalfeldt et al. 2001), and PEA15 (Lee et al. 2012) that correlate with the progression of the disease.
5.3.1 Parallels in Other Diseases

MMPs are implicated in the pathology of arthritis, cancer metastasis, and Alzheimer’s disease (Malemud 2006, Chakraborti et al. 2003). The ability of PEA15 to regulate levels of MMPs by altering the translocation of ERK, could give it considerable significance in other pathologies. ERK phosphorylates the transcription factors AP-1, Elk-1, Sp-1, and Sp-3 which in turn play roles in activating transcription of MMP1, MMP2, MMP9 and MMP13 (Malemud 2006, Chakraborti et al. 2003, Vincenti and Brinckerhoff 2002, Szalad et al. 2009). MMP1 and MMP13 are two collagenases seen increased in arthritis that are known to play major roles in the degradation of cartilage (Vincenti and Brinckerhoff 2002, Szalad et al. 2009). MMP2 and MMP9 have been implicated in many types of cancers as they degrade the basement membrane allowing tumour migration to blood vessels (Malemud 2006). In addition, Alzheimer’s patients have displayed increased levels of MMP2 and MMP9 in senile plaques (Mizoguchi et al. 2011).

Increased levels of mechanical strain and PEA15 upregulation also mark arthritis (Radin et al. 1984, Giusti et al. 2006, Hopwood et al. 2009), cancer (Eckert et al. 2008, Dong et al. 2001, Bartholomeusz et al. 2008), and Alzheimer’s disease (Thomason et al. 2013). High levels of strain on joints characterize arthritis. The role of biomechanical insult in cancer has only recently become prominent with strain revealed to be important for cancer progression (Menon and Beningo 2011). Likewise, characterization of the rigidity of amyloid plaques present in Alzheimer’s disease have revealed extreme stiffness of the fibrils in the plaques leading to the distortion of cell membranes and exertion of mechanical insult on the cells around them (Fitzpatrick et al. 2013).

Although studied here in the context of glaucoma, this trio of increased levels of mechanical insult, MMPs, and PEA15 emerges as a common altered pathway in several diseases.
It appears that mechanical insult induces elevated levels of phosphorylated PEA15, which in turn upregulate levels of MMPs through increased levels of ERK activation. This could be a common pathway between glaucoma, arthritis, cancer, and Alzheimer’s. The findings in this project may be useful to elucidate some of the underlying pathological mechanisms caused by mechanical insult induced deregulation of PEA15, and the downstream consequences.

5.4 Conclusions

This project identified a novel protective role for PEA15 in suppressing the level of MMP2 in ONH astrocytes. It also explored, for the first time, the effect of mechanical insult on the protective functions of PEA15 in apoptosis and ECM remodeling. Interestingly, mechanical insult was observed to uncouple the protective functions of PEA15, which lead to increases in ONH astrocyte apoptosis and the expression of MMP2 that contributes to pathological ECM remodeling in glaucoma.

Phosphorylation of serine104 during mechanical insult was observed to be responsible for the loss of anti-apoptotic activity of PEA15, since mutation of this residue rescued protective function. This may be because phosphorylation of serine104 blocks the interaction between ERK and PEA15. Increased phosphorylation of PEA15 during mechanical insult releases ERK, which could allow for the induction of apoptosis.

This increased phosphorylation of PEA15 and release of ERK was also responsible for PEA15 mediating increases in MMP2. During mechanical insult, ERK was able to translocate into the nucleus and activate targets responsible for transcription of MMP2. This was confirmed when an ERK inhibitor and a mutant plasmid blocking the PEA15-ERK interaction restored suppressed levels of MMP2.
The overall findings of this project show that under normal conditions, PEA15 is protective to two different pathogenic processes found during glaucoma; apoptosis and ECM remodeling. However, glaucomatous mechanical insult applied at previously modeled levels was shown to uncouple the protective functions of PEA15, and change its function to contribute to pathological ECM remodeling by increasing levels of MMP2 in the ONH astrocytes. A schematic of this proposed pathological process can be seen in Figure 28.
Figure 28. Schematic of mechanical insult uncoupling the protective functions of PEA15 on apoptosis and ECM remodeling. In untreated cells, serine104 on PEA15 is not phosphorylated allowing PEA15 to bind ERK and prevent downstream transcription of MMP2, and inhibition of the extrinsic apoptotic pathway. Upon exposure to mechanical insult, both of these protective functions are inhibited. Serine 104 becomes phosphorylated preventing PEA15 from inhibiting ERK transcriptional activation of MMP2, and apoptosis is induced.
6 References


Appendix 1. Verification of PEA15 siRNA knockdown. (A) Transfection efficiencies were determined by transfection of cells with siGLO Indicator alone as a positive control (denoted by siGLO) and PEA15 siRNA with siGLO indicator (denoted by siRNA). (B) Levels of PEA15 24 hours after a non-targeting (NT) siRNA and PEA15 siRNA were determined. (C) Levels of PEA15 protein were determined 24 hours after siRNA knockdown.

Appendix 2. Verification of PEA15 overexpression. (A) Levels of V5-tagged PEA15 plasmid were measured 24 hours after transfection using a V5 antibody. (B) Image of HEK293 cells and (C) A7 cells 24 hours after transfection of GFP-tagged PEA15 plasmid. Localization of PEA15 to the cytoplasm can be observed as identified by arrows.