Biomarkers of Optic Nerve Head Glial Cell Activation Following Biomechanical Insult

Ronan Rogers

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Institute of Medical Science
University of Toronto
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Abstract

Glaucoma is a leading cause of irreversible blindness worldwide. Primary Open Angle Glaucoma is the most common form of the disease and can be characterized by the slow and irreversible apoptotic death of retinal ganglion cells, a unique optic nerve neuropathy resulting in loss of vision. Increased intra-ocular pressure is known to be a leading risk-factor for glaucoma, and lowering IOP is currently the only evidence based method for the clinical management of the disease. However the exact mechanism by which an elevated IOP leads to the death of the retinal ganglion cells is still poorly understood.

By using previous finite element models of glaucoma to quantify the biomechanical environment within the optic nerve head we have built human primary cell culture models in an attempt to replicate aspects of early glaucomatous optic neuropathy. In these models we mimic the in vivo biomechanical environment in the lamina cribrosa by growing human optic nerve head astrocytes.
and lamina cribrosa cells on compliant substrates and subjecting the cells to deformation. Specifically, a global protein scan using isobaric tags for relative and absolute quantitation (iTRAQ) was performed on all the experiments to identify potential biomarkers for glaucoma. A secondary analysis using enzyme-linked immunosorbent assay (ELISA) identified extracellular proteins of interest. Over 520 proteins were identified in response to biomechanical strain from both cell types. Many of these proteins centred on TGF-β, p53 and TNF, which have previously been shown to play a role in the pathogenesis of glaucoma. Proteins found in astrocytes were astrocytic phosphoprotein (PEA15), UDP-glucose dehydrogenase (UGDH), and annexin A4 (ANXA4). LC proteins were bcl-2-associated atnanogene 5 (BAG5), nucleolar protein 66 (NO66) and Eukaryotic translation initiation factor 5A (eIF-5A).

These proteomic results will enable a series of functional studies looking into the role select markers play in ONH glial cell activation, a process still not well understood. Candidates for this work will be prioritized based on novelty and relevance to mechanisms of cellular stress and death. We hypothesize that study of these molecular pathways will provide insight into this process, as well as improve our understanding of how glial activation contributes to the development of glaucomatous optic neuropathy.
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For my family: Mum and Dad, Eam, Mel and Caggs, you all know exactly what these years have entailed and I cannot express how much love and respect I have for everything you have done for me. You are all amazing.

A special thanks to the one who brought my heart back from the brink…I now have everything I need.

Sincerely,
Ronan Rogers
# Table of Contents

Acknowledgments........................................................................................................ iv

Table of Contents........................................................................................................... v

List of Tables ................................................................................................................ vii

List of Figures ................................................................................................................ viii

Abbreviations ................................................................................................................ x

1 Introduction .................................................................................................................. 1

1.1 Glaucomatous Optic Neuropathy .......................................................................... 1

1.2 Finite Element Modelling ..................................................................................... 7

1.3 Glaucoma and Proteomics .................................................................................... 12

1.4 References ............................................................................................................. 26

2 Culture and Stretch of Human Optic Nerve Head Lamina Cribrosa Cells and Astrocytes .... 39

2.1 Abbreviations Page .............................................................................................. 40

2.2 Introduction .......................................................................................................... 41

2.3 Results .................................................................................................................. 44

2.4 Discussion ............................................................................................................ 48

2.5 Acknowledgements .............................................................................................. 49

2.6 References ............................................................................................................ 50

3 Proteomics Analyses of Human Optic Nerve Head Astrocytes Following Biomechanical Strain .............................................................................................................. 52

3.1 Abbreviations Page .............................................................................................. 53

3.2 Summary .............................................................................................................. 54

3.3 Introduction .......................................................................................................... 55

3.4 Experimental Procedures ..................................................................................... 58

3.5 Results .................................................................................................................. 64

3.6 Discussion ............................................................................................................ 80
3.7 Acknowledgements........................................................................................................... 85
3.8 References.......................................................................................................................... 86

4 Proteomics Analyses of Activated Human Optic Nerve Head Lamina Cribrosa Cells
Following Biomechanical Strain................................................................................................. 95
4.1 Abstract.................................................................................................................................. 96
4.2 Introduction............................................................................................................................ 97
4.3 Methods................................................................................................................................ 101
4.4 Results.................................................................................................................................. 108
4.5 Discussion............................................................................................................................. 117
4.6 Acknowledgements.............................................................................................................. 121
4.7 References............................................................................................................................. 122

5 Extracellular Protein Analysis From Human Optic Nerve Head Glia Following
Biomechanical Insult Reveals Increased Matrix Remodelling and bFGF Signalling............ 129
5.1 Abstract................................................................................................................................ 130
5.2 Introduction............................................................................................................................ 131
5.3 Methods................................................................................................................................ 132
5.4 Results.................................................................................................................................. 135
5.5 Discussion............................................................................................................................. 137
5.6 Conclusion............................................................................................................................... 142
5.7 Acknowledgements.............................................................................................................. 143
5.8 References............................................................................................................................. 144

6 Conclusion................................................................................................................................. 149
6.1 References............................................................................................................................. 155
List of Tables

Top Proteins Differentially Expressed in 3% 2 hour, Astrocytes ................................................. 66
Top Proteins Differentially Expressed in 3% 24 hour, Astrocytes ............................................. 67
Top Proteins Differentially Expressed in 12% 2 hour, Astrocytes.................................................. 69
Top Proteins Differentially Expressed in 12% 24 hour, Astrocytes ............................................. 70
Top Proteins of Interest Confirmed Using Gene Ontology Analysis, Astrocytes ......................... 72
Proteins Found Without Meeting Gene Ontology Criteria, Astrocytes ........................................ 73
Top Proteins Differentially Expressed in the 12% for 2 Hour Stretch, LC Cells......................... 108
Top proteins of Interest Found (confirmed and unconfirmed) Using GO analysis, LC Cells.... 110
Protein Levels discovered of seven target proteins, LC Cells ..................................................... 135
List of Figures

Finite Element Models Depicting Levels of Strain Within Tissue of the Optic Nerve Head...... 9
Model of the Lamina Cribrosa........................................................................................................... 42
Magnitude of Computed Distributions of Strains on the Optic Nerve Head......................... 42
Explantation Protocol.......................................................................................................................... 43
Fluorescence Microscope Images of Cells From Human Optic Nerve Heads .................... 46
Image of Dots on the Silastic Membrane......................................................................................... 47
Diagram of Percentage Change ........................................................................................................ 48
Characterization Panel of Astrocytes............................................................................................... 59
ROC Plot Analysis of Astrocytes .................................................................................................... 65
Trends in Major Protein Pathways of Astrocyte Stretch ............................................................. 74
Ingenuity pathways Analysis of Astrocytes - 3% for 2 Hours..................................................... 75
Ingenuity pathways Analysis of Astrocytes - 3% for 24 Hours.................................................... 76
Ingenuity pathways Analysis of Astrocytes - 12% for 2 Hours................................................... 77
Ingenuity pathways Analysis of Astrocytes - 12% for 24 Hours................................................... 78
Validation of iTRAQ Proteomic Results by Western Blot............................................................. 79
Validation of iTRAQ Proteomics by GFAP Staining........................................................................ 80
Characterization Panel of Lamina Cribrosa Cells........................................................................ 99
Workflow of iTRAQ Proteomics Analysis ...................................................................................... 103
Trends in Major Protein Pathways of Lamina Cribrosa Stretch................................................... 114
Ingenuity pathways Analysis of Lamina Cribrosa Cells - 12% for 2 Hours .................................... 116

Bar chart of normalized protein levels of ELISA Astrocyte Cell Culture Media ...................... 136

Bar chart of normalized protein levels of ELISA Lamina Cribrosa Cell Culture Media ........... 137

Ingenuity pathways Analysis of Lamina Cribrosa Cells Highlighted - 12% for 2 Hours .......... 141

Ingenuity pathways Analysis of Astrocytes Highlighted - 12% for 2 Hours ............................. 142
Abbreviations

\( \alpha \text{-smA} \) – Alpha Smooth Muscle Actin

AGM – Astrocyte Growth Media

CASP3 – Caspase 3

DPBS – Dulbecco’s Phosphate Buffered Saline

DMEM – Dulbecco’s Modified Eagles Serum

FBS – Fetal Bovine Serum

GO – Gene Otology

GFAP – Glial Fibrillary Acidic Protein

IPA – Ingenuity Pathways Analysis

iTRAQ – isobaric Tag for Relative and Absolute Quantitation

LC – Lamina Cribrosa

MS – Mass Spectrometry

MMTS – Methyl MethaneThioSulphate

m/z – Mass to Charge Ratio

NCAM – Neural Cell Adhesion Molecule

OCBN – Ontario Cancer Biomarker Network

ONH – Optic Nerve Head

Pax-2 – Paired Box Gene 2

POAG – Primary Open Angle Glaucoma
PCR – Polymerase Chain Reaction

RIPA – Radioimmunoassayprecipitation Buffer

SCX – Strong Cation Exchange

TGFβ1 – Transforming Growth Factor Beta 1

TNF – Tumour Necrosis Factor

TP53 – Tumor Protein 53
1 Introduction

1.1 Glaucomatous Optic Neuropathy

Glaucoma is currently the leading cause of irreversible blindness worldwide. Primary Open Angle Glaucoma (POAG) is the most common form of the disease and can be characterized by the slow and irreversible apoptotic death of retinal ganglion cells, a unique optic nerve neuropathy resulting in loss of vision. Increased intra-ocular pressure (IOP) is known to be a leading risk-factor for glaucoma, and lowering IOP is currently the only evidence based method for the clinical management of the disease. However the exact mechanism by which an elevated IOP leads to the death of the retinal ganglion cells is still poorly understood.

There are two main theories involved in the pathogenesis of glaucoma. The biomechanical theory hypothesizes that elevated IOP deforms (stretches) the optic nerve head tissues, and in particular, the lamina cribrosa, leading to several effects. First, it causes misalignment of pores between lamellae, which in turn causes kinking and distortion of nerve fibre bundles as they traverse the LC. Second, there is evidence for both retrograde and antegrade blockage of axonal transport. Third, LC deformation may alter local cellular function. This hypothesis is consistent with histologic and other studies showing that elevated IOP causes retrograde bowing of the LC, can disrupt the organization of the LC, and that there is decreased connective tissue density in the superior and inferior poles of the ONH. The latter observation correlates with clinical findings that the arcuate nerve fibre bundles that enter the ONH at the superior and inferior poles are usually first affected in glaucoma. In this theory, differences in the mechanical characteristics of ONH tissues are hypothesized to account for differences in susceptibility to IOP-induced damage. In other words, individuals with “weak” ONHs are at increased risk of optic neuropathy due to elevated IOP.
The second theory of glaucomatous damage is the vasogenic theory, which proposes that glaucomatous optic neuropathy is due to insufficient vascular perfusion at the level of the LC, resulting in ischemic injury. Inadequate autoregulatory function in the branches of the short posterior ciliary arteries supplying the laminar region, complications in the hemodynamics between the peripapillary choroidal flow and the anterior optic nerve flow, or other hematologic factors could then account for differences in susceptibility to IOP-induced damage.

Nickells et al.\textsuperscript{11} proposed a 5-stage model that attempts to elucidate the some of these important events that lead to glaucoma. In stage one it is believed that an elevation of IOP leads to the activation of optic nerve glia in the lamina cribrosa. This takes into account the ‘mechanical damage model’ depicted earlier, and goes on to explain how for decades glia have been thought of nothing more than ‘brain glue’, but more and more evidence is pointing to their essential role in neuronal homeostasis and disease.\textsuperscript{29} Under normal conditions these cells provide a variety of functional support including regulation of extracellular K\textsuperscript{+} levels, removal of glutamate, ammonium detoxification, regulation of extracellular pH levels and osmolality, and energy support to neurons.\textsuperscript{29} When these glia are damaged however, they dramatically alter their expression profile, \textit{i.e.} they become ‘activated’.

In order to fully understand these current theories, it is important to have knowledge of the major glial cell types in the optic nerve head (ONH) and associated with the retinal ganglion cell axons. Within the nerve head is a small section of mesh-like tissue called the lamina cribrosa (LC) through which the ganglion cell axons exit the eye. It this region that is thought to be affected by changes in pressure gradients between the intra-ocular pressure and the cerebrospinal fluid pressure. Knowledge of their cellular functions and how the various models of glaucoma may affect them, leading to retinal ganglion cells death is vital to understanding this disease. The first
cell type, astrocytes, form supportive tubes around each axon leading from the retina through the optic nerve head to the laterate geniculate nucleus. They also surround the cribrosal beams of the lamina cribrosa. Lamina cribrosa cells are found only in the lamina and are believed to have a structural role, supporting both the cribrosal and laminar beams, with actin-rich areas ideal to resisting biomechanical strains in both the normal and abnormal ONH environment.

One of the earliest signs that astrocytes have become activated is the increased expression of glial fibrillary acidic protein (GFAP). The interaction that occurs between the activated glial cells and neurons is still not very well understood, but a number of theories have been postulated. One belief is that the microglia of the optic nerve head become pathogenic directly to these cells through the release of nitric oxide, which is compelling but has yet to be verified.

Another theory suggests that activated astrocytes may induce mini-strokes in the optic nerve head by stimulating vasoconstriction of regional small capillaries. Axons may then be starved of energy sources through the effects of ischemia, which takes into account the vasoconstrictive model, also previously mentioned. The normal activity of astrocytes is to provide fuel to the axons through the breakdown of glycogen, and their stress-induced activation may alter or impede their role as a fuel supplier either mimicking or exacerbating the energy demands caused by local ischemia.

The second stage of glaucoma is the damage to the axon and the process of degeneration. Even though it is unclear what stimulates these axons to degenerate, it is known that once it has been damaged, it will execute a self-destruction program that will disassemble its cytoskeleton and other organelles. This includes the breakdown of microfilaments and microtubules and the swelling of the mitochondria.
The third stage of glaucoma is the signalling of ganglion cell death apoptosis. Apoptosis is defined as the molecular process by which cells kill themselves. Retinal ganglion cells and neurons execute a complex series of molecular events that lead to the activation of a cascade of proteases (caspases and calpains) that will digest the internal contents of the dying cell. This process is very efficient at reducing the amount of extracellular debris that may incite an inflammatory response. There is evidence that retinal ganglion cells and neurons go through both the intrinsic and extrinsic apoptotic pathway. It is not exactly clear what activates this particular pathway in these cells although it is believed that it may be caused by neurotrophin deprivation.

Neurotrophins are a group of growth factors that act on neurons. These factors are acquired from a group of neurons in the lateral geniculate nucleus and the superior colliculus in the brain, and specific molecules such as brain-derived neurotrophic factor (BDNF), glial-cell derived neurotrophic factor (GDNF) and nerve growth factor beta (NGF-β) are known to act upon retinal ganglion cells. A lack of these neurotrophins is believed to cause developmental programmed cell death of retinal ganglion cells that are not properly innervated to their target neurons. It has been proposed that glaucoma could be caused by a similar mechanism.

The fourth stage of glaucoma is proposed to be a secondary degeneration of retinal ganglion cells popularized by a theory of increased levels of glutamate in the vitreous. Glutamate at high doses can damage neurons for it can stimulate NMDA receptors, leading to an influx of toxic extracellular Ca++. Ganglion cells contain a high concentration of NMDA receptors, which has led to the suggestion that a second phase of ganglion cell death is occurring after a partial lesion of the optic nerve. However research concerning the elevated levels of glutamate in glaucomatous primate models has been called into question by Kwon et al. The original author
of the glutamate study has since been found guilty of scientific misconduct for fabricating evidence.\textsuperscript{50} This has been further reinforced after the trial of memantine, an open channel blocker of the NMDA receptor, failed to show any significant neuroprotective qualities.

The final proposed stage of glaucoma, glial activation in response to neurodegeneration, is still not well understood as the lack of appropriate temporal evaluation of this occurrence has been one of the more difficult issues to address. The difference between stage one and stage five of this model is that in the final stage, the cells are responding to the degeneration of RGCs as opposed to the initial stage, which is in response to high IOP and ischemic disruption. The response to the degeneration appears to be quite complex and is directed by a variety of factors. Muller cells of the retina respond by secreting neurotrophic factors, possibly in an effort to protect the local environment from damaged ganglion cells.\textsuperscript{51, 52} Within the optic nerve, astrocytes may initiate similar protective mechanisms such as sequestration of $K^+$ and $Ca^+$ ions and release lactate and glutamate to axons as neuronal energy is depleted. Both the retina and optic nerve most likely react to the activation of the astrocytes all through the various stages of this disease.

The five stages of glaucoma as laid out by Nickells et al.\textsuperscript{11} discussed the progression of damage from the optic nerve head to the death of retinal ganglion cells. A recent study by Dai et al.\textsuperscript{53} furthers the research behind the primary phase of insult in glaucoma. It is known that an increase in intraocular pressure damages retinal ganglion cells as they pass through the optic nerve head. As has been shown previously by Sigal et al.\textsuperscript{54-56} the connective tissues of the optic nerve head is highly complex and the method by which these interact is only now being fully realized. Using a rat model, which has no lamina cribrosa, Dai et al.\textsuperscript{53} show how the astrocytes of the optic nerve head are ‘fortified’ by dense cytoskeletal fibres which they state would make them ideal
transducers of distorting mechanical forces. Previous studies have shown that these murine models are still susceptible to glaucoma,\textsuperscript{57, 58} hence by demonstrating the presence of these particular astrocytes, and their cytoskeletal structure and arrangement, it is proposed that they could act as mechanical transducers. They propose two new mechanisms for the pathogenesis of glaucoma, first, that the specialized astrocytes of the ONH are the elements that are damaged by the mechanical stresses of raised IOP, and second, that the damage to the axon is metabolic in nature, caused by the loss of the astrocytic processes important to the support of energy metabolism of the axon. The ‘fortified’ astrocytes are depicted as having stout end feet anchored around the midventral “hilar” surface facing the ophthalmic surface.\textsuperscript{53} In the images provided, it can be seen that the astrocytic processes radiate out from these ventral attachments, separated by longitudinal channels of unmyelinated RGC axons. These processes then continue to the dorsal circumferential cap where they branch repeatedly, eventually converging in the axon free preterminal layer, eventually terminating in a fine mesh at the dorsal surface\textsuperscript{53}. The authors put forth that the astrocytes of the ONH are a unique glial cell type with specific characteristics, including orientation, a fibrous nature, a low proportion of orthogonal membrane particles in the subpial feet and enclosing wide perivascular spaces where abluminal surfaces of the apposed endothelial cells are furnished with abundant pinocytotic pits.\textsuperscript{53}

A significant factor that may influence the susceptibility of the non-myelinated axons to pressure-related damage may also have to do with the association of these ONH astrocytes as a barrier to the rostral migration of oligodendrocyte precursors.\textsuperscript{59, 60} As these cells run orthogonally across the ONH, they are more susceptible to an increase in pressure as opposed to the axons of the RGCs, which run longitudinally and are quite flexible. As this was a study in rat ONH architecture, efforts to correlate this work to that of human glaucomatous optic neuropathy was a primary concern for the authors. The collagen and fibroblasts of the perivascular spaces of the
ONH were greatly expanded to form the connective tissue of the lamina cribrosa. It has been generally assumed that this tissue structure acts as a transducer of increased pressure, causing axonal loss.\textsuperscript{61, 62} Since the presence of IOP induced damage is seen in rat and mouse models without this extracellular matrix in the perivascular spaces of the ONH indicates that the effects of pressure transduction is still being exerted. Their model puts forth that there are unique astrocytes of the optic nerve head arranged in a radial array that surround the axons of RGCs. It is these astrocytes, which have been depicted previously\textsuperscript{63} that may be causing the death of RGC axons through an indirect metabolic-based attack, as opposed to the direct approach of damage to the axons as previously thought.

It is clear, however, that the theories surrounding mechanical, vasogenic and metabolic insult are not mutually exclusive, and in fact, are likely to be synergistic. Throughout this introduction, greater analysis will be placed on the cellular models that have led to the development of these theories, the method of activation for these major glial cells of the optic nerve head, and the proteomics research that has led to our current understanding of this disease.

1.2 Finite Element Modelling

A tool that has helped advance the development of the profiles for this disease is finite element modeling. These models are a mathematical method for solving complex physical problems on domains with complicated geometries. The eye being one such domain, computers are proving to be a viable alternative to complicated, practically unfeasible or unethical experiments. When specifically looking at glaucoma, the increase in IOP results in stresses being applied to the all the various tissues within the globe, including the trabecular meshwork and the optic nerve head. Research by Sigal et al.\textsuperscript{64} used finite element modeling to quantify the various stresses and strains caused by an increase in IOP. Specifically they researched the effects of peripapillary
sclera and prelaminar and postlaminar neural tissue on the overall biomechanical environment within the ONH. Three models were constructed in order of increasing realism and complexity. They discovered a number of factors that affected the results, and came to draw four main conclusions from this research.

First, that deformation of the vitreoretinal interface does not necessarily correspond to the deformation of the anterior surface of the LC. Second, they note that biologically significant levels of strain are produced at the ONH when the IOP increases from normal to high, even when the microarchitecture of the lamina is not taken into account (figure 1) Thirdly, they discovered that strain across the laminar region is more affected by compliance of the sclera, less affected by compliance of the lamina, and least affected by neural and pia mater compliance. Essentially, that the scleral rigidity had the greatest effect on laminar deformation under high IOP. Finally they note that the addition of a simplified central retinal vasculature to the model does not have any major effect on biomechanics of the region. The authors note that, as the results of this study are obtained from simplified models, there are certain aspects of ONH biomechanics that are impossible to capture. These initial models were developed in an attempt to identify the fundamental elements involved in ONH biomechanics and not to detail individual-specific aspects.
Through the development of these models, various assumptions and simplifications are taken into account when analyzing any outcomes. As shown previously,\textsuperscript{55} property values for ONH tissues must be assigned, such as stiffness and compression, however data reported for these tissue properties have demonstrated a large range. In a follow up study, Sigal et al.,\textsuperscript{55} systemically analyzed through the use of finite element modeling, which anatomical and biomechanical factors more influenced the biomechanical response of the ONH to changes in IOP. The goal of this research was to help guide future modeling and experimental studies by understanding how sensitive model predictions are to these assumptions and simplifications. Ideally, they would be able to determine which input factors have the largest influence on ONH biomechanics, eventually providing insights into why some individuals tolerate elevated IOP better than others. The main conclusion of the work is that stiffness of the sclera is the most influential input factor. This result was found to be very robust across a wide spectrum out outcome measure for different ranges of input factors. A number of other factors were also shown to be very influential input factors, including IOP, eye globe radius, LC stiffness, scleral

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Finite element models depicting levels of strain within tissue of the optic nerve head at an IOP of 55mmHg. (reprinted with permission from Sigal et al., 2004)}
\end{figure}
shell thickness, and scleral canal radius. The authors summarize how the complexity of ONH anatomy and material properties, along with the inter-individual variety, even over that individuals own lifetime, makes it a difficult region to understand biomechanically. However as they report, relatively few factors account for most of the biomechanical effects, which has greatly simplified the problem of understanding this regions biomechanics.

Following up these finite element models, Sigal et al.\textsuperscript{65} described a method for the construction of patient specific models that could be used to evaluate the biomechanical environment of the optic nerve head. The reason they followed with this line of research was due to the mechanical damage theory of glaucoma, which hypothesizes that retinal ganglion cell damage is a result of increased IOP-induced strain in the lamina cribrosa, and differences in anatomy and composition of the ONH between individuals leads to different susceptibilities. Through the use of histomorphometric data from donated eyes, the goal was to develop a foundation for the etiology of glaucoma and the different sources for susceptibility. They discovered that through reconstruction, they could successfully model patient specific optic nerve heads, which were then validated against a virtual eye, which represented a comparison point. Deformation and strain between the reconstructed model and the virtual eye allowed the authors to state that their technique is viable and can be used to accurately compute human optic nerve head biomechanics. In a follow up study,\textsuperscript{56} this group again constructed computational models that would focus not only on the magnitude of IOP-deformation of the ONH, but specifically on the mode of deformation, be it stretch, compression or shear. When they increased the IOP, the tissues of the ONH were subjected to an extremely complex strain environment, characterized by all three modes of strain having varying effects on the different tissues types. They found that the highest levels of strain were found in the neural tissue region, and the largest strain magnitudes were found to be compression, then shear, then extension. A two part paper was subsequently
published\textsuperscript{66, 67} which summarized all of the research up to this point, indicating the usefulness and applicability of finite element modeling within glaucoma.

A recent review\textsuperscript{2} examined the current literature in an attempt to understand the biomechanical basis for glaucomatous cupping,\textsuperscript{68, 69} in particular looking more in depth into the biomechanics driving connective tissue remodelling from a normal to diseased state. Taking into account all the previously highlighted finite element models, they focused on the laminar extracellular matrix and feedback mechanisms of glaucomatous changes within this region on an anatomic, structural, cellular, and sub-cellular level. The premise for this review stems from the basis that glaucomatous optic neuropathy, through the ONH’s susceptibility to IOP insult is a function of both acute and long-term response of the constituent tissues.\textsuperscript{70} All of the modeling done by these groups\textsuperscript{5, 55, 56, 64-67, 70-74} have hypothesized that IOP-related deformations cause the anterior laminar beams to either acutely yield or fail, which transfers the damage to adjacent beams, eventually causing the cupping. This is thought to be the result of chronic injury, as experiments in monkeys have shown that there was no posterior cupping in response to an acute rise in IOP\textsuperscript{75} This matches what is seen in modeling studies, which demonstrate that even if the lamina is not displaced anteriorly, there is still an appreciable level of strain.\textsuperscript{54, 76, 77} Similarly, when looking at laminar connective tissue remodelling, evidence was put forward to suggest that this remodelling results in a progressive posterior movement of the laminar insertion, eventually into the pia mater\textsuperscript{2}, similar to other research both modeled and in vivo.\textsuperscript{72, 78} Numerical modeling has been used to study how ONH biomechanics are affected by the changes that occur in the sclera and laminar regions as associated with early experimental glaucoma. Stress-lowering effects of a thicker lamina have been discussed previously using axisymmetric generic models of the human ONH\textsuperscript{55, 66, 67} as well as a parameterized models of the monkey eye.\textsuperscript{79}
This review brings into context the importance of finite element models with regard to ONH biomechanics and the glaucomatous changes that occur at the laminar extra-cellular matrix at the anatomic to sub-cellular levels. Overall, this method of analysis allows for research within the eye to be a viable alternative to complicated, practically unfeasible or unethical experiments. In terms of the research within our lab, the research of Sigal et al.\footnote{56} has directed our hypotheses in terms of the amount of strain to apply to our cell cultures, as well as to the expected outcome measure to be expected. The following section details the emerging importance of proteomic research, eventually leading to the experimental chapters that have combined both the results of these models and modern bimolecular techniques to further elucidate the pathophysiology of glaucoma.

### 1.3 Glaucoma and Proteomics

Over the past decade, proteomics research has become an area of interest across many fields, including vision research. Previous reviews within this area have focused upon specific tissues of the eye, including the retina and trabecular meshwork.\footnote{80,81} Then, as now, there are complications regarding the availability of tissues and the difficulty in culturing ocular cell types,\footnote{80} hence having techniques that allow for the minor usage of protein is helpful. Historically, the methods used to determine the protein composition of total protein samples was through the use of 2D gels.\footnote{82} Essentially proteins were isolated from total tissue or specific cell cultures, digested then isolated depending on their charge.\footnote{83} These proteins are then separated based on mass on a traditional polyacrylamide gel. These spots were then extracted from the gel and run through a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI/TOF MS). As the fragments pass through the machine they are compared to a database and identified. Methods today still use mass spectrometry to identify the various proteins,
however the use of 2D gels is slowly being replaced by newer methods. This new approach is called quantitative proteomics and relies on either labelling each protein with stable isotopes or utilizes protein cross-linkers such as D4-BS3, to cause a mass shift in the spectrum. Techniques such as stable isotope labelling of amino acids in cell culture (SILAC), tandem mass tagging (TMT), isobaric tags for relative and absolute quantification (iTRAQ), and isobaric peptide termini labelling (IPTL) are all used to quantify levels of protein in very low protein levels. SILAC is a method that allows for peptides of different physiological conditions to be quantified at the MS level, while the other three options require MS/MS data to reveal quantitative information for the peptides. The main advantage of tagging methods using iTRAQ and TMT is the ability to compare eight different samples. The way in which iTRAQ works is through the chemical tagging of the N-terminus of peptides generated from protein digests that have been isolated from samples of up to eight different states. These samples are then combined, fractionated by nanoLC and analyzed by tandem mass spectrometry. The data that results from the fragmentation are processed though a database in order to identify the various labelled peptides, and the resulting proteins. The reporter ion that is attached to each specific sample allows for the measurement of the intensity of these ions, allowing for the relative quantitation of the peptides in each digest, indicating exactly from which proteins they originated.

Within the past 10 years since the Steely and Clark review, there have been a number of studies that have further analyzed various tissue types and diseases within ocular tissues. Within this review we shall focus on proteomic analysis of glaucomatous tissues, or models related to this specific neurodegenerative disease.
The Trabecular Meshwork

Of the tissues within the eye that are targets for glaucoma proteomics, the trabecular meshwork has been the most popular. When this region is compromised either biochemically or structurally, the normal outflow through the meshwork is reduced causing an increase in IOP. Zhao et al.\textsuperscript{90} attempted to determine the genomic and proteomic expression changes in human trabecular meshwork cells after they are treated with transforming growth factor beta (TGF-\(\beta\)). It is known that various subtypes of this growth factor can have profound effects on several tissues of the eye.\textsuperscript{91-93} Two hypotheses were tested, first, that changes in the cytoskeleton of the TM caused an increased rigidity, which in turn caused an increase in resistance, and second, that increased amounts of sheath-derived plaques and changes within the extracellular matrix were observed in the TM of patients with POAG, indicating a link between TGF-\(\beta\) and cellular function.\textsuperscript{94-98} What they observed after treating human TM cell cultures to specific levels of TGF-\(\beta1\) or TGF-\(\beta2\) was a substantial upregulation of genes that were related to secreted proteins or the extra-cellular matrix. Signalling pathways that were disrupted are related to ErbB and Wnt, while changes in the prostaglandin pathway indicated that they may have a different cellular profile in the presence of glaucoma. Two genes that were highly upregulated in the presence of TGF-\(\beta\) were osteoblast-specific factor 2 and corneal derived transcript 6. Through the course of their proteomics evaluation, an increase in vimentin and tropomyosin-1\(\alpha\) was seen which is believed to have an effect on cytoskeletal remodelling. This paper showed how human trabecular meshwork cells can be subjected to increased levels of TGF-\(\beta\) over long periods of time which may contribute to the development of glaucoma.

Another study by Bhattacharya and colleagues looked at the presence of cochlin, which is a protein associated with a deafness disorder, DFNA9.\textsuperscript{99} They discovered that this protein was
uniquely associated with glaucomatous TM, as determined through SDS-PAGE and mass spectrometry. Cochlin was of interest at the time due to the manner in which mutations in this gene were of late onset and progressive in nature, and match closely the clinical manifestations of POAG. This protein compromises the major non-collagen component of the ECM of the inner ear. The authors reported that cochlin expression within the TM increased with age, and that acidophilic cochlin deposits increase with glaucoma. These deposits may interact with various fibrillary collagens, which may trigger collagen degradation. These results, along with a decrease in type II collagen that was observed in glaucoma, were consistent with an altered ECM.

Zhang et al. determined the molecular changes in membrane proteins in TM cells of glaucomatous patients as compared to normal controls. Using 2-D gels they found that copine1 was highly upregulated in glaucomatous tissue. This protein is a highly conserved membrane-binding protein, linked to growth regulation, apoptosis, gene transcription, and cytoskeletal organization and defence. The exact role of this protein in the progression of glaucomatous optic neuropathy is still not well understood.

Amelinckx and Bhattacharya investigated the mechanism by which laser trabeculoplasty lowers intraocular pressure. Cells of the TM possess a wide range of structural and biomechanical properties, including the ability to make glycosaminoglycans and various other connective tissue proteins. Previous research had implicated tightening of the inner TM lamella, changes to the synthesis of the extracellular matrix and the cellular makeup of the TM. They found an up-regulation of various glycoproteins, such as biglycan, proargin, keratocan, and co-filin-1, in treated eyes when compared to the controls. The role of these proteins in the TM is purported to regulate aqueous outflow resistance through modulation of the
extracellular matrix and further research is needed to better understand potential therapeutic applications.

**Optic Nerve**

Bhattacharya and colleagues produced one of the first papers to apply proteomics research to the optic nerve head, specifically identifying the role of peptidyl arginine deiminase 2 (PAD2), an enzyme that converts the protein arginine to citrulline, and is calcium modulated. PADs have previously been implicated in demyelinating diseases while citrullination has been implicated in various other degenerative diseases. Through the use of gel based protein separation followed by tandem mass spectrometry, over 250 proteins were detected with 68 being specific to the glaucomatous tissue. Specifically they detected PAD2 in four of the eight glaucomatous optic nerve heads and none in the controls. Through further analysis using immunoblot assays and westerns they were able to confirm that this protein was expressed in all 12 glaucomatous tissue samples but none of the controls. This research was unable to discern whether or not PAD2 expression and citrullination cause neurodegeneration or are consequential to damage in glaucomatous optic nerve.

**Pseudoexfoliation Glaucoma**

Proteomic analysis has also been used to identify proteins of interest associated with pseudoexfoliative syndrome. Lens capsules were analyzed and found to contain higher levels of extracellular matrix and basement membrane structural and metabolic proteins such as fibulin-2, versican, syndecan-3, laminin, fibronectin, and fibrillin-1. Proteins that are associated with the complement system, e.g. C1q were also identified. Low molecular weight proteins had previously been identified. Together with the work by Lee et al. it is now believed that
they are hemoglobin type molecules. It was proposed that pseudoexfoliative material is a complex mix of proteins that preferentially precipitate out of the aqueous humor and deposit in the eye, concentrating in the angle and on the lens capsule.\textsuperscript{115} Direct analysis of this material through mass spectrometry has also led to the detection of lysyl oxidase-like 1 and apolipoprotein E\textsuperscript{119} as potential targets.

**Aqueous Humor and Glaucoma**

Specific studies have targeted with aqueous humor of patients with glaucoma in order to determine potential biomarkers to further understand its pathogenesis and develop new techniques for patient monitoring. A novel protein-bound microarray study\textsuperscript{120} was one of the first to look at changes in expression between these two groups. The primary pathways discovered were related to oxidative stress, mitochondrial alterations, apoptosis, tissue disaggregation and neuronal damage. They also found a number of proteins that relate to tissue integrity and cellular maintenance; catenins, junctional plaque proteins, dynein and cadherin. It is believed that the tissues with which the aqueous humor interacts are undergoing major repair mechanisms due to the presence of a number of cytoplasmic chaperones including calnexin.\textsuperscript{120} They also noted an increased level of nitric oxide synthase 2 along with the downregulation of other antioxidant proteins, including total glutathione S-transferases and superoxide dismutase. A more traditional approach was used by Duan et al.\textsuperscript{121} who performed 2-D gel analysis followed by mass spectrometry to determine differences between patients with POAG and those without. Seven spots from the 2-D gels were isolated for further analysis, and a significant increase in levels of transthyretin and cystatin C was observed in POAG patients. Similar results have been reported previously in patients with Alzheimer’s disease.\textsuperscript{122, 123} They also observed high levels of transferrin, which is indicative of inflammation.\textsuperscript{124} The aqueous humor of patients suffering from
congenital glaucoma has also been analyzed\textsuperscript{125} in an attempt to correlate contributing proteins to known gene loci. Through the use of a global proteomics screen, Apolipoprotein A-IV, albumin and antithrombin III were detected as being significantly higher in patients with glaucoma, as opposed to the controls. Proteins that were found to be significantly lower than controls included transthyretin, prostaglandin-H2 D-isomerase, opsin and interphotoreceptor retinoid. A number of these proteins play a significant role in the regulation of retinoic acid which plays a major role in tissue development and maintenance.\textsuperscript{126, 127} It was also noted that the proteins of interest discovered in this study very closely matched those found in Alzheimer’s disease.\textsuperscript{128, 129}

Retinal Proteomics

Tezel et al\textsuperscript{130} looked at the oxidatively modified retinal proteins in a rat-model of glaucoma. The goal of this research was to perform proteomic analysis to determine oxidative modification of retinal proteins after experimental elevation of intraocular pressure. This analysis is based upon previous research that found mitochondrial dysfunction was associated with neuronal apoptosis in experimental rat glaucoma models.\textsuperscript{131, 132} They discovered novel evidence that oxidative modification may be occurring, and targeted three potential proteins, including glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme; HSP72, a stress protein; and glutamine synthetase, an excitotoxicity-related protein\textsuperscript{130} They also noted the power of proteomics in large scale identification of changes in protein complement, which may characterize pathogenic mechanisms.

Tezel and colleagues continued their proteomics research by analyzing 14-3-3 proteins to determine their cellular localization and functional role in glaucomatous degeneration.\textsuperscript{133} Their work followed previous research implicating phosphorylation cascades in RGC signalling during glaucomatous degeneration.\textsuperscript{134, 135} They chose to focus on this family of proteins since they are
known to bind to multiple protein ligands, and they can alter the subcellular localization, stability, phosphorylation state, activity, and molecular interactions of many target proteins. This implicates them as key regulators in diverse intracellular signal transduction pathways. They found various proteins interacted with 14-3-3, including calmodulin and a proapoptotic member of the Bcl-2 family, Bad. This protein was found to be sequestered in the cytoplasm by a 14-3-3 scaffold, which prevented its mitochondrial translocation to induce apoptosis. They conclude that 14-3-3 serves as a key integration point of stress and survival in RGCs, thereby contributing to the life-and-death cycle.

Induced ocular hypertension by the addition of dexamethasone (DEX) was another technique used to investigate global protein expression of rat retinas. Injected rats showed raised IOPs, which were then compared to controls. Four proteins showed a significant change in regulated as compared to control: apolipoprotein A1 (apoA1), a lipid-binding protein, upregulated 1.9-fold; alpha A crystallin (CRYAA), a molecular chaperone, downregulated 2.7-fold; superoxide dismutase 1 (SOD1), an antioxidant enzyme, downregulated 2.3-fold; and triosephosphate isomerase 1 (TPI1), a glycolytic enzyme, downregulated 2.3-fold. In the discussion, it is explained that a reduction in SOD1 may increase the risk of the retina to oxidative stress, as reported previously. CRYAA is another protein that is directly involved in the protection of the retina against oxidative stress, acting as molecular chaperone and preventing protein aggregation from cellular stress, again suggesting a potential vulnerability when it is downregulated. TPI1 has been implicated in the production of advanced glycation end-products which have been linked to neurodegenerative diseases such as Alzheimer’s. The role of the upregulated ApoA1 is not yet fully understood. They note that these effects have not been identified as the result of the hypertension or from the DEX.
Kanamoto et al.\textsuperscript{141} used DBA/2J mice which have a genetic disposition for elevated IOP, to determine the function of proteins associated with the death of RGCs. At three different age points (5-, 7- and 11-months), the retinas were collected and analyzed to look for proteomic differences. They found a total of 18 potential targets including cell membrane receptors and proteins associated with intracellular signalling pathways. Integrin β7 gave a decrease of ~90\% from the 5 month time point to the 7 month time point. They suggested that expression of this protein was related to glutamate toxicity within the retina, a possible cause of RGC death. Previous studies had demonstrated higher levels of glutamate present within the vitreous of glaucomatous eye,\textsuperscript{44} and the induction of RGC cell death through N-methyl-D-aspartate (NMDA) treatment in an experimental model.\textsuperscript{142} They proposed that a down-regulation of Integrin β7 before the presentation of cell death at 11 months could be a compensatory mechanism meant to protect cells from a potential increase in glutamate production and the resulting toxicity through glutamine transaminase expression. It is noted that further research is needed to determine whether or not a downregulation of this protein has protective effects against other agents toxic to RGCs.

Using another animal model of glaucoma, Carter-Dawson et al.\textsuperscript{143} investigated retinal extracts from laser scarred rhesus monkeys. They used 2-D gels to determine the differentially expressed proteins. They reported a 2.3 fold up-regulation in albumin, and a positive correlation with the severity of glaucoma. In extra-ocular tissues, albumin has numerous roles from the maintenance of osmotic pressure, transport of hormones, fatty acids, bilirubin, binding to toxins and metal ions rendering them inactive, along with antioxidant properties.\textsuperscript{144} There have been a few studies that support the idea of albumin acting as a neuroprotectant and aiding in the recovery from oxidative stress.\textsuperscript{145} The manner by which the albumin gets into the retina is one for debate. It may be derived from retinal vasculature as a result of a breakdown in blood-retinal barrier,
however it cannot be ruled out that it may still be derived from the vitreous or the de novo synthesis. They still concluded that albumin was a neuroprotectant against the oxidative damage following elevated IOP.

Huang et al. injected hypertonic saline into aqueous veins of rats to raise the IOP and study the role of calcium dyshomeostasis in the pathogenic events leading to the loss of RGCs.\textsuperscript{146} They proposed that a calcium induced activation of calpain, a protein that has been implicated in a number of other neurodegenerative diseases,\textsuperscript{147, 148} is having a similar effect in glaucoma. They discovered that calpain was being activated by caspase 3 in experimental glaucoma, and that spectrin and calcineurin, two well established calpain substrates, were only present in eyes that had an elevated IOP. Previous research has pointed to the activation ability of caspase 3\textsuperscript{149} and the authors of this paper hypothesize that calpain activation may amplify calcineurin activation by creating a non-regulatable form of the enzyme, which in turn may have multiple downstream consequences, including dephosphorylation of the a specific proapoptotic protein pBad. The stated goal of this research was to develop therapeutic strategies using inhibitors of these targets during early glaucomatous neurodegeneration.

Another group of proteins targeted for neuroprotection were crystallins, which have recently been shown to increase in regulation in response to cellular stress.\textsuperscript{150, 151} In a study by Chiu et al.,\textsuperscript{152} they focused on the role of \textit{L. barbarum polysaccharide} (LBP), which is a derivative of an eastern medicine medicinal berry, and the way it interacts with various members of the crystallin family. LBP had previously been reported to have protective effects following cerebral ischemia and reperfusion, and possibly improving the cognitive function by enhancing spontaneous electrical activity of the hippocampus. They also claimed neuroprotective effects on RGCs in a rat model of glaucoma.\textsuperscript{153} Using 2-D gels, LBP resulted in an up-regulation of βB2-crystallin.
They found this protein to be expressed in the ganglion cell layer, the inner nuclear layer, as well as the nerve fibre layer, using immunohistochemistry. They concluded that βB2-crystallin, as upregulated by LBP, possibly acts as a neuroprotectant that may lead to greater survival of RGCs in vivo.

Proteomics-based research up to this point relied upon 2-D gels, with differentially regulated spots excised and analyzed by mass spectrometry. In order to get a more robust list of proteins, a global protein analysis using iTRAQ tagging techniques was used by Crabb et al.\textsuperscript{154} to isolate differences in retinal ganglion cells in unilateral experimental glaucoma of mature rats. Immunopanning was used to separate the RGC layer of cells from 22 animals. A total of 268 total proteins were found, with 22 upregulated and 36 downregulated. Three upregulated proteins were isolated: “voltage-dependent anion channel protein 2, a regulator of ion transport in the mitochondrial outer membrane; aldosereductase, acyto-plasmic NADPH-dependent oxidoreductase associated with signalling and cell proliferation pathways; and ubiquitin, a cytoplasmic and nuclear protein modifier that generates many functional consequences, including proteasomal degradation and regulation of gene expression”\textsuperscript{154}. Only one protein was significantly down regulated in the glaucomatous sample, prothymosin α, which is implicated in the reduction of immune function through anti-apoptosis and cell proliferation pathways. This paper demonstrated the use of a global protein quantitative analysis in ophthalmic research and the different conclusions that can be drawn when compared to gene expression research and the variability in overlap has been seen between transcription and protein levels.\textsuperscript{155}

Tezel et al. followed up this study with another gel free mass spectrometric analysis of human retinal protein samples, both with and without glaucoma.\textsuperscript{156} The focus of this research was upon the complement system, with a primary aim of determining whether oxidative stress may be
involved in the regulation of the activation of this system. *In vitro* studies were also carried out using primary cultures of RGCs in the presence and absence of oxidative stress. Through the proteomic analysis, it was found that the glaucomatous human retina synthesizes various complement components that are involved in the classic and lectin pathway. The authors are quick to point out that it still remains unclear whether or not this activation is a “physiological complement-mediated tissue clearance process or a complement-mediated component of neurodegenerative injury”. Specifically, six different complement components (C1s, C1r, C1q, C3, C4b, C7–9) and receptors (CR1, CR2, C5aR) were discovered. Bioinformatics analysis using Ingenuity Pathways Analysis (IPA) in order to determine protein interaction networks. The major hubs that were interacting with the discovered proteins revolved around complexes involved in the ERK, NFκB and the MAPK pathway. All three of which are involved in the complement regulation in glaucoma. Particular attention was also paid to Complement factor H (CFH) and the way it responded to oxidative stress in vitro. H₂O₂-induced oxidative stress led to the CFH down-regulation. The study proposed that complement activation may serve as an intrinsic signal for the elimination of dying RGCs, a down-regulation of CFH through oxidative stress may lead to an increased vulnerability of adjacent neurons to complement-mediated injury. Further research is required to explore the importance of the complement system in glaucoma and the mechanism of interactions between complement proteins, immune system cells, and immune mediators.

This group used the same proteomics assay to determine the regulation of immune system activation as it is associated with Toll-like receptor (TLR) signalling in glaucoma. Their theory was based upon various studies that support a prominent immune response during glaucomatous neurodegeneration. TLRs are receptors that recognize molecular patterns of pathogens such as viral double-stranded RNA, however growing evidence supports the idea that
they also detect non-pathogen stimuli, which would initiate an immune response. Samples of glaucomatous and non-glaucomatous retinal tissues were isolated and gel-free quantitative proteomics was used, as well as immunohistochemical analysis to determine cellular localization. Results for this study indicated that a consistent upregulation of TLRs was detected in the glaucomatous retina, specifically citing TLR2, -3, and -4 as being localized to retinal microglia and astrocytes. Through the use of bioinformatic analysis, they were able to highlight a number of potential molecules in the TLR signalling pathway which may result in a diverse immune response. They found that Heat Shock Proteins and H₂O₂-induced oxidative stress can activate these receptors and stimulate the antigen-presenting function of glial cells though their activation. They list thirteen different TLRs and explain the way they interact, through modulation, translocation and various cross-regulatory stressors. They summarized that danger signals to the immune system can be transmitted by HSPs and oxidative stress-related products via TLR signalling.

Researchers from the Devers Eye Institute also utilized a label-free quantitative mass spectrometry analysis to compare changes in non-human primates at the onset of early experimental glaucoma (EEG) and 3 weeks after optic nerve transection (ONT) in order to monitor potential biomarkers. They found that there was a substantial difference between models, from low and high IOP EEGs and ONT. They also noticed a prominent alteration in cytoskeletal architecture between the two models due to an upregulation of tubulins. There was a general trend of down-regulation seen in the low IOP EEG proteome as opposed to an upregulation seen in the other two groups. The authors did not highlight any particular protein for further analysis or specific identification. Listed were approximately 200 proteins from each group that were identified and then compared intra-experimentally in order to determine overlap.
or overarching trends. The results imply there is condition-specific regulation of retinal processes depending on the method of insult.

In the ten years since the Steely and Clark review, the most accurate and readily available technique for quantitative assessment of a proteome went from 2D gels to the use of labelled and label-free mass spectrometric analysis. Of the labelled proteomic screen, a number of different stable isotope techniques have surfaced including SILAC, iTRAQ, GIST and ICAT. The primary advantage of this form of analysis is the ability to further identify the proteins and pathways that are playing a role in disease pathogenesis. Future technologies are emerging that will allow for more detailed analysis of specific proteins, such as being able to detect and quantify post-translational modifications which are highly abundant proteins that are currently being overlooked. As greater amounts of data are submitted to the various journals publishing these studies, a system that can easily compare and contrast results, identifying the similarities and disparities between the research will be an increasing useful tool. However, there are potential artefacts associated with cell culture research that need to be taken into consideration, as cells may express proteins differently than when in vivo. An insightful point made in the conclusion of the Steely review indicates how ophthalmic genomics and proteomics are not mutually exclusive, and in the last decade the explosion of research that is attempting to tie together potential genetic abnormalities with protein pathways is leading us ever closer to the molecular causes of disease and eventually develop novel therapeutic approaches to glaucoma.
1.4 References


2 Culture and Stretch of Human Optic Nerve Head Lamina Cribrosa Cells and Astrocytes

Ronan Rogers MSc\textsuperscript{1, 2}

Inka Tertinegg\textsuperscript{2}

John Flanagan, PhD\textsuperscript{1, 2, 3}

1. Institute of Medical Science, University of Toronto, Toronto
2. Department of Ophthalmology & Vision Science, Toronto Western Hospital, Toronto
3. School of Optometry, University of Waterloo, Waterloo, Ontario, Canada

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Corresponding Author:
Ronan Rogers
Institute of Medical Science, University of Toronto, Department of Ophthalmology & Vision Science, Toronto Western Hospital, Ontario, Canada
Phone: (416) 603-5800 ext. 2850
Email: ronan.rogers@utoronto.ca
2.1 Abbreviations Page

a-smA – Alpha Smooth Muscle Actin

AGM – Astrocyte Growth Media

DAPI – 4’,6-diamidino-2-phenylindole

DMEM – Dulbecco’s Modified Eagles Serum

FBS – Fetal Bovine Serum

GFAP – Glial Fibrillary Acidic Protein

IOP – Intraocular Pressure

LC – Lamina Cribrosa

NCAM – Neural Cell Adhesion Molecule

ONH – Optic Nerve Head

Pax-2 – Paired Box Gene 2

PBS – Phosphate Buffered Saline

POAG – Primary Open Angle Glaucoma
2.2 Introduction

Primary open angle glaucoma (POAG) is a disease that is characterized by the slow and irreversible death of retinal ganglion cells. As these cells undergo apoptosis, vision loss is the unfortunate consequence. There are numerous risk-factors that are associated with POAG, first and foremost being high intraocular pressure (IOP).\textsuperscript{1-3} It has been shown that a reduction in IOP can dramatically reduce the progression of the disease in the clinical management of patients with glaucoma.\textsuperscript{3-5} At this time however, the exact mechanism by which an increase in IOP results in a progressive loss of vision is not completely understood. One of the leading hypotheses is that the increase in IOP deforms (stretches) the tissues of the optic nerve head (ONH) and in particular, places undue stress upon the Lamina Cribrosa (LC) and its capillary vascular supply.\textsuperscript{6} This is a region within the ONH that acts as a sieve-like interface between the myelinated optic nerve and the unmyelinated nerve fibre layer of the retina. The belief is that individuals with ‘weak’ optic nerve heads, and those with poor vascular perfusion, are at increased risk of optic neuropathy due to an elevation of IOP.\textsuperscript{6}

We have previously modelled the biomechanical effect of raised IOP on the human optic nerve\textsuperscript{7-13} using finite element modeling. The purpose of this paper is to develop an \textit{in vitro}, primary, human, ONH glial cell model of activation following biomechanical insult, using the Flexercell 4000 (Flexcell International Corporation, Hillsborough, North Carolina). The Flexercell system is capable of performing controlled experimental equiaxial stretch.\textsuperscript{14,15}
Figures 1 and 2 illustrate the lamina cribrosa, the target tissue of interest, as well as the specific forces of principle strain that are being exerted upon it when an IOP of 50 mm Hg is applied. Figure 3 shows a histological section of a human Lamina Cribrosa (LC). The highest percentage strain calculated for this generic model was at the scleral/laminar interface and was approximately 10% to 15%, while the average across the entire region was 5.5%.
Lei et al\textsuperscript{16} recently investigated the effects of hydrostatic pressure and concluded that the resulting changes in cell migration, morphology and $\alpha$-tubulin architecture were due to changes in oxygen tension rather than biomechanical strain. The Flexercell system has been used previously to stretch ocular cell types,\textsuperscript{15, 17} and appears to offer the potential of inducing mechanical insult.

\textbf{Figure 3 –} Explantation protocol. Optic nerves (ON) were dissected using a Zeiss stereo dissecting microscope (10-50x mag.).

Panel A&B: ON and a small ring of surrounding sclera were dissected from the eye and incubated for 1 hour in Explant Media.

Panel C: The dura mater was completely removed.

Panel D: The retina was removed from around the ONH.

Panel E: Tangential cuts were made around the circumference of the ONH, to carefully cut away the remaining ring of sclera and choroid. Lines indicate the position and direction of the cuts made to the ON tissue

Panel F: The pia mater and superficial nerve fibre layer of the optic nerve are removed.

Panel G: The prelaminar and laminar region is dissected away and carefully cut into 4 or 5 small pieces. Panel I: Any visible blood vessels are carefully removed. (C, Choroid; R, Retina; Sc, Sclera; DM, dura mater ; PM, pia mater ; V, vessels ; PL/L, prelaminar/laminar tissue).
2.3 Results

Human eyes that were donated for research to the Eye Bank of Ontario, if less than 24 hours old and with an enucleation time of less than 8 hours, were used to harvest optic nerves for subsequent cell culture. The optic nerve and a small ring of surrounding sclera are dissected from the eye and incubated for one hour in explant media (DMEM/F12, supplemented with penicillin/streptomycin gentamicin, fungizone, L-gluatamine, and 10% fetal bovine serum). Fine dissection was then performed in explant media under the dissecting microscope. The dura mater was completely removed and the retina was similarly removed from around the optic nerve head. Tangential cuts were made around the circumference of the ONH, to carefully cut away all remaining sclera and choroid. The pia mater and superficial nerve fiber layer of the optic nerve were removed, leaving a clear delineation between the unmyelinated prelaminar and laminar regions and the myelinated optic nerve. The prelaminar and laminar region was dissected away and carefully cut into four or five small pieces, after which all visible blood vessels were carefully removed (figure 3). The optic nerve head pieces were allowed to attach to a T25 Primaria flask (BD Biosciences, San Jose, California) in an upright position for 20 minutes while in a 37°C incubator with 5% CO₂ with a minimal amount of media (1-2ml) then laid flat. The explant flask was allowed to incubate for a further 10 days without disturbance. Regular media changes then commenced every 3-4 days until confluent.

The cells were then trypsinized off the flask using TrypeLE Express Stable Trypsin-like Enzyme (Invitrogen, Carlsbad, California) and split into a T75 flask. At this point, the cells that have come off the T25 were a co-culture of astrocytes and lamina cribrosa cells and in need of being selected. In order to select for lamina cribrosa cells, the explants were placed in LC media (DMEM, low glucose, supplemented with 10% FBS, L-gluatamine and a 1%
Penicillin/Streptomycin mixture). Due to the high level of serum and nature of LC cells, they outgrow the astrocytes and create a mono-culture.

In order to select for astrocytes, the explant culture was placed in a serum free astrocyte growth media (AGM –serum, Lonza, Switzerland; astrocyte basal media plus bullet kit (rhEGF, 0.5 ml; Insulin, 1.25 ml; Ascorbic Acid, 0.5 ml; GA-1000, 0.5 ml, L-Glutamine, 5.0 ml)) for two weeks until morphologically homogenous in appearance. At the two week point the culture was maintained in AGM complete with 3% FBS, for another two weeks. The media was then changed to DMEM/F12 (supplemented with 10% FBS, 1% penicillin/streptomycin). When the flasks are confluent, they are again trypsinized and split in a 1:3 ratio and maintained in the DMEM/F12 media until ready for experimentation.

In order to ensure that cell differentiation has occurred and the appropriate cell types were being identified, cell characterization was performed using immunochemistry. At the time of the last trypsinization, an aliquot of cells was reserved and seeded onto glass cover slips in a 24-well plate and allowed to grow to and maintain an 80% confluence. They were fixed with 4% paraformaldehyde and permeabilized overnight with 0.2% Triton X-100. After blocking in 1% BSA, the cells were incubated with a primary anti-body overnight at 4°C. The secondary antibody incubation continued for 1 hour at room temperature. After the final wash in PBS, the cells were rinsed in distilled water and the slides were allowed to air dry. The cover slips were mounted using Prolong® Gold antifade reagent with DAPI and the slides were examined under a Zeiss Axioplan 2 microscope with Axiocam, Axiovision and Deconvolution. The primary stain for the astrocytes was the positive reaction for glial fibrillary acidic protein, which is an intermediate filament protein specific for these cell types. The lamina cribrosa cells were GFAP negative (figure 4).
**Figure 4** – Fluorescence microscope images of cells from human Optic Nerve Heads (hONHs).

Left Panel: Astrocyte cells show positive staining for the intermediate filaments GFAP, Desmin and Vimentin, for S-100, the paired box gene Pax-2, and the neural cell adhesion molecule (NCAM). They exhibit negative staining for A2B5, and α-smooth muscle actin (α-smA).

Right Panel: LC cells show positive staining for NCAM, Vimentin, Desmin, Pax-2, S-100, and α-smA. There is negative staining for GFAP, and A2B5. This panel would indicate that these are not Astrocytes based primarily on their lack of GFAP reactivity. (40 X magnification on a Zeiss Axioplan deconvolution microscope)

We wanted to calibrate the Flexercell system to determine if the percentage of strain requested from the Flexcell interface was the actual percentage being applied to the silastic membrane of the six-well plate. This was tested up to a maximum stretch of 15% at 1Hz. We developed a method to determine the real equiaxial stretch being applied in order to objectively determine the percentage strain. The first step was to apply markers to the membrane through the use of a circular stencil of equal size. A circular grid with small uniform perforations was used to allow a fine-tipped marker to place a dot on the silastic membrane. The marked plate was then placed into the Flexercell machine. The machine was set to a 1Hz cycle (one full waveform of 0%-15% and back within one second) and a high quality digital camera was positioned directly above and perpendicular to the well of interest. The Flexercell system was started and a video recording
(30fps) of the silastic membrane being stretched to its maximum and minimum positions was recorded. Upon analyzing the video, still images were taken at the exact video frame when the membrane was at both the maximum and minimum stretch position. These images were imported into ImageJ (version 1.42q) and manipulated. First, the image was circularly cropped so that only the membrane was analyzed. The image was then reduced to 8-bit and the brightness and contrast manipulated so that only the black dots on the membrane were apparent. The image threshold was set at automatic, and the “analyze particle” feature was used, with size set at 0-infinity, circularity set at 0.00-1.00 and outputting the exact x and y coordinates for each dot on the membrane. A similar process was followed for the extended membrane, and the x/y coordinates were double checked to ensure that each dot was matching the dot from the previous stretch image. The data of all the x/y coordinates from the before and after stretch was analyzed in order to determine the percent movement of each dot as compared to all other dots. This was computed for the remaining dots to give the stretch of the whole membrane (figure 5).

Figure 5 – Image of dots upon the silastic membrane and seated within the Flexercell 4000 machine. Left panel shows 0% stretch, while right image shows 15%. Right panel demonstrates the overlay of the two images with white spots representing the stretched position of the black spots. Blue circle is the centre of the stretch and blue lines show displacement example.

All six wells per plate were used (N=6) to determine the mean and standard deviation of stretch for the percent chosen. Using a 15% stretch at 0.5Hz resulted in 15.9%±3.4, however at 1Hz, this
dropped to 12.3%±1.2. When a value of 12% was set within the Flexercell the result was 11.4%±0.7, which is represented in the output image from the plotting program used. (Tecplot, Bellevue, WA, USA) (figure 6).

![Diagram of percentage change for each point on two different wells when subjected to a 12% strain at 1Hz](image)

**Figure 6** – Diagram of percentage change for each point on two different wells when subjected to a 12% strain at 1Hz

### 2.4 Discussion

Optic nerve head astrocytes and lamina cribrosa cells were successfully isolated from LC explants (figure 3). Cell characterization has been established and was consistent with previous literature.\(^{18-20}\) The Flexercell system has been used previously\(^{15}\) however it was unclear until now the exact maximum strain capable at a specific frequency. Kirwan et al\(^{15}\) claimed to be using 15% stretch at 1 Hz, but it is likely that they only achieved approximately 12% stretch. We are also confident that this method of strain is more physiologically relevant than hydrostatic pressure, which has been previously used. Lei et al\(^{16}\) have reported that hydrostatic pressure likely caused a lowering of oxygen tension but did not provide a mechanical insult.\(^{16}\) Our methods can be used to determine the exact percentage of strain capable at various frequencies and for different waveforms and is applicable across all fields of research and cell culture types.

We required a 1Hz cycle in order to mimic the *in vivo* ocular pulse, however a faster or slower
cycle may be required for different cell types. This in turn affects the maximal strain that is possible when using the FX-4000. It is possible that there is a minimal amount of shear stress induced by movement of the media over the cells. We propose to use 12% stretch at 1 Hz to further investigate the response of ONH glial cells to biomechanical insult.

2.5 Acknowledgements

Support for this work is acknowledged from the Canadian Institutes of Health Research, the American Health Assistance Foundation, the Glaucoma Research Society of Canada, and scholarships to RR from the Peterborough K.M. Hunter Studentship and the Vision Science Research Program of the Toronto Western Research Institute.
2.6 References


3 Proteomics Analyses of Human Optic Nerve Head Astrocytes Following Biomechanical Strain

Ronan S. Rogers, MSc\textsuperscript{1, 2, 3}

Moyez Dharsee, BSc\textsuperscript{4}

Suzanne Ackloo, PhD\textsuperscript{4}

Jeremy M. Sivak PhD\textsuperscript{2, 3}

John G. Flanagan, PhD, MCOptom\textsuperscript{1, 2, 3, 5}

1. Institute of Medical Science, University of Toronto, Toronto
2. Vision Science Research Program, Toronto Western Research Institute, University Health Network
3. Department of Ophthalmology & Vision Science, Toronto Western Hospital, Toronto
4. Ontario Cancer Biomarker Network, Toronto, Ontario, Canada
5. School of Optometry, University of Waterloo, Waterloo, Ontario, Canada

Number of Tables: 6
Number of Figures: 9
Corresponding Author:
Ronan Rogers
Institute of Medical Science, University of Toronto, Department of Ophthalmology & Vision Science, Toronto Western Hospital, Ontario, Canada
Phone: (416) 603-5800 ext. 2850
Email: ronan.rogers@utoronto.ca

3.1 Abbreviations Page

α-smA – Alpha Smooth Muscle Actin

AGM – Astrocyte Growth Media

CASP3 – Caspase 3

DPBS – Dulbecco’s Phosphate Buffered Saline

DMEM – Dulbecco’s Modified Eagles Serum

FBS – Fetal Bovine Serum

GO – Gene Otology

GFAP – Glial Fibrillary Acidic Protein

IPA – Ingenuity Pathways Analysis

iTRAQ – isobaric Tag for Relative and Absolute Quantitation

LC – Liquid Chromatography

LCr – Lamina Cribrosa

MS – Mass Spectrometry

MMTS – Methyl Methane ThioSulphate

m/z – Mass to Charge Ratio

NCAM – Neural Cell Adhesion Molecule

OCBN – Ontario Cancer Biomarker Network

ONH – Optic Nerve Head

Pax-2 – Paired Box Gene 2
3.2 Summary

We investigate the role of glial cell activation in the human optic nerve caused by raised intra-ocular pressure, and their potential role in the development of glaucomatous optic neuropathy (GON). To do this we present a proteomics study of the response of cultured, optic nerve head (ONH) astrocytes to biomechanical strain, the magnitude and mode of strain based on previously published quantitative models. In this case, astrocytes were subjected to 3% and 12% stretches for either 2 hours or 24 hours. Proteomic methods included nano-liquid chromatography (LC), tandem mass spectrometry (MS/MS), and iTRAQ labelling. Using controls for both stretch and time, a six-plex iTRAQ LC/MS/MS experiment yielded 573 proteins discovered at a 95% confidence limit. The pathways included transforming growth factor β1 (TGFβ1), tumour necrosis factor (TNF), caspase 3 (CASP3), and tumour protein p53 (TP53), which have all been implicated in the activation of astrocytes and are believed to play a role in the development of GON. Confirmation of the iTRAQ analysis was performed by Western blotting of various proteins of interest including ANXA 4, GOLGA2 and αB-Crystallin.
3.3 Introduction
Glaucoma is the world’s most common neurodegenerative disease, affecting an estimated 60 million people, double the number affected by all other neurodegenerative diseases combined. It is also the second leading cause of blindness worldwide.\(^1\) Primary open angle glaucoma (POAG), the most common type of glaucoma, can be characterized by the slow and irreversible apoptotic death of retinal ganglion cells, a unique optic nerve neuropathy and loss of visual function.\(^2\) Intra-ocular pressure (IOP) is a major risk-factor\(^3\)\(^4\) for the development of glaucoma, and reducing the IOP has been shown to be unequivocally beneficial in the clinical management of patients with the disease.\(^4\)\(^6\) Nickells\(^7\) proposed a 5-stage model of glaucoma that unifies much of the clinical, animal and cell based research. The primary stage is described as the ‘elevation of IOP and the activation of optic nerve glia in the lamina cribrosa’ and includes disruption of both retrograde and anterograde axonal transport, including neurotrophins and motor proteins. The research presented here is intended to contribute to our knowledge of stage 1 of this disease, the ‘activation of the optic nerve glia in the lamina cribrosa’\(^7\).

Astrocytes are the cell type of interest in this study as they are the major glial cell within the optic nerve head (ONH), providing a supportive role to the surrounding axons, while communicating with connective tissues and surrounding blood vessels.\(^8\) Normally the astrocytes remain ‘quiescent’, but following insult through injury or disease, they become reactive and can either reduce or exacerbate the damage to the neural tissue.\(^9\) They support the tissue through the release of neurotrophic factors and antioxidants, and through the degradation of abnormal extracellular protein deposits.\(^10\) The role they play in the degeneration of surrounding tissue is believed to occur by release of reactive oxygen species, proteases, cytokines and nitric acid\(^11\)\(^16\) (for review see\(^17\)\(^21\)). Glial fibrillary acidic protein (GFAP) has been shown to be up regulated
with astrocyte activation, and is associated with an increase in cell surface molecules important to cell-cell interactions, as well as cell adhesion substrates, cytokines and growth factors. To evaluate the effects of IOP on ONH biology, understanding the forces and deformations experienced by cells in the ONH is of the utmost importance. This is problematic as the tissue of primary interest, the laminar cribrosa (LCr), is small, relatively inaccessible and difficult to visualize. Moreover, it is a relatively compliant (mechanically weak) tissue that is surrounded by the much stiffer sclera, making it difficult to isolate the mechanical properties of the LCr. Our research, and that of others has therefore applied finite element modelling to better understand the biomechanical environment within the ONH. We know that astrocytes, and other cells, are sensitive to mechanical stretch, and that the viability of retinal ganglion cells depend on normal astrocyte function.

From these numerical models, we developed cell culture models to replicate the conditions experienced by astrocytes within the LCr. In these models we mimic the in vivo biomechanical environment in the LCr by growing human ONH astrocytes on flexible, silastic membranes and subjecting the cells to deformation. A similar approach has been used previously on LCr cells. Other studies have analysed the protein regulation of cells from the ONH using hydrostatic pressure. However, this is the first time that equiaxial stretch has been applied to human ONH astrocytes. Lei et al recently investigated the effects of hydrostatic pressure and the resulting changes in oxygen tension on cell migration, morphology and α-tubulin architecture. They reported that an increase in hydrostatic pressure had no effect, and that the biological effects previously reported were most likely artefacts due to hypoxia within the medium. We are confident that our approach of inducing biomechanical strain using equi-axial stretch is a more realistic model of the conditions found within the human lamina cribrosa. However potential
artefacts associated with all cell culture research needs to be taken into consideration, as cells may express proteins differently than when in vivo. For proteomics, the proteins from the cell lysate were analyzed in collaboration with the Ontario Cancer Biomarker Network (OCBN). Previous eye related proteomic research have been reviewed by Steely and Clark\textsuperscript{54} and Tezel.\textsuperscript{14} Steely and Clark\textsuperscript{55} characterized the human trabecular meshwork (TM) proteome through the use of a transformed TM cell line, which was compared to healthy cells. Zhao et al\textsuperscript{56} and Fuchshofer et al\textsuperscript{57} investigated the role of TGF-β, proposing its importance in the pathogenesis of glaucoma. Bhattacharya et al\textsuperscript{52} found a positive link between the presence of PAD2 and glaucoma and in more recent research this group has outlined the occurrence of retinal deiminisation,\textsuperscript{58, 59} further confirming the post-translational effects and their role in glaucoma. Tezel and colleagues\textsuperscript{14, 60} have used proteomics to determine the role of oxidative stress on retinal proteins in glaucoma, the influence of haemoglobin expression and regulation,\textsuperscript{14} and the importance of the complement pathway.\textsuperscript{14} Tezel\textsuperscript{14} has recently proposed a unifying theory of oxidative stress and its importance to the immune response in the pathogenesis of glaucoma.

We present the total protein analysis of ONH astrocytes, stressed in a manner similar to that modelled to occur following raised IOP, with the ultimate goal of better understanding astrocyte activation and how this may lead to the loss of retinal ganglion cells. Proteins of interest are isolated and presented based on gene ontology (GO) analysis, including the role that they play in relating pathways from physical stress to transcriptional changes.
3.4 Experimental Procedures

Cell Lines

The lamina cribrosa (LCr) from 4 healthy human donors (Eye Bank of Canada, Ontario Division) were dissected into explants and grown in Dulbecco Modified Eagle Serum (DMEM) Nutrient Mixture F12 (4 mM L-glutamine; 1g/L glucose; 1.5 g/L sodium bicarbonate; 10 % FBS; penicillin/ streptomycin) until confluent in accordance with the human biosafety requirements of the University Health Network, Toronto, Canada. The astrocytes were isolated from other cell types using a technique previously described. Briefly, explants were split into serum-free astrocyte growth media (AGM – serum, Lonza, Switzerland; astrocyte basal media plus bullet kit (rhEGF, 0.5 ml; Insulin, 1.25 ml; Ascorbic Acid, 0.5 ml; GA-1000, 0.5 ml, L-Glutamine, 5.0 ml)) for a period of two weeks, when the media was replaced with DMEM/F12. The cells were grown to confluence and split in this media until there were enough viable cells to conduct an experiment. A fourth cell line was grown to be used for validation purposes using Western blotting. Cultures were maintained in sterile incubators at 37°C and 5% CO₂, and media was changed twice a week. Morphologically, the astrocytes were cultured in a monolayer and were similar to those previously reported. See figure 1 for cell characterization and morphology. All cultures are of at least 95% purity based on morphology and staining.
Figure 1 – This characterization panel shows that these cells are astrocytes through positive staining for the intermediate filaments GFAP, desmin and vimentin, S-100, the paired box gene Pax-2, and the neural cell adhesion molecule (NCAM). Negative staining for A2B5, and a-smA is also shown. (40 X magnification on a Zeiss Axioplan deconvolution microscope)

Immunohistochemistry

Cells from each cell line were grown on 35mm plates after the 3rd passage and allowed to grow to confluence after which they were washed twice with DPBS (+Mg and Ca). The cells were then fixed in formalin, permeabilized in Triton-X and the specific primary and secondary antibodies. Primary human astrocytes were characterized by positive staining for glial fibrillary acidic protein (GFAP), desmin, vimentin, S-100, neural cell adhesion molecule (NCAM), and negative staining for A2B5, α-smooth muscle actin (α-smA) and Pax-2. (figure 1)
**Stretch**

For the stretch experiments, cells at the 4\textsuperscript{th} passage were seeded onto 6-well flexcell plates (Flexcell International Corporation, Hillsborough, NC) and allowed to grow to confluence. Using the Flexercell® Tension Plus FX-4000T system, a programmable amount of equi-axial strain was applied to the cells through the use of a vacuum pump and a custom base-plate. The astrocytes were seeded onto pre-coated collagen type IV 6-well plates, which were additionally coated with collagen type I (Rat Tail Collagen, BD Biosciences, Franklin Lakes, NJ, USA). Four plates per experiment were stretched while 4 control plates were placed in the same incubator. Control cells were also grown on the coated BioFlex culture plates. All cells were serum deprived for 24 hours prior to stretching, which was performed at a 1Hz cycle of 0% to 3%, or 0% to 12%, for either 2 hours or 24 hours. This gave rise to a total of 6 experimental conditions for each cell line.

**Protein Isolation**

Total protein was isolated from three experimental cell lines and combined to be used in proteomics analysis through the use of a RIPA buffer. A volume of RIPA was added to each well (300ul) and allowed to sit at room temperature for 15 minutes. The 6 wells from each plate were scraped down, combined into an Eppendorf tube, aspirated and centrifuged at 10,000 rpm for 10 minutes. The cleared protein lysate was then prepared using the Total Protein Clean-up Kit (Norgen Biotek Corp., Thorold, ON). The protein required for the western validation was collected through the use of a RNA/Protein purification kit (Norgen), and kept at -80°C until required.

**Proteomics Analysis**

Proteomic screening was performed by the Ontario Cancer Biomarker Network (OCBN, Toronto, ON), using 6 astrocyte cell lysates; a 2 hour control, a 24 hour control, a 12\% for 2 hour
stretch, a 12% for 24 hour stretch, a 3% for 2 hour stretch, and a 3% for 24 hour stretch (each lysate was a combination of the respective treatment from the three cell lines).

**Digestion and Labelling:** Protein was extracted from cell lysates using a Norgen Total Protein Clean-up kit. The concentration was determined using a micro-BCA assay kit (Thermo Scientific, USA). One hundred micrograms of protein from each condition was processed for iTRAQ\(^{64,65}\) labelling. Briefly, the proteins were de-natured, reduced, alkylated, trypsin digested and then labelled with the appropriate iTRAQ tags. After labelling, the 100 microgram aliquots were pooled.

**Strong-Cation Exchange (SCX) Chromatography:** Each pooled sample was fractionated using SCX chromatography and a Thermo BioBasic SCX column, 0.2mm internal diameter and 10cm long. Each sample was diluted with the loading buffer (15 mM KH\(_2\)PO\(_4\) in 25% acetonitrile, pH 3.0) to a total volume of 2 mL and the pH adjusted to 3.0 with phosphoric acid. Samples were filtered using a 0.45μm syringe filter (Millipore, Canada) before loading onto the column. Two millilitres of diluted sample were injected into the SCX system. Separation was performed using a linear binary gradient from 0% solvent B to 50% solvent B in 40 min. The gradient was ramped to 100% Solvent B in 2 min. and held for 60 min.. Buffer A was identical in composition to the loading buffer, while Buffer B was the same as Buffer A with 350 mM KCl. After a 2 min. delay to evacuate the void volume, fractions were manually collected every 2 min. to the end of the gradient. The last fraction was the 2 min. block after the UV signal returned to baseline. The fractions were dried by speed vacuuming and re-suspended in 0.1% formic acid. The contents of each SCX fraction were resolved by C18 reversed-phase (RP) liquid chromatography.

**Reversed-Phase Chromatography:** The Nano LC-Ultra (Eksigent Technologies, USA) consists of a trap column (300μm ID) and an analytical column (75 μm ID) packed with 5μm, 300Å
Zorbax SB-C18 beads. The analytical column is made at OCBN. Separation was performed using a linear binary gradient where solvent A is 98% H₂O: 2% CH₃CN and 0.1% formic acid and solvent B is 2% H₂O : 98% CH₃CN and 0.1% formic acid, and at a flow-rate of 300 nL/min with a 60 minute gradient to 30% B. The equivalent of 2 µg of protein was injected.

*LC/MS/MS:* The eluant from the Nano LC was coupled to a quadrupole time-of-flight mass spectrometer (QSTAR® Elite, AB Sciex, USA), through an electrospray ionization source equipped with a 15 µm ID emittor tip, for use in a single-run iTRAQ analysis. After each survey scan, from m/z 400 to m/z 1500, three of the most intense ions with charge state 2 to 4 were selected for MS/MS analysis. These ions were then placed in a dynamic exclusion list for 3 minutes in order to avoid further selection of the same ions. For this proposed project, each protein will be quantified by a minimum of two peptides and each peptide will be monitored by a minimum of three transitions. The iTRAQ workflow on Analyst® QS software was employed.

**Western Blotting**

The protein was purified using the Total Protein/RNA Clean-up Kit (Norgen Biotek Corp., Thorold, ON). The targeted proteins were ANXA 4 (Proteintech Group, Inc., rabbit antihuman polyclonal, 1:2000), GOLGA2 (Abcam, rabbit monoclonal, 1:2000) and αB-Crystallin (Crystalin, Abcam, mouse monoclonal, 1:750). Equal volumes of protein are resolved on SDS-PAGE gels and then transferred to PVDF membrane by electroblotting at 4°C. Membranes were blocked with 3% BSA in PBST and antibodies were diluted in 1% BSA in PBST. Incubation with the primary antibody proceeded overnight at 4°C. After washing, blots were incubated with HRP-conjugated secondary antibody incubated for 1 hour at room temperature (Cedarlane’s anti-rabbit IgG HRP (Cat # 12-348; 1:1000) or anti-mouse IgG HRP ( Cat # 12-349; 1:2000)). Antigens were visualized using the West Pico System (Pierce) and the Bio-Rad Fluor-S Max
MultiImager. Densitometry was performed using both ImageJ and Quantity One Software and the bands were equilibrated to the protein concentration in the samples.

**Data Processing and Analysis**

Relative quantification and protein identification were performed with the ProteinPilot™ software version 2.0 (AB SCIEX, USA) using the Paragon™ algorithm as the search engine. Each MS/MS spectrum was searched against a concatenated forward and reverse database of human protein sequences containing >500,000 sequence entries comprising ~190 million amino acids abstracted from ~200,000 references (Swiss-Prot, 22/07/2008). The search parameters allowed for 8-plex iTRAQ, QSTAR Elite ESI, trypsin digestion, cysteine modification by methyl methanethiosulfonate (MMTS), homo sapiens, and biological modifications programmed in the algorithm (which include phosphorylations, amidations, and semi-tryptic fragments). The detected protein threshold (unused protscore (confidence)) in the software was set to 0.05 to achieve 10% confidence, and identified proteins were grouped by the ProGroup algorithm (AB SCIEX, USA) to minimize redundancy. The bias correction option was executed. Proteins without quantitative information, i.e. only one iTRAQ ratio, were deleted from the list of identified proteins. Differentially expressed proteins were defined as those showing an absolute fold-change of at least 1.5 relative to time-matched controls. Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com, version 8.7) was used to determine pathways and functions implicated in stretching the cells for the prescribed amount of time. For each condition, the IPA Core Analysis feature was used to construct molecular interaction networks based on relationships between observed proteins and with other molecules, as annotated in the Ingenuity Knowledge Base. The resulting networks were then merged (using the Merge Networks feature) through the introduction of additional relationships. This analysis also provided a mapping of observed proteins to known cellular functions and processes; a p-value based on a right-tailed
Fisher Exact Test was associated with each functional category found to be enriched in the expression dataset. In the right-tailed Fisher's Exact Test, only over-represented functions or pathways are significant (i.e. those that have more Functions/Pathways/Lists of eligible molecules than expected by chance). Under-represented functions or pathways ('left-tailed' p-values) which have significantly fewer molecules than expected by chance are not shown. Gene Ontology categorizing was also used on the resulting proteins. This type of analysis allowed for the grouping of proteins into relevant functional groups that are pertinent to our research, specifically, apoptosis, activation, neurodegeneration, DNA damage/repair, cellular remodelling or stress responses.

3.5 Results

Prior to samples being injected, a 5 fmol bovine serum albumin (BSA) digest was used to determine the quality of the LC/MS/MS system. The sample had to record at least 20% sequence coverage with scores per peptide ≥ 20. The identification of the protein was performed using ProteinPilot against a database with concatenated target and decoy sequences. Using this method, a false discovery rate (FDR) was estimated to identify proteins with a probability of being correct 95% or more of the time. Based on the results of this search, a numeric receiver operating characteristic (ROC) plot (figure 2) showing absolute numbers of correct and incorrect protein identifications indicated how well proteins were correctly discriminated from false positives. The ROC curve showed a steep initial slope, angling towards the top left of the graph, indicating a high degree of sensitivity and specificity. ProteinPilot reports a p-value and an error factor associated with each protein ratio, and an error percentage associated with each peptide ratio. Since the protein ratio statistics were not provided for all proteins, we report the maximum peptide ratio error percentage associated with each protein as an estimate of protein
quantification error. The peptide error percentage is a measure of the error in the calculated peptide ratio, derived from the error for each of the reporter ion peak areas used in the ratio calculation. Of the 1683 proteins found through all experiments, 86 were false positives. Based on the 95% confidence value, 573 proteins were discovered. In the most conservative estimation, 86 of the 573 proteins detected were false positives, as opposed to being equally spread across all discovered proteins.

Figure 2 – ROC plot analysis indicating a high number of true positives and a relatively low level of false positives. Note the unequal scales on each axis. This demonstrates that the samples had a high degree of sensitivity and specificity.

The database search yielded 573 proteins identified at a 95% confidence limit. All proteins provided in supplemental material, provided on web page. Differentially expressed proteins for each experimental condition were processed by IPA in order to determine the pathways and functions involved in a specific stretch. The distribution of the fold-changes for the top differentially expressed proteins associated with known molecular interactions and functions according to IPA bioinformatics are listed in tables 1-4. Table 5 lists proteins that were found in more than one stretch/time condition, and met the GO criteria; involved in apoptosis, activation, neurodegeneration, DNA damage/repair, cellular remodelling or stress responses. Table 6
presents the proteins that were differentially expressed in three different stretch groups, but did not meet the GO criteria.

Table 1 – Top proteins differentially expressed through the 3% 2 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (%Cov95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.

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<tr>
<th>Gene Symbol</th>
<th>Accession</th>
<th>Protein Name</th>
<th>Number of Peptides</th>
<th>Peptide Conf.</th>
<th>Cellular Location</th>
<th>%Cov</th>
<th>%Cov (95)</th>
<th>Fold Ratio</th>
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<td>13.4</td>
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Table 2 — Top proteins differentially expressed through the 3% 24 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (% Cov 95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Accession</th>
<th>Protein Name</th>
<th>Number of Peptides</th>
<th>Peptide Conf.</th>
<th>Cellular Location</th>
<th>%Cov</th>
<th>% Cov (95)</th>
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<td>%Cov</td>
<td>%Cov (95)</td>
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<td>Nucleus</td>
<td>30.9</td>
<td>3.3</td>
<td>-1.65</td>
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Table 3 — Top proteins differentially expressed through the 12% 2 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (%Cov95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.
<table>
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<th>Gene Symbol</th>
<th>Accession</th>
<th>Protein Name</th>
<th>Number of Peptides</th>
<th>Peptide Conf.</th>
<th>Cellular Location</th>
<th>%Cov</th>
<th>%Cov (95)</th>
<th>Fold Ratio</th>
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<td>S-phase kinase-associated protein 1</td>
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<td>P54709</td>
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<td>95</td>
<td>Plasma membrane</td>
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<td>95</td>
<td>Cytoplasm</td>
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<td>NEK7</td>
<td>Q8TDX7</td>
<td>Serine/threonine-protein kinase Nek7</td>
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<td>99</td>
<td>Nucleus</td>
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<td>40S ribosomal protein S5A</td>
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<td>-1.56</td>
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Table 4 – Top proteins differentially expressed through the 12% 24 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (%Cov95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.
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<th>Protein Name</th>
<th>Description</th>
<th>Expression</th>
<th>Location</th>
<th>Fold Change</th>
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<td>Cytochrome P450</td>
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<td>97</td>
<td>Cytoplasm</td>
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<td>40S ribosomal protein 5A</td>
<td>2</td>
<td>99</td>
<td>Plasma Membrane</td>
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<tr>
<td>TWF1</td>
<td>Q12792</td>
<td>Twinfilin-1</td>
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<td>Cytoplasm</td>
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<td>Q9UDY4</td>
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<td>Nucleus</td>
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<td>ANXA4</td>
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<tr>
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<td>Accession</td>
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<td>Cellular Location</td>
<td>Stretch (%)</td>
<td>%Cov</td>
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<td>Cytoplasm</td>
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Table 5 – Top proteins of interest found in multiple stretch/time conditions and confirmed using Gene Ontology analysis
### Table 6 – Proteins found in three stretch/time conditions without meeting Gene Ontology criteria

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<th>Cellular Location</th>
<th>Stretch% for 24 hours</th>
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The top cellular functions that were discovered from the pathways of each time/stretch experiment identified various cellular reactions to biomechanical insult (figure 3). The
relationship of the specific function charts showed change at a cellular level as time or intensity was increased.

**Figure 3** – Values (-log (P)) representing trends in major protein pathways discovered by Ingenuity pathways Analysis. 3A shows the top functional group for the 3% 2 hour stretch, metabolic activity. 3B shows the top group for 3, 24 hours which was cellular activity and organization. 3C shows cell cycle was at the highest level in the 3% stretch for 24 hours, while DNA replication, recombination and repair was the highest at the 12% for 24 hour stretch.

Interaction networks from the 3% for 2 hour stretch have TP53, TNF and TGFβ (figure 4) as primary hubs. Of the differentially expressed proteins observed in this condition, 17 were specific to this stretch condition alone, 6 were found in one other stretch condition and 3 were found in 3 stretch conditions (Tables 1,5,6).
Figure 4 – Integration of the identified proteins into the canonical pathways for astrocyte cell stretch of 3% for 2 hours using Ingenuity Pathway Analysis. Proteins were identified as being within the nucleus, the cell membrane, extracellular or intracellular. Solid lines indicate direct interaction. Dashed lines indicate indirect interactions. Red molecules were upregulated and green molecules were downregulated. White molecules were not user specified, but were incorporated into the network through relationships with other molecules. Of particular note were the network hubs centered on TP53, TNF and TGFβ1.

The cells that were stretched at 3% for 24 hours produced merged networks again showing the same three principle hubs; TP53, TNF and TGFβ (figure 5). 15 proteins were found to be specific to this stretch condition. 9 proteins were confirmed in two conditions and 2 in three conditions (Tables 1, 5, 6).
Figure 5 – Integration of the identified proteins into the canonical pathways for astrocyte cell stretch of 3% for 24 hours using Ingenuity Pathway Analysis. Proteins were identified as being within the nucleus, cell membrane, extracellular or intracellular. Solid lines indicate direct interaction. Dashed lines indicate indirect interactions. Red molecules were upregulated and green molecules were downregulated. White molecules were not user specified, but were incorporated into the network through relationships with other molecules. Of particular note were the network hubs centered on TP53, TNF, TGFβ1 and CASP3.

When the cells were stretched at 12% for 2 hours the resulting networks created by the proteins detected in these samples again had hubs at TP53, TNF and TGFβ. In addition, there was a
greater role played by specific caspases (CASP3 and CASP8) (figure 6). 8 proteins were found in this stretch condition, with 5 in two conditions, and 2 were found in three conditions (Tables 1, 5, 6).

Figure 6 – Integration of the identified proteins into the canonical pathways for astrocyte cell stretch of 12% for 2 hours using Ingenuity Pathway Analysis. Proteins are identified as being within the nucleus, the cell membrane, extracellular or intracellular. Solid lines indicate direct interaction. Dashed lines indicate indirect interactions. Red molecules were upregulated and green molecules were downregulated. White molecules were not user specified, but were incorporated into the network through relationships with other molecules. Of particular note were the network hubs centered on TP53, TNF, TGFβ1, CASP3 and CASP8.
When the cells were stretched at 12% for 24 hours, as in the previous conditions, the primary hubs involved in these networks were TGFβ, TNF and TP53 (figure 7). In this final group, 18 of the differentially expressed proteins were unique to this condition, 14 were present within two conditions, while 2 were observed in three conditions (Tables 1, 5, 6).

**Figure 7** – Integration of the identified proteins into the canonical pathways for astrocyte cell stretch of 12% for 24 hours using Ingenuity Pathway Analysis. Proteins were identified as being within the nucleus, the cell membrane, extracellular or intracellular. Solid lines indicate direct interaction. Dashed lines indicate indirect interactions. Red molecules were upregulated and green molecules were downregulated. White molecules were not user specified, but were incorporated into the network through relationships with other molecules. Of particular note were the network hubs centered on TP53, TNF, TGFβ1, and CASP3.
Western blotting of lysate using the 12%, 2 hour stretch condition for ANXA4, GOLGA2 and αB-Crystallin (figure 8) were performed to validate the protein screening results. These proteins were chosen for validation purposes as they demonstrated regulation and antibodies were readily available. Western blots showed changes in regulation that closely matched those found using iTRAQ: ANXA4 was upregulated 1.5 fold as compared to iTRAQ’s 1.99 fold increase; GOLGA2 was upregulated 2.3 fold compared to iTRAQ’s 2.58; and αB-Crystallin was downregulated 1.7 fold compared to a 2.77 fold decrease with iTRAQ. Normally, the astrocytes remain in a ‘quiescent’ mode, however when insulted through injury or disease, they can become ‘reactive’, as evidenced by changes in cell morphology and increased staining of marker proteins, most prominently the intermediate filament component GFAP. The results show an increase of 1.9 times the level of GFAP as compared to the control (figure 9).

**Figure 8** – Validation of iTRAQ proteomic results by Western blot for specific markers. A) Cultured human ONH astrocytes were submitted to 0%, 3%, OR 12% stretch for 2 hours. Total proteins were collected in lysis buffer, concentrated, and quantified by Bradford assay. Equal amounts of protein from each condition were probed by Western blot for three markers that were differentially regulated in the iTRAQ results; GOLGA2, ANXA4, and...
CRYAB. Detection and band densitometry was performed with a Li-Cor infrared imager and marker bands were normalized to actin. B) Fold change was calculated from 4 separate cell lines by dividing the normalized result from each condition by the 0% stretch result and averaged, as alongside the original iTRAQ stretch results for comparison. (WB: western blot, iTRAQ: preliminary iTRAQ results)

**Figure 9** – Increased staining of GFAP in ONH astrocytes submitted to increasing amounts of stretch. A) Cultured human ONH astrocytes were submitted to 0%, 3%, OR 12% stretch for 2 hours. Total proteins were collected in lysis buffer, concentrated with ultrafiltration columns, and quantified by Bradford assay. Equal amounts of protein from each condition were probed by Western blot for GFAP and actin. B) Detection and band densitometry was performed with a Li-Cor infrared imager and GFAP bands were normalized to actin. Fold increase was calculated by dividing the normalized result from each condition by the 0% stretch result.

### 3.6 Discussion

iTRAQ-based proteomics analysis enables the investigation of the mechanisms of disease using a multiplexed approach. This is the first time that the iTRAQ technique has been used to explore optic nerve head glial cell activation, believed to be involved in the very earliest stages of the development of glaucomatous optic neuropathy. In this study we investigated the differential protein expression of ONH astrocytes that were stretched by either 3% or 12%, for 2 or 24 hours. The stretch magnitudes were based on finite element models from our previous research. These models directly contribute to the various stretch and time combinations that were chosen for our experiments. Sigal et al. predicted a peak strain within the lamina cribrosa approaching 15% when IOP was raised to 50mmHg. The 3% strain was used to represent a much lower insult, and may in future be considered as a baseline level of strain. Our use of the 2-hour and 24-hour time points was to compare the cellular reactions following different exposure to
insult, a model which has been used previously.\textsuperscript{46, 47} Due to the experimental design, the resulting proteins were not expected to be similar across all conditions (Tables 5,6). Wanner et al.\textsuperscript{69} stretched brain astrocytes in order to investigate glial scar formation and axonal growth inhibition. They used rat cortical astrocytes and stretched the cells for 50ms. This is very different to our own models that use human optic nerve head astrocytes stressed over periods of hours using differential levels of strain. However our work is similar with respect to the level of cellular activation and GFAP expression. Further refining this work through proteomic research we have been able to detect biomarkers for glial cell activation.

Over 1600 total proteins were found across all stretch/time combinations, of which 573 were discovered at a 95\% confidence limit. The receiver operating characteristic (ROC) plot, depicting true and false positive rates for the iTRAQ technique, indicated a high level of sensitivity and specificity, supporting the use and experimental design of this technique in our study.

A number of observed proteins were potential markers of astrocyte activation. These proteins were chosen primarily based upon their ontology, as well as for their potential role in mediating the response of the physical stress on the cellular membranes to actual transcriptional changes within the nucleus. This included astrocytic phosphoprotein (PEA15) which was up-regulated 1.5 fold in the 12\%, 2 hour stretch condition. PEA15 was first identified by Araujo et al.\textsuperscript{70} Renault et al.\textsuperscript{71} described its role in the control of apoptosis and cell cycle within astrocytes. It has also been shown to play a significant role in mitogen-activated protein (MAP) kinases, which respond to extracellular stimuli. Specifically, it activates the extra-cellular signal receptor – activated kinases (ERK1/2).\textsuperscript{72} This is an indicator that PEA15 is an early response protein to physical stretch. It is predominantly expressed within the central nervous system and,
considering its role within cells targeted for apoptosis and the fact that it was up-regulated within our experiments, it may provide an important target of future research.

UDP-glucose dehydrogenase was characterized by Spicer et al.\(^7^3\) and is noted for its role in converting UDP-glucose to UDP-glucoronate, which is an essential component to the glycosaminoglycans, such as hyaluronans which are needed for cell proliferation and migration. It was found upregulated 1.72 fold in the 3% for 24 hour stretch. It is known that in the absence of this enzymes activity, embryogenesis fails to occur.\(^7^4\) Further research has indicated that defects in the production, activity or expression of this protein may lead to more general defects in proteoglycan or glycosaminoglycan function.\(^7^3\) It has also been shown that cells that are treated with TGFβ gave an increase in production of UDP-glucose dehydrogenase.\(^7^5\) Similar results were reported following hypoxia.\(^7^6\) Clarkin et al.\(^7^7\) went on to implicate the role of UDP-glucose dehydrogenase in mitogen-activated protein kinase (MAPK) pathways and demonstrated how it may be a good therapeutic target due to its role in the conservation of the extracellular matrix.

Annexin A4 (ANXA4) was up-regulated 2.0 and 1.6 fold in 3% and 12% stretch for 2 hours, and 1.58 fold in the 12% stretch for 24 hours. This protein is a member of the lipocortin family of calcium-dependent phospholipid-binding proteins\(^7^8\) and is interesting as other members of its family have previously been reported to be up-regulated in animal models of glaucoma.\(^7^9\) In this latter study, ANXA1 and ANXA3 were proposed to play a role in membrane repair or in the aggregation of vesicles known to occur in axonal transport blockade. ANXA2 has also been shown to play a role in angiogenesis through co-localization and binding with a member of the s100 family.\(^8^0\) Although ANXA4 function is not well documented, it may play a role similar to others in its family. It has been shown to have numerous other cellular functions including cell
division, Ca2+ signalling, growth regulation and inflammation, and to co-localize on cell membrane surfaces, which may indicate a role in the reaction to physical stress. It also plays an important role in modulating the NF-κB signalling pathway, which is important in regulating numerous genes involved in the immune response, cell proliferation, differentiation, survival and apoptosis.

Another protein of interest belongs to the family of s100 proteins and is known to regulate the progression of inflammation, innate immunity, tissue damage and wound healing. In particular, s100-a13, is a protein that has been reported to participate directly in the angiogenic process, particularly of cancerous tissue types. This protein was upregulated 2.00 fold and 1.52 fold in the 3% for 2 hour and 12% for 2 hour stretch respectively. Previous research on this protein in the eye showed an up-regulation following an inflammation-associated corneal neovascularization. S100-a13 has also been shown to play a role in damage-associated molecular patterns (DAMPs), which interact with the NF-κB pathway and various inflammatory cytokines such as IL-1B and IL-8, which implicates this important pathway in our model.

The functional pathways in figure 3 describe how an increase in strain percentage or duration can affect the regulation of the pathway. Normal protein patterns are associated with low percentage strain, with cellular repair and DNA repair associated with the higher percentage strain and longer exposure. In figure 3A metabolic activity is the most regulated pathway in the 2 hour stretch models, with the highest levels seen following 3% stretch. This would indicate that at 3%, 2 hours, the cells are adjusting to the initial insult. There was no major increase of other diseased or damaged cell functional pathways following 3% stretch. In cellular assembly and organization (figure 3B), the largest change was seen following the 3% for 24 hour stretch. Regulated proteins, such as golgi proteins (GOLGA2) and collagens (COL1A1, COL1A2), are associated
with normal cellular processes such as cellular division\textsuperscript{91} and cellular structure of the eye.\textsuperscript{92} Cell cycle functions (figure 3C) following 12\% strain resulted in proteins that are associated with both normal and diseased cellular processes. Normal functioning proteins include kinesin family 14 (KIF14)\textsuperscript{93} and kinase anchor proteins (AKAPs), both of which are important in microtubule organization.\textsuperscript{94} However AKAPs are also associated with cancer\textsuperscript{95-97} and is an important anaphase promoting complex.\textsuperscript{98} Figure 3D demonstrated that following 12\% stretch for 24 hours, DNA replication, recombination and repair showed the greatest functional response. Of the top proteins involved in DNA replication, recombination and repair, promyelocytic leukemia (PML) is one of the most important and has been shown to play in role in apoptosis, senescence and cell death. It has recently been proposed as a possible target for therapeutic approaches to a variety of diseases\textsuperscript{99} and has also been associated with single nucleotide polymorphisms in glaucoma.\textsuperscript{100} Together figure 3, demonstrates an increasing response to increasing biomechanical strain.

In summary, we investigated multiple stress interactions of time and stretch on the activation of human optic nerve head astrocytes. Lysates from each time/stretch condition were prepared as part of a 6-plex iTRAQ quantitative proteomic analysis with the goal of better understanding the activation of astrocytes and the role astrocyte activation plays in the pathogenesis of glaucoma. An additional aim was to identify potential biomarkers for ONH astrocyte activation. We investigated proteins involved in cellular differentiation and morphogenesis that may have a role in the early activation of astrocytes, eventually leading to the apoptotic death of retinal ganglion cells in glaucoma. The proteomics strategy used has been previously validated to identify proteins of interest and potential biomarkers.\textsuperscript{101-103} We have identified a number of proteins of potential interest, including PEA15, UDP-glucose dehydrogenase, ANXA4 and s100a13.
3.7 Acknowledgements

Support for this work is acknowledged from the Canadian Institutes of Health Research, the American Health Assistance Foundation, the Glaucoma Research Society of Canada, and scholarships to RR from the Peterborough K.M. Hunter Studentship and the Vision Science Research Program of the Toronto Western Research Institute. Special thanks to Darren Chan and Cindy Guo for their technical support of the Western blots.
3.8 References


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Proteomics Analyses of Activated Human Optic Nerve Head Lamina Cribrosa Cells Following Biomechanical Strain

Ronan Rogers, MSc 1, 2, 3
Moyez Dharsee, BSc 4
Suzanne Ackloo, PhD 4
John Flanagan, PhD 1, 2, 3, 5

6. Institute of Medical Science, University of Toronto, Toronto
7. Vision Science Research Program, Toronto Western Research Institute, University Health Network
8. Department of Ophthalmology & Vision Science, Toronto Western Hospital, Toronto
9. Ontario Cancer Biomarker Network, Toronto, Ontario, Canada
10. School of Optometry, University of Waterloo, Waterloo, Ontario, Canada

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Number of Figures: 4

Corresponding Author:
Ronan Rogers
Institute of Medical Science, University of Toronto, Department of Ophthalmology & Vision Science, Toronto Western Hospital, Ontario, Canada
Phone: (416) 603-5800 ext. 2850
Email: ronan.rogers@utoronto.ca
4.1 Abstract

Purpose: To determine protein regulation following activation of human, optic nerve head (ONH), lamina cribrosa (LC) cells in response to mechanical strain.

Methods: LC cells were isolated and grown from donor tissue in specific media at 37°C and 5% CO₂ humidified incubator. Cells were grown to confluence on collagen I coated BioFlex culture plates, rinsed with DPBS and left for 24 hours in serum-free media. They were subjected to 3% or 12% cyclic equi-axial stretch for 2 or 24 hours using the Flexercell FX-4000. Control cells were also grown on the coated BioFlex culture plates, serum-deprived and incubated without stretch for 24 hours. Nano LC-MS/MS analysis using iTRAQ labelling was used to determine protein regulation.

Results: 526 proteins were discovered at a 95% confidence limit. Analysis of associated pathways and functional annotation indicated that the LC cells reacted in vitro to mechanical strain by activating pathways involved in protein synthesis, cellular movement, cell-to-cell signalling and inflammation. These pathways indicated consistent major protein hubs across all stretch/time conditions involving transforming growth factor-β1 (TGFβ1), tumour necrosis factor (TNF), caspase-3 (CASP3) and tumour protein-p53 (p53). Amongst proteins of particular interest, also found in multiple stretch/time conditions, were bcl-2-associated athanogene 5 (BAG5), nucleolar protein 66 (NO66) and eukaryotic translation initiation factor 5A (eIF-5A).

Conclusion: Pathway analysis identified major protein hubs (TGFβ1, TNF, CASP3, p53) and pathways all previously implicated in cellular activation and in the pathogenesis of glaucomatous optic neuropathy. Several specific proteins of interest (BAG5, NO66, eIF-5A) were identified for future investigation as to their role in ONH glial activation.
4.2 Introduction

The main characteristic of primary open angle glaucoma (POAG) is the progressive death of retinal ganglion cells. Loss of vision results as these cells undergo apoptosis. A rise in intracocular pressure (IOP) is a known risk-factor for glaucoma,\(^1\)\(^2\) and reducing the pressure within the eye has been shown to be beneficial to the clinical management of the disease.\(^1\)\(^3\)\(^4\) Currently, the basic mechanisms by which elevated IOP leads to the apoptotic death of retinal ganglion cells is poorly understood.

Lamina cribrosa (LC) cells are one of the major glial cell types found within the optic nerve head,\(^5\)\(^6\)\(^7\) and their primary role, although still not clearly understood, is thought to revolve around maintenance and protection of retinal ganglion cell function.\(^7\) They are glial-like with staining characteristics that are similar to astrocytes, \(i.e.\) positive for neural cell adhesion molecule (NCAM), vimentin, desmin and paired box gene -2 (Pax-2), but differ in that they stain negatively for glial acidic fibrillary protein (GFAP) (Fig. 1). They have been shown to react to cyclical strain by up-regulating tumour growth factor-\(\beta\)1 (TGF\(\beta\)1) and matrix metalloproteinase-2 (MMP2).\(^7\)\(^8\) Release of these mediators indicates that these cells may have a mechano-sensory function which responds to physical strain. It has been known for some time that LC cells are very similar in morphology and characterization to that of trabecular meshwork (TM) cells.\(^9\)\(^10\) There has been a considerable amount of work published on the cytoskeleton and functional properties of TM cells\(^11\)-\(^14\) however there has been relatively little research concerning LC cells.\(^10\) A sign of glaucoma is the loss of neural rim tissue in the optic nerve head along with compression, stretching and rearrangement of the cribriform plates of the lamina cribrosa.\(^15\)-\(^19\) There are a number of connective tissues that are remodelled within the glaucomatous ONH, and the activation of the glial cells may be playing a role in this process. After injury, reactive glial cells have been shown to synthesize extra-cellular matrix (ECM) proteins such as tenascin,
laminin and chondroitin sulfate proteoglycan, which can remodel the microenvironment of the neural tissue, possibly providing boundaries to isolate damaged neurons or prevent the migration of inflammatory cells.\textsuperscript{20} A number of immunohistochemical and molecular biological techniques have documented the changes in the macromolecular components of the ECM. These changes have been documented in various collagen types, basement membrane components, glycosaminoglycans, elastin, tenascin and fibrillin which are believed to be playing a role in the modification of the ONH.\textsuperscript{21-28} Actin has been shown to arrange in a polygonal nature, named cross-linked actin networks (CLANS), as a possible precursor to the development of glaucoma. This has been particularly noted in the TM.\textsuperscript{27} In an ongoing parallel study, we are looking into the modification of CLANS of the ONH following insult. The stress fibres of LC cells appear to have properties similar to those of the TM, in that they respond to and possibly resist the biomechanical strain that occurs in the normal and abnormal environment of the optic nerve head.\textsuperscript{29} The organization of all the tissues that make up the lamina cribrosa contribute to the ability of that tissue to adapt to normal changes in IOP, however this organization is disrupted in those with glaucomatous optic neuropathy. Lamina Cribrosa cells were initially characterized by Hernandez et al.,\textsuperscript{5} found to be a flat and polygonal in shape, and grow in a monolayer. These differ from fibroblasts which multilayer and have a much more prominent intracellular space.\textsuperscript{5} They stain positive for vimentin and desmin, which differentiates them from desmin-negative staining fibroblasts.\textsuperscript{30} For a recent review on glia refer to the supplemental issue in Nature Insight.\textsuperscript{31-35}
Figure 1 – Lamina Cribrosa Cells, dissected from post-mortem human optic nerves, were characterized and seeded onto flexible culture plates. These cells show positive staining for NCAM, Vimentin, Desmin, Pax-2, S-100, and α-smA. There is negative staining for GFAP, and A2B5. This panel would indicate that these are not Astrocytes based primarily on their lack of GFAP reactivity. (40 X)

We have developed models used to mimic the in vivo biomechanical environment in the LC by growing human ONH LC cells on flexible, silastic membranes and subjecting the cells to deformation. The level of strain used in these models was previously calculated by Sigal et al.\textsuperscript{16, 17, 36} using finite element modelling that are experienced by cells of the optic nerve head.
depending on the level of intraocular pressure. This is accomplished using the Flexercell® Tension Plus FX-4000T system (Flexcell International Corporation, Hillsborough, NC) which allows for the controlled cyclical stretch, between 1% and 12% deformation up to 1Hz, for chosen periods of time. Higher degrees of deformation are possible but only at a lower frequency. We wanted to ensure that 1 Hz was used. Research similar to this has been conducted previously with the Flexercell system. Other studies have analysed the protein pathways of cells from the ONH following exposure to hydrostatic pressure, although it is not clear from a biomechanical perspective, what type of stress hydrostatic pressure will induce. Ethier et al. compared the effects of hydrostatic pressure and gas tension within the culture medium on cell migration, morphology and α-tubulin architecture. They reported that an increase in hydrostatic pressure had no effect, and that the biological effects previously reported were most likely artefacts due to hypoxia within the medium. We are confident that our approach of inducing biomechanical strain using equi-axial stretch is a more realistic model of the conditions found within the human lamina cribrosa. However, potential artefacts associated with cell culture research in general needs to be taken into consideration, as cells may express proteins differently than when in vivo.

Proteomics research is a highly effective approach to the study of disease mechanisms. This is the first time that proteins from primary, ONH LC cells have been analyzed using isobaric tag for relative and absolute quantitation (iTRAQ) based proteomics. We have recently published research using this method to characterize biomarkers and pathways associated with optic nerve head astrocytes. Crabb and colleagues have recently used iTRAQ to investigate the proteomic characterization of ganglion cells in a rat glaucoma model. An advantage of this technique is its ability to determine the relative protein quantity of up to 8 different samples simultaneously, which makes it an ideal method for comparative studies. It has a large dynamic range, being able
to detect both high and low abundance proteins. The main disadvantages are the increase in mass spectrometry time required due to the increased number of peptides and the strict guidelines required for sample preparation. The present work was performed in collaboration with the Ontario Cancer Biomarker Network (OCBN; Ontario, Canada).

We present the total protein analysis of lamina cribrosa cells that had been stressed in a way to reproduce the level of strain predicted by our previous finite element models following a rise in IOP. The ultimate goal was to better understand, through protein analysis, the process of LC cell activation following biomechanical strain.

4.3 Methods

The LC from three healthy human donors (Ages 18, 19, 27) (Eye Bank of Canada, Ontario Division) were dissected into explants and grown in Dulbecco Modified Eagle Serum (DMEM) F12 (4 mM L-glutamine; 1g/L glucose; 1.5 g/L sodium bicarbonate; 10 % FBS; penicillin/streptomycin) until confluent, and in accordance with the human biosafety requirements of the University Health Network. When these cells reached confluence, they were split into T75 flasks and fed with a basic DMEM media. Cultures were maintained in sterile incubators at 37°C and 5% CO₂ and media was changed twice a week. Morphologically, the LC cells are large, flat and stellate and grow in a monolayer. There was a high degree of cell purity (>95% cell type). Cells were grown on 35mm plates after the 3rd passage for the purpose of characterization and allowed to grow to confluence, after which they were washed twice with DPBS (plus Mg and Ca). The cells were then fixed in formalin, permeabilized in Triton-X, and specific primary and secondary antibodies were added. Primary human lamina cribrosa cells were characterized in a similar way to previous publications with negative staining for GFAP, and positive for α-smooth muscle actin (α-smA), Pax-2, vimentin, desmin and S100. There was also negative
staining for A2B5, which indicates that the cells are non-neuronal. However, these cells are distinct from myofibroblasts by having a positive stain for desmin. They do not multilayer like scleral fibroblasts which we have grown in our lab. They are distinct morphologically by being broad, flat and polygonal, as opposed to stellate like the astrocytes (Figure 1).

**Stretch Parameters:** For the stretch experiments, cells at the 4th passage were seeded onto 6-well, uncoated flexcell plates (Flexcell International Corporation, Hillsborough, NC), which were coated with collagen type I (Rat Tail Collagen, BD Biosciences, Franklin Lakes, NJ, USA), and allowed to grow to confluence. Using the Flexercell® Tension Plus FX-4000T system, a programmable amount of equi-axial strain was applied to the cells through the use of a vacuum pump and a custom base-plate. Four plates per experiment were stretched while four control plates were placed in the same incubator beside the base plate. Control cells were also grown on the coated BioFlex culture plates. All cells were serum deprived for 24 hours prior to stretching, which was performed at a 1Hz cycle of 0% to 3%, or 0% to 12% for either 2 hours or 24 hours. This produced six experimental results for each cell line.

**Protein Isolation:** Total protein was isolated from experimental cells to be used in proteomics analyses through the use of a radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris pH 7.5; 150 mM sodium chloride (NaCl); 1% Nonidet P-40; 0.5% Sodium Deoxycholate; 0.1% SDS; Complete Protease Inhibitors (Roche)). A volume of RIPA was added to each well (300ul) and allowed to sit at room temperature for 15 minutes. The six wells from each plate were scraped down, combined into an eppendorf tube, aspirated and centrifuged at 10,000 rpm for 10 minutes. The cleared protein lysate was then prepared using a Total Protein Clean-up Kit (Norgen Biotek Corp., Thorold, ON) according to the manufacturer’s directions.
**Proteomics Analysis:** Proteomics were performed by the OCBN. Six LC cell lysates were analyzed; a 2 hour control, a 24 hour control, a 3% for 2 hour stretch, a 3% for 24 hour stretch, a 12% for 2 hour stretch and a 12% for 24 hour stretch (each lysate was a combination of the respective treatment from the three cell lines). Figure 2 represents the workflow of the iTRAQ analysis.

**Figure 2** – Workflow of the iTRAQ proteomic analysis. The six cell samples were labelled with their respective isobaric tag and analyzed by Liquid Chromatography/MS/MS. The peak MS/MS charge from each tag indicates which sample is being measured and its quantity. The subsequent MS/MS fragmentation pattern identifies the peptides.
Digestion and Labelling: Protein was extracted from cell lysates using a Norgen kit. The concentration was determined using a micro-bicinchoninic acid (BCA) assay kit (Thermo Scientific, USA). One hundred micrograms of protein from each condition was processed for iTRAQ labelling using a six-plex approach. Briefly, the proteins from each condition were de-natured, reduced, alkylated, trypsin digested and then labelled with the appropriate iTRAQ tags. After labelling, the 100 μg aliquots were pooled into one sample.

Strong-Cation Exchange Chromatography: Each pooled sample was fractionated using strong cation exchange (SCX) chromatography using a Thermo BioBasic SCX column, with 0.2mm internal diameter and 10cm length. Each sample was diluted with the loading buffer (15 mM potassium dihydrogen phosphate (KH₂PO₄) in 25% acetonitrile (CH₃CN), pH 3.0) to a total volume of 2 mL and the pH adjusted to 3.0 with phosphoric acid. Samples were filtered using a 0.45μm syringe filter (Millipore, Canada) before loading onto the column. Two millilitres of diluted sample were injected into the SCX system. Separation was performed using a linear binary gradient from 0% solvent B to 50% solvent B in 40 minutes (solvent B: 2% H₂O : 98% CH₃CN and 0.1% formic acid). The gradient was ramped to 100% Solvent B in 2 min. and held for 60 min.. Buffer A was identical in composition to the loading buffer, while Buffer B was the same as Buffer A but with the addition of 350 mM potassium chloride (KCl). After a 2 min. delay to evacuate the void volume, fractions were manually collected every 2 min. to the end of the gradient. The last fraction was the 2 min. block after the ultra-violet (UV) signal returned to baseline. The fractions were dried by speed vacuuming and re-suspended in 0.1% formic acid. The contents of each SCX fraction were resolved by C18 reversed-phase (RP) liquid chromatography.
**Reversed-Phase Chromatography:** The Nano Liquid Chromatography-Ultra (Eksigent Technologies, USA) consists of a trap column (300μm ID) and an analytical column (75 μm ID) packed with 5μm, 300Å Zorbax SB-C18 beads. The analytical column is home-made at OCBN. Separation was performed using a linear binary gradient where solvent A is 98% H₂O: 2% CH₃CN and 0.1% formic acid and solvent B is 2% H₂O : 98% CH₃CN and 0.1% formic acid, and at a flow-rate of 300 nL/min with a 60 minute gradient to 30% B. The equivalent of 2 µg of protein was injected.

**Liquid Chromatography-Mass Spectrometry (Liquid Chromatography/MS/MS):** The eluant from the Nano Liquid Chromatography system was coupled to a quadrupole time-of-flight mass spectrometer (QSTAR® Elite, AB Sciex, USA), through an electrospray ionization source equipped with a 15 μm ID emitter tip. After each survey scan, from m/z 400 to m/z 1500, three of the most intense ions with a charge state of 2 to 4 were selected for MS/MS analysis. These ions were then placed in a dynamic exclusion list for 3 minutes in order to avoid further selection of the same ions. The iTRAQ workflow used Analyst® QS software, which is an integrated validation and quality control program found in the Liquid Chromatography/MS/MS.

**Data Processing and Analysis:** Relative quantification and protein identification were performed with the ProteinPilot™ software version 2.0 (AB SCIEX, USA) using the Paragon™ algorithm as the search engine. Each MS/MS spectrum was searched against a concatenated forward and reverse database of human protein sequences (Swiss-Prot, 22/07/2008). The search parameters allowed for 8-plex iTRAQ, QSTAR Elite ESI, trypsin digestion, cysteine modification by methyl methanethiosulfonate (MMTS), homo sapiens, and biological modifications programmed in the algorithm (which include phosphorylations, amidations, and semi-tryptic fragments). The detected protein threshold (unused protscore (confidence)) in the
software was set to 0.05 to achieve 10% confidence, and identified proteins were grouped by the ProGroup algorithm (AB SCIEX, USA) to minimize redundancy. A minimum protein confidence threshold of 10% was used for the ProteinPilot database search and only those proteins with at least 95% confidence and at most 5% false discovery rate (FDR) were considered in subsequent analysis of putative differential expression. The bias correction option was executed. Proteins without quantitative information, i.e. only one iTRAQ ratio, were deleted from the list of identified proteins. Differentially expressed proteins were defined as those showing an absolute fold-change of at least 1.5 relative to time-matched controls. Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com, version 8.7) was used to determine pathways and functions implicated in stretching the cells for the prescribed amount of time. For each condition, the IPA Core Analysis feature was used to construct molecular interaction networks based on relationships between observed proteins and with other molecules, as annotated in the Ingenuity Knowledge Base. The resulting networks were then merged (using the Merge Networks feature) through the introduction of additional relationships. This analysis also provided a mapping of observed proteins to known cellular functions and processes; a p-value based on a right-tailed Fisher Exact Test was associated with each functional category found to be enriched in the expression dataset. In the right-tailed Fisher's Exact Test, only over-represented functions or pathways (those that have more Functions/Pathways/Lists Eligible molecules than expected by chance) are significant. Under-represented functions or pathways ('left-tailed' p-values) which have significantly fewer molecules than expected by chance are not shown. Pathways analyses such as these allow for the creation of networks based on specific cell and tissue types, such as neural or organ related. Unfortunately at this time there is not specific database for glial cells. These pathways represent the most current scientific literature available and are changing on a weekly basis as more research is published. The pathways constructed are
the most up to date at time of publication, but are liable to change as new literature becomes available. Gene Ontology (GO) analysis was also used on the resulting proteins. This type of analysis allowed for the grouping of proteins into relevant functional groups that are pertinent to our research, specifically apoptosis, activation, neurodegeneration, DNA damage/repair, cellular remodelling and/or stress responses. The importance of using this analysis was to reduce the large number of discovered proteins to those that were most likely the result of cell activation.

**iTRAQ Calibration:** Before samples were analysed, 5 fmol of a bovine serum albumin (BSA) digest was used to determine the quality of the Liquid Chromatography/MS/MS system. The sample was required to show at least 20% sequence coverage with peptide scores ≥20 (ProteinPilot 2.0). Protein sequence identification was assigned with ProteinPilot 2.0 using a concatenated database of target and decoy sequences. This method enabled the calculation of false discovery rate (FDR), and the number of proteins identified with greater than a 95% probability of being correct at an FDR of 0.05. A stringent error tolerance, represented by the minimum 95% protein confidence score, was used to identify proteins with a high likelihood of correct sequence identification. In addition, the decoy database search was conducted to derive a robust estimate of local FDR, as described in Tang et al.. From the results of this search, a numeric receiver operating characteristic (ROC) plot showing absolute numbers of correct and incorrect protein identifications was generated to indicate correctly identified sequences in relation to false positive identifications. The false positive rate was approximately one in twenty.

ProteinPilot reports a p-value and an error factor associated with each protein ratio, and an error percentage associated with each peptide ratio. Since the protein ratio statistics were not provided for all proteins, the maximum peptide ratio error percentage associated with each protein as an estimate of protein quantification error was used. The peptide error percentage is a measure of
the error in the calculated peptide ratio, derived from the error for each of the reporter ion peak areas used in the ratio calculation.

4.4 Results

The ProteinPilot database search yielded 526 proteins identified at a 95% confidence level at an FDR threshold of 0.05. Of the 1792 proteins found through all experiments, 90 were false positives. In the most conservative estimation, 90 of the 526 proteins detected were false positives, as opposed to being equally spread across all discovered proteins. Differentially expressed proteins for each experimental condition were processed by Ingenuity Pathways Analysis (IPA) software to determine pathways and functions involved in a specific stretch. The distribution of the fold-changes for the top 20 differentially expressed proteins associated with known and unknown molecular interactions and functions for the 12% stretch for 2 hours are listed in Table 1. Table 2 lists proteins that were found in more than one stretch/time condition, and either met the GO criteria (involved in apoptosis, activation, neurodegeneration, DNA damage/repair, cellular remodelling or stress responses) or did not. The appendix contains the proteins discovered in all remaining time/stretch conditions.

Table 1 – Top differentially expressed proteins associated with known and unknown molecular interactions (IPA analysis) and functions for 12% stretch at 2 hours.

<table>
<thead>
<tr>
<th>Lamina Cribrosa Cells</th>
<th>Insult</th>
<th>Protein Name Abbreviation</th>
<th>Protein Name</th>
<th>Cellular Location</th>
<th>Fold Ratio</th>
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</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td>12% for 2 Hours</td>
<td>IMPDH2</td>
<td>IMP (inosine monophosphate) dehydrogenase 2</td>
<td>Cytoplasm</td>
<td>2.834</td>
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**Down-regulated**

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The top cellular functions that were discovered from the pathways of each time/stretch point identified various cellular reactions to stress (Figure 3). The relationship of the specific function charts indicated what was occurring on a cellular level as time or intensity was increased. These functions are the result of our discovered iTRAQ proteins that are associated with specific trends based on peer-reviewed publications. These trends are automatically compiled and given confidence values from Ingenuity Pathways Analysis. A trend of increasing protein synthesis can be seen as the cells were subjected to greater degrees of stretch as well as longer times of exposure. A similar trend is seen regarding cellular movement, indicating that proteins discovered were involved in physical reorganization. Cell-to-cell signalling and the inflammatory response are slightly different in that they exhibit a greater response during the 24 hour stretch compared to the 2 hour stretch.
The results for the LC cells at the 3% for 2 hour stretch showed the top scoring network included a number of proteins that were either up- or down-regulated with interactions concentrated around tumour necrosis factor (TNF) and tumour protein 53 (p53). Of all differentially regulated proteins in this stretch, 9 were specific to the stretch, 13 were found in two stretches, while 2 were found in three of the stretch conditions. Proteins found in multiple conditions were further analyzed using gene ontology (GO) annotation, and highlighted if known to be involved in
apoptosis, activation, neurodegeneration, DNA damage/repair, and cellular remodelling or stress response.

The results of the 3% stretch for 24 hours produced networks primarily interacting with TNF and p53. 22 proteins were found to be specific to this stretch, 11 were found in 2 stretch conditions and 2 were found in 3 stretch conditions.

The 12% stretch for 2 hours generated merged networks that indicated transforming growth factor beta 1 (TGFβ1), TNF, p53 and caspase-3 (CASP3) continue to play a significant role (Figure 4) 20 proteins were unique to this stretch, while 13 were found in 2 conditions and 2 were found in 3 distinct stretch conditions.

Molecular and cellular functions from the 12% stretch for 24 hours once again centred on TNF, TGFβ1 and p53. A total of 21 proteins were found to be unique to this condition; 9 were found in 2 conditions and 3 were found in 3 conditions.
Figure 4 – Integration of the identified proteins into the canonical pathways for LC cell stretch of 12% for 2 hours using Ingenuity Pathway Analysis. Proteins were identified as being within the nucleus, the cell membrane, extracellular or intracellular. A solid line indicates a direct protein-protein interaction; a dashed line indicates an indirect interaction; a dotted line indicates a protein-DNA or protein-RNA interaction. Direct interactions involving observed proteins were displayed as bold lines. Red molecules are up-regulated and green molecules are down-regulated. White coloured molecules were not observed in the experiments, but were incorporated into the network through relationships with observed proteins. Of particular note were the network hubs centered on TGFβ, TNF, p53 and CASP3.
4.5 Discussion

iTRAQ-based proteomics analyses enables researchers to identify potential biomarkers and better understand disease mechanisms using relative quantification and multiplexing approaches.\textsuperscript{38, 51-53} This study, in conjunction with our previously published research\textsuperscript{41} is the first time that a global protein analysis using this technique has been used to examine stress induced glial cell activation. In this study we investigated the differential protein expression of ONH lamina cribrosa cells that were stretched by either 3% or 12% for 2 or 24 hours.

Over 1700 proteins were found in each stretch/time combination, of which 526 were discovered outside the 95% confidence limit. Of the top proteins found for the lamina cribrosa cells within each test condition, based on highest fold-change and confidence, most were located within the cytoplasm, while a few were nuclear or were bound to the plasma membrane. The remaining were either in the extracellular space or the location was not clearly annotated. These results are most likely due to our use of cell lysate rather than cellular media and the analysis of secreted proteins. The receiver operating characteristics (ROC) plot depicting true and false positive protein identification rates for the iTRAQ technique indicated a high level of sensitivity and specificity, supporting its use and the experimental design of our study.

A number of proteins expressed could be considered potential indicators of cellular activation. Here we focus upon the proteins which satisfied the GO-based functional refinement procedure (Table 2) and their putative involvement in the activation of the surrounding astrocytes and the remodelling of the lamina cribrosa. The protein bcl-2-associated athanogene 5 (BAG5) is of interest and was up-regulated 2.2 fold in the 3% and 12% stretch for 24 hours. This protein is a member of the family of molecular chaperone regulators that affect numerous cellular pathways,\textsuperscript{54} and has been shown to enhance dopaminergic neuronal degeneration in tandem with
the chaperone activity of heat shock protein 70 (HSP70).\textsuperscript{55} Specifically it interacts with HSP70 by inhibiting its ability to refold misfolded proteins. One of the most significant findings from the Kalia et al. study\textsuperscript{55} is that over-expression of this protein significantly enhances cell death of neurons within specific areas of the affected brain. It has also been implicated in other neurodegenerative diseases such as Parkinson’s.\textsuperscript{55,56}

Nucleolar protein 66 (NO66) was up-regulated 1.8 and 1.5 fold in the 3\% and 12\% for 2 hours stretch conditions, respectively. This protein is found in the nucleolus and has been shown through various proteomics studies to be highly conserved and play a role in the biogenesis of ribosomes, and in the replication or silencing of certain heterochromatic regions.\textsuperscript{57}

Eukaryotic translation initiation factor 5A (eIF-5A) was the most down-regulated signal within the 12\% for 24-hour stretch condition, at -3.8 fold. eIF5A is the only cellular protein that contains the amino acid hypusine.\textsuperscript{58,59} Vertebrates carry two genes that encode two eIF5A isoforms, eIF5A-1 and eIF5A-2, which, in humans, are 84\% identical.\textsuperscript{60} It is highly conserved in mammals\textsuperscript{61} and has been implicated in numerous disease processes including cancer,\textsuperscript{60,62} dengue fever,\textsuperscript{63} diabetes\textsuperscript{64} and HIV.\textsuperscript{65} We have previously shown an association between eIF-5A and apoptosis\textsuperscript{66} and more recently, that apoptosis induction by eIF-5A involves activation of the intrinsic mitochondrial pathway.\textsuperscript{67}

An important finding through all time and stretch combinations were the primary protein hubs involved in all pathways. TGFβ1, Tumour protein 53 (p53), TNF-α and caspase-3 connected the majority of the discovered proteins. These hubs have previously been linked to inflammatory conditions and matrix remodelling. These hubs were also significant in our study examining the effect of these stressors on ONH astrocytes.\textsuperscript{41} TGFβ1 has been found to be differentially regulated in glaucomatous lamina cribrosa tissue\textsuperscript{68} and is known to play a significant role in the
Thrombospondin is thought to be involved in modulating this protein, and was downregulated 2-fold in the 12% for 2 hour stretch. TGFβ1 mRNA levels were measured in lamina cribrosa cells using a stretch model similar to the one presented here. They found significant increases after both 12-hours and 24-hours of stretch. Another major protein hub was p53. Gene expression of this protein was found to be significantly upregulated in the rat retina in an ischemia/reperfusion model. They also found caspase-3, another major hub in the 12% for 2 hour stretch, to be upregulated. Caspase-3 was found to be upregulated in the retina at both the protein and gene level, again following an ischemic-reperfusion model. Tezel and Wax reported how the apoptotic process involved the caspase-3 pathway, and used caspase inhibitors to block the apoptotic cascade. They also proposed that TNFα was associated with retinal ganglion cell death. All of the major protein hubs listed have been shown to play an important role in the apoptotic cascade that effect in the retinal ganglion cells and their axons in glaucoma.

Figure 3 illustrates the changes in pathway activity experienced by the LC cells with an increase in time or magnitude of strain. The level of protein synthesis increased logarithmically from the initial 3% stretch for 2 hours, up to the 12% stretch for 24 hours. A number of the proteins associated with this trend relate to the metabolism, biosynthesis, and translation of the protein. Specifically several eukaryotic translation initiation factors were involved, which have a direct role in regulating proteolysis and are likely to increase with stress. The level of proteins associated with cellular movement increased in a similar manner, with the highest level seen in the 12% for 24 hour stretch. The cells are likely attempting to accommodate to the increased levels of insult by producing proteins that will allow them to mitigate the stresses being exerted upon them. Specific proteins such as thrombin, insulin-like growth factor binding protein and
integrin alpha have previously been associated with this type of activity.\textsuperscript{78, 79} Within our study, the protein prothrombin was found in three of the four stretches. A slightly different trend was seen for both the cell-to-cell signalling and inflammatory response. The highest levels of change were seen in both the 24 hour stretches as compared to the 2 hour stretches, regardless of the magnitude. It is possible that the cells that are stressed for a longer period of time are more likely to produce proteins that are associated with both of these pathways. In particular, a number of coagulation factors and integrins, associated with cell signalling and cell adhesion, were upregulated.\textsuperscript{80, 81} More time points with the same percentage of stretch will be analyzed in the future in order to better visualize the trends and interactions that are occurring.

We examined the stress response induced by stretch of different magnitude and time course, on human lamina cribrosa cells cultured in vivo from the human optic nerve head. Lysates from four stress conditions were prepared as part of a 6-plex iTRAQ quantitative proteomic analysis with the goal of better understanding LC remodelling and activation. These cells are an important part of the structural anatomy of the optic nerve head, and how they respond to mechanical strain is potentially important in understanding the early pathogenesis of glaucoma.\textsuperscript{29} Determining the role of proteins implicated in this response may lead to a greater understanding of the mechanisms involved in the apoptotic death of the retinal ganglion cells and the remodelling of the lamina cribrosa. We have identified seventeen proteins of potential interest, of which we propose BAG5, NO66 and eIF-5A to be of particular interest in the pathogenesis of glaucoma, the second leading cause of blindness in the world.
4.6 Acknowledgements

Support for this work is acknowledged from the Canadian Institutes of Health Research, the American Health Assistance Foundation, the Glaucoma Research Society of Canada, and scholarships to RR from the Peterborough K.M. Hunter Studentship and the Vision Science Research Program of the Toronto Western Research Institute.
4.7 References


Extracellular Protein Analysis From Human Optic Nerve Head Glia Following Biomechanical Insult Reveals Increased Matrix Remodelling and bFGF Signalling

Ronan Rogers, MSc $^{1,2}$

Jeremy M. Sivak, PhD $^{2,4}$

John Flanagan, PhD, MCOptom$^{1,2,3,4}$

11. Institute of Medical Science, University of Toronto, Toronto
12. Vision Science Research Program, Toronto Western Research Institute, University Health Network
13. School of Optometry, University of Waterloo, Waterloo, Ontario, Canada
14. Department of Ophthalmology, University of Toronto, Toronto, Ontario, Canada

Number of Tables: 1
Number of Figures: 4
Corresponding Author:
Ronan Rogers
Institute of Medical Science, University of Toronto, Department of Ophthalmology & Vision Science, Toronto Western Hospital, Ontario, Canada
Phone: (416) 603-5800 ext. 2850
Email: ronan.rogers@utoronto.ca
5.1 Abstract

**Purpose:** To investigate regulated concentrations of key extracellular signalling proteins in isolated human optic nerve head (ONH) astrocytes and lamina cribrosa (LC) cells in response to pathologically relevant equi-axial stretch.

**Methods:** Human ONH astrocytes and LC cells were isolated and cultured from healthy human donor tissue in specific media at 37°C in a 5% CO\textsubscript{2} humidified incubator. Each cell type was seeded onto coated BioFlex culture plates and grown to confluence. Based on previous modelling, the cells were then subjected to 12% cyclic (1 Hz, sinusoid), equi-axial stretch for 2 hours. Control cells were also grown on the coated BioFlex culture plates and incubated without stretch for the duration of the experiment. Conditioned culture media was collected from each group and analyzed for concentrations of selected signalling proteins by multiplex ELISA. The proteins chosen for analysis were based on pathways implicated by previous work, and included MMP2, MMP9, and TIMP1, related to remodelling of the extracellular matrix, and growth factors BDNF, GDNF, NGF-β, and bFGF.

**Results:** All selected proteins were identified by ELISA. Protein levels ranged from low (NGF-β at 1-2 pg/ml) to high (TIMP1 at 5000-15000pg/ml). For both astrocytes and LC cells, there was an increase in matrix remodelling enzymes and a decrease in growth factors following stretch. bFGF was an exception for the growth factors as it increased following stretch, compared to control.

**Conclusion:** Extracellular proteins within culture supernatant of both astrocytes and LC cells were differentially expressed following 12% stretch for 2 hours. These results suggest a general increase in extracellular matrix remodelling activity and a decrease in growth factors following
glial cell stretch provocation, with the exception of a striking increase in bFGF. bFGF may play an important role in the regulation of matrix remodelling and related injury responses following glial cell activation as a result of biomechanical insult.

5.2 Introduction

Glaucoma is the world’s most common neurodegenerative disease, as well as the second leading cause of blindness worldwide. An elevation of intraocular pressure (IOP) is considered the leading risk factor and is associated with the development and progression of glaucoma. IOP modulation has been established as an effective treatment for the disease (for recent review see Caprioli et al.\textsuperscript{1}). The mechanisms that associate elevated IOP with retinal ganglion cell death are poorly understood, but involve activation of optic nerve and nerve fiber layer (NFL) glia through biomechanical strain and ischemia. Our lab has previously used finite element modeling to quantify the biomechanical environment within the optic nerve head (ONH).\textsuperscript{2-4} Part of this modeling process was a systematic sensitivity analysis that determined the anatomical and biomechanical factors with greatest influence on the response of the ONH to acute changes in IOP. We subsequently developed an \textit{in vitro} human model whereby ONH astrocytes and laminar cribrosa (LC) glial cells, are grown on deformable substrates. In these models we mimic the \textit{in vivo} biomechanical environment in the LC using specific forms of strain, including stretch and compression. Importantly, we are able to generate controlled magnitudes of physiologically and pathologically relevant strain, thus permitting investigations into the early mechanisms modulating glial cell activation. With this approach, we have previously identified differential intracellular protein expression profiles for human ONH cells subjected to various degrees of equi-axial stretch.\textsuperscript{5} Through our analysis, several intracellular protein interactions were discovered that were of interest for both ONH cell types. These profiles were then analyzed by
Ingenuity Pathways Analyses (IPA), which connects relevant proteins into pathways to provide insight into implied molecular interactions. IPA predicted a number of extracellular proteins to be involved in equi-axial stretch responses that may be present in culture supernatant. The purpose of the work presented is to investigate regulation of these extracellular proteins from human ONH astrocytes and LC cells in response to equiaxial stretch.

We chose seven targets of interest, divided into two general categories. The first category is involved in remodelling the extra-cellular matrix, including matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of metalloproteinases 1 (TIMP1). The second category are growth factors including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor beta (NGF-β), and basic fibroblast growth factor (bFGF; FGF2) which has been implicated in a number of neurodegenerative diseases\textsuperscript{6, 7} as well as a modifier of tissue matrices.\textsuperscript{8} However, there are potential artefacts associated with cell culture research that need to be taken into consideration, as cells may express proteins differently than when \textit{in vivo}.

5.3 Methods

Cell Lines

The lamina cribrosa (LC) from 6 healthy human donors (Eye Bank of Canada, Ontario Division) were dissected into explants and grown in Dulbecco Modified Eagle Serum (DMEM) Nutrient Mixture F12 (4 mM L-glutamine; 1 g/L glucose; 1.5 g/L sodium bicarbonate; 10 % FBS; penicillin/ streptomycin) until confluent in accordance with the human biosafety requirements of the University Health Network, Toronto, Canada. The astrocytes were isolated from other cell types using a technique previously described.\textsuperscript{9} Briefly, three explants were split into serum-free astrocyte growth media (AGM –serum, Lonza, Switzerland; astrocyte basal media plus bullet kit
(rhEGF, 0.5 ml; Insulin, 1.25 ml; Ascorbic Acid, 0.5 ml; GA-1000, 0.5 ml, L-Glutamine, 5.0 ml)) for a period of two weeks, when the media was replaced with DMEM/F12. The remaining three cell lines were split directly into DMEM/F12 in order to achieve an LC cell population. The cells were grown to confluence and split in this media until there were enough viable cells to conduct an experiment. Cultures were maintained in sterile incubators at 37°C and 5% CO₂, and media was changed twice a week. Morphologically, the astrocytes and lamina cribrosa cells were cultured in a monolayer and were similar to those previously reported.⁵ ¹⁰-¹² All cultures are of at least 95% purity based on morphology and staining.

**Immunofluorescence**

Cells from each cell line were grown on 35mm plates after the 3rd passage and allowed to grow to confluence after which they were washed twice with DPBS (with Mg²⁺ and Ca²⁺). The cells were then fixed in formalin, permeabilized in Triton-X and the specific primary and secondary antibodies. Primary human astrocytes were characterized by positive staining for glial fibrillary acidic protein (GFAP), desmin, vimentin, S-100, neural cell adhesion molecule (NCAM), and negative staining for A2B5, α-smooth muscle actin (α-smA) and Pax-2. Primary human lamina cribrosa cells were characterized by negative staining for GFAP, and positive for α-smooth muscle actin (α-smA) and Pax-2.

**Stretch**

For the stretch experiments, cells at the 4th passage from three different lines were seeded onto 6-well flexcell plates (Flexcell International Corporation, Hillsborough, NC) and allowed to grow to confluence. Using the Flexercell® Tension Plus FX-4000T system, a programmable amount of equi-axial strain was applied to the cells through the use of a vacuum pump and a custom base-plate. The LC cells and astrocytes were seeded onto pre-coated collagen type IV 6-well plates,
which were additionally coated with collagen type I (Rat Tail Collagen, BD Biosciences, Franklin Lakes, NJ, USA) for astrocytes. Four plates per experiment were stretched while 4 control plates were placed in the same incubator. Control cells were also grown on the coated BioFlex culture plates. All cells were serum deprived for 24 hours prior to stretching, which was performed at a 1Hz cycle at 12% for 2 hours. This gave rise to a total of 2 experimental conditions for each cell line.

**Protein Isolation**

Intracellular proteins were isolated and subjected to iTRAQ proteomics as described previously\(^5\). The resulting list of differentially regulated proteins was further analyzed by Ingenuity Pathways Analysis (Ingenuity Systems Inc., Redwood City, CA, USA). Pathways that demonstrated the connection from intracellular to extracellular proteins were highlighted in green or red depending on up or down regulation.

Extracellular proteins from individual cell lines were left in the cell culture media for direct processing by sandwich multiplexed ELISA performed by Aushon Biosystems (Aushon Biosystems, Inc., Billerica, MA). Briefly, each sample (in duplicate) was incubated for one hour on the array plates that are pre-spotted with capture antibodies specific for each protein biomarker. Plates are decanted and washed four times before adding a cocktail of biotinylated detection antibodies to each well. After incubating with detection antibodies for 30 minutes, plates are washed four times and incubated for 30 minutes with streptavidin-horseradish peroxidase conjugate. All incubations were performed at room temperature with shaking at 200 rpm. Plates were again washed four times before adding a chemiluminescent substrate. The plates were immediately imaged using the Aushon CCD imaging system, and data was analyzed using Aushon Array Analyst software. The amount of luminescent signal produced is
proportional to the amount of each protein present in the original standard or sample. Concentrations were interpolated from a standard curve (run in triplicate). Statistical analysis between control and stretched media samples was performed using a student t-test (p < 0.05).

5.4 Results

The results of the ELISA are presented in table 1 and illustrated in figures 1 and 2. The analysis of both cell types demonstrated similar protein patterns, specifically the matrix remodelling proteins and bFGF. Levels of both MMP2 and MMP9 ranged from 100pg/ml to 600pg/ml. TIMP1 gave approximately ten times the level protein for the two cell types. The growth factors also showed an increase, although at a lower magnitude when compared to the matrix remodelling proteins. The most interesting trend seen in both cell types was the increase of bFGF. However, no statistical significance was detected between stretch and control.

Table 2 – Amount of protein discovered of seven targets found in the extracellular space as determined by three different samples of stressed and control media. Each sample was tested twice through a multiplexed sandwich-ELISA approach. Values are in pg/ml.

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<td>4.82±1.54</td>
<td>2.70±0.66</td>
<td>1.74±0.64</td>
</tr>
<tr>
<td>Astrocyte Control</td>
<td>523.59±362.83</td>
<td>85.70±6.09</td>
<td>14991.40±7133.32</td>
<td>5.31±1.41</td>
<td>5.96±0.34</td>
<td>2.84±0.67</td>
<td>0.96±0.33</td>
</tr>
<tr>
<td>Astrocyte Stretch</td>
<td>625.70±383.46</td>
<td>170.61±63.13</td>
<td>19017.17±10998.33</td>
<td>16.54±7.03</td>
<td>4.98±1.87</td>
<td>2.32±0.58</td>
<td>1.46±0.35</td>
</tr>
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</table>
Figure 1 – Bar chart representing normalized protein levels of stretch vs. control of astrocyte cell culture media. An increasing trend was seen for the MMP2, MMP9, NGF-β and GDNF. The largest change was seen in bFGF, increasing in the stretch sample as compared to the control.
**Figure 2** – Bar chart representing normalized protein levels of stretch vs. control of lamina cribrosa cell culture media. MMPs tended to increase while TIMP1 was decreased. A decreasing trend was seen in all of the growth factors except for bFGF which again showed a large increase compared to other proteins.

### 5.5 Discussion

MMPs are zinc endopeptidases which are typically secreted as pro-enzymes that become active in the extracellular environment.\(^\text{13}\) In primary open angle glaucoma, it has been proposed that remodelling of the extracellular matrix of the lamina cribrosa occurs through the increased expression of cell adhesion molecules and neurotoxic mediators by reactive astrocytes.\(^\text{14}\) MMP2 specifically degrades gelatin, type IV collagen and elastin.\(^\text{15, 16}\) It was noted by Agapova et al.\(^\text{17}\) that similar levels of this protein were found in both normal and glaucomatous optic nerve head tissue, indicating that it may play an important role in tissue homeostasis. This would correlate
with our findings, which did not show a significant increase in MMP2 levels between stretched and control cells from both tissue types. MMP9, also called gelatinase B, is very similar to MMP2 in its substrate specificity. First detected as a product of neutrophils and macrophages, it has subsequently been associated with the opening of the blood-brain barrier and inflammation. In multiple sclerosis patients, it has been shown that an increase in MMP9 serum levels leads to an increase in disease activity. It is involved in various physiological processes including tissue remodelling and organ development. A reduced risk of scarring is correlated with a coding polymorphism in MMP9, which indicates its role in the pathogenesis of various ocular diseases. Modulation of MMP9 levels and activity has been shown during corneal repair and neovascularization. Research into age-related macular degeneration has demonstrated a reduction in the active isoforms of both MMP2 and MMP9 in Bruch’s membrane, which the authors indicate may again be responsible for impaired matrix degradation. MMP9 has also been implicated in diabetic retinopathy, where the active form is upregulated through guanine nucleotide-binding protein pathways (H-Ras). Within the retina, it has been demonstrated that astrocyte-associated MMP9 expression is appropriately localized to cause degradation of the extra-cellular matrix in the nerve fiber layer. This group also went on to implicate the role of MMP9 and plasminogen activators might also play a role in excitotoxic retinal damage. To further this research, Ganesh et al. used two glial toxins to inhibit reactive gliosis and measure the effect on protease-mediated death of retinal ganglion cells (RGC). They found that they were able to reduce levels of MMP9, which lead to a higher survivability of RGCs. Our analyses demonstrated no significant difference in concentration between stretch and control of both cell types, however a trend towards increasing levels was seen in astrocytes. This indicates that MMP9 may play a role in the optic nerve head similar to previous studies. Tissue inhibitor of metalloproteinases 1 (TIMP1) is another glycoprotein that plays an important
role in modifying the behaviour of a number of MMPs. Our results showed a lowering trend in the level of this protein from control to stretch for both cell types, was although it did not reach significance. Previous research in this area has shown that a decrease in the level of TIMP1 can cause an increase in matrix degradation and an impairment of cell growth and survival. TIMP3, while not assessed in this study, has been previously linked to Sorsby’s fundus dystrophy, an age-related macular degeneration (AMD) like disease with choroidal neovascularization. It also has numerous roles in wound healing and regeneration, cell morphology and survival, tumour metastasis, angiogenesis, and the inflammatory response. As reported previously, the levels of TIMP1 found within optic nerve head astrocytes was also markedly higher in both stretch and control tissues as compared to MMPs. This model of decreasing TIMPs and increasing MMPs is in line with the traditional view of their roles in tissue remodelling.

Growth factors were the second group of extracellular proteins detected in the media of both the LC cells and astrocytes. bFGF is a member of the heparin-binding growth factors that has been implicated in retinal neovascularization, primarily located in the ganglion cells and inner layer of the retina. Presence of this protein promotes epithelial migration during wound healing and developmental morphogenesis. Isolating this protein for analysis is important for the way it may work to coordinate the various MMPs in restructuring the optic nerve head. Our results show a marked increase in bFGF in the stretch group of both cell types, however the difference was not significant (n=3). It has been shown that bFGF increases the activity of MMP9 as well as the activation of MMP2, while it has been shown to inhibit TIMP1, MMP2 is upregulated at the same time. This protein may modulate MMPs and growth factors (see Ingenuity Pathways Analysis; figures 3 and 4). GDNF is a growth factor that has been shown to promote the survival and differentiation of dopaminergic neurons in culture, as well as being able to prevent apoptosis of motor neurons by axotomy. This protein has also been
implicated in numerous neurodegenerative diseases,\textsuperscript{51, 52} and has been shown to be amplified by the presence of bFGF.\textsuperscript{53} There was no statistical change in the regulation of GDNF between the groups or cell types, however the presence of this protein in the extracellular media warrants its interest and further analysis. NGF-b is another target of importance and is involved in regulation of growth and the differentiation of sympathetic and certain sensory neurons. It has an important role in rescuing damaged neural cells from apoptosis,\textsuperscript{54} bFGF controls levels of this protein in neural tissue by decreasing the regulation and response when present in higher levels,\textsuperscript{55} which perhaps may explain the low levels we detected in our samples. BDNF was not found to be significantly changed however it has been shown to be increased in the presence of bFGF.\textsuperscript{56} In a number of studies, an increase in NGF-b levels has been associated with an increase in BDNF.\textsuperscript{57-59} The similar trends that were detected for both cell types indicate that they may have similar roles in their reaction to biomechanical insult. There were subtle differences, with more obvious regulation of bFGF and matrix remodelling protein levels in LC cells, but the trends remain similar.
Figure 3 – Integration of the identified proteins into the canonical pathways for LC cell stretch of 12% for 2 hours using Ingenuity Pathway Analysis. Proteins were identified as being within the nucleus, the cell membrane, extracellular or intracellular. A solid line indicates a direct protein-protein interaction; a dashed line indicates an indirect interaction; a dotted line indicates a protein-DNA or protein-RNA interaction. Direct interactions involving observed proteins were displayed as bold lines. Red molecules were up-regulated and green molecules were down-regulated. White coloured molecules were not observed in the experiments, but were incorporated into the network through relationships with observed proteins. Proteins of interest are gradated and found in the extracellular space. The connection of discovered intracellular proteins, major hubs and proteins of interest has been highlighted with red lines for upregulation and green for downregulation.
Figure 4 – Integration of the identified proteins into the canonical pathways for astrocyte cell stretch of 12% for 2 hours using Ingenuity Pathway Analysis. Protein colour and line legend is the same as figure 1. Proteins of interest are gradated and found in the extracellular space. The connection of discovered intracellular proteins, major hubs and proteins of interest has been highlighted with red solid lines.

5.6 Conclusion

Proteins from the remodelling group (MMP2, MMP9, TIMP1) were all found in higher levels in both the control and stretch when compared to the growth factor group (bFGF, BDNF, GDNF, NGF-β). Much higher levels of TIMP1 were found compared to MMP levels which are in line
with the findings of others. The level of the various growth factors may be at lower levels as the optic nerve head is not in a cellular growth, proliferation or differentiation mode at this time, but is attempting to remodel in order to mitigate the strain to which the cellular environment has been subjected. We propose a potential link between bFGF and the matrix remodelling by ONH glia in response to biomechanical strain. This protein could be an important regulatory factor in the degenerative process leading to development of glaucomatous optic neuropathy. Further research is required to better understand these interactions and how their manipulation may affect glial cell injury responses.

5.7 Acknowledgements

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5.8 References


6 Conclusion

Glaucoma is the world’s most common neurodegenerative disease and the second leading cause of blindness worldwide. The most common form of the disease, primary open angle glaucoma, is characterized by the slow and irreversible death of retinal ganglion cells, and eventually the loss of visual function. A rise in intra-ocular pressure has been associated with RGC death, however the mechanisms are still poorly understood. A number of theories have pointed to the activation of the optic nerve and glia of the nerve fiber layer through biomechanical strain and ischemia as the initiator of this disease. The biomechanical theory hypothesizes that the elevation in IOP deforms the ONH tissues, in particular the lamina cribrosa, leading to several deleterious effects. The second theory of glaucomatous damage is the vasogenic theory, which proposes that glaucomatous optic neuropathy is due to insufficient vascular perfusion at the level of the LC, resulting in ischemic injury. In following the five stages of glaucoma as laid out by Nickells et al., this disease progresses from the activation of the glia of the optic nerve head to damage to the axons of RGCs, eventually leading to their death causing secondary degeneration neighbouring cells. Research by Dai et al. has recently attempted to answer the question of primary insult by introducing special ‘fortified’ astrocytes of the optic nerve head which are the transducers of the increased IOP, resulting in a metabolic-based attack as opposed to the direct approach of damage to the axons as previously thought.

In order to better understand the actual process of degeneration within the lamina cribrosa, various models were first needed in order to give direction to the research. Studies by our lab and others have developed finite element models of the ocular environment and the way the numerous tissues interact. These models, acting as a mathematical method of solving the complex physical interactions and geometry of the eye, has proved to be a viable alternative to
complicated, practically unfeasible or unethical experiments. The models, developed by Sigal et al., were used as the basis for research in this thesis by determining the percent strain being affected upon the lamina cribrosa as IOP was increased. With knowledge of stress levels acting upon the optic nerve head, a cellular in vitro model of primary human ONH cells was pursued.

Optic nerve head astrocytes and lamina cribrosa cells were successfully isolated from LC explants and cell characterization was established. The Flexercell system was calibrated to determine the maximum strain capable at a specific frequency. We required a 1Hz cycle in order to mimic the in vivo ocular pulse. We are also confident that this method of strain is more physiologically relevant than hydrostatic pressure, which has been previously used.

Advances in the field of proteomics made this avenue of research an extremely viable option to examine how these levels of stress perturbed the astrocytes and lamina cribrosa cells of the optic nerve head. A number of proteomics-based articles have been published previously on various diseased tissues of the eye, however none have examined the effects of IOP on the optic nerve head. Our research focused on the whether or not mechanical stress, in the form of equi-axial stretch, caused human ONH glial cells, specifically the astrocytes and LC cells, to activate. We were determined to identify the specific protein regulation that is a consequence of this activation. Following the validation of the methodology and results, the ensuing proteomic screen presented a number of intracellular and extracellular proteins that are believed to be important to this activation process.

The first experimental chapter of this thesis details how ONH astrocytes responded to strains of 3% and 12 % over periods of 2 and 24 hours. Astrocytes, as a major supportive cell type, are extremely important in providing a supportive role for axons, and communication for surrounding connective tissues and blood vessels. The results of our iTRAQ based proteomics
study resulted in over 570 proteins being identified at a 95% confidence limit, which were then further analyzed to have a fold-change of at least 1.5. When these top proteins were examined for their molecular interactions using Ingenuity Pathways Analysis, specific hubs and targets began to emerge from the formed networks. Traditional targets of apoptosis and degeneration were seen to be involved in proteins such as TP53, TNF, and TGFβ for all stretch and time condition pairings, while various caspases were seen in the 12%, 2 hour stretch. From the networks and protein database, specific molecular targets were isolated due to their regulatory response and gene ontology. Astrocytic phosphoprotein (PEA15) was chosen due to its role as an early response protein, previously found to respond to physical stressors within the central nervous system. UDP-glucose dehydrogenase is another protein that was found and is known to be involved in the MAPK pathway and has been shown to conserve the extracellular matrix. Annexin A4 (ANXA4) was chosen for the way it interacts with a number of proteins within the NF-κB signalling pathway which has been shown to be important in regulating numerous genes involved in the immune response, cell proliferation, differentiation, survival and apoptosis. Finally, S100-A13 was targeted for it also has a role in the NF-κB signalling pathway, while modulating various inflammatory cytokines such as IL-1B and IL-8. This study was the first of its kind and was meant to determine the global protein profile of astrocytes of the optic nerve head. This profile was used to isolate the proteins that may be responsible for the activation of the optic nerve head and glia of the nerve fiber layer, leading to the apoptosis of retinal ganglion cells.

The second cell type that was identified as possibly playing a role in the activation of these glia were the lamina cribrosa cells, whose protein profile and biomarkers were the subject of the second experimental chapter of this thesis. These cells are known to be involved in building cross-linked actin networks which are found in the trabecular meshwork and have been found to
resist biomechanical strains that occur in the normal and abnormal environment of the optic nerve head. They are also a major cell type of the lamina cribrosa, further implicating them in a role of RGC apoptosis. The results of the iTRAQ study found over 520 proteins, again with a number of them falling into the 1.5-fold requirement. These cells produced different proteins from the astrocytes, however they were still interacting around the primary hubs of TGFβ1, TNF and TP53. This sort of interaction is logical as the major hubs of both cell types are affecting a relatively small area within the optic nerve head, however each cell type may have different results based on the biomarkers released. The specific proteins isolated for further research include bcl-2-associated athanogene 5 (BAG5) which has been shown to interact with various heat shock proteins and when over-expressed has enhanced the cell death of neurons within specific affected areas of the brain. It is also a known molecule in other neurodegenerative diseases such as Parkinson’s. Nucleolar protein 66 (NO66) was chosen for its role in the biogenesis of ribosomes and in the replication or silencing of certain heterochromatic regions. Eukaryotic translation initiation factor 5A (eIF-5A) is a significant finding for previous research in our lab and elsewhere has demonstrated its role in a number of diseases as well as apoptosis and the activation of the intrinsic mitochondrial pathway.

The functional pathways analysis of astrocytes and LC cells described how an increase in strain percentage or duration can affect the regulation of a specific pathway. For astrocytes, normal protein patterns are associated with low percentage strain, while cellular repair and DNA repair were associated with the higher percentage strain and longer exposure. Lamina cribrosa cells illustrated that the level of protein synthesis increased logarithmically with a number of proteins associated with metabolism, biosynthesis, and translation of the protein. The level of proteins associated with cellular movement increased in a similar manner, with the highest level seen in the 12% for 24 hour stretch. A subtly different pattern of behaviour is seen with both the cell-to-
cell signalling and inflammatory response. The highest levels of change are seen in both the 24 hour stretches as compared to the 2 hour stretches, regardless of the magnitude. From the functional analyses of both cell types, it appears that the lamina cribrosa cells are attempting to remodel their cellular structure in order to cope with the stress, while the astrocytes are attempting to repair damage. This may be another indicator of the fundamental differences and roles of these two cell types. Future Research may consider time course analysis assessing the hypothesis that LC cells react earlier to stress given their bias towards remodelling.

The proteins that were isolated from both the astrocytes and LC cells were selected based on gene ontology, regulation, and previous research. A total of 1074 unique proteins were found in the LC cells, and 968 in astrocytes, with a 700 protein overlap. However these proteins were primarily isolated to the intracellular space as protein lysates were used, which excludes any extracellular proteins and their possible interactions. The third experimental chapter of this thesis focused on the extracellular proteins from the 12% for 2 hour stretches for both cell types. By targeting potentially important proteins that were a part of the networks for both conditions, a multiplexed-ELISA approach was taken to confirm proteins purported to be present by iTRAQ and IPA network analysis. Two groups of proteins were chosen, comprising seven proteins in total. The first group was three matrix remodelling proteins which were all found by the ELISA. The levels of these proteins did not vary significantly between controls and stretched however a trend towards their upregulation was seen. The second group of proteins was growth factors, where again the amounts of protein did not vary between the two groups for both cell types, however their levels were significantly lower than those of the remodelling proteins. This relates to previous research that demonstrated low levels of these proteins are believed to cause the targeting of retinal ganglion cells for programmed cell death.²
Proteomic analysis of these cells has provided a list of putative biomarkers which may suggest novel regulatory elements and signalling pathways that is driving the initial cellular injury response, as well as potential new therapeutic strategies. The research within this thesis has delved deeper into the fundamental question of ONH glial activation and the instigation of the apoptotic cascade that leads to the development of glaucomatous optic neuropathy. In line with the research by Nickells\textsuperscript{2} and Dai\textsuperscript{3}, we feel that the proteins discovered here, both intra- and extracellular, are important to the activation of glial cells. As an aspect of the primary phase of glaucoma or as products of specialized ONH astrocytes, this research furthers the overall understanding of how these cells are activated and the potential pathways involved. As research within this field continues, a greater emphasis will be placed on these and other biomarkers and the way they interact. These interactions will lead to the development of therapeutics with the ultimate goal of stopping one of the leading causes of blindness worldwide.
6.1 References

