THE ROLE OF TRANSFORMING GROWTH FACTOR BETA 2 SIGNALING AND MICRORNAS IN OPTIC NERVE HEAD REMODELING

DISSERTATION

Presented to the Graduate Council of the University of North Texas Health Science Center of Fort Worth in Partial Fulfilment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fort Worth, TX

22nd July 2020

ACKNOWLEDGEMENTS

My deepest gratitude to my mentors, Dr. Tara Tovar-Vidales and Dr Abbot Clark for their encouragement, continuous dedication to graduate student education and support during my training. Dr Clark, thank you for the opportunity to be a part of NTERI and allowing me to develop into a critical thinking and independent scientist. Your unwavering enthusiasm for science constantly engaged my research- you are an inspirational scientist. Dr. Tovar-Vidales, thank you for shaping the foundation of my career as a scientist, welcoming all my questions and your friendship. I will cherish the time we shared.

I am grateful for my advisory committee members Dr Iok-Hou Pang, Dr. Yang Liu and university member Dr Nicole Phillips. Dr. Pang your insight and dedication to research is motivational, and I am appreciative of your guidance and humour over the years. Dr. Liu, I'll always be grateful for your wealth of knowledge, guidance towards my research and kindness. Dr. Phillips, thank you for always being available to help and your valuable input towards my research and education. I have also been fortunate to have additional mentors who have dedicated their time to teaching. My sincerest appreciation to my fellow Brit, Dr. J. Cameron Millar and Dr. Gulab Zode. Thank you to my Neurobiology of Aging family, Dr. Nathalie Sumien, Elizabeth Baker and Dr. Michael Forster; your mentorship and guidance through the years have helped in every aspect of my training. I would also like to thank the Graduate School of Biomedical Sciences administration, you have all been instrumental in my time here at UNTHSC.

I am only successful as the team and collaborators whom I've had the privilege of working with at NTERI. My lab family, past and present, have been a significant part of growth

since joining the lab in 2016, each always willing to help. It has been a great pleasure to start this journey and interact with emerging scientists. To Gita Patak, Shruti Patil, Hannah Webber, Alex Marciante, Carmen Claudio, Frank Wendt, Nicole Novroski, Avani Modi, Pinkal Patel and Guarang Patel, I am very grateful for your friendship and that we experienced graduate school together. I would also like to thank my friends in Texas and London- Amanda Birch, Alyson Taylor, Waiza Kadri, Jigna and Matthew Pappas, Nisha Patel, Dani and Kelly Young and Lauren Meyer.

I am forever grateful to my family. Mum and Dad (Rossi and Vinod), thank you for your unrelenting support throughout my life and graduate career. Even though you are thousands of miles away, you have always encouraged me, accepted my calls at any hour of the day, and believed in me. You've been present in every aspect of my life, even now listening to me practise my talks in graduate school. I am privileged to be your daughter. To my brother Sanjay and cousins Rubin and Anushka and many more cousins, aunts and uncles, I miss you dearly and thank you for your love, confidence in me and support throughout this journey. I am also grateful to the family that I inherited- Jaime, Bertha, Donna and Nancy thank you for being my family away from home. Most importantly I would like to thank my husband James Thomas. You and Cookie (our dog) are my whole heart and have fulfilled me with so joy and positive energy. Thank you for your love, patience and being my support system throughout this journey- you are truly the kindest person I know.

Lastly, thank you to all the funding sources including the T32 Neurobiology of Aging predoctoral fellowship and Glaucoma Research Foundation grant, the department of Pharmacology and Neuroscience and UNTHSC.

TABLE OF CONTENTS PAGE

LIST OF FIGURES PAGE

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF TABLES PAGE

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER I

INTRODUCTION

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Glaucoma

Glaucoma is a prevalent age-related disease that causes progressive neurodegenerative damage to the visual system. Currently, glaucoma is the leading cause of irreversible vision loss worldwide- over 70 million people are affected by glaucoma. In an ageing population, this number is expected to increase 47% to 111.8 million by the year 2040[1], underscoring the global impact of glaucoma.

Glaucoma is characterised by thinning of the neuro-retinal rim, degeneration of retinal ganglion cell (RGC) axons, cupping of the optic disc and decreased visual field sensitivity. Glaucoma is a heterogeneous group of optic neuropathies, broadly categorized as primary and secondary glaucomas. Primary glaucoma refers to an unknown source of pathology and include: open-angle glaucoma (OAG), angle closure glaucoma (ACG), normal tension glaucoma and congenital glaucoma. The angle referred to in OAG and ACG is between the iris and cornea, the iridocorneal angle. Secondary glaucoma refers to an identifiable source of pathology and include steroid-induced glaucoma, uveitic glaucoma, neovascular glaucoma, pigmentary glaucoma and exfoliation glaucoma. All forms of glaucoma result in neurodegeneration of RGC axons, leading to an irreversible loss of visual function; however, a major concern is the variability of case definitions. A condition known as ocular hypertension is the only modifiable risk factor associated with glaucoma. There is a subset of patients with normal ocular pressure levels, normal-tension, that develop glaucoma; therefore, increased susceptibility to neuronal damage may be due to structural differences in the optic nerve head.

Primary open-angle glaucoma

Primary open-angle glaucoma (POAG) is the most common form of glaucoma, accounting for greater than 70% of all glaucomas[1]. Patients with mild to moderate POAG can often remain asymptomatic or may experience a gradual loss in peripheral vision; therefore, undetected for decades. Regular eye exams are paramount to diagnose and follow glaucoma progression through visual field testing, a dilated eye exam, tonometry and optic nerve imaging. Currently, there is no cure for POAG; however, through early detection and medication, the disease can be controlled to slow the rate of vision loss.

POAG is associated with several risk factors, including elevated intraocular pressure (IOP), increasing age, a family history of glaucoma and ethnicity. IOP is considered elevated if it is > 21mmHg. Aqueous humor, a fluid that nourishes the anterior segment of the eye, is produced by the ciliary body epithelium and drains to distal regions primarily through the trabecular meshwork and Schlemm's canal. Progressive resistance within aqueous humor outflow pathways results in an increase in IOP. The incidence of POAG increases after the age of 40[2]; age-related changes to the biochemical structure of the optic nerve head[3,4] likely increase susceptibility to IOP-related damage. Ethnicity is also a risk factor for POAG; African Americans are 4-5 times more likely to develop POAG compared to Caucasians[5]. Approximately 50% of people with POAG, have a family history of glaucoma, reflecting a genetic susceptibility to POAG[6,7]. Elevated IOP remains the most prominent etiologic risk factor for development and progression of glaucomatous injury and the only modifiable factor in the treatment of POAG.

The optic nerve head is the primary site of IOP-related injury and RGC damage[8]. There is structural deformation and extensive tissue remodelling of the glaucomatous optic nerve head[8-10]. Experimentally induced models of ocular hypertension have been shown to cause progressive damage and biochemical changes to the optic nerve head[10-14]. The anatomy of the optic nerve and optic nerve head is an important in terms of understanding the mechanism of axonal damage in glaucoma.

The eye is a unique structure with many similarities to the brain and spinal cord in terms of anatomy, function and response to damage. During development, the retina and optic nerve extend from the diencephalon; thus, is considered an extension of CNS. The retina is formed of layers of specialised neurons; the outermost layer, the RGCs, project their axons, coursing the vitreous to the optic disc. This is known as the nerve fibre layer. Axons are surrounded by and interact with glial cells in the nerve fibre layer. Retinal arterial vasculature and capillaries nourish the glia and axons in this region. Within the nerve fibre layer, RGC axons complete a ninety degree turn at the optic disc and enter the optic nerve head.

The optic nerve head

The optic nerve head is an opening in the posterior sclera, where more than a million RGC axons converge at the optic disc, exit the intraocular space and form the optic nerve. RGC axons project to the lateral geniculate nucleus and superior colliculus, which is then relayed to visual centres of the brain.

There are three histologically distinct regions of the optic nerve head: the pre-laminar region, the lamina cribrosa and the post laminar region. RGC axons that pass through the pre-laminar and lamina cribrosa are unmyelinated and in the post laminar region they are myelinated. The central retinal artery traverses through the optic nerve supplying all the nerve fibres that constitute the optic nerve head. Long and short posterior ciliary arteries, derived from the ophthalmic arteries, surround the optic nerve [15]. In the prelaminar region, few choroidal vessels also provide circulation. One possible mechanism of IOP-related axon damage, is neural ischaemia.

The lamina cribrosa is the initial site of glaucomatous injury including posterior displacement; compression; and disorganisation of the axon bundles[16]. The lamina cribrosa is a ~200-400um layer of fibro-elastic connective tissue which mechanically supports the optic nerve head[8]. The pressure gradient across this region influences the physiology of RGC axons; therefore, it is important that the connective tissue supports and protects unmyelinated axon bundles from mechanical injury[17]. Sagittal and cross-sections of the lamina cribrosa show layers of connective tissue plates (lamellae), which align to form trabeculae and channels for axon bundles to pass. It is thought that structural differences in the lamina cribrosa, including thickness, pore size and extracellular matrix structure, may determine susceptibility to glaucoma[18-20].

Extracellular Matrix of the Lamina Cribrosa

The major constituents of the lamina cribrosa are collagen (24-45%) and elastin (7-28%) [21,22]. Fibrillar collagen type I is the most abundant collagen within the stroma of the lamina cribrosa connective tissue plates [23]. Collagen type III is a fibrillar collagen within the stroma of plates and walls of blood vessels [23,24]. Together collagens I and III are the main structural components and

provide tensile strength to the lamina cribrosa. Collagen type V is located between collagen types I and III and is essential for fibrillogenesis [23-25]. The network forming collagen type VI binds to other collagens and thickens collagen fibres [24,26,27]. Fibronectin is a glycoprotein with diffuse expression between collagen fibrils and may help in adherence of cells to structural collagens [23,24,28,29]. α -elastin fibres within the stroma, extend longitudinally, parallel to fibrillar collagens and provide resilience and elasticity to the lamina cribrosa [30]. This is particularly important to withstand changes in IOP. Proteoglycans (0.8-2.3%) have an important role in the binding and organisation of these extracellular matrix macromolecules [31]. Proteoglycans consist of a core protein covalently bonded to one or more sulphated glycosaminoglycan side chains: dermatan sulphate, keratin sulphate, heparin sulphate and chondroitin sulphate. The largest and most abundant in the lamina cribrosa is the chondroitin-4-suplahte proteoglycan, which loosely assemble around and between collagen fibrils [32,33]. Dermatan-sulphate proteoglycans are associated with chondroitin-4 sulphate and arranged perpendicular to collagen fibres [32]. Chondoritin-6 sulphate proteoglycans are within the lamina cribrosa plates and walls of blood vessels [32]. Decorin, a core protein which binds a single chondroitin or dermatan sulphate glycan, associates with collagen type I possibly influencing assembly and spacing of fibrils [32,34]. A basement membrane surrounds the stroma of plates which is composed of collagen type IV, laminin (α1, α2, β1, β2, γ1 subunits) and heparan-sulphate proteoglycans [23,28,32,35,36]. Collagen type IV fibres branch out extending into the stroma binding other extracellular matrix macromolecules, forming a fibrous network [37,38]. This highly organized extracellular matrix structure forms a protective framework for RGC axons and regulates cell adhesion and signalling.

Cells of the optic nerve head

Cells present in the optic nerve head include astrocytes, lamina cribrosa cells, microglia, vascular endothelial cells, and vascular pericytes[39,40]. These cells can be distinguished morphologically and by intracellular and extracellular markers.

Astrocytes have large cell somas, with multiple processes and express the intermediate filament, glial fibrillary acidic protein (GFAP). Optic nerve head astrocytes are the predominant glia in the optic nerve head, and provide structural and neurotrophic support for RGC axons. There are two main classes of optic nerve head astrocytes: type 1 and type 2 astrocytes. Type 1 astrocytes are present in the pre-laminar and lamina cribrosa and type 2 astrocytes in the post-laminar region[41,42]. Type 1 astrocytes are further divided into subtypes: type 1A and type 1B. Astrocytes in the lamina cribrosa have a different cell signature compared to astrocytes in the pre-laminar region. Astrocytes in the lamina cribrosa express neural cell adhesion molecule, indicative of type 1B astrocytes[43]. The different subtypes of astrocytes are thought to be responsible for regional differences in the optic nerve head regions. Optic nerve head astrocytes in the pre-laminar region are arranged in columns surrounding unmyelinated RGC axons bundles, which provide neurotrophic support, and help guide and transition axons through the lamina cribrosa [41,42]. Astrocytes in the lamina cribrosa line connective tissue plates and synthesise extracellular matrix molecules to support the lamina cribrosa and also provide structural and neurotrophic support to RGC axons[44].

Lamina cribrosa cells are broad, flat cells and express alpha smooth muscle actin (α-SMA). Lamina cribrosa cells are localized to the lamina cribrosa region of the optic nerve head, within and between connective tissue plates [45]. Lamina cribrosa cells synthesise extracellular matrix

molecules which support the structure of the lamina cribrosa[46]. Scleral fibroblasts are spindle-shaped cells, grow in multiple layers and can be distinguished from lamina cribrosa cells by negative expression of basement membrane proteins, collagen type IV and laminin[8].

In normal physiological conditions, microglia are stellate shaped with a small nucleus and thin processes[47]. Microglia express HLA-DR and CD-45; however, are negative for GFAP, which can be used to distinguish from astrocytes in the optic nerve head[47]. Pericytes surround blood vessels and are embedded within the basal lamina and express 3G5[8]. Their processes are oriented along the axis of each capillary enabling cell-cell contact with capillary endothelial cells [48]. Vascular endothelial cells are positive for Factor VIII[8].

Extracellular matrix changes in POAG

Increases in IOP exert mechanical stretch-stress on the optic nerve head tissue and cells which leads to laminar connective tissue rearrangement [16,49,50]. Analysis of the lamina cribrosa in experimental monkey models shows at least 42% increase in the connective tissue volume compared to contralateral control eyes [14]. Human POAG optic nerve head sections and an experimental model of primate glaucoma, show thickened elastin deposits that appear curled, disorganised and fragmented, an indication of elastosis [10,51-53]. There is also an increase in expression of collagen type IV and VI surrounding connective tissue plates [54,55]. These extracellular matrix changes result in pathogenic tissue remodeling of the optic nerve head in glaucoma, which affect how the tissue responds to changes in IOP and increases the

susceptibility of RGCs to progressive damage. Cells in the optic nerve head may be the source of this extracellular matrix remodelling [56].

Optic nerve head cells support the structure of the optic nerve head and provide trophic and immune support to RGC axons. In glaucomatous conditions, optic nerve head cells increase synthesis of growth factors, extracellular matrix proteins and pro-inflammatory mediators that contribute to tissue remodeling and neurodegeneration of RGC axons. Optic nerve head astrocytes become reactive, increase expression of GFAP, migrate from lamina connective tissue plates and secrete inflammatory factors that may be harmful to RGC axons [39,57-60]. Glaucomatous optic nerve head astrocytes upregulate expression of genes involved in signal transduction, cell adhesion and proliferation, extracellular matrix synthesis and degradation[57]. This suggests a pivotal role in tissue remodeling[57]. There are differences that exist between optic nerve head astrocytes from Caucasian Americans and African Americans and the genes differentially expressed in the African Americans may be responsible for their increased susceptibility to optic nerve head damage[61,62]. Using electron microscopy, Hernandez demonstrated cell-cell contact for optic nerve head astrocytes and lamina cribrosa cells in the in the lamina cribrosa[39]. Lamina cribrosa cells are thought to have mechanosensitive properties and increased IOP may lead to membrane stretch and activate ion channels [63]. Stretch activated channels influence gene transcription, which may stimulate a profibrotic response and remodeling of the optic nerve head [64,65]. Microarray analysis showed upregulated expression of profibrotic genes including TGFβ, collagen types I, V, VI and lysyl oxidase, the crosslinking enzyme, in human lamina cribrosa cells from POAG eyes compared to normal control eyes [64]. Microglia become activated in injury, re-tract their processes, migrate and upregulate the expression of cytokines. In the glaucomatous

optic nerve head, activated microglia expressed TGFβ and TNFα[66]. Microglial activation has been suggested to occur prior to RGC loss and can predict the severity of optic nerve degeneration[67]. These studies suggest that optic nerve head astrocytes and lamina cribrosa cells most likely contribute to the extracellular matrix tissue remodeling of the glaucomatous optic nerve head.

The lamina cribrosa extracellular matrix and cells have an important role in cell signaling and regulating the dynamics of the lamina cribrosa. Integrins and glycoproteins connect extracellular matrix fibrils to intracellular cytoskeletal actin filaments; therefore, cells can respond to extracellular biochemical or mechanical changes. The extracellular matrix can capture and localize growth factors near cell-surface receptors and activate intracellular signaling pathways. A central growth factor associated with cell signaling and tissue remodeling in POAG is transforming growth factor beta (TGFβ).

Transforming growth factor β and micrornas (miRNAs)

The TGFβ superfamily is comprised of over 30 members which include TGFβ isoforms, BMPs, inhibins, activins, and growth differentiation factors which regulate a range of homeostatic processes including proliferation, differentiation, motility, adhesion and ECM synthesis and secretion [68-70]. In human tissue, three isoforms have been identified: TGFβ1, TGFβ2 and TGFβ3. In the optic nerve head, the predominant isoform is TGFβ2[71,72]. TGFβ2 activates the SMAD-dependent TGFβ signaling pathway. The TGFβ2 ligand binds to its transmembrane serine/threonine cell surface receptor, TGFβ type II receptor (TGFβRII) and leads to TGFβ type

I/II receptor dimerization, and phosphorylation of intracellular receptor-specific SMAD proteins, SMAD2 and 3 (SMAD2/3) [73]. Phosphorylated SMAD2/3 interacts with SMAD4, which then translocates to the nucleus. In the nucleus, the heteromeric p-SMAD2/3-SMAD4 complex interacts with co-factors and transcription factors, and binds to specific DNA sequences, SMAD binding elements (SBE), in the promoter region of target genes. Activation of TGFβ2 signaling regulates the transcription of specific genes, including proteins and non-protein coding microRNAs (miRNAs) [74-76].

Human genomes contain a number of genes that produce non-coding RNAs which have structural, enzymatic and regulatory functions. Those with regulatory activity include microRNAs (miRNAs). miRNAs were first discovered in C.elegans with the identification of lin-4, which contained a sequence complimentary to the repeated sequence element of lin-14 mRNA and led to a decrease in lin-14 protein [77]. Hundreds of miRNAs have since been identified that have roles in a range of physiological processes such as fetal development and differentiation.

miRNA biosynthesis and the spatial and temporal expression pattern is extremely important in the homeostatic control of cellular activities. miRNAs are single stranded, short RNA sequences, 18-22 nucleotides in length, that often are transcribed from introns, although some are transcribed from an exonic location [78]. miRNAs are initially transcribed as long primary miRNA (pri-miRNA) transcripts in the nucleus, which are then cleaved by RNase III Drosha to from hairpin loop structures termed precursor miRNA (pre-miRNA) [79]. This intermediate structure is exported from the nucleus to the cytoplasm by the Ran-GTP dependent receptor, exportin-5 [80,81]. The pre-miRNA is processed by RNase III Dicer into a short double stranded ~ 22 nucleotide

sequence [82]. The cleavage site is an important determining step as the strand with the less stable 3' end usually proceeds as the mature miRNA, while the other is degraded by a nuclease [83,84]. There can be different splice variants of mature miRNAs (miR) such as miR-29a, b or c. Mature miRNAs can also be derived from different arms of the hairpin loop structure, the 5' arm or 3' arm, and are denoted a '-3p' or '-5p', for example miR-29a-3p and miR-29a-5p.

miRNAs regulate post-transcriptional gene expression by anti-sense RNA-RNA interactions with target mRNA, which leads to translational repression or degradation of mRNA [77,85-87]. Specifically, gene silencing is elicited by imperfect base-pairing of the 5'end of miRNA and 3' untranslated region (UTR) of mRNA [77,86]; however, the seed region at the 5'end of the miRNA must have perfect complimentary base pairing with the mRNA. A single miRNA can target several mRNAs and a single mRNA can be targeted by several miRNAs, therefore pairing is highly complex[88]. Since miRNAs pair with target mRNA with imperfect base pairing, identification of miRNA targets can be a challenge. Bioinformatics tools such as TargetScan, miRanda, MiRWalk2.0 and RNA Hybrid use algorithms to search for complimentary seed sequences to predict target mRNA. Several miRNAs have thus been characterized as pro- or anti-fibrotic.

miRNAs are essential to the SMAD-dependent TGFβ pathway. TGFβ activation of SMADs, cofactors, and transcription factors can lead to transcriptional activation or inhibition of miRNA genes. miRNAs can also be modulated at the post-transcriptional level by R-SMAD proteins, which recruit and interact with members of the miRNA processing complex, DROSHA and p68,

and enhance cleavage of pri-miRNAs to mature miRNA [75]. Therefore, important to achieve homeostatic regulation of TGFβ signalling.

Histopathological analysis shows that levels of TGFβ2 are increased in the POAG lamina cribrosa and co-localises to optic nerve head astrocytes[71,89]. Increased TGFβ2 is associated with a disruption in the collagen and elastin structure, loss of axon bundles, and excess accumulation of basement membrane proteins [10,13,54,90]. Accumulating evidence shows that optic nerve head astrocytes and lamina cribrosa cells are responsible for extracellular matrix changes in the lamina cribrosa [89,91,92]. TGFβ is secreted by optic nerve head astrocytes and lamina cribrosa cells[89]. Optic nerve head astrocytes and lamina cribrosa cells respond to TGFβ2 by increasing the gene and protein expression of fibronectin, collagen type I and IV, connective tissue growth factor and crosslinking enzymes[89,91-93]. It is important to understand the cell-specific responses to TGFβ2 and what leads to increase expression of extracellular matrix genes, proteins and disorganized fibrils. It is possible that miRNAs have a role in regulating TGFβ2 signaling and aberrant TGFβ2 signaling may inhibit select miRNAs to cause tissue remodeling in glaucoma.

Our exploratory study focused on the role of TGFβ2 and miRNAs in the optic nerve head. Since TGFβ signaling can directly influence the expression of miRNAs, and miRNAs regulate translation of genes, we hypothesized **that TGFβ2 deregulates miRNA expression and contributes to lamina cribrosa remodeling** (Figure 2)**.** To address this hypothesis, we carried out three specific aims:

Aim 1. miRNA expression in optic nerve head astrocytes and lamina cribrosa cells

We dissected human donor eyes and isolated optic nerve head astrocytes and lamina cribrosa cells. Cells were characterized by cell morphology and intracellular and extracellular markers. Using miRNA PCR arrays, we studied the expression of miRNAs in TGFβ2 treated optic nerve head astrocytes and lamina cribrosa cells. The mRNA targets of differentially expressed miRNAs were predicted using a seed sequence algorithm and signaling pathways associated using miRnet. These approaches will help us understand cell-specific changes in response to TGFβ2

Aim 2. The Role of differentially expressed miRNAs in optic nerve head astrocytes and lamina cribrosa cells

Cells were transfected with differentially expressed miRNA mimics and inhibitors to experimentally validate target genes and optimum concentrations. We also determined the effects of overexpression or inhibition of candidate miRNAs on TGFβ2 induced extracellular matrix protein synthesis by western blot and immocytochemical analysis. These approaches will help us understand how dysregulation of TGFβ2 and miRNAs stimulates extracellular matrix synthesis in the optic nerve head.

Aim 3. Determine the expression of ECM components normal and glaucomatous human optic nerve head tissue

We will use immunofluorescence to determine the expression of extracellular matrix components in human normal and glaucomatous optic nerve head tissue sections.

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Figures

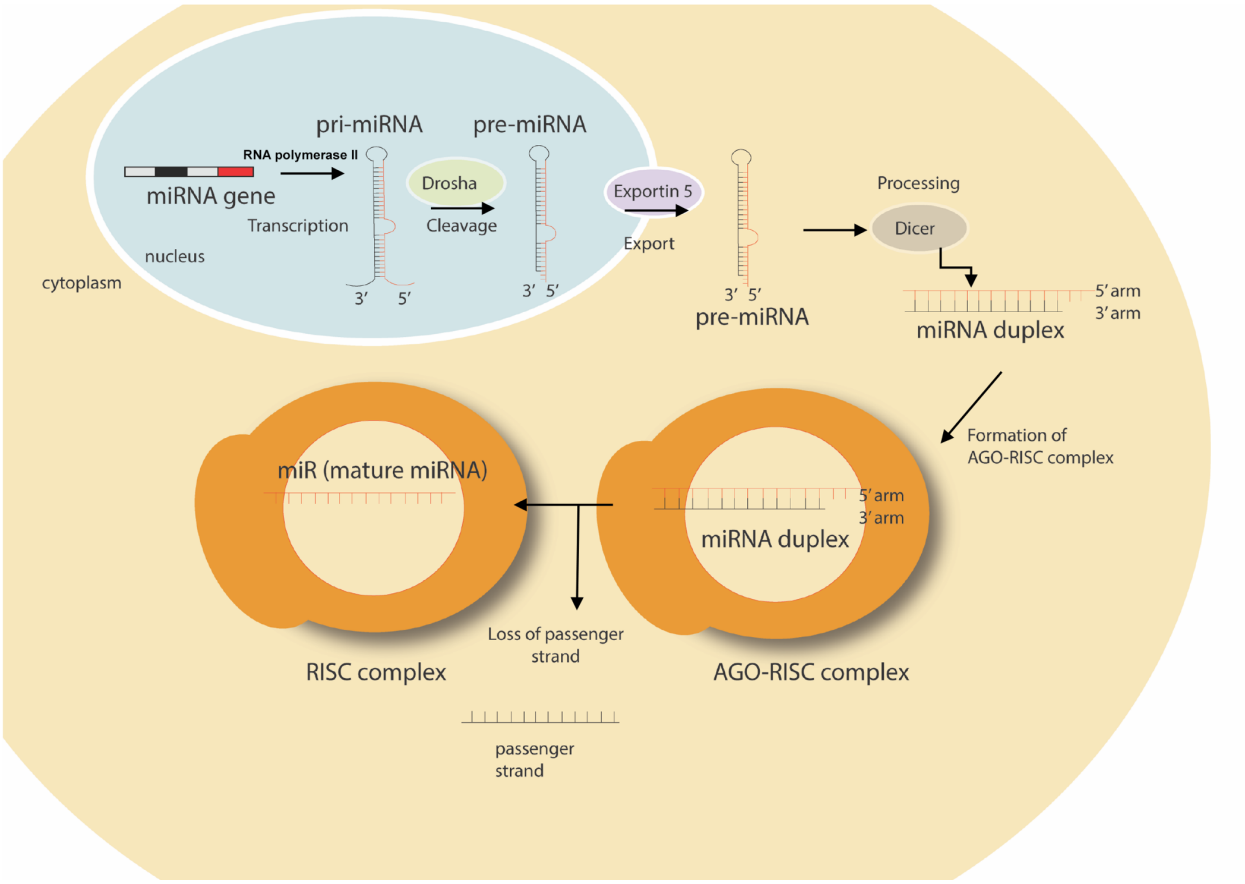

Figure 1. Biosynthesis of miRNAs
miRNAs are transcribed by RNA polymerase II in the nucleus and cleaved by Drosha to form precursor miRNA (pre-miRNA). This is exported from the nucleus by Exportin-5 and undergoes further processing to from the miRNA duplex. The miRNA duplex is loaded onto the AGO-RISC complex and one strand is selected as the mature miRNA, while the other strand is degraded. The mature miRNA targets mRNA by binding most commonly to the 3'UTR leading to translational repression or miRNA degradation.

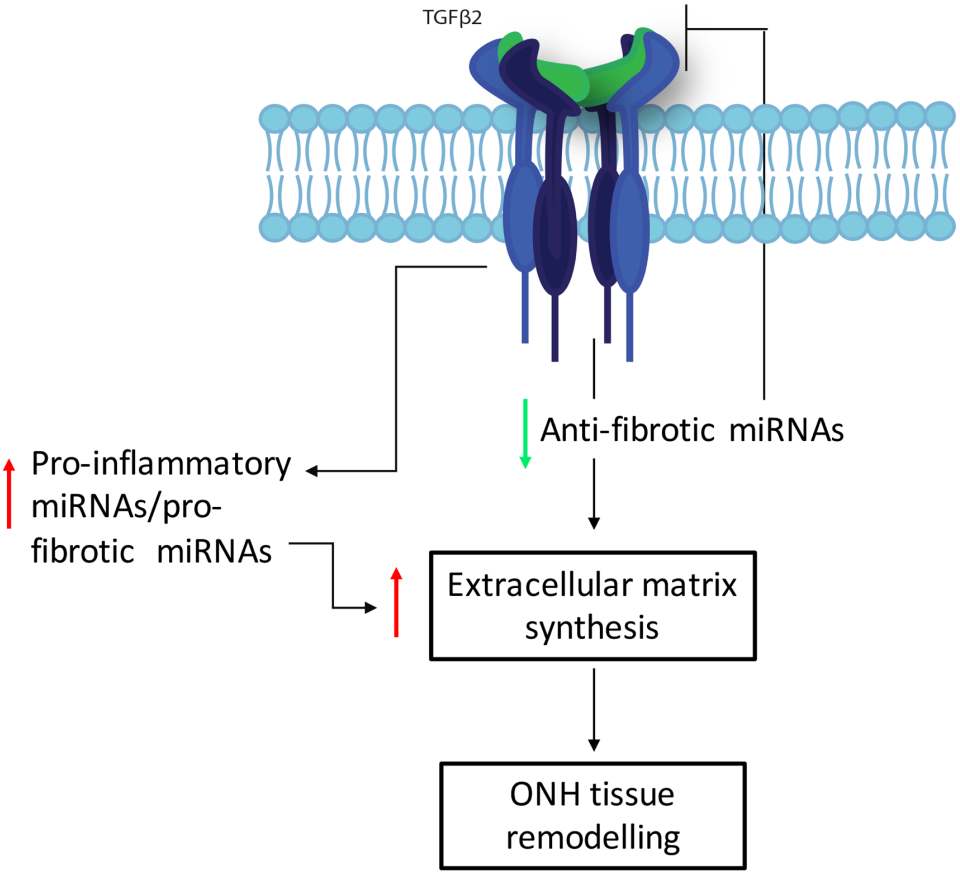

Figure 2. Hypothesis.
Increased TGFβ2 signaling dysregulates the expression of miRNAs in optic nerve head astrocytes and lamina cribrosa cells.

CHAPTER II

ISOLATION AND CHARACTERISATION OF HUMAN OPTIC NERVE HEAD ASTROCYTES AND LAMINA CRIBROSA CELLS

Published: Experimental Eye Research 2020

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Abstract

The lamina cribrosa is the initial site of glaucomatous injury. Pathological changes to the lamina cribrosa include posterior displacement of the lamina cribrosa, loss of trophic support, and remodeling of the extracellular matrix. Optic nerve head (ONH) astrocytes and lamina cribrosa cells synthesize extracellular matrix proteins to support and maintain the lamina cribrosa under physiological conditions. During glaucoma, these cells respond to mechanical strain and other stimuli, which leads to pathological remodeling of the ONH. Although ONH astrocytes and lamina cribrosa cells have been previously cultured, there is no well-accepted, straightforward technique to isolate both cell types from a single dissected human ONH. To better understand the pathophysiology of glaucoma, we obtained and cultured lamina cribrosa explants from human donor eyes. Initially, cells that grew out from the explant were ONH astrocytes and lamina cribrosa cells. Using a specialized medium, we isolated pure populations of lamina cribrosa cells and ONH astrocytes. ONH astrocytes expressed glial fibrillary acidic protein (GFAP). Lamina cribrosa cells expressed alpha-smooth muscle actin (α-SMA), but were negative for GFAP. This method of ONH cell isolation and cell-culture will provide a technique to better understand the molecular and cell-specific changes in glaucomatous damage to the ONH.

Keywords

- Human ONH, astrocytes, lamina cribrosa cells, explant

Highlights

- Dissection of human optic nerve head (ONH)
- A technique to isolate human ONH astrocytes and lamina cribrosa cells from a single ONH explant
- Characterization of ONH cells

1. Introduction

The optic nerve head (ONH) is an opening in the posterior sclera, where more than a million, unmyelinated retinal ganglion cell axons converge at the optic disc and exit the eye to form the optic nerve. Histological analysis of the ONH shows distinct regional differences recognized as the surface nerve fiber layer, pre-laminar, lamina cribrosa, and post-laminar myelinated optic nerve. For all types of glaucoma, the primary site of injury is the lamina cribrosa region of the ONH (Quigley and Addicks, 1981; Quigley et al., 1983). Cells in the lamina cribrosa actively respond to physiological and pathophysiological conditions; therefore, isolating and culturing these cells may help understand normal lamina cribrosa function and glaucoma pathophysiology.

Cells of the human ONH include five cell types: astrocytes, lamina cribrosa cells, microglia, vascular endothelial cells, and vascular pericytes (Hernandez, 2000; Hernandez et al., 1988). The lamina cribrosa explant yields two primary cell types, ONH astrocytes and lamina cribrosa cells (Hernandez et al., 1988). ONH astrocytes are glial fibrillary acidic protein (GFAP) positive and the major glial cell type found throughout the ONH, which mechanically supports and provides neurotrophic support to retinal ganglion cell axons (Hernandez et al., 2008; Lambert et al., 2001; Tovar-Vidales et al., 2016; Vecino et al., 2016; Yang and Hernandez, 2003). Lamina cribrosa cells are broad, polygonal-shaped cells, localized within and between connective tissue plates in the lamina cribrosa (Hernandez et al., 1988; Tovar-Vidales et al., 2016). Lamina cribrosa cells express alpha-smooth muscle actin (α-SMA), and similar to ONH astrocytes, these cells synthesize extracellular matrix proteins that form the cribriform network (Hernandez et al., 1988; Lambert et al., 2001; Zode et al., 2011). Glaucomatous ONH astrocytes and lamina cribrosa cells show increased expression of growth factors and fibrotic genes; these differences are likely

responsible for the fibrotic remodeling of the glaucomatous lamina cribrosa (Hernandez, 2000; Hernandez et al., 2002; Kirwan et al., 2009; Pena et al., 1999; Schneider and Fuchshofer, 2016; Wallace and O'Brien, 2016; Zode et al., 2011).

Our lab aims to gain a better understanding of the role of ONH cells and remodeling of the extracellular matrix. Previous methods have been described to isolate astrocytes and lamina cribrosa cells from human ONH tissue (Hernandez et al., 1988; Lambert et al., 2001; Rogers et al., 2012a; Rogers et al., 2012b; Yang and Hernandez, 2003). Here, we describe a modified method where both lamina cribrosa cells and ONH astrocytes can be isolated from a single ONH explant from human donor eyes. We characterize lamina cribrosa cells and ONH astrocytes by cell morphology, intracellular, and extracellular matrix markers.

2. Materials and Supplies

2.1 Human donor eyes

Human donor eyes without a history of ocular or neurodegenerative diseases were obtained within 24 hours of death from the Lions Eye Institute for Transplant and Research (Tampa, FL).

2.2 Equipment for dissection of the eye

I. NUNC cell culture petri dishes (100x21mm; Thermo Fisher Scientific, USA; Cat # 172931)

II. NUNC cell culture treated flasks with filter caps (T-25; Thermo Fisher Scientific, USA; Cat #150628)

III. NUNC treated 12 well cell culture multi-dish (Thermo Fisher Scientific, USA; Cat #136196)

IV. NUNC 15mL conical tube (Thermo Fisher Scientific, USA; Cat # 339651)

V. Eppendorf Centrifuge 5810R 15 amp (Eppendorf, NY; Cat # 022625501)

VI. Olympus 1.7mL microtubes (Genesee Scientific, USA; Cat # 24-282)

VII. 1000uL reach barrier tips (Genesee Scientific, USA; Cat # 24-430)

VIII. 10mL serological pipets (Genesee Scientific, USA; Cat # 12-104)

IX. Surgical scissors (Fine science tools, USA; Cat # 14002-16)

X. Fine scissors (Fine science tools, USA; Cat # 14106-09)

XI. Graefe curve forceps (Fine science tools, USA; Cat # 11051-10)

XII. Graefe straight forceps (Fine science tools, USA; Cat # 11050-10)

XIII. Wescott spring scissors (Fine science tools, USA; Cat # 15015-11)

XIV. Vannas spring scissors (Fine science tools, USA; Cat # 15000-10)

XV. Surgical knife (Surgical Specialties Corporation; Cat # 72-2201)

XVI. AirClean Systems ductless microscope enclosure (AC648TMIC)

XVII. SMZ-800 Zoom Stereo Microscope System (Nikon Instruments, Inc, USA; Cat# MNA41000) that includes the following components: (Binocular tube (MNB42100); Widefield Eyepiece (MMK30102); Plan Achromat Objective (MNH43100), Plain stand (MMD31000), Beam splitter (MNB45901); LV-TV Tube for Ti3 (MBB63430); Cover type 104-lab/OptE400/E600/SMZ (MXA22061); C-NI-150 Fiber optic light source with heat filament 115V (83365); C-dual gooseneck light pipes (83371); C-

focusing lens for gooseneck light pipes (83373); DS-L2 Camera control unit (MQA21010); DS-Vi1 color digital camera head (MQA120-10), DS camera I/F cable (MQF11000); AC adapter for 55iLED microscope (MQF52055); power cord (79035); C-0.7x DXM relay lens (MQD42070); DS-L2 Deluxe support kit (97049); (Nikon Instruments, Inc, USA)

XVIII. Gauze sponges (Fisher Scientific, USA; Cat # 22-037-902)

2.3 Chemicals and reagents for cell culture

I. Betadine (Thermo Fisher Scientific, USA; Cat # 19-027136)

II. Nutrient mixture Ham's F-10 (Sigma-Aldrich, St Louis, MO; Cat # N6013)

III. Astrocyte basal medium complete kit (ScienCell Research Laboratories Cat #1801, Carlsbad, CA)

IV. Fetal bovine serum (Atlas Biologicals, USA; Cat # FP-0500-A)

V. L-glutamine (Thermo Fisher Scientific, USA; Cat # SH3003402)

VI. Penicillin streptomycin (Sigma-Aldrich, St Louis, MO; Cat #P4333)

VII. Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO; Cat # D8537)

VIII. TrypLE Express (Thermo Fisher Scientific, USA; Cat # 12605028)

IX. Transforming Growth Factor Beta 2 (R&D Systems, Minneapolis, MN; Cat # 302-B2-010)

2.4 Antibodies for immunocytochemistry and western blot (see Table 1)

Table 1. List of antibodies

Antibody	**Dilution and application**	**Source**	**Cat #**
Mouse monoclonal α-SMA-FITC conjugated	1:200 ICC	Sigma	F3777
Rabbit α-SMA	1:500 WB 1:100 IHC	Abcam	Ab5694
Mouse monoclonal GFAP	1:500 WB 1:100 ICC	Thermo Fisher	MA5-12023
Mouse monoclonal NCAM	1:100 ICC	Sigma	C9672
Rabbit s100β	1:100 ICC	Abcam	Ab52642
Rabbit Laminin	1:100 ICC	Sigma	L9393
Anti-Mouse HRP	1:1000 WB	Cell Signalling	7076S
Anti-Rabbit HRP	1:1000 WB	Cell Signalling	7074S
Donkey Anti Mouse 594	1:200 ICC	Invitrogen	A-21207
Donkey Anti- Rabbit 488	1:200 ICC	Invitrogen	A-21206
Mouse monoclonal GFAP	1:100 IHC	NeoMarkers	Ab-6

ICC=immunocytochemistry, IHC=immunohistochemistry, WB=western blot

3. Detailed Methods

3.1 Human eye donor source, time of enucleation and age

Human donor eyes were obtained from the Lions Eye Institute for Transplant and Research (Tampa, Florida) within 24 hours of death. All donor eyes obtained were negative for the human immunodeficiency virus 1 and 2, hepatitis B virus, and the hepatitis C virus. The eyes were obtained and managed in compliance with the Declaration of Helsinki. The human eyes used for each experiment ranged from 56 to 99 years old.

3.2 Dissection of the lamina cribrosa from human tissue

Eye globes were sterilized in a specimen container with Betadine (Thermo Fisher Scientific, USA) for 1 minute and then submerged in PBS into a new sterile container. Next, the straight Graefe forcep was used to transfer the eye from the PBS specimen container into a 10cm petri dish lined with a PBS-moist mesh gauze (Figure 2 A). Extraneous tissue from the eye globe was removed by using both the Graefe forceps and surgical scissors. A surgical knife was used to make a small incision into the sclera at the equator and surgical scissors were used to bisect the eye (Figure 2 B). The posterior eye segment was secured with straight Graefe forceps with one hand, and with the other hand, the curved Graefe forceps were used to remove the vitreous, retinal pigment epithelium and choroid from the posterior segment of the eye (Figure 2 C-D). With the posterior segment facing down (Figure 2B), the sheath surrounding the optic nerve should be gently grasped and removed using the surgical scissors and dissected up to the scleral canal surrounding the ONH. As the posterior segment faces up, grasp the stalk of the optic nerve

with the Graefe forceps, and use the surgical scissors to trim up to the peripapillary sclera. Under a dissecting microscope, the peripapillary sclera should be removed using the Wescott spring scissors and the prelaminar region of the ONH delicately trimmed using the Vannas spring scissors (Figure 1 and Figure 3 B and D). At this point, use the fine or Westcott spring scissors to carefully dissect the lamina cribrosa region of the ONH, approximately 1mm down and section into 2 or 3 segments for explant cell culture. If desired, section the optic nerve, and culture the explant in ABM with supplements to culture optic nerve astrocytes. We did not separetly culture the prelaminar region, which should contain both type 1A and type 1B astrocytes. We choose to specifically look at the lamina cribrosa since this tissue is remodeled during glaucomatous optic neuropathy.

3.3 Tissue culture of the lamina cribrosa explant

The dissected explants should be placed into a 12-well plate without medium for approximately 1-2 minutes to allow adhesion to the well. Ham's F-10 (500µL-1000µL) growth media, containing10% FBS L-glutamine (0.292 mg/ml), and penicillin (100units/ml)/ streptomycin (0.1mg/ml) was used to incubate the explants in a humidified chamber at 37°C in 5% CO_2. ONH cell outgrowth and migration from the explant can take up to 3-4 weeks. The medium was replaced once per week until cells migrated out of the explant; after that, the medium was changed every 2-3 days. We recommend leaving approximately 10-20% of the conditioned medium in the wells when changing medium to ensure cells have constant contact with growth factors that were secreted into the culture medium.

3.4 Culturing ONH astrocytes and lamina cribrosa cells

To obtain both ONH astrocytes and lamina cribrosa cells from the same explant, we cultured the explant in Ham's F-10 medium supplemented with 10% FBS L-glutamine (0.292 mg/ml), penicillin (100units/ml)/streptomycin (0.1mg/ml) in a 12-well culture plate. Cell confluency takes about 8-12 weeks in a single well. Initially, cells were passaged with TrypLE Express reagent and diluted 1:2 ratio (passage 1) into a new 12-well culture plate. One well in the 12-well plate was used to maintain lamina cribrosa cells in Ham's F-10 medium with 10% FBS and supplements. The other well was used to obtain ONH astrocytes. For astrocyte isolation, the trypsinized cells were resuspended and cultured in astrocyte basal medium (ABM) without FBS for 72 hours, and then switched to ABM with 5% FBS and supplements. Note, this step is essential to isolate astrocytes since lamina cribrosa cells fail to attach in serum-free conditions (Lambert et al., 2001; Yu et al., 2008). Cells were grown to confluency and the passage protocol repeated in their respective mediums until cells can be expanded into a T25 or T75 flask. We then used Cytodex 3 microcarrier beads instead of trypsin for subsequent cell passage. We have observed that Cytodex 3 microcarrier beads allow these cells to be carried to about 8 passages. We recommend continuing the passaging of cells until these cells begin to become senescent or unhealthy. Cells serially passaged with trypsin become senescent more quickly at lower passages. We generally perform experiments on cells from passages 3-6. Be sure to cryopreserve early passage numbers of each cell strain.

3.5 Characterization of ONH cells using western blot

Whole cell lysates were collected from cultured ONH astrocytes and lamina cribrosa cells using MPER lysis buffer with Halt protease inhibitor cocktail (Pierce Biotech, Rockford, IL). The protein concentration was determined using the BioRad Dc Protein Assay kit (Bio-Rad Laboratories; Hercules, CA) according to manufacturers' instructions. After protein estimation, 15-30μg total protein from each sample was loaded and separated on an SDS-PAGE denaturing 10% acrylamide gel, and then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were subsequently blocked with 5% non-fat dry milk in Tris-Buffered Saline, 0.1% Tween-20 (TBST) for 1 hour at room temperature, and then incubated with specific primary antibodies (Table 1) at 4°C on a rotating shaker overnight. The membranes were washed three times using TBST and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour on a rotating shaker at room temperature. The signal was detected using a chemiluminescence substrate and blots were exposed to an imager (Bio-Rad, USA).

3.6 Characterisation of ONH cells using immunocytochemistry

To document the presence of GFAP, α-SMA, NCAM, s100β, and laminin in human ONH cells, ONH astrocytes and lamina cribrosa cells were cultured on round glass coverslips in 24 well plates until confluent. ONH cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and permeabilized with 0.2% Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA) in PBS at room temperature. Donkey serum in superblock PBS was used for 1 hour to prevent non-specific binding of the antibody. The cells were incubated with or

without the primary antibody (negative control) overnight at 4°C. After the incubation period, the cells were washed three times with PBS and then incubated with the appropriate fluorescent conjugated secondary antibody for 1 hour in dark conditions at room temperature. The cells were washed three times with PBS, followed by two quick H_2O rinses. Coverslips were mounted to glass slides with mounting medium containing 4'6'-diamino-2-phenylindole (DAPI; Prolong with DAPI; Invitrogen Molecular Probes) to stain nuclei. Slides were kept in dark conditions and dried overnight at room temperature before imaging using the Nikon Eclipse TieU Microscope (Melville, NY) containing the Nuance FX imaging system (CRI Burlington, MA).

3.7 Localization of ONH cells using immunohistochemistry

Immunocytochemistry details for ONH tissues have been published previously (Tovar-Vidales et al., 2016). Human ONH tissues were fixed and immunostained with α-SMA and GFAP, followed by the appropriate secondary antibodies. Immunofluorescence images were taken using a Zeiss 410 confocal imaging system (Carl Zeiss, Thornwood, NY).

4. Results

4.1 Primary cell culture from the ONH

Initial cell migration from the lamina cribrosa explant appeared after approximately three weeks in culture. Cells grew as a monolayer surrounding the lamina cribrosa explant (Figures 4 A-B). Cells were a mixed population of ONH astrocytes and lamina cribrosa cells with few to multiple processes. After passage 1, we cultured the cells in either Ham's F-10 medium for optimal

growth of lamina cribrosa cells or ABM for astrocytes, as described in the detailed methods. Lamina cribrosa cells appeared flat, polygonal-shaped with few processes, and grew as a confluent monolayer (Figure 4C). ONH astrocytes have several long thin processes with star-shaped morphology and do not grow as a confluent monolayer; however, as soon as cell processes contact neighboring processes, then cell proliferation ceases (Figure 4D). It is important to note that these cells cultured were not microglia cells. Microglia cells have a different morphology, do not express GFAP, and are difficult to isolate and grow under the conditions we used (Hernandez, 2000).

4.2 Characterisation of lamina cribrosa cells and ONH astrocytes

Western blot analysis (Figure 5) and immunofluorescent staining (Figure 6 -7) using cellular markers were used to characterize the ONH cells. ONH astrocytes stained for the intermediate filament marker GFAP, NCAM, s100β (Figure 6), but were negative for α-SMA. In contrast, lamina cribrosa cells were positive for α-SMA, NCAM (data not shown), s100β, but negative for GFAP (Figure 7). The vast majority of ONH astrocytes stained GFAP positive and the vast majority of lamina cribrosa cells were a-SMA positive (i.e. nearly all of the DAPI stained lamina cribrosa cells were α-SMA positive and DAPI stained ONH astrocytes were GFAP positive). Treatment with TGFβ2 resulted in more pronounced α-SMA fibers compared to control treated lamina cribrosa cells (Figure 7). Lamina cribrosa cells were positive for laminin, confirming they were not scleral fibroblasts. Scleral fibroblasts do not express the basement membrane proteins laminin or collagen type IV, have a long spindle-shaped morphology, and generally grow in multiple layers (Clark et al., 1995; Hernandez et al., 1987; Hernandez et al., 1991). Lamina

cribrosa cells grow as a monolayer and are considered as a unique cell type within the lamina cribrosa (Hernandez, 2000).

4.3 Localization of lamina cribrosa cells and ONH astrocytes in human ONH tissue

We used immunofluorescent staining of human donor eyes to identify ONH astrocytes and lamina cribrosa cells within the lamina cribrosa (Figure 8). We used antibodies against α-SMA (red) and GFAP (green) to show expression and localization of GFAP positive ONH astrocytes and α-SMA positive lamina cribrosa cells. As previously described, lamina cribrosa cells were localized within lamina cribrosa plates, while ONH astrocytes were present within the lamina cribrosa and their processes seem to surround RGC axon bundles (Hernandez, 2000; Tovar-Vidales et al., 2016). Nuclei of ONH cells were visualized by DAPI.

4.4 Summary of ONH cell characterization (See Table 2)

Previously, our laboratory has shown that both ONH astrocytes and lamina cribrosa cells express laminin *(Clark et al., 1995; Lambert et al., 2001)*. We have summarized the markers used to characterize these two ONH cell types in Table 2.

Table 2. Summary of ONH cell characterization. ONH astrocytes isolated from lamina cribrosa expressed GFAP, NCAM, and s100β, but were negative for α-SMA. Lamina cribrosa cells expressed α-SMA, laminin, and s100β, but were negative for GFAP.

Intracellular/extracellular marker	**ONH astrocytes**	**Lamina Cribrosa cell**
α-SMA	-	+
GFAP	+	-
NCAM	+	+
s100β	+	+
Laminin	+ (Clark et al., 1995; Lambert et al., 2001)	+

6. Potential Pitfalls and Trouble Shooting

5.1 Presence of scleral fibroblasts

Fibroblast contamination is avoided by careful dissection of the lamina cribrosa explant. This procedure involves the removal of the scleral tissue, pigment, and nerve sheath surrounding the ONH. Incomplete removal of scleral tissue may result in scleral fibroblast proliferation from the explant. We recommend distinguishing between lamina cribrosa cells and scleral fibroblasts by using cell morphology (i.e. scleral fibroblasts grow in multiple layers) and immunocytochemical

analysis of collagen type IV or laminin (Clark et al., 1995; Hernandez et al., 1987; Hernandez et al., 1991).

5.2 Location of the lamina cribrosa

Determining the location of the lamina cribrosa may be challenging. Nerve fibers at the posterior margins (post laminar region) of the lamina cribrosa are myelinated and increase in thickness; therefore, we recommend using the unmyelinated and myelinated regions of the optic nerve as a landmark for dissection. We recommend making the posterior cut approximately 1mm down from the anterior surface of the ONH before the increased thickness of the myelinated region (Figure 1).

5.3 Passage number and isolation of ONH astrocytes

Choosing the correct passage to culture ONH astrocytes can influence the growth and proliferation of astrocytes. At passages 2-3, there is a co-culture of lamina cribrosa cells and ONH astrocytes; at later passages of 4 or more, the ability to isolate ONH astrocytes declines.

5.4 Adherence of the lamina cribrosa explant

Try to be certain that the lamina cribrosa explant pieces adhere to the plate before the addition of medium so that the explants do not float in the medium. We recommend not to add more than 1mL of medium to the 12-well culture plate and avoid any disturbances while moving the plate.

The explant must adhere to the culture dish to increase the likelihood of cell migration from the explant. The explant may need to be cultured for up to 4 weeks in vitro to generate ONH cell outgrowth. It may help to cut the explant into 2-3 segments before culturing or use a collagenase enzyme to digest the collagen extracellular matrix to enhance cell migration from the explant. We have not used collagenase to digest the ONH explant; however, other researchers were successful in using collagenase to dissociate cells from the trabecular meshwork explants (Keller et al., 2018; Stamer et al., 1995).

5.5 Type 1B astrocytes

Astrocytes can be categorized into subclasses within the ONH: type 1 (A and B) and type 2. Type 1 astrocytes are located in the pre-laminar region and lamina cribrosa, whereas type 2 astrocytes are located in the myelinated optic nerve. Type 1 B is the major astrocyte subtype present in the lamina cribrosa and are NCAM positive, while type 1 A astrocytes are NCAM negative.

5.6 Age of donor

Age of human donor eyes may have implications in cell isolation. The infant lamina cribrosa is composed mostly of collagen type III, in contrast to the adult lamina, which is mostly collagen type I, suggesting that the lamina cribrosa is not fully developed and organized (Hernandez et al., 1991; Morrison et al., 1989). The margins may not be evident in infant lamina cribrosa, and there

could be contamination from other cell types. We recommend obtaining eyes from donors > 1 year.

5.7 Time of enucleation

The time of death to culture can influence the growth rate and yield of cells from the explant. The use of eyes enucleated and received within 24 hours of death is recommended for increased recovery of viable cells. However, we have successfully cultured cells from lamina cribrosa explants up to 72 hours post enucleation.

7. Discussion

The lamina cribrosa is a region of dense fibroelastic connective tissue, forming the border between the intraocular and retrobulbar tissues. Retinal ganglion cell axons traverse this region from a relatively high pressure to a low-pressure environment. The lamina cribrosa is the initial site of glaucomatous damage- the earliest detectable change is compression of connective tissue plates (Quigley et al., 1983). Isolating cells that populate this region will increase our understanding of cellular and extracellular matrix changes that occur in glaucoma.

Previous methods have been described isolating human, rat, and porcine ONH astrocytes using immunopanning or astrocyte selective medium (Lukas and Wang, 2012; Murphy et al., 2011; Obazawa et al., 2004; Rogers et al., 2012b; Yang and Hernandez, 2003; Yu et al., 2008; Yu et al., 2007). Techniques to isolate human ONH astrocytes have been published from other research

laboratories. Previously, investigators have isolated ONH astrocytes from lamina cribrosa cells by using serum-free astrocyte growth medium (Rogers et al., 2012b; Yu et al., 2007). Yang and Hernandez have previously cultured ONH explants for up to four weeks and selected ONH astrocytes by immunopanning using specific antibodies to remove non-astrocytes (Yang and Hernandez, 2003).

Our lab has developed a modified technique using a selective medium to isolate both lamina cribrosa cells and ONH astrocytes from a single lamina cribrosa explant from human donor eyes. Initially, cells that grew from the explant were a co-culture of ONH astrocytes and lamina cribrosa cells. For ONH astrocytes, we serum-deprive co-cultures to isolate astrocytes from non-adhered lamina cribrosa cells. Researchers have shown that ONH astrocytes can be isolated from lamina cribrosa cells after trypsinization and placed in astrocyte serum-free medium for 24 hours from a co-culture consisting of ONH astrocytes and lamina cribrosa cells (Yu et al., 2008). To ensure complete removal of lamina cribrosa cells, we have used serum-deprivation for up to 72 hours and then ONH astrocytes were placed in astrocyte medium with 5% FBS. The 72-hour time point was determined empirically as providing the best astrocyte yield without lamina cribrosa cell contamination. The ability to isolate ONH astrocytes decreases as the co-culture cell passage increases. We recommend isolating ONH astrocytes no later than passage 3 from ONH co-cultures. Lambert and co-workers used high serum (10%) Ham's F-10 Nutrient Mixture to isolate lamina cribrosa cells from ONH astrocytes (Lambert et al., 2001). Passaging cells a few times may be necessary to remove all ONH astrocytes to obtain a pure lamina cribrosa cell population.

We used both western blot and immunocytochemistry to characterize ONH cells using markers previously described (Hernandez et al., 1988; Lambert et al., 2001; Rogers et al., 2012a; Rogers et al., 2012b; Yang and Hernandez, 2003). Astrocytes isolated from the lamina cribrosa expressed GFAP, but were negative for α-SMA. In contrast, lamina cribrosa cells expressed α-SMA, but were negative for GFAP (Table 2). ONH astrocytes also expressed NCAM, suggesting these cells are type 1B astrocytes (Kobayashi et al., 1997; Ricard et al., 1999).

With this cell culture isolation technique, we can study cell-specific changes that occur during disease pathophysiology, such as glaucoma. Primary open-angle glaucoma is associated with elevated intraocular pressure. Ocular hypertension may lead to mechanical deformation of the ONH, including compression and stretching. Increased pressure within the eye may directly affect pressure-sensitive cells in the ONH, including ONH astrocytes and lamina cribrosa cells. Using this technique, we can study gene expression changes and activated pathways in response to glaucomatous conditions such as mechanical stretching or activated growth factors.

ONH astrocytes are the focus of many studies because they are the main glial cell type in the ONH. ONH astrocytes provide structural and trophic support to RGC axons, as well as communicate with the surrounding extracellular matrix, tissues, cells, and vasculature. Although ONH astrocytes have many normal homeostatic functions, they also are responsible for many pathological changes in the glaucomatous ONH (Hernandez, 2000; Hernandez et al., 2002; Hernandez et al., 2008; Schneider and Fuchshofer, 2016). Several investigators have utilized biomechanical strain to mimic the effects of pressure induced strain on ONH astrocytes in order to identify early cellular events/changes in glaucoma (Rogers et al., 2012b). Lamina cribrosa

cells also respond to stretch and investigators have used the Flexercell system to induce cyclical strain and evaluate changes in gene expression (Kirwan et al., 2005; Rogers et al., 2012a). Key upregulated pathways in response to strain are transforming growth factor-beta (TGFβ) and extracellular matrix synthesis (Kirwan et al., 2005; Quill et al., 2011; Rogers et al., 2012a; Rogers et al., 2012b). The pro-fibrotic cytokines TGFβ2 and gremlin are elevated in the glaucomatous ONH and promote extracellular matrix synthesis (Pena et al., 1999; Zode et al., 2009; Zode et al., 2011). Our lab has previously used recombinant TGFβ2 protein to replicate endogenous conditions for glaucoma in cultured ONH cells to determine the effects on extracellular matrix proteins (Zode et al., 2011). These techniques and experimental approaches will allow more scientists to isolate both ONH astrocytes and lamina cribrosa cells to improve our understanding of cell-specific responses to glaucomatous conditions and potential treatments. For example, we have used ONH cells isolated from the human lamina cribrosa to identify the expression and secretion of neurotrophins and trk receptors, suggesting they may provide neurotrophic support for RGC neurons (Lambert et al., 2001). Also, we have shown using isolated ONH astrocytes and lamina cribrosa cells that TGFβ2 and the bone morphogenetic protein antagonist gremlin is implicated in the pathology of the glaucoma ONH (Zode et al., 2009; Zode et al., 2011).

8. Conclusions

The lamina cribrosa is progressively remodeled in glaucoma. Cells within the lamina cribrosa may be responsible for this pathological remodeling. Two major cell types have been identified in the lamina cribrosa: GFAP positive ONH astrocytes and α-SMA positive lamina cribrosa

cells. There may be cell specific changes during glaucoma pathology; therefore, isolating and culturing these cell types may help understand the cellular and molecular changes that occur in glaucoma.

Acknowledgments

The authors would like to thank the Lions Eye Institute for Transplant and Research. This research was supported by the NIH training grant T32 AG 020494 (NL).

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Figures

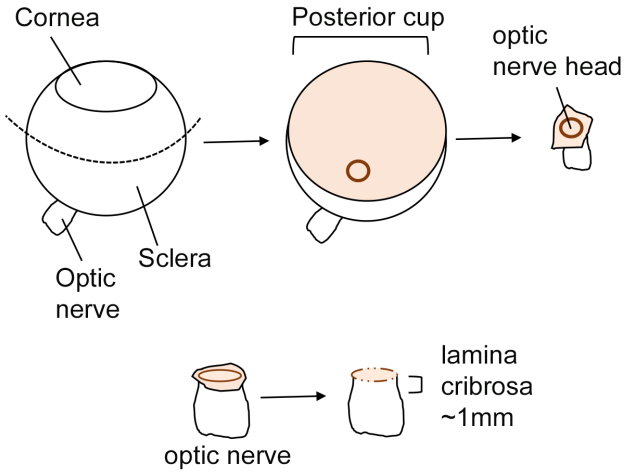

Figure 1. Schematic of eye dissection

A schematic diagram illustrating the different steps involved in ONH dissections from a human eye. An incision at the equator is made to remove the posterior segment of the eye, the scleral of the ONH up to the scleral canal is removed, removal of the peripapillary sclera, scleral canal and the prelaminar region. *Dotted line*: the equator.

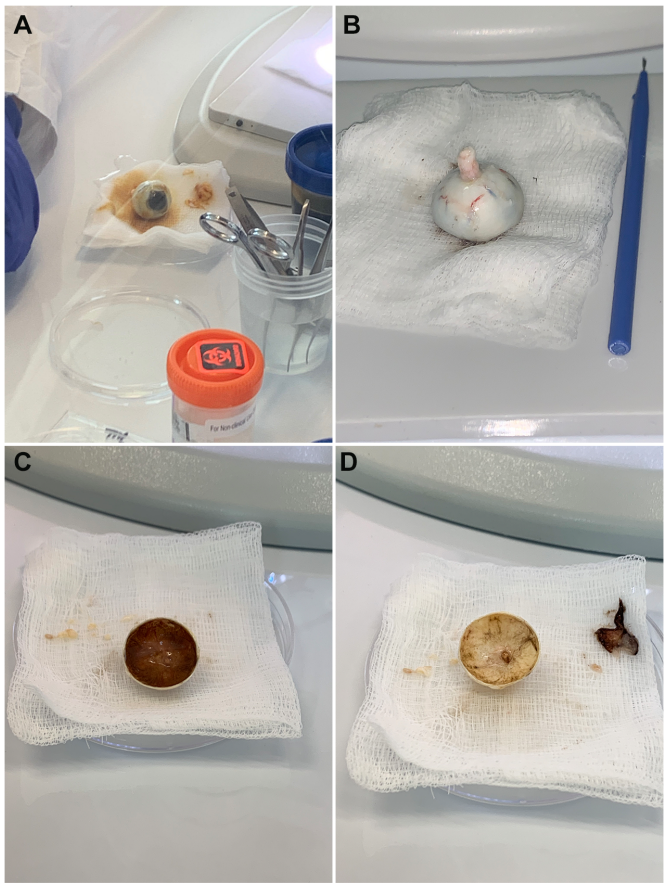

Figure 2. Dissection of the human ONH. Dissection images of the human ONH. (A) A whole human eye globe, (B) the posterior segment with the lens and vitreous humour removed, (C) lastly, the choroid and RPE are removed from the posterior segment of the human eye, leaving the scleral shell, optic nerve head, and the optic nerve (D).

Figure 3. Light microscopy images of the ONH with the optic nerve. Progressive removal of the RPE and peripapillary sclera from the ONH. (A-B) Peripapillary sclera with pigment surrounding the ONH, (C-D) removal of the peripapillary sclera with the pigment is trimmed. Scale bar: 100µm. Note: Pigment remains are visible for identification of the ONH, but was removed prior to dissection.

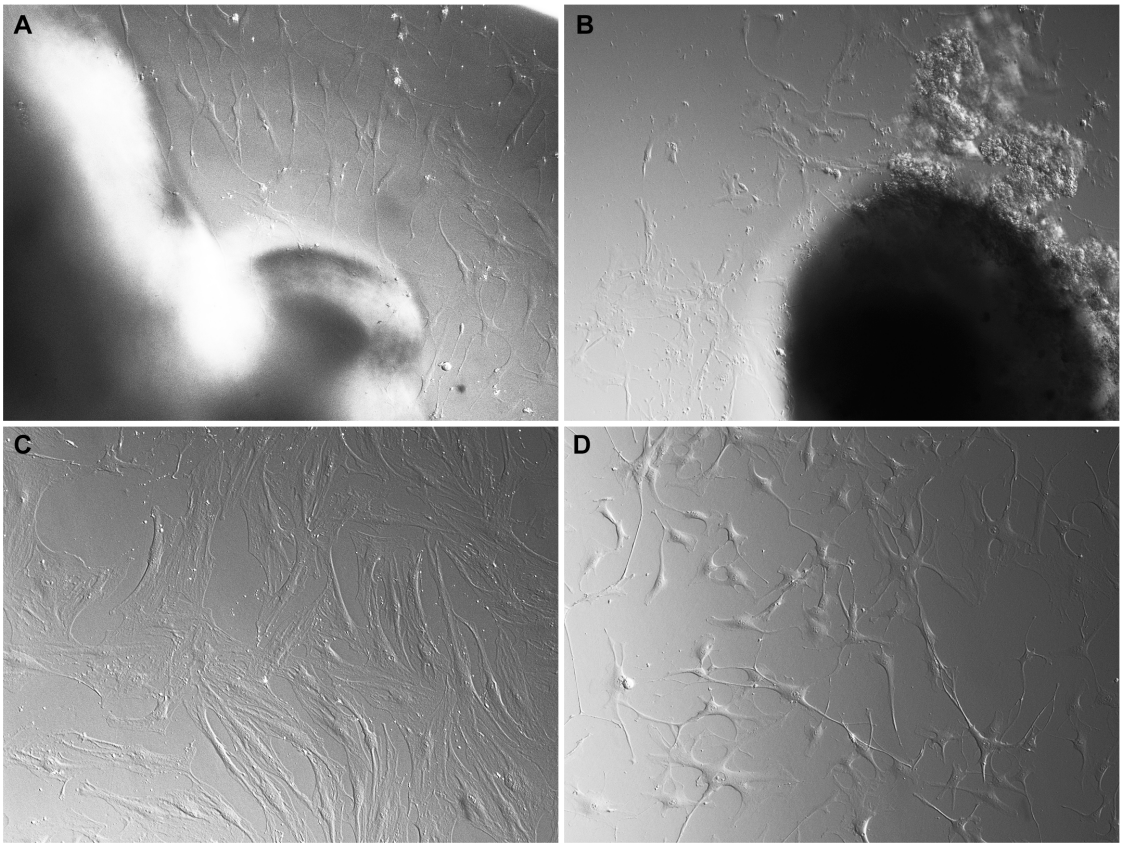

Figure 4. Morphology of human lamina cribrosa explant and migrating cells. (A-B) ONH cells migrating from lamina cribrosa explant using Hoffman modulus optics (Nikon, Inc.), (C) lamina cribrosa cells appeared as flat, broad shaped cells, (D) ONH astrocytes had several processes with star shape morphology.

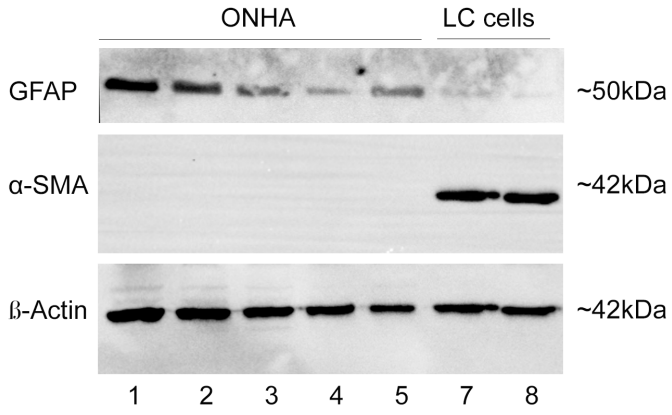

Figure 5. Western blot image of GFAP and α-SMA in ONH cells. (Lanes 1-5) ONH astrocytes were positive for GFAP and negative for α-SMA. In contrast, lamina cribrosa cells (Lanes 7-8) were negative for GFAP and positive for α-SMA. β-actin was used as a loading control.

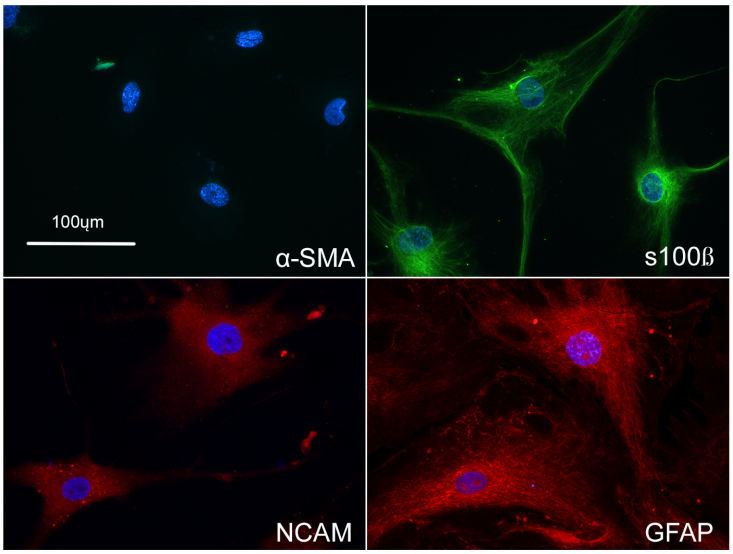

Figure 6. Characterization of ONH astrocytes. Immunostaining of in ONH astrocytes for GFAP, s100β, and NCAM, and α-SMA. ONH astrocytes expressed GFAP, s100β, and NCAM; however, were negative for α-SMA. Nuclei (blue) were stained with DAPI. Negative control consisted of PBS-BSA without primary antibody (data not shown) (Scale bar: 100µm)

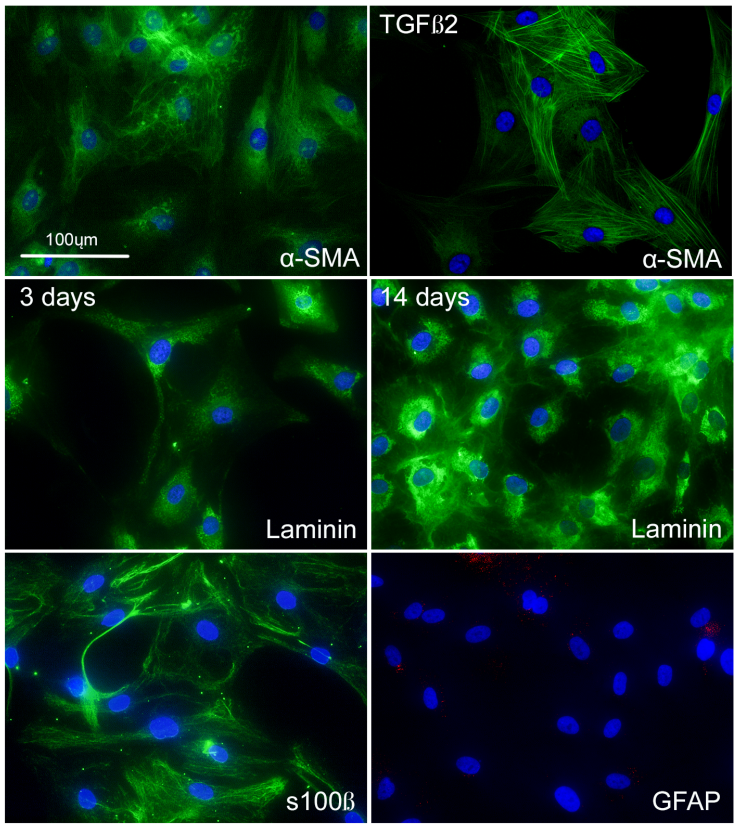

Figure 7. Characterization of lamina cribrosa cells. Immunostaining of lamina cribrosa cells for α-SMA, s100β, laminin, and GFAP. Lamina cribrosa cells treated with or without TGFβ2 expressed α-SMA. Also, lamina cribrosa cells expressed s100β, laminin; however, they were negative for GFAP. Nuclei (blue) were stained with DAPI. The Negative control consisted of PBS-BSA without the primary antibody (data not shown). (Scale bar: 100µm)

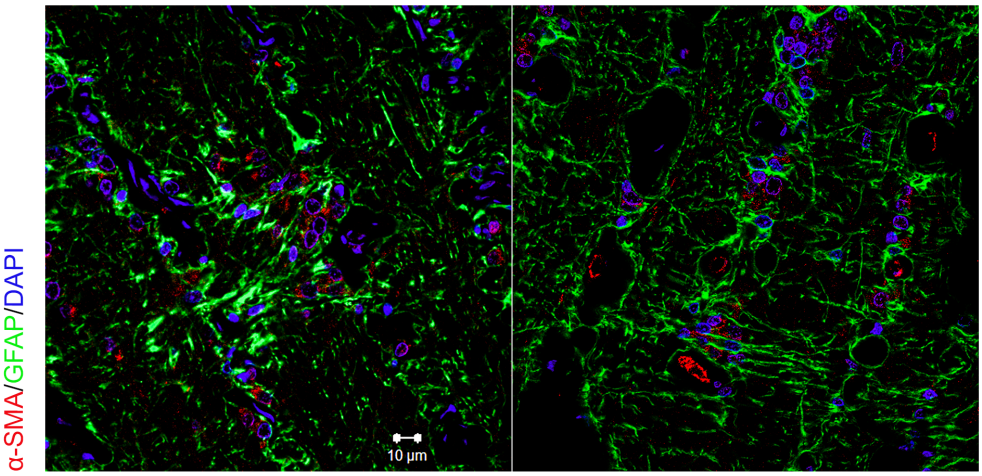

Figure 8. Immunohistochemical localization of GFAP and α-SMA in the human ONH. ONH tissues were fixed, sectioned and stained with antibodies for GFAP and α-SMA. Immunohistochemical analysis shows the presence of α-SMA positive lamina cribrosa cells and GFAP positive astrocytes within the lamina cribrosa region. Nuclei (blue) was stained with DAPI. The negative controls consisted of PBS-BSA without primary antibody, IgG, and mouse ascites (data not shown). (Scale bar: 10µm)

CHAPTER III

MIRNA EXPRESSION IN TGFβ2 TREATED OPTIC NERVE HEAD ASTROCYTES AND LAMINA CRIBROSA CELLS

Submitted: IOVS

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Abstract

Purpose: TGFβ2 is a pro-fibrotic cytokine that is elevated in the glaucomatous lamina cribrosa and leads to increased extracellular matrix synthesis in optic nerve head (ONH) astrocytes and lamina cribrosa cells. In this study, we aimed to investigate the intermediary mechanisms that lead to increased extracellular matrix gene expression in response to TGFβ2 signaling. microRNAs (miRNAs) regulate gene expression by inhibiting protein translation. Deregulation of miRNAs is implicated in diseases associated with perturbed TGFβ2 signaling and extracellular matrix synthesis. We hypothesized that TGFβ2 signaling influences the expression of miRNAs in ONH astrocytes and lamina cribrosa cells.

Methods: We isolated ONH astrocytes and lamina cribrosa cells from non-glaucomatous human donor eyes and treated these cells with TGFβ2 or vehicle control. We used miRNA PCR arrays to determine differentially expressed miRNAs. We utilized the miRNet database to determine signal pathways and biological processes associated with differentially expressed miRNAs.

Results: We analyzed the expression of 88 mature miRNAs in ONH astrocytes and lamina cribrosa cells. There was a significant upregulation of miR-744-5p and miR-208a-5p and downregulation of miR-199a-5p in ONH astrocytes. We also observed an increase in miR-155-5p in ONH astrocytes, which is associated with regulation of pro-inflammatory cytokines. Although not statistically significant, downregulation of miR-29c-3p in lamina cribrosa cells may be of biological significance.

Conclusions: This exploratory study determined that TGFβ2 signalling resulted in differential expression of miRNAs in ONH astrocytes and lamina cribrosa cells. Dysregulated miRNAs may have important roles in ONH extracellular matrix and inflammatory pathways.

Keywords: optic nerve head astrocytes, lamina cribrosa cells, microRNAs, TGFβ2

Abbreviations:

POAG- Primary open-angle glaucoma

ONH- Optic nerve head

RGC- Retinal ganglion cell

GFAP- Glial fibrillary acidic protein

α-SMA- Alpha smooth muscle actin

NCAM- Neural cell adhesion molecule

s100β- s100 calcium binding protein beta

miRNA- microRNA

TGFβ2- Transforming growth factor beta 2

Introduction

Primary open-angle glaucoma (POAG) is a neurodegenerative disease caused by dysfunction and degeneration of retinal ganglion cells (RGCs), which leads to an irreversible loss of visual function. In POAG, there is structural damage to the optic nerve head (ONH), including compression of the lamina cribrosa and progressive remodelling of the extracellular matrix [1,2].

Altered expression of fibrogenic growth factors contributes to gene expression changes, increased extracellular matrix synthesis, disorganization of collagen and elastin fibrils, and remodelling of the glaucomatous lamina cribrosa [3-8]. An important growth factor associated with extracellular matrix tissue remodelling is transforming growth factor-beta 2 (TGFβ2), an activator of the canonical SMAD-dependent TGFβ pathway [9]. TGFβ2 binds to the TGFβ type II receptor (TGF βRII), leads to TGFβ type I/II receptor dimerization, and phosphorylation of intracellular receptor-specific SMAD proteins SMAD2 and 3 (SMAD2/3) [10]. Phosphorylated SMAD2/3 interacts with SMAD4, and this complex translocates to the nucleus. In the nucleus, the heteromeric p-SMAD2/3-SMAD4 complex interacts with co-factors and transcription factors and binds to specific DNA sequences, SMAD binding elements (SBE), in the promoter region of target genes. Activation of TGFβ2 signaling regulates the transcription of specific genes, including proteins and non-protein coding microRNAs (miRNAs) [11-13].

miRNAs are regulatory RNAs that inhibit the expression of select proteins and are essential to the SMAD-dependent TGFβ pathway. miRNAs are initially transcribed as long primary miRNA (pri-miRNA) transcripts, which are then cleaved by RNase III DROSHA to form hairpin loop structures, the precursor of miRNAs (pre-miRNA) [14]. This intermediate structure is exported

from the nucleus to the cytoplasm by the Ran-GTP dependent receptor, exportin-5 (EXP-5) [15,16] and processed by RNase III DICER into a short double stranded ~ 22 nucleotide sequence [17]. The double stranded miRNA is loaded into argonaute (AGO) proteins, and the guide strand is selected and presented to the RNA-induced silencing complex (RISC). The mature miRNAs generated select and bind most commonly to the 3' untranslated region (UTR) of target mRNAs, and lead to translational repression or degradation of these mRNAs [18-21]. Due to the inhibitory action of miRNAs, the spatiotemporal expression is important to regulate the physiological function of cells and tissues. It is not surprising that deregulation of miRNAs is implicated in disease.

The expression of miRNAs is sensitive to growth factor signaling, including TGFβ [22]. TGFβ activation of SMADs, cofactors, and transcription factors can lead to transcriptional activation or inhibition of miRNA genes. For example, TGFβ signalling induces differentiation of myoblasts to myofibroblasts via SMAD3 binding to the miR-29 promoter and downregulation of miR-29 expression [23]. miRNAs can also be modulated at the post-transcriptional level by R-SMAD proteins, which recruit and interact with members of the miRNA processing complex, DROSHA and p68, and enhance cleavage of pri-miRNAs to mature miRNAs [12]. It is therefore important to achieve homeostatic regulation of TGFβ signalling. In POAG however, levels of TGFβ2 are increased in the lamina cribrosa[8,24] and histopathological analysis shows there is a disruption in collagen and elastin structures, loss of axon bundles, and excess accumulation of basement membrane proteins [1,25-28]. Accumulating evidence shows that the ONH cells, ONH astrocytes and lamina cribrosa cells are responsible for these structural extracellular matrix changes in the lamina cribrosa [8,29,30]. Since TGFβ signaling can directly influence the expression of miRNAs,

and miRNAs regulate translation of proteins, we hypothesized that TGFβ2 deregulates miRNA expression in ONH astrocytes and lamina cribrosa cells and contributes to lamina cribrosa extracellular matrix remodelling. In this preliminary study, we treated normal ONH astrocytes and lamina cribrosa cells with TGFβ2 to determine whether TGFβ2 alters the expression of miRNAs using miRNA PCR arrays.

Materials and Methods

Cell Culture and TGFβ2 treatment

Primary ONH astrocyte cell strains (n=4) and lamina cribrosa cell strains (n=3) without history of glaucoma were generated from human lamina cribrosa explants and characterized as previously described[31]. Briefly, ONH astrocytes and lamina cribrosa cells were characterized by cell markers including GFAP, α-SMA, NCAM, s100β and laminin[31]. ONH astrocytes were maintained in astrocyte basal medium (ABM) supplemented with 5% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin and streptomycin (Thermofisher, Waltham, MA). lamina cribrosa cells were maintained in Ham's F10 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin and streptomycin (Thermofisher, Waltham, MA). We seeded cells into wells of a 6-well plate in serum medium and when the cells reached 100% confluency, we replaced the medium with serum-free medium for 24 hours. The next day the cells were treated with recombinant TGFβ2 (5ng/ml, R&D Systems, Minneapolis, MN) or vehicle control in serum free medium.

Patient Demographics

The baseline characteristics of the patient groups are summarized in Table 1. ONH astrocytes and lamina cribrosa cells were derived from normal human donor eyes as previously described[31]. The mean age of the of the patients were 73± 4.68 (mean±SD).

Table 1. Baseline Demographics

Subject ID	Cell strain	Age, yr	Sex	Race	Normal/ POAG	notes	History of Neurological/ ocular disease
1	ONHA	74	M	C	Normal	cardiac arrest	No
2	ONHA	70	M	C	Normal		No
3	ONHA	70	M	C	Normal		No
4	ONHA	56	F	C	Normal		No
5	LC cells	74	M	C	Normal	cardiac arrest	No
6	LC cells	56	F	C	Normal		No
7	LC cells	82	M	C	Normal		No

ONHA= ONH astrocytes; LC cells= lamina cribrosa cells M = Male; C =. Caucasian

Isolation of miRNA and reverse transcription

After 24 hours of TGFβ2 treatment, total RNA was isolated using QIAzol and the miRNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's guidelines. RNA was quantified using the Nanodrop 2000 (Thermofisher Scientific, Waltham, MA) and purity/quality of RNA assessed by 260/280 and 260/230 ratios. miRNA cDNA was synthesized using the HiSpec buffer and miScript II RT kit (Qiagen). The thermoprofile parameters used were 37°C for 60 mins and 95°C for 5 mins.

miRNA PCR Arrays

cDNA was diluted to 200µL and we performed mature miRNA profiling using the miScript miRNA PCR array for Human Fibrosis (Figure 1, MIHS-117Z, Qiagen) that includes for 88 mature human miRNA primers. PCR was performed on the CFX96 real-time PCR system (Bio-Rad Laboratories). The thermoprofile parameters were 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and concluded with a melting curve step. Primary human ONH astrocyte (n=4) and primary lamina cribrosa cell (n=3) strains were studied and representative data shown on volcano plots. Statistical analysis was performed using Qiagen's data anlaysis software https://dataanalysis.qiagen.com/mirna/arrayanalysis.php. The miRNAs included in this array target the mRNA of pro-fibrotic, anti-fibrotic, signal transduction, epithelial mesenchymal transition and extracellular matrix genes. For the full miRNA list, visit Qiagen's website (https://geneglobe.qiagen.com/product-groups/miscript-mirna-pcr-arrays).

Reactome pathway analysis of differentially expressed miRNAs

We used miRNet to identify significant Reactome pathways and GO terms biological processes associated with differentially expressed miRNAs (https://www.mirnet.ca/miRNet/upload/MirUploadView.xhtml). This uses a hypergeometric distribution of gene targets using the whole human genome and generates P values.

Results

Differentially expressed miRNAs in ONH astrocytes and lamina cribrosa cells in response to TGFβ2 signaling

Mature miRNA expression was analysed across ONH astrocytes in response to TGFβ2 compared with vehicle control (Figure 1). The expression of miRNAs in ONH astrocytes is shown in a volcano plot (Figure 1). Several miRNAs were upregulated and downregulated at least 2-fold difference in ONH astrocytes (Supplementary Table 1). Those that were upregulated included miR-141-3p, miR-150-3p, miR-155-5p, miR-208a-3p ($P<0.05$), miR-372-3p, miR-375, miR-5011-5p, miR-503-5p, miR-661 and miR-744-5p ($p<0.05$) and downregulated included miR-199a-5p ($p<0.05$), miR-200b-3p, miR-223-3p, miR-29a/b/c-3p, miR-30a, miR-324, miR338-5p and miR-377-3p ($n=4$). miRNA expression was also analysed in lamina cribrosa cells in response to TGFβ2 treatment; miRNA expression is shown in a volcano plot (Figure 2.). miRNAs upregulated included miR-146b-5p, miR-20a-5p, miR-217, miR-324-5p, miR-328-3p and miR-377-3p (Supplementary Table 1). miRNAs downregulated included miR-10a-5p, miR-122-5p, miR-146a-5p, miR-19b-3p, miR-200a3p, miR29b/c-3p and miR-449a (Supplementary Table 1). The miRNA expression changes in lamina cribrosa cells were not statistically significant; however, may be of biological relevance.

Signal pathway analysis of downregulated miRNAs

miRNAs inhibit the translation of a set of proteins, and therefore affect cellular activities and signaling pathways associated with those proteins. To investigate the function of miRNAs in tissue remodelling and fibrosis, we utilized miRNet. miR-199a-5p, which was downregulated in ONH astrocytes ($p<0.05$; Supplementary Table 1), is associated with SMAD2/3 and SMAD4

signaling and regulation of phosphorylation (Table 2). miR-29 was downregulated in ONH astrocytes and lamina cribrosa cells and is associated with extracellular matrix synthesis, organization and degradation (Table 3). Downregulation of these miRNAs in ONH astrocytes and lamina cribrosa cells may lead to aberrant TGFβ-SMAD signaling, increased extracellular matrix synthesis and disorganization of extracellular matrix fibrils. These miRNA could have therapeutic implications in regulation of the TGFβ2-SMAD signalling pathway.

miRNAs upregulated in ONH astrocytes and lamina cribrosa cells

Several miRNAs were upregulated in ONH astrocytes and lamina cribrosa cells. miR-146b-5p and miR-155-5p have pro-inflammatory roles and are associated with the regulation of cytokines (Supplementary Table 2). miR-744-5p is associated with translation initiation and miR-208a-5p associated with NOTCH signalling and apoptosis (Supplementary Table 2). miR-150-5p also has a role in regulating NOTCH signaling and the cellular response to stress, while miR-141-3p regulates protein kinase activity (Supplementary Table 2.).

Discussion

We have found the first analyses of miRNA expression in human ONH astrocytes and lamina cribrosa cells. We have shown that TGFβ2 deregulates miRNA expression in ONH cells and may contribute to the lamina cribrosa remodeling. We also used miRNet, a web-based tool, to investigate the function of miRNAs in tissue remodelling and fibrosis. We identified miRNAs associated with the TGFβ2 canonical signaling pathway, extracellular matrix synthesis and degradation, and pro-inflammatory roles.

Extracellular matrix remodelling of the lamina cribrosa contributes to the pathophysiology of glaucoma [32-34]. Lamina cribrosa cells and ONH astrocytes secrete extracellular matrix molecules to support RGC axons, and both ONH cell types also regulate neuronal function by secreting neurotrophic factors and cytokines [30,35,36]. TGFβ2 is a profibrotic cytokine that is elevated in the glaucomatous ONH [8,24] and is associated with excessive extracellular matrix synthesis in the lamina criborsa[1,27,28]. We aimed to elucidate if TGFβ2 signaling induces these extracellular matrix changes in the ONH by modulating miRNA expression. In this exploratory study, we used miRNA PCR arrays to analyze changes in miRNA expression. We treated human lamina cribrosa cells and ONH astrocytes with TGFβ2 and analysed the expression of 88 mature miRNAs involved in signal transduction, extracellular matrix synthesis and epithelial-mesenchymal transition. This provides a unique advantage to identify cell-specific miRNA changes that may contribute to ONH tissue remodeling.

Using miRNet we identified potential signal pathways and biological processes affected by differential expression of miRNAs. The miR-29 family is predicted to regulate extracellular matrix organization, assembly of collagen fibrils, collagen biosynthesis, proteoglycans and degradation of extracellular matrix (Table 3). The observed decrease of miR-29 in both lamina cribrosa cells and ONH astrocytes may increase extracellular matrix synthesis and lead to disorganization of fibrils, which may alter the structure of the lamina cribrosa. Decreases in miR-199a-5p expression in ONH astrocytes may deregulate phosphorylation and SMAD signalling and lead to increased expression of genes influenced by SMAD signaling (Table 2).

miRNAs significantly upregulated in ONH astrocytes treated with TGFβ2 included miR-744-5p and miR-208a-5p. While not much is known about the role of these miRNA in ONH astrocytes, enrichment analysis showed that miR-744-5p is associated with initiation of translation and miR-208a-5p is associated with regulation of NOTCH signaling and apoptosis. We also identified pro-inflammatory miRNAs that were upregulated in response to TGFβ2. Enrichment analysis showed that miR-155 is associated with cellular responses to stress, VEGF signaling, immune regulation and interleukin-6 (IL-6) signaling. miR-155 regulates the inflammatory cytokine microenvironment by targeting suppressor of cytokine signalling 1(SOCS1), to enhance the production of pro-inflammatory mediators, including IL-6 [37,38]. Interestingly, astrocyte activation is associated with the release of pro-inflammatory cytokines and IL-6 is upregulated near RGC axons in a rat model of experimental glaucoma [39,40]. Although IL-6 contributes to increased RGC survivability in culture, excess production of IL-6 may amplify the immune response by recruiting other inflammatory mediators and cells; thus, cause structural degeneration of RGC axons and dysfunction in glaucoma [41,42]. Recent evidence shows that miR-155 may also contribute to chronic fibrosis since it is overexpressed in fibrotic tissues [43].

To summarize, miRNA expression differed in lamina cribrosa cells and ONH astrocytes treated with TGFβ2 compared to vehicle control. These preliminary observations suggest that these differentially expressed miRNAs may regulate the extracellular matrix and inflammatory pathways in normal and pathologic ONH. Although our sample size is small, this comparative analysis will enhance our understanding of how ONH astrocytes and lamina cribrosa cells respond to increased TGFβ2 signalling in the ONH and contribute tissue remodelling. Further in-vitro and in vivo studies are needed to elucidate the specific roles of these miRNAs in the ONH.

Acknowledgements

The authors would like to thank the Lions Eye Institute for Transplant and Research. This research was supported by the NIH training grant T32 AG 020494 (NL) and Glaucoma Research Foundation grant (TTV).

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Figures

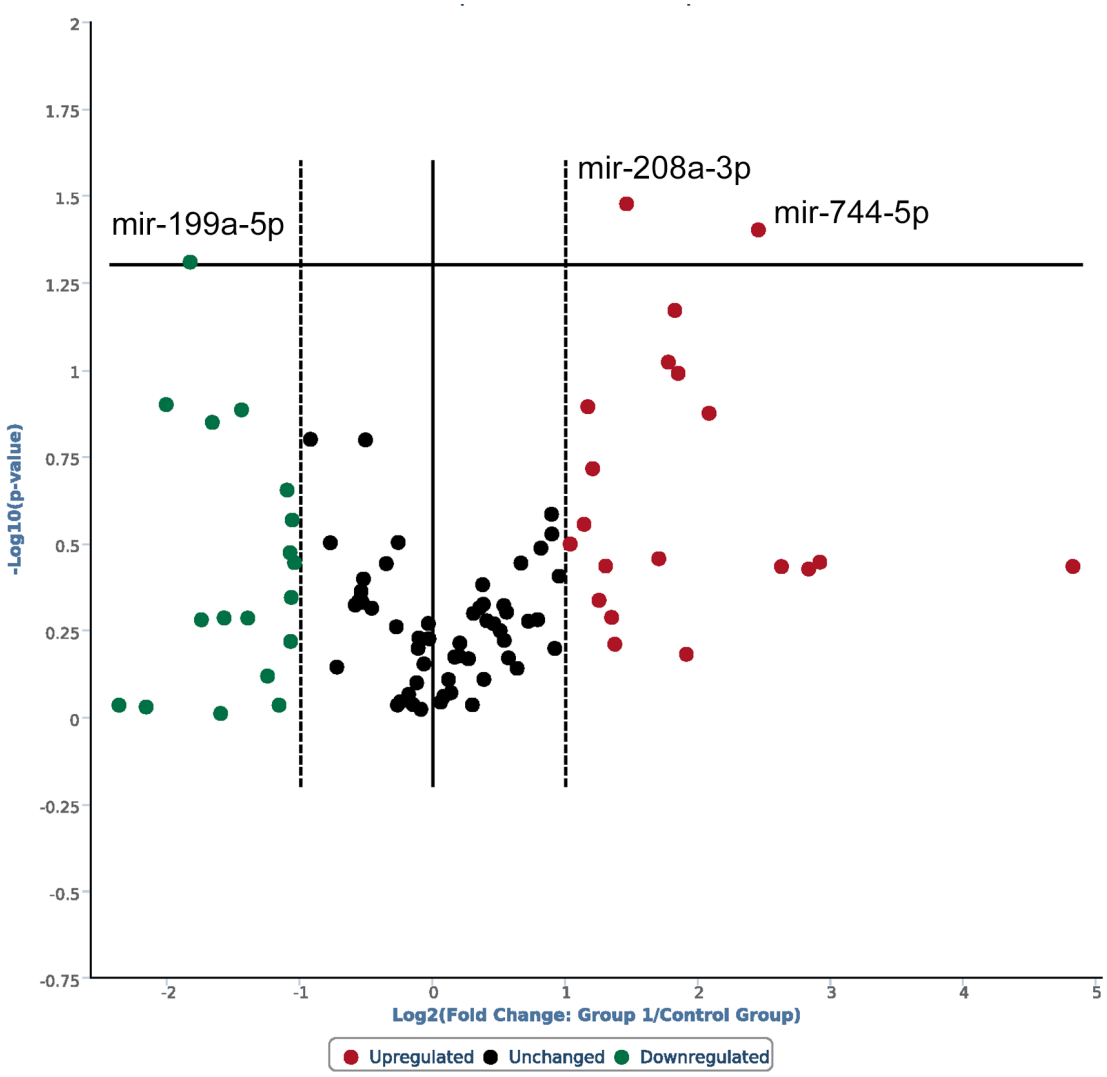

Figure 1. TGFβ2-induced miRNA expression changes in ONHA. Upregulated miRNAs are shown in red to the right and downregulated miRNAs are shown in green to the left. The horizontal bar indicates the threshold significance of P<0.05. miRNAs were considered upregulated if they passed the threshold significance of P<0.05 (horizontal line) or if the difference in expression was greater than 2-fold change (right vertical line). miRNAs were considered downregulated if they passed the threshold significance of P<0.05 (horizontal line) or if the difference in expression was greater than negative 2-fold change (left vertical line).

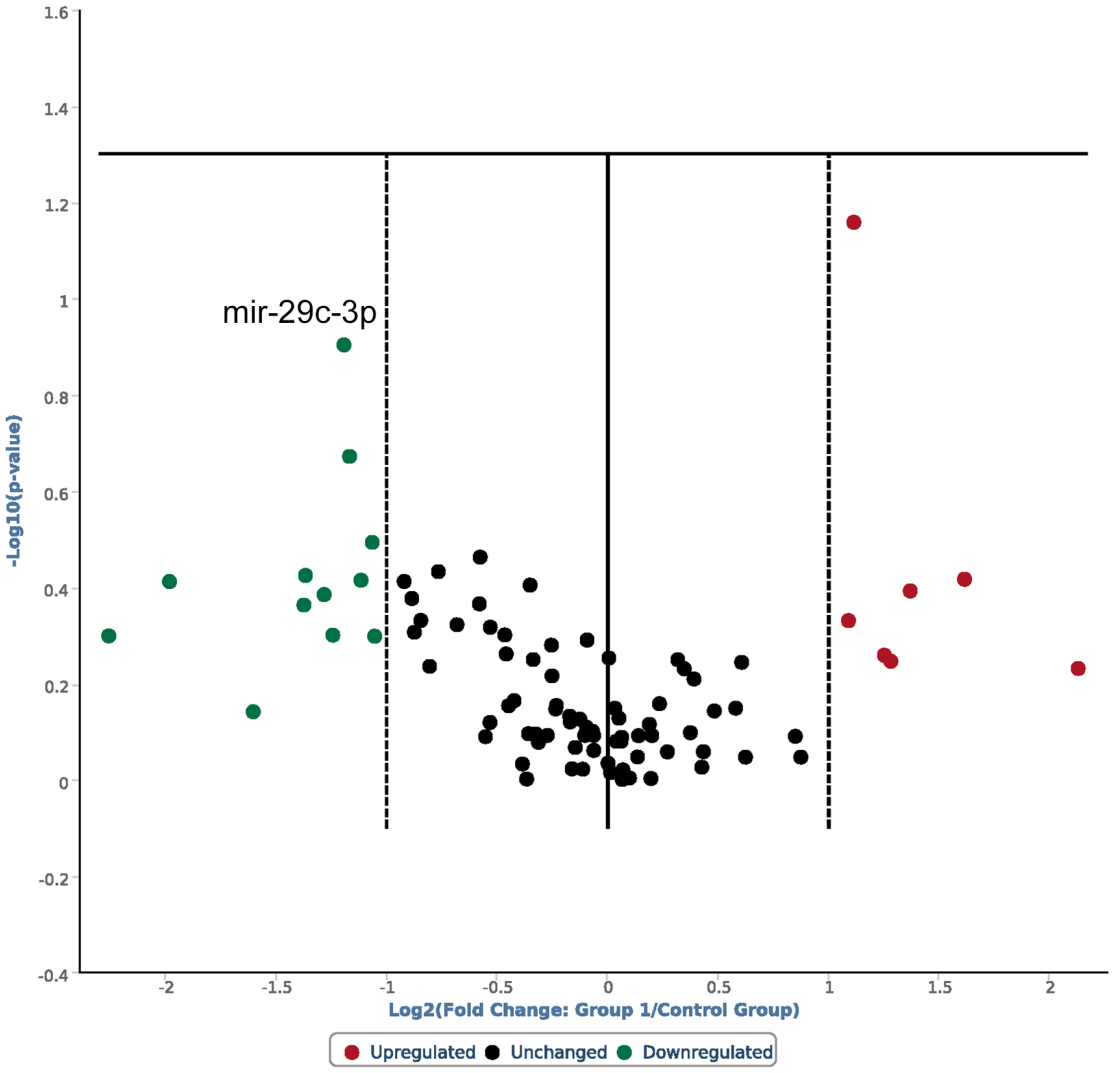

Figure 2. TGFβ2-induced miRNA expression changes in lamina cribrosa cells. Upregulated miRNAs are shown in red to the right and downregulated miRNAs are shown in green to the left. The horizontal bar indicates the threshold significance of P<0.05. miRNAs were considered upregulated if they passed the threshold significance of P<0.05 (horizontal line) or if the difference in expression was greater than 2-fold change (right vertical line). miRNAs were considered downregulated if they passed the threshold significance of P<0.05 (horizontal line) or if the difference in expression was greater than negative 2-fold change (left vertical line).

Reactome Pathway	Hits	p value	adjusted p value
SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription	5	0.0000233	0.001685
Diseases of signal transduction	12	0.0000373	0.001685
Loss of Function of SMAD2/3 in Cancer	3	0.0000674	0.001685
SMAD2/3 MH2 Domain Mutants in Cancer	3	0.0000674	0.001685
Signaling by TGF-beta Receptor Complex in Cancer	3	0.000107	0.00214
Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer	5	0.000197	0.003142857
Downstream signaling events of B Cell Receptor (BCR)	9	0.00022	0.003142857
Fc epsilon receptor (FCERI) signaling	9	0.000275	0.003177778
Signaling by TGF-beta Receptor Complex	6	0.000286	0.003177778
Signaling by Activin	3	0.000405	0.00405
Signaling by the B Cell Receptor (BCR)	9	0.000651	0.005918182
GO:BP (biological processes)			
phosphorylation	46	3.28E-09	1.92E-07
positive regulation of protein phosphorylation	27	7.69E-09	1.92E-07
protein phosphorylation	42	8.07E-09	1.92E-07
regulation of protein phosphorylation	33	9.14E-09	1.92E-07
positive regulation of cellular metabolic process	58	1.13E-08	1.92E-07
positive regulation of phosphorylation	27	1.26E-08	1.92E-07
vasculature development	26	1.35E-08	1.92E-07
positive regulation of metabolic process	60	1.66E-08	1.92E-07
regulation of phosphorylation	34	1.73E-08	1.92E-07
regulation of protein modification process	37	2.37E-08	2.37E-07

Table 2. Reactome pathways and biological processes associated with miR-199a-5p. miR-199a-5p was significantly downregulated in ONH astrocytes. Reactome pathways associated with miR-199a-5p are mainly associated with TGFβ and intracellular SMAD proteins. Analysis of biological processes indicate miR-199a-5p may regulate phosphorylation of these proteins.

Reactome Pathway	Hits	p value	adjusted p value
Extracellular matrix organization	40	1.41E-22	8.20E-21
Assembly of collagen fibrils and other multimeric structures	20	1.64E-22	8.20E-21
Collagen formation	23	1.21E-20	4.03E-19
Collagen biosynthesis and modifying enzymes	20	2.24E-18	5.60E-17
ECM proteoglycans	20	3.94E-17	7.88E-16
Degradation of the extracellular matrix	24	5.43E-17	9.05E-16
Non-integrin membrane-ECM interactions	18	1.18E-16	1.69E-15
Collagen degradation	19	1.45E-16	1.81E-15
Integrin cell surface interactions	21	2.64E-16	2.93E-15
Anchoring fibril formation	9	1.11E-11	1.11E-10
GO: BO (bioloical proceses)			
extracellular structure organization	29	1.39E-14	1.39E-12
response to endogenous stimulus	61	2.97E-09	1.13E-07
positive regulation of DNA metabolic process	15	3.38E-09	1.13E-07
negative regulation of cell differentiation	34	5.30E-09	1.33E-07
regulation of developmental process	74	1.08E-08	2.16E-07
regulation of cell differentiation	57	1.66E-08	2.77E-07
negative regulation of developmental process	37	4.07E-08	5.81E-07
regulation of DNA metabolic process	20	7.67E-08	8.84E-07
positive regulation of nucleobase-containing compound metabolic process	61	7.96E-08	8.84E-07
positive regulation of metabolic process	92	1.12E-07	0.00000112

Table 3. Reactome pathways and biological processes associated with miR-29c-3p. miR-29c-3p was downregulated in lamina cribrosa cells. Analysis of pathways and processes indicate miR-29c-3p is associated with extracellular matrix structure, primarily synthesis, degradation and organization of extracellular matrix components.

Supplementary Table 1. Complete list of differentially expressed miRNAs and p values. miRNA expression in ONH astrocytes and lamina cribrosa cells treated with TGFβ2 compared to vehicle control.

Mature ID	ONHA		LC cells	
	Fold Change	p value	Fold Change	p value
hsa-let-7d-5p	1.227	0.920793	0.9299	0.806758
hsa-miR-1-3p	0.8436	0.901507	1.2054	0.87327
hsa-miR-101-3p	0.9243	0.633339	1.3426	0.939207
hsa-miR-107	0.5265	0.158913	0.8393	0.607084
hsa-miR-10a-5p	0.9785	0.594419	0.461	0.384335
hsa-miR-10b-5p	1.888	0.633871	1.0427	0.829382
hsa-miR-122-5p	0.6862	0.434746	0.5561	0.465858
hsa-miR-125b-5p	0.6656	0.475572	0.6229	0.475203
hsa-miR-126-3p	7.118	0.374795	0.5877	0.368771
hsa-miR-129-5p	0.9528	0.702523	0.7269	0.546826
hsa-miR-132-3p	1.0833	0.779642	1.0269	0.830048
hsa-miR-133a-3p	1.645	0.529593	0.8939	0.947085
hsa-miR-141-3p	28.3227	0.368482	0.9565	0.806661
hsa-miR-142-3p	6.1737	0.369014	1.1482	0.807149
hsa-miR-143-3p	0.7253	0.485678	0.5451	0.492738
hsa-miR-145-5p	1.5501	0.723462	0.4448	0.212594
hsa-miR-146a-5p	1.1439	0.666627	0.2528	0.386749
hsa-miR-146b-5p	1.1191	0.671276	2.5815	0.404274
hsa-miR-148a-3p	1.0397	0.903992	0.4776	0.320628
hsa-miR-150-5p	4.2288	0.133604	1.101	0.807785
hsa-miR-155-5p	2.5413	0.516374	0.7751	0.994141
hsa-miR-15b-5p	1.756	0.326453	1.2453	0.561942
hsa-miR-16-5p	0.7021	0.159379	0.8872	0.735969
hsa-miR-17-5p	0.9378	0.948173	1.2707	0.586659
hsa-miR-18a-5p	1.2345	0.502853	1.0083	0.964153
hsa-miR-192-5p	1.4496	0.601096	1.0701	0.988899
hsa-miR-194-5p	0.928	0.591619	0.903	0.854547
hsa-miR-195-5p	0.8247	0.549405	0.9567	0.867223
hsa-miR-196a-5p	1.7269	0.523801	0.691	0.758418
hsa-miR-199a-5p	0.2808	0.049176	0.838	0.524132

hsa-miR-199b-5p	0.8297	0.922128	0.8885	0.756327
hsa-miR-19a-3p	0.6757	0.465543	1.5396	0.894943
hsa-miR-19b-3p	0.6901	0.466576	0.209	0.501557
hsa-miR-200a-3p	1.274	0.485513	0.3854	0.432428
hsa-miR-200b-3p	0.4747	0.604831	1.4932	0.708232
hsa-miR-203a-3p	0.1936	0.923273	1.0484	0.95184
hsa-miR-204-5p	1.3033	0.777629	0.7836	0.393218
hsa-miR-208a-3p	2.748	0.033417	0.9299	0.806758
hsa-miR-20a-5p	0.833	0.31445	2.4292	0.565851
hsa-miR-211-5p	1.2028	0.678696	0.937	0.511555
hsa-miR-215-5p	1.2994	0.47327	0.7452	0.683567
hsa-miR-21-5p	1.4469	0.477206	1.0218	0.708429
hsa-miR-216a-5p	0.2231	0.934583	0.5278	0.386474
hsa-miR-217	1.4192	0.563696	2.1272	0.466205
hsa-miR-223-3p	0.4844	0.359251	0.4813	0.502056
hsa-miR-23a-3p	1.3729	0.538288	1.1388	0.765268
hsa-miR-25-3p	1.8595	0.297246	1.3103	0.616104
hsa-miR-26a-5p	1.5819	0.36038	1.0356	0.743386
hsa-miR-26b-5p	2.0468	0.317738	1.176	0.693438
hsa-miR-27a-3p	0.4762	0.452198	0.8489	0.710919
hsa-miR-27b-3p	0.9177	0.795646	1.0005	0.921867
hsa-miR-29a-3p	0.3673	0.130709	0.9532	0.79144
hsa-miR-29b-3p	0.4733	0.33622	0.7911	0.561297
hsa-miR-29c-3p	0.2478	0.126246	0.437	0.124716
hsa-miR-302b-3p	2.4634	0.367331	0.8043	0.834101
hsa-miR-30a-5p	0.4662	0.222413	0.7324	0.700633
hsa-miR-31-5p	0.5843	0.31492	0.5408	0.419091
hsa-miR-324-3p	0.3293	0.974937	0.3873	0.375465
hsa-miR-324-5p	0.6946	0.400072	2.3805	0.549989
hsa-miR-325	2.3793	0.461184	1.8005	0.810469
hsa-miR-32-5p	1.2951	0.415629	1.2954	0.796304
hsa-miR-328-3p	1.9307	0.393169	4.3718	0.585733
hsa-miR-335-5p	0.9007	0.920563	0.6813	0.811995
hsa-miR-338-5p	0.4207	0.76134	0.7796	0.800294
hsa-miR-34a-5p	0.3153	0.142076	0.6916	0.481111
hsa-miR-372-3p	2.5839	0.616522	0.9299	0.806758
hsa-miR-375	3.4179	0.095301	0.5717	0.580046
hsa-miR-377-3p	0.3795	0.51873	2.1632	0.069378
hsa-miR-378a-3p	1.0572	0.869371	0.9243	0.948687

hsa-miR-382-5p	2.3012	0.192846	1.3964	0.717899
hsa-miR-449a	0.6043	0.717313	0.3287	0.720958
hsa-miR-449b-5p	3.2522	0.34996	0.4107	0.411446
hsa-miR-451a	0.975	0.537458	0.7967	0.800861
hsa-miR-491-5p	1.8566	0.260758	3.061	0.382456
hsa-miR-5011-5p	3.5365	0.067649	1.0033	0.557469
hsa-miR-503-5p	3.5979	0.102626	1.5216	0.569011
hsa-miR-5692a	1.1496	0.611782	1.832	0.895405
hsa-miR-590-5p	0.3353	0.518331	0.851	0.699686
hsa-miR-661	3.7562	0.658835	0.422	0.499481
hsa-miR-663a	0.4469	0.923184	1.0976	0.894716
hsa-miR-744-5p	5.4716	0.039736	1.3494	0.872277
hsa-miR-7-5p	2.2001	0.278811	1.1448	0.991175
hsa-miR-874-3p	0.2978	0.524282	0.7649	0.924679
hsa-miR-92a-3p	1.4678	0.498494	0.9348	0.776151

ONHA= ONH astrocytes; LC cells= lamina cribrosa cells

Supplementary Table 2. Pathways associated affected by differentially expressed miRNAs. Pathways and biological processes associated with differentially expressed miRNAs in ONH astrocytes and lamina cribrosa cells.

miR-208a-5p			
Reactome Pathway	**Hits**	**p value**	**adjusted p value**
SUMO is proteolytically processed	2	0.000506	0.0506
Processing and activation of SUMO	2	0.0012	0.06
Factors involved in megakaryocyte development and platelet production	4	0.00562	0.1873333
NOTCH1 Intracellular Domain Regulates Transcription	2	0.0232	0.1916667
Signaling by NOTCH1 PEST Domain Mutants in Cancer	2	0.0301	0.1916667
Signaling by NOTCH1 in Cancer	2	0.0301	0.1916667
Constitutive Signaling by NOTCH1 PEST Domain Mutants	2	0.0301	0.1916667
Signaling by NOTCH1 HD+PEST Domain Mutants in Cancer	2	0.0301	0.1916667
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants	2	0.0301	0.1916667
Transport and synthesis of PAPS	1	0.0351	0.1916667
SUMO is transferred from E1 to E2 (UBE2I, UBC9)	1	0.0351	0.1916667
GO:BP (biological processes)			
negative regulation of transferase activity	6	0.00103	0.0965
developmental growth	7	0.00193	0.0965
negative regulation of protein metabolic process	9	0.00551	0.1428
regulation of growth	9	0.00605	0.1428
negative regulation of cellular protein metabolic process	8	0.00714	0.1428
negative regulation of binding	3	0.0119	0.1545455
negative regulation of phosphorylation	5	0.0171	0.1545455

negative regulation of DNA binding	2	0.0213	0.1545455
blood coagulation	8	0.0214	0.1545455
apoptotic signaling pathway	5	0.0215	0.1545455

miR-155-5p			
Reactome Pathway	**Hits**	**p value**	**adjusted p value**
Gene Expression	87	0.0000063	0.0003745
Cellular responses to stress	36	0.0000074 9	0.0003745
Signaling by VEGF	19	0.0000185	0.000505
VEGFA-VEGFR2 Pathway	18	0.0000202	0.000505
VEGFR2 mediated vascular permeability	8	0.0000618	0.001236
Cytosolic tRNA aminoacylation	8	0.0000875	0.001458333
Fatty acid, triacylglycerol, and ketone body metabolism	20	0.000115	0.001642857
Interleukin-6 signaling	5	0.000218	0.001813333
Fc epsilon receptor (FCERI) signaling	24	0.000219	0.001813333
Constitutive Signaling by EGFRvIII	6	0.000221	0.001813333
GO:BP (biological processes)			
negative regulation of transcription from RNA polymerase II promoter	59	6.53E-07	0.0000653
aging	27	0.0000104	0.000242
negative regulation of transcription, DNA-dependent	86	0.0000113	0.000242
regulation of transcription from RNA polymerase II promoter	86	0.0000113	0.000242
regulation of cell cycle	127	0.0000121	0.000242
positive regulation of transcription from RNA polymerase II promoter	78	0.0000208	0.000304286
negative regulation of nucleobase-containing compound metabolic process	72	0.0000213	0.000304286
protein transport	94	0.0000285	0.000324706
epidermal growth factor receptor signaling pathway	111	0.0000408	0.000324706
regulation of myeloid cell differentiation	23	0.0000427	0.000324706
negative regulation of RNA metabolic process	21	0.000043	0.000324706

miR-744-5p

Reactome Pathway	Hits	p value	adjusted p value
Gene Expression	58	1.96E-10	1.96E-08
L13a-mediated translational silencing of Ceruloplasmin expression	16	7.79E-08	2.59667E-06
3' -UTR-mediated translational regulation	16	7.79E-08	2.59667E-06
Eukaryotic Translation Initiation	16	2.16E-07	0.00000432
Cap-dependent Translation Initiation	16	2.16E-07	0.00000432
GTP hydrolysis and joining of the 60S ribosomal subunit	15	5.42E-07	9.03333E-06
Influenza Infection	15	0.0000023	0.000033125
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	13	0.0000026 5	0.000033125
Nonsense-Mediated Decay (NMD)	14	0.0000034 1	0.0000341
Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)	14	0.0000034 1	0.0000341
GO:BP (biological processes)			
RNA catabolic process	20	0.000016	0.000716667
macromolecule catabolic process	51	0.0000289	0.000716667
translational initiation	51	0.0000289	0.000716667
epidermal growth factor receptor signaling pathway	17	0.0000324	0.000716667
macromolecular complex disassembly	15	0.0000375	0.000716667
viral reproductive process	16	0.000043	0.000716667
cellular protein complex disassembly	33	0.0000529	0.000755714
translation	14	0.0000901	0.00111
cellular macromolecule catabolic process	36	0.0000999	0.00111
protein complex disassembly	41	0.000138	0.001290909

miR-141-3p			
Reactome Pathway	**Hits**	**p value**	**adjusted p value**
Negative regulation of the PI3K/AKT network	3	0.0000118	0.00118
Generic Transcription Pathway	9	0.0000285	0.001425
Gene Expression	19	0.000051	0.0017
Transcriptional Regulation by TP53	4	0.00121	0.02216667
TP53 Regulates Metabolic Genes	4	0.00121	0.02216667
Signaling by ERBB4	6	0.00133	0.02216667

Cyclin D associated events in G1	3	0.00207	0.025875
G1 Phase	3	0.00207	0.025875
Fc epsilon receptor (FCERI) signaling	6	0.00311	0.02845455
Activation of PPARGC1A (PGC-1alpha) by phosphorylation	2	0.00313	0.02845455
GO:BP (biological processes)			
regulation of protein kinase activity	16	0.000263	0.006741667
positive regulation of cellular metabolic process	37	0.000358	0.006741667
regulation of kinase activity	16	0.000525	0.006741667
positive regulation of metabolic process	38	0.000606	0.006741667
positive regulation of nucleobase-containing compound metabolic process	25	0.000623	0.006741667
G1 phase of mitotic cell cycle	4	0.000659	0.006741667
embryo development	20	0.000746	0.006741667
regulation of growth	13	0.000748	0.006741667
regulation of transferase activity	16	0.00075	0.006741667
G1 phase	4	0.000773	0.006741667

miR-150-5p			
Reactome Pathway	**Hits**	**p value**	**adjusted p value**
Metabolism of water-soluble vitamins and cofactors	8	0.00151	0.057
Metabolism of vitamins and cofactors	8	0.00151	0.057
RNA Polymerase I, RNA Polymerase III, and Mitochondrial Transcription	7	0.00171	0.057
Pre-NOTCH Expression and Processing	4	0.00336	0.05869565
Regulation of Hypoxia-inducible Factor (HIF) by oxygen	4	0.00391	0.05869565
Cellular response to hypoxia	4	0.00391	0.05869565
Advanced glycosylation endproduct receptor signaling	3	0.00431	0.05869565
Signaling by NOTCH	7	0.00757	0.05869565
Organelle biogenesis and maintenance	15	0.00796	0.05869565
RNA Polymerase I Transcription Termination	4	0.0086	0.05869565
GO:BP (biological processes)			
DNA catabolic process	7	0.00389	0.1936667
ovulation cycle	8	0.00416	0.1936667
rRNA metabolic process	9	0.00581	0.1936667
rRNA processing	8	0.0113	0.2231884

cell projection assembly	13	0.0123	0.2231884
endothelial cell proliferation	7	0.0144	0.2231884
cofactor transport	3	0.0198	0.2231884
positive regulation of defense response	14	0.0214	0.2231884
regulation of peptidyl-tyrosine phosphorylation	10	0.0219	0.2231884
peptidyl-tyrosine phosphorylation	12	0.0267	0.2231884

miR-146b-5p			
Reactome Pathway	**Hits**	**p value**	**adjusted p value**
Fc epsilon receptor (FCERI) signaling	10	0.00000137	0.000110667
Signalling by NGF	12	0.00000254	0.000110667
NF-kB is activated and signals survival	4	0.00000332	0.000110667
p75NTR signals via NF-kB	4	0.0000828	0.000207
PI3K/AKT activation	7	0.0000168	0.000336
Interleukin-1 signaling	5	0.000037	0.000616667
Downstream signaling events of B Cell Receptor (BCR)	8	0.0000699	0.000823529
p75 NTR receptor-mediated signalling	6	0.0000855	0.000823529
DAP12 interactions	8	0.0000938	0.000823529
Signaling by SCF-KIT	7	0.000128	0.000823529
GO:BP (biological processes)			
ositive regulation of I-kappaB kinase/NF-kappaB cascade	8	0.0000117	0.000945
regulation of I-kappaB kinase/NF-kappaB cascade	9	0.0000189	0.000945
I-kappaB kinase/NF-kappaB cascade	9	0.0000654	0.0021275
positive regulation of sequence-specific DNA binding transcription factor activity	8	0.0000851	0.0021275
positive regulation of NF-kappaB transcription factor activity	6	0.000171	0.0028625
cytokine biosynthetic process	6	0.000197	0.0028625
cytokine metabolic process	6	0.000226	0.0028625
regulation of nucleocytoplasmic transport	7	0.000229	0.0028625
regulation of protein import into nucleus	6	0.000441	0.0049
positive regulation of signal transduction	17	0.000662	0.00662

CHAPTER IV

CROSSTALK BETWEEN MIR-29 AND TRANSFORMING GROWTH FACTOR-BETA IN HUMAN LAMINA CRIBROSA CELLS

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Abstract

Purpose: Primary open-angle-glaucoma (POAG) is the most prevalent form of glaucoma and is associated with pathological extracellular matrix remodeling of the lamina region of the optic nerve head. TGFβ2 is a pro-fibrotic cytokine that is elevated in the glaucomatous lamina cribrosa. Activated TGFβ2 signaling leads to increased expression of extracellular matrix proteins in lamina cribrosa cells, suggesting lamina cribrosa cells contribute to pathological extracellular matrix changes in glaucoma. The miR-29 family regulates the expression of genes associated with extracellular matrix synthesis, organization and degradation. We hypothesized that TGFβ2 downregulates the expression of miR-29, to induce the synthesis of extracellular matrix proteins and remodeling of the glaucomatous lamina cribrosa.

Method: Non-glaucomatous primary human lamina cribrosa cells were grown to 100% confluency and treated with TGFβ2 (5ng/ml) or control for 24hours. Differences in expression of miR-29 were analyzed by Q-PCR. Lamina cribrosa cells were transfected with miR-29c-3p mimic, inhibitor or non-targeting controls and analysed by Q-PCR to confirm overexpression or knockdown of miR-29c-3p. mRNA targets of miR-29c-3p were determined by Q-PCR and immunocytochemistry analysis. The effects of miR-29c-3p and TGFβ2 on extracellular matrix protein expression were evaluated by immunocytochemistry.

Result: TGFβ2 treatment downregulated the expression of miR-29c-3p in lamina cribrosa cells. Inhibition of miR-29c-3p leads to increased collagen types I and IV expression. Overexpression of miR-29c-3p decreased TGFβ2-induced collagen types I and IV expression.

Conclusion: TGFβ2-induced downregulation of miR-29c-3p may stimulate a pro-fibrotic response and pathogenic remodeling of the optic nerve head. The inhibitory effects of miR-29c-3p on TGFβ2 suggest that miR-29c-3p regulates extracellular matrix protein synthesis and TGFβ2 signaling, indicating some level of cross-talk.

Introduction

The glaucomas are a heterogeneous group of optic neuropathies defined by an irreversible loss of visual function. The cellular and molecular pathophysiology of glaucoma are complex, given multiple factors contribute to the etiology; however, a universal characteristic of all glaucomas is damage to the optic nerve head (ONH) and degeneration of retinal ganglion cell (RGC) axons.

Primary open-angle glaucoma (POAG) is the most prevalent subtype of glaucoma. Although it is a multifactorial disease, elevated intraocular pressure (IOP) is a strong risk factor for the development of POAG[1-3]. Electron micrographs of the ONH of POAG eyes show that the earliest detectable damage is compression of the lamina cribrosa[4]. The lamina cribrosa is a distinct region of the ONH formed by successive, connective tissue plates that provide essential scaffolding for unmyelinated RGC axons. The connective tissue plates are composed of extracellular matrix macromolecules collagen, elastin, proteoglycans and glycoproteins, which together provide the strength and elasticity of the lamina cribrosa[5-7]. The glaucomatous lamina cribrosa has notably increased collagen type IV deposition as well as disorganisation of collagen type I fibrils and fragmentation of elastin[8-13]. A weakened lamina cribrosa can result in excessive forces acting on RGC axons that trigger degeneration and RGC apoptosis.

In addition to the structural properties, the extracellular matrix directs the physiological function of the lamina cribrosa by binding growth factors that when released interact with cell-surface receptors to induce intracellular signaling, and regulate gene transcription. Lamina cribrosa cells are mechanosensitive cells that interact with the surrounding extracellular matrix[14] and respond to mechanical strain by upregulating gene expression of growth factors including transforming

growth factor beta 2 (TGFβ2) and extracellular matrix proteins including collagen type IV[15]. Our research group and others have shown that the level of TGFβ2 is higher in glaucomatous lamina cribrosa compared to normal age matched controls [16,17] and is implicated in altered ONH gene expression and increased extracellular matrix synthesis in glaucoma[12,15,16,18]. Cultured lamina cribrosa cells secrete TGFβ2 and respond to exogenous TGFβ2 by activating the canonical smad signaling pathway that increases the synthesis and secretion of extracellular matrix proteins[16], suggesting lamina cribrosa cells have an active role in the pathological remodeling of the glaucomatous lamina cribrosa.

Many cells respond to TGFβ2 to induce fibrosis, increasing extracellular matrix deposition through increased protein synthesis, decreased degradation, and increased extracellular matrix cross-linking[16,19-21]. Here, we aimed to further explore the effects of TGFβ2 on lamina cribrosa cells and determine the intermediary mechanisms involved in regulating extracellular matrix gene expression. microRNAs (miRNAs) are known to mediate post-transcriptional regulation of protein-coding genes, including the extracellular matrix. They bind predominately to the 3' untranslated region (3'UTR) of target mRNAs leading to translational repression and/or mRNA degradation. miRNAs are tightly regulated to maintain homeostasis; however, their expression is altered in fibrotic disease conditions, including glaucoma. The miR-29 family, which includes miR-29a, miR-29b and miR-29c, is a widely studied family of miRNAs that regulate fibrosis, including the trabecular meshwork, which is responsible for regulating IOP [22,23]. We hypothesized that elevated TGFβ2 signaling and altered expression miR-29 leads to extracellular matrix remodeling of the lamina cribrosa. In this study, we determined the expression of miR-29

in POAG, normal and TGFβ2 treated lamina cribrosa cells. We also investigated the effects of miR-29 on TGFβ2-induced extracellular matrix protein expression.

Methods

Cell culture

Primary human lamina cribrosa cell strains from normal (n=3) and glaucoma (n=1) donors were characterized as previously described[24]. In brief, human donor eyes were bisected and the ONH was isolated from the neighboring tissues. The lamina cribrosa was cut into two or three explants and placed in a 12-well plate with Ham's F10 medium (Sigma-Aldrich, St Loius, MO) supplemented with 10% fetal bovine serum (Atlas Biologicals, USA), Penicillin streptomycin (Sigma-Aldrich, St Loius, MO) and L-glutamine (Thermo Fisher Scientific, USA) and maintained at 37^{o}C in a humidified atmosphere of 5% CO_2. As cells grew from the explant to 100% confluency, they were passaged using 500μL of TrypLE Express (Thermo Fisher Scientific, USA). Lamina cribrosa cells were maintained in Ham's F10 medium and subsequently passaged to remove other cell populations such as astrocytes. Lamina cribrosa cells were characterized as α-SMA (Sigma) and laminin (Sigma) positive and GFAP (Thermofisher) negative[24]. Human donor eye information: 5295-17 (74y Male), 1349-18 (56y Female) and 5603-17 (70y Male).

miRNA PCR Arrays

We seeded POAG and normal lamina cribrosa cells into a 6-well plate and when cells were confluent we isolated RNA using miScript RT kit II (Qiagen). miRNA cDNA was synthesized using the HiSpec buffer and miScript II RT kit (Qiagen). The thermoprofile parameters used were 37°C for 60 mins and 95°C for 5 mins. cDNA was diluted to 200uL and we performed

mature miRNA profiling using the miScript miRNA PCR array for Human Fibrosis (Figure 1, MIHS-117Z, Qiagen) that includes for 88 mature human miRNA primers. PCR was performed on the CFX96 real-time PCR system (Bio-Rad Laboratories). The thermoprofile parameters were 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and concluded with a melting curve step. Normal(n=3) and glaucomatous (n=1) primary lamina cribrosa cell strains were studied and data shown on scatter plots. The miRNAs included in this array target the mRNA of pro-fibrotic, anti-fibrotic, signal transduction, epithelial-mesenchymal transition and extracellular matrix genes. For the full miRNA list, visit Qiagen's website (https://geneglobe.qiagen.com/product-groups/miscript-mirna-pcr-arrays).

Transforming Growth Factor β2 treatment

We seeded cells into a 6 well plate and once confluent lamina cribrosa cells were serum-deprived for 24 hours, followed by treatment with 5ng/mL TGFβ2 (R&D Systems, Minneapolis, MN) or vehicle control for 24 hours to analyze miRNA and mRNA expression. Lamina cribrosa cells were seeded into 24-well plates with coverslips and treated with or without TGFβ2 for 48hours for protein analysis.

Transfection

Lamina cribrosa cells were plated 24 hours before transfection in Ham's F10 medium and transfected at a density of ~50-60% confluency (HiPerFect; Qiagen), following the manufacturer's instructions. In brief, 10nM of mimic, inhibitor or non-targeting miRNA and 2.5uL HiPerFect were diluted in 1mL serum free OPTIMEM medium (Invitrogen), incubated for 15 minutes at room temperature (RT), and transferred to the appropriate well with lamina

cribrosa cells. The cells were then incubated overnight at 37°C in 5% CO_2-95% air. The efficiency of the transfection was confirmed by Q-PCR.

RNA Isolation and Q-PCR

Isolation of total RNAs was performed using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using the Nanodrop 2000 (Thermofisher Scientific, Waltham, MA) and purity/quality of RNA assessed by 260/280 and 260/230 ratios. cDNA was synthesized from total RNA (500ng) by reverse transcription using the iScript supermix (BioRad) according to the manufacturer's instructions. Q-PCR reactions were performed in 20µL mixture containing 1µL of the cDNA preparation (1X SYBR Green Supermix; Bio-Rad, Hercules, CA), using the following parameters: 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds, 65°C for 15 seconds, and 72°C for 15 seconds (CFX96 System and CFX Manager Sofware; Bio-Rad Laboratories; CITY, ST). Primers were designed using Primer3 (Table 1). The expression of mRNAs was normalized to *GADPH* using the ΔΔ cycle threshold (CT) method. For miRNA analysis, total RNA was isolated using a miRNeasy isolation kit (miRNeasy mini kit, Qiagen) according to the manufacturer's guidelines. Using 200ng RNA, miRNA cDNA was synthesized by reverse transcription (HiSpec buffer and miScript II RT kit, Qiagen). The thermoprofile parameters used were 37°C for 60 mins and 95°C for 5 mins. cDNA was diluted to 200uL to perform Q-PCR reactions in 25µL mixture containing miScript SYBR Green, miScript primer, miScript Universal Primer, RNase free water and cDNA. Specific primers for miR-29a-3p and miR-29c-3p are listed in Table 1. The expression of miRNAs was normalized to SNORD 95 using the ΔΔ cycle threshold (CT) method.

Table 1. List of Primers

Primer	**Primer Sequence**
hsa-miR-29a-3p	5'UAGCACCAUCUGAAAUCGGUUA
hsa-miR-29c-3p	5'UAGCACCAUUUGAAAUCGGUUA
COLLAGEN IA1	Forward 5'-AGCCAGCAGATCGAGAACAT-3' Reserve 5'-TCTTGTCCTTGGGGTTCTTG-3'
COLLAGEN IA2	Forward 5'-TGCAAGAACAGCATTGCATAC-3' Reverse 5'-GGCAGGCGAGATGGCTTATTTGTT-3'
COLLAGEN IVA1	Forward 5'-ATAGACGGATATCGGGGGCCT-3' Reverse 5'- GGATTTGAAAAAGCAATGGCACTC-3'
COLLAGEN IVA2	Forward 5′-AACCAGGTTTTCGTGGGGCT-3′ Reverse 5′-TTCCGGCTGGCATAGTAGCA-3′
LYSYL OXIDASE	Forward 5'-CGACCCTTACAACCCCTACA-3' Reverse 5′-AAGTAGCCAGTGCCGTATCC-3'
GAPDH	Forward: 5′-GGTGAAGGTCGGAGTCAAC-3′ Reverse: 5′-CCATGGGTGGAATCATATTG-3′

Immunocytochemistry

Lamina cribrosa cells were plated at a density of 8,000 cells/well in a 24 well plate with glass coverslips and incubated in Ham's F10 medium at 37°C and 95% air. The following day the cells were transfected with 10nM of miRNA mimics, inhibitors or controls in OptiMem. The next day the cells were treated without or with TGFβ2 for 48 hours in Ham's F10 medium. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO) for 10 mins at room temperature. Cells were permeabilized with 0.2% Triton-X 100 at room temperature for 20 mins and then blocked with 10% donkey serum in PBS superblock for 1 hour at room temperature, followed by primary antibody (Table 2) (diluted 1:100 in Superblock PBS) incubation overnight at 4°C in dark conditions. The next day the cells were washed three times with PBS for 5 mins

each, followed by appropriate secondary antibody incubation conjugated to a fluorescent 594 or 488 dye (Invitrogen; diluted 1:200 in Superblock PBS) for 1 hr at room temperature in dark conditions. Cells were then washed three times with PBS for 5 mins each, followed by two quick rinses with dH_2O. Coverslips were mounted carefully with DAPI prolonged gold (Invitrogen) and left to dry at room temperature for 24 hrs in dark conditions. Controls consisted of omission of primary antibodies. Images were captured using the SMZ-800 Zoom Stereo Microscope System.

Table 2. Antibody list

Antibody	**Source**	**Dilution/application**
Rabbit Collagen I	Abcam	1:200 ICC
Rabbit Collagen IV	Abcam	1:200 ICC
Donkey anti-Rabbit 488	Invitrogen	1:500 ICC
Donkey anti-Rabbit 594	Invitrogen	1:500 ICC

ICC= Immunocytochemistry

Results

TGFβ2 signaling decreased miR-29 expression in lamina cribrosa cells.

Using miRnet we determined predicted genes and pathways associated with miR-29. The miR-29 family targets genes that regulate the synthesis, organization and degradation of extracellular matrix proteins including *COL1A1, COL1A2*, COL4A1, COL4A2 and LOX (Figure 1). We analyzed the expression of miR-29 in POAG compared to normal lamina cribrosa cells. miR-

29a, miR-29b and miR-29c were downregulated in POAG (n=1) lamina cribrosa cells compared to normal (n=3) (Figure 2 and Supplementary Table 1).

TGFβ2 is a regulator of extracellular matrix proteins and miR-29 is a negative regulator of extracellular matrix synthesis[22,23]. Since TGFβ2 is elevated in the glaucomatous lamina cribrosa, we analysed the effects of TGFβ2 on the expression of the miR-29 family. We treated lamina cribrosa cells with or without 5ng/mL TGFβ2 for 24 hours. The expression of miR-29a and miR-29c was analysed by Q-PCR (Figure 3). TGFβ2 did not significantly affect the expression of miR-29a (Figure 3A); however, we found that TGFβ2 decreased the expression of miR-29c-3p in lamina cribrosa cells (Figure 3B).

Effects of miR-29c on extracellular matrix genes

We validated predicted targets of miR-29c from Figure 1 using Q-PCR. We transfected lamina cribrosa cells with miR-29c-3p and determined the transfection efficiency (Figure 4A). Transfection of miR-29c-3p mimic resulted in an increase in miR-29c-3p expression and a decrease with the inhibitor in lamina cribrosa cells (Figure 4A). To determine the gene targets of miR-29c-3p, we overexpressed miR-29c-3p in lamina cribrosa cells. This resulted in a decrease in collagen 1a1, collagen 4a1 and collagen 4a2 gene expression (Figure 4B). We also analyzed protein expression by immunocytochemistry and found decreased expression of collagen type I and IV with the miR-29c-3p mimic in lamina cribrosa cells. To analyse the potential relevance of the downregulation of miR-29 mediated by *TGFβ2*, we analyzed target protein expression. Inhibition of miR-29c-3p resulted in an increase in collagen type I and IV expression (Figure 4C-D).

Effects of miR-29c on TGFβ2 induced-extracellular matrix proteins

We next analyzed the potential effects of miR-29c-3p on extracellular matrix proteins increased by TGFβ2 (Figure 5). TGFβ2 increased the expression of collagen type I and IV in lamina cribrosa cells. Transfection of miR-29c-3p mimic resulted in downregulation of collagen type I and IV protein that were increased by TGF*β*2. To determine the relevance of downregulation of miR-29c-3p, we inhibited miR-29c-3p and treated with TGFβ2. There was no inhibitory effect on collagen type I and IV, suggesting that miR-29c-3p is necessary to regulate TGFβ2 signaling and synthesis of extracellular matrix proteins.

Discussion

Our results show that TGFβ2 regulates the expression of miRNAs. We found that TGFβ2 decreased expression of miR-29c-3p and increased collagen type I and IV expression in lamina cribrosa cells. We also show that overexpression of miR-29c-3p downregulated the expression of extracellular matrix proteins. TGFβ2-induced downregulation of miR-29c-3p in the optic nerve head may therefore contribute to glaucomatous tissue remodeling.

In POAG, there is increased expression of TGFβ2 and collagen type IV in the lamina cribrosa[11,16,17,25]. Experimental and histopathological analysis also shows there is a degradation of elastin fibres, and disorganization of collagen fibrils in the glaucomatous lamina cribrosa [9,12,13]. TGFβ2 is thought to drive extracellular matrix remodeling of the lamina cribrosa and lead to progressive damage. We aimed to identify the intermediary mechanisms that lead to increased expression of extracellular matrix proteins in the lamina cribrosa. Isolated cells from the human lamina cribrosa provide a unique advantage to study cellular responses to growth factors.

Lamina cribrosa cells have been successfully cultured from human optic nerve head explants [18,24,26,27] and have been shown to secrete TGFβ2[16]. Cultured primary lamina cribrosa cells respond to activated TGFβ2 signaling by upregulating the expression of extracellular matrix proteins[16]. Though there has not been a study to show the *in-situ* expression of miR-29 in POAG compared to normal optic nerve head tissue sections, we show that in POAG lamina cribrosa cells and cells treated with TGFβ2, miR-29c-3p expression is downregulated compared to controls. We are yet to determine whether TGFβ2 signaling directly inhibits transcription of miR-29c-3p or affects processing of miR-29c-3p[28,29]. Investigating whether TGFβ2 signaling, specifically intracellular Smad proteins, leads to inhibitory transcription factors in the nucleus binding to the promoter region of miR-29 would be an important next step. To determine whether TGFβ2 signaling affects the processing of miR-29c, we could analyse the expression of primary miRNAs and compare this to mature miRNA expression in lamina cribrosa cells treated with TGFβ2.

miRNAs have been shown to regulate TGFβ2 signaling[30]. miR-29c is an anti-fibrotic miRNA that may be important to extracellular matrix and TGFβ2 signaling homeostasis. We show that miR-29c downregulated the gene expression of collagen1a1, collagen 4a1 and collagen 4a2 in lamina cribrosa cells. We also show that the induction of extracellular matrix proteins by TGFβ2 is decreased by miR-29c indicating that miR-29c also regulates TGFβ2 signaling. It is possible that that miR-29c exerts its effects by targeting TGFβ2 ligands, TGFβ2 receptors, intracellular SMAD proteins or directly targeting the mRNA of extracellular matrix genes[30].

In summary, TGFβ2 signaling and miR-29 regulate extracellular matrix synthesis in lamina cribrosa cells. Downregulation of miR-29c by TGFβ2 and upregulation of collagen type I and IV may be important pathological cellular events that contribute to tissue remodeling in glaucoma. Our data suggest that restoration of miR-29c expression in the ONH may help regulate TGFβ2 signaling and extracellular matrix turnover.

Acknowledgements

NIH training grant T32 AG 020494, Gluacoma Research Foundation

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Figures

A

Signaling Pathway	Hits	P value	Adjusted P value
Extracellular matrix organization	43	3.94E-21	3.94E-19
Assembly of collagen fibrils and other multimeric structures	20	2.34E-20	1.17E-18
Collagen formation	23	3.26E-18	1.09E-16
Integrin cell surface interactions	23	2.34E-16	5.10E-15
Collagen biosynthesis and modifying enzymes	20	2.82E-16	5.10E-15

B

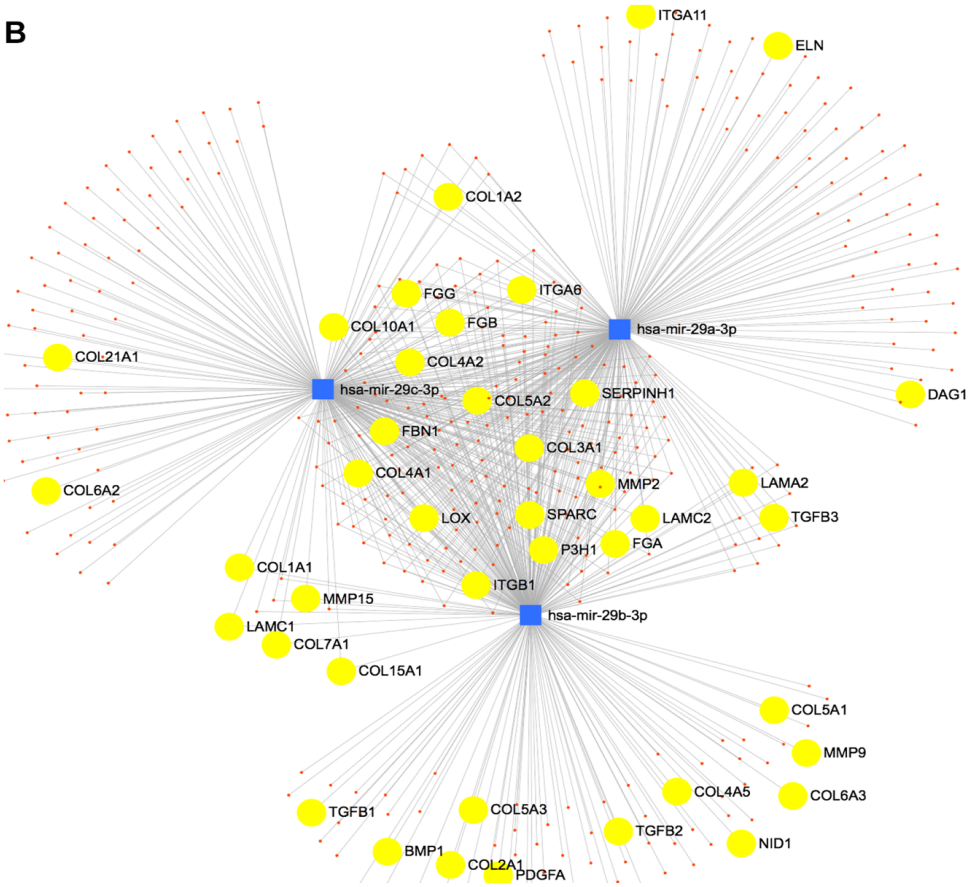

Figure 1. Signaling pathways and genes associated with miR-29 family

We utilized miRNet to determine genes and significant pathways associated with miR-29. A) Significant Reactome pathways which are regulated by miR-29. B) Genes associated with significant pathways.

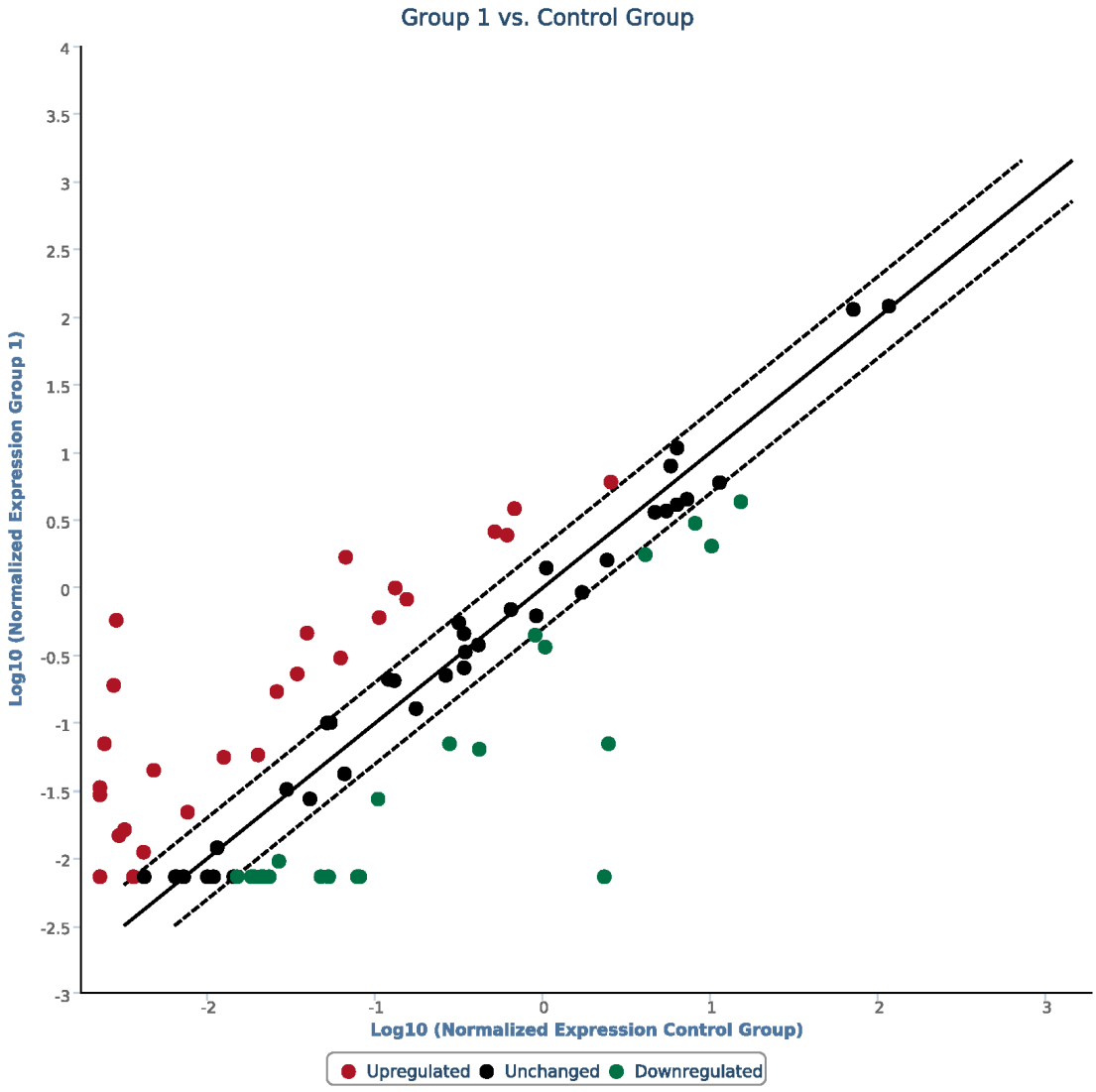

Figure 2. miRNA expression in POAG compared to normal lamina cribrosa cells

Upregulated miRNAs are shown in red and downregulated miRNAs are shown in green. The diagonal lines indicate the 2-fold change threshold. miRNAs were considered upregulated if the difference in expression was greater than 2-fold change. miRNAs were considered downregulated if the difference in expression was greater than negative 2-fold change. POAG (n=1), Normal (n=3). Group 1=POAG group.

Supplementary Table 1. miRNA fold change in POAG compared to normal lamina cribrosa cells. miRNA expression in POAG (Group 1) lamina cribrosa cells treated with TGFβ2 compared to vehicle control (Control group).

		Up-Down Regulation (comparing to control group)
		Group 1
Position	Mature ID	Fold Regulation
A01	hsa-let-7d-5p	-2.3265
A02	hsa-miR-1-3p	3.2019
A03	hsa-miR-101-3p	1.0488
A04	hsa-miR-107	-1.4838
A05	hsa-miR-10a-5p	-6.4951
A06	hsa-miR-10b-5p	-2.9012
A07	hsa-miR-122-5p	14.5725
A08	hsa-miR-125b-5p	-3.5114
A09	hsa-miR-126-3p	6.7274
A10	hsa-miR-129-5p	-1.5565
A11	hsa-miR-132-3p	1.7541
A12	hsa-miR-133a-3p	3.2019
B01	hsa-miR-141-3p	-2.5954
B02	hsa-miR-142-3p	3.2019
B03	hsa-miR-143-3p	-1.1713
B04	hsa-miR-145-5p	5.0303
B05	hsa-miR-146a-5p	-3.1946
B06	hsa-miR-146b-5p	-2.0556
B07	hsa-miR-148a-3p	4.4544
B08	hsa-miR-150-5p	1.7439
B09	hsa-miR-155-5p	1.3532
B10	hsa-miR-15b-5p	-1.857
B11	hsa-miR-16-5p	-1.0302
B12	hsa-miR-17-5p	1.089
C01	hsa-miR-18a-5p	-2.4942
C02	hsa-miR-192-5p	-2.8019
C03	hsa-miR-194-5p	-2.9692
C04	hsa-miR-195-5p	-1.3249
C05	hsa-miR-196a-5p	2.8811
C06	hsa-miR-199a-5p	1.5851
C07	hsa-miR-199b-5p	-1.4886

C08	hsa-miR-19a-3p	-1.9604
C09	hsa-miR-19b-3p	4.8602
C10	hsa-miR-200a-3p	3.2019
C11	hsa-miR-200b-3p	28.9218
C12	hsa-miR-203a-3p	201.3834
D01	hsa-miR-204-5p	-6.5242
D02	hsa-miR-208a-3p	3.2019
D03	hsa-miR-20a-5p	-11.0783
D04	hsa-miR-211-5p	2.0218
D05	hsa-miR-215-5p	4.9768
D06	hsa-miR-21-5p	1.6036
D07	hsa-miR-216a-5p	9.362
D08	hsa-miR-217	2.0171
D09	hsa-miR-223-3p	12.8818
D10	hsa-miR-23a-3p	1.0382
D11	hsa-miR-25-3p	1.0686
D12	hsa-miR-26a-5p	-1.6083
E01	hsa-miR-26b-5p	-2.7017
E02	hsa-miR-27a-3p	-1.5108
E03	hsa-miR-27b-3p	-1.2889
E04	hsa-miR-29a-3p	-4.9903
E05	hsa-miR-29b-3p	-3.9496
E06	hsa-miR-29c-3p	-1.537
E07	hsa-miR-302b-3p	6.587
E08	hsa-miR-30a-5p	-1.0932
E09	hsa-miR-31-5p	2.3731
E10	hsa-miR-324-3p	25.1698
E11	hsa-miR-324-5p	5.3182
E12	hsa-miR-325	5.0954
F01	hsa-miR-32-5p	1.0132
F02	hsa-miR-328-3p	-35.0402
F03	hsa-miR-335-5p	-7.2175
F04	hsa-miR-338-5p	3.2019
F05	hsa-miR-34a-5p	-1.476
F06	hsa-miR-372-3p	-318.0966
F07	hsa-miR-375	5.6868
F08	hsa-miR-377-3p	2.6713
F09	hsa-miR-378a-3p	68.8839
F10	hsa-miR-382-5p	4.0002
F11	hsa-miR-449a	7.562
F12	hsa-miR-449b-5p	2.9047

G01	hsa-miR-451a	3.2019
G02	hsa-miR-491-5p	1.7515
G03	hsa-miR-5011-5p	1.9344
G04	hsa-miR-503-5p	-3.7987
G05	hsa-miR-5692a	-1.3659
G06	hsa-miR-590-5p	1.1371
G07	hsa-miR-661	1.3359
G08	hsa-miR-663a	5.6704
G09	hsa-miR-744-5p	1.7097
G10	hsa-miR-7-5p	1.8594
G11	hsa-miR-874-3p	11.7676
G12	hsa-miR-92a-3p	-2.0194

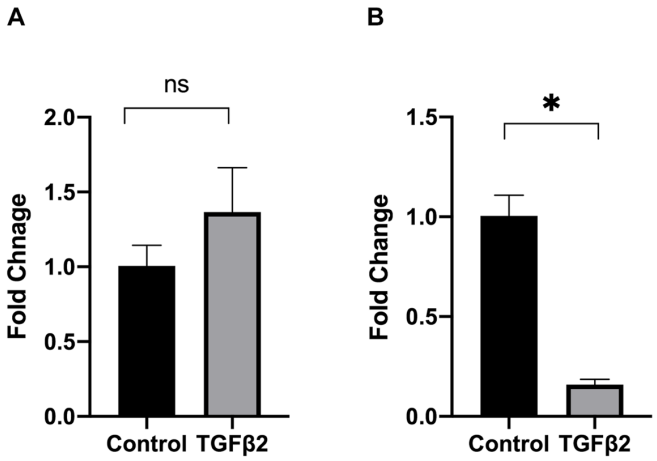

Figure 3. Activation of TGFβ2 signaling decreased the expression of miR-29c-3p in lamina cribrosa cells. Primary lamina cribrosa cells were treated with TGFβ2 or vehicle control for 24hours. The expression of miR-29a-3p and miR-29c-3p was analysed by Q-PCR. The graph represents fold change miRNA expression in TGFβ2 treated cells compared to control. A) miR-29a-3p expression was not affected by TGFβ2 treatment. B) TGFβ2 signaling led to a decreased expression of miR-29c-3p in lamina cribrosa cells (n=1). Bars represent SD from one cell strain. *P≤0.05, technical triplicates.

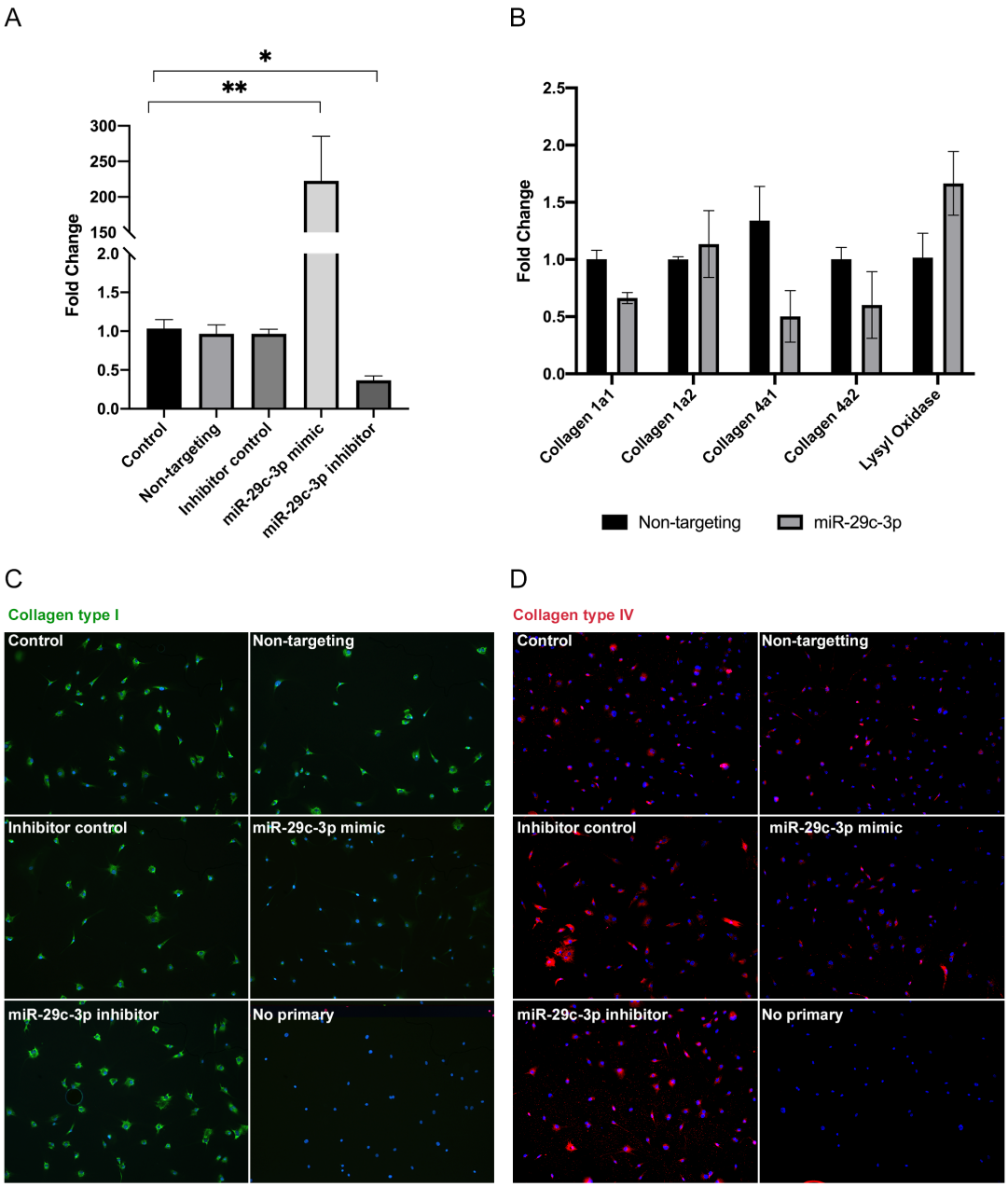

Figure 4. miR-29c targets include collagen type I and IV. A) Lamina cribrosa cells transfected with miR-29c-3p mimic resulted in overexpression of miR-29c, whereas transfection with miR-29c inhibitor resulted in knockdown of miR-29c expression (n=3). B-D) Lamina cribrosa cells transfected with miR-29c-3p mimic decreased collagen type I and IV expression (n=2).

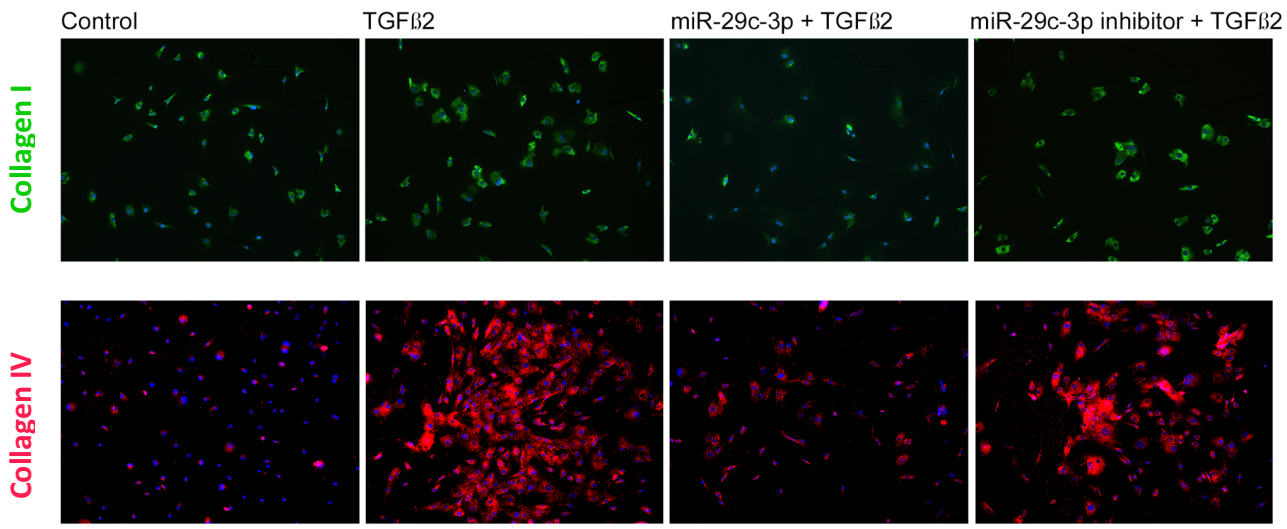

Figure 5. Effects of miR-29c on TGFβ2-induced include collagen type I and IV expression. TGFβ2 treatment increased collagen type I and IV expression in cultured lamina cribrosa cells. Lamina cribrosa cells transfected with miR-29c mimic and treated with TGFβ2 resulted in a decrease in TGFβ2-induced collagen type I and IV expression (n=2).

CHAPTER V

MIR-200B-3P REGULATES TGFβ2 INDUCED FIBRONECTIN EXPRESSION IN OPTIC NERVE HEAD ASTROCYTES

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Abstract

Purpose: Primary open-angle-glaucoma (POAG) is a neurodegenerative disease that causes an irreversible loss of vision. The primary site of injury in POAG is the lamina cribrosa; increased TGFβ2 signaling contributes to excess extracellular matrix synthesis and tissue remodeling. In this study, we analysed the expression of fibronectin in POAG and the effects of miR-200b-3p, a negative regulator of TGFβ2 signaling, in optic nerve head astrocytes.

Methods: Normal and POAG optic nerve head tissue sections were analysed for expression of fibronectin. Non-glaucomatous primary human optic nerve head astrocytes were grown to 100% confluency and transfected with miR-200b-3p mimic, inhibitor or non-targeting controls and analysed by Q-PCR to confirm overexpression or knockdown of miR-200b-3p. mRNA targets of miR-200b-3p were determined by western blot and immunocytochemistry analysis. The effects of miR-200b-3p and TGFβ2 on extracellular matrix protein expression were evaluated by western blot and immunocytochemistry.

Results: The levels of fibronectin were increased in POAG optic nerve heads compared to age-matched controls and co-localized with optic nerve head astrocytes**.** TGFβ2 induced the expression of fibronectin compared to control treated astrocytes. Inhibition of miR-200b-3p increased fibronectin expression. Overexpression of miR-200b-3p decreased TGFβ2-induced fibronectin expression.

Conclusion: The inhibitory effects of miR-200b-3p on TGFβ2-induced downregulation fibronectin suggest that miR-200b-3p regulates TGFβ2 signaling. It would be informative to

determine if miR-200b-3p targets the mRNA of TGFβ2 ligands, receptors or intracellular smads to regulate fibronectin or by direct binding to the mRNA of fibronectin.

Introduction

Glaucoma causes morphological and functional changes in the retina and optic nerve head including neurodegeneration of retinal ganglion cells (RGC), thinning of the neuro-retinal rim and structural deformation of the optic nerve head, which leads to an irreversible loss of vision. Histopathological studies show us axonal loss in glaucoma is associated with reactivation of optic nerve head astrocytes and upregulation of glial fibrillary acidic protein (GFAP)[1,2].

The primary site of glaucomatous injury in the optic nerve head is the lamina cribrosa region[3]. The lamina cribrosa is a connective tissue structure with pores that permit the passage of RGC axons. The lamina cribrosa undergoes extensive extracellular matrix remodeling in glaucoma[4-8]. Transforming growth factor (TGFβ2) is thought to play a pivotal role in driving pathological tissue remodeling in glaucoma. TGFβ2 has been found to be elevated in the lamina cribrosa of glaucoma eyes compared to aged-matched control eyes[9,10]. In vitro experiments show that optic nerve head astrocytes treated with TGFβ2 increase the synthesis of several extracellular matrix components including fibronectin[9,11].

miRNAs are small non-coding RNAs that regulate gene expression. miRNAs control the activity of protein-coding genes to regulate cellular processes including extracellular matrix synthesis[12,13]. A preliminary study analysing miRNAs showed that miR-200b is downregulated by TGFβ2 treatment in optic nerve head astrocytes (data not published). miR-200b belongs to the miR-200b family of microRNAs (miRNAs) which includes miR-200a, miR-200b, miR-200c, miR-141, and miR-429. miR-200b prevents TGFβ-induced increases in fibronectin[14], however

the role of miR-200b is unknown in optic nerve head astrocytes. In this study, we investigated the relationship between miR-200b and TGF-β2-induced extracellular matrix protein expression.

Methods

Cell culture, transfection and TGFβ2 treatment

Optic nerve head cell astrocytes were isolated from human donor eyes and characterised as previously described (n=3)[15]. ONH astrocytes were maintained in astrocyte basal medium (ABM) supplemented with 5% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin and streptomycin (Thermofisher, Waltham, MA). We seeded cells into wells of a 6-well plate or 24-well plate with coverslips. The next day cells were transfected at 60% confluency using 3 μL HiPerFect (qiagen) transfection reagent and 10nM miR-200b-3p mimic, inhibitor or non targeting controls. (Qiagen) in Opti-MEM serum free medium (ThermoFisher scientific). Cells transfected with NTsiRNA should not induce or minimally induce changes in target gene expression when compared with the miRNA mimic and inhibtior. These negative controls gives us an idea of the effects of miR-200b-3p on its target gene expression. Following 24hours transfection, the cells were treated with or without recombinant TGFβ2 (5ng/ml, R&D Systems, Minneapolis, MN) or vehicle control for 24 hours.

Isolation and Quantification of miRNA and mRNA

Total RNA was isolated using the miRNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using the Nanodrop 2000 (Thermofisher Scientific, Waltham, MA) and purity/quality of RNA assessed by 260/280 and 260/230 ratios. Using 200ng RNA, miRNA cDNA was synthesized by reverse transcription (HiSpec buffer and miScript II RT kit,

Qiagen). The thermoprofile parameters used were 37°C for 60 mins and 95°C for 5 mins. cDNA was diluted to 200uL to perform Q-PCR reactions in 25µL mixture containing SYBR Green, miRNA primer, water and cDNA. The expression of miRNAs was normalized to SNORD 95 using the ΔΔ cycle threshold (CT) method.

Immunocytochemistry

Cells were cultured on glass coverslips in a 24-well plate. Following the experiment, the cells were fixed with 4% para-formaldehyde for 15 mins at room temperature. After washing with PBS, the cells were incubated with 0.5% Triton X-100 (Fisher Scientific, Pittsburgh, PA) for 20 mins at room temperature. The cells were then washed twice with PBS and incubated with primary fibronectin (1:200, EMD Millipore, Billerica, MA, USA; catalog # AB1945) and collagen type IV (abcam) (1:100) antibodies at 4 °C overnight. Cells incubated without primary antibody served as a negative control. Following the incubation, cells were washed three times with PBS and further incubated for 1 hour at room temperature with the secondary antibody (Alexa donkey anti-rabbit 488; 1:500; Thermo Scientific, Rockford, IL). After PBS washing, glass coverslips with cells were then mounted on ProLong gold anti-fade reagent with DAPI (Invitrogen-Molecular Probes, Carlsbad, CA, USA). All images were taken with a Nikon Eclipse microscope with Nuance imaging system.

Immunohistochemistry

Optic nerve head sections from normal and POAG donor eyes were deparaffinized in xylene and rehydrated twice each with 100%, 95%, 70%, and 50% ethanol for 3 minutes. Tissue sections were blocked (10% donkey serum + 0.2% Triton-X 100) for 2 hrs in a dark and humid chamber.

Tissue sections were then washed with PBS and immunolabeled with rabbit polyclonal fibronectin antibody (1:100; Millipore) and GFAP (ASTRO6 ThermoFisher) and incubated overnight at 4 °C. Tissue sections incubated without primary antibody served as a negative control. Following the incubation, tissue sections were then washed three times with PBS and further incubated for 1.5 hrs at room temperature with the appropriate secondary antibodies (Alexa Donkey anti-rabbit 488; 1:500; Thermo Fisher Scientific, Inc.). Tissue sections were washed three times with PBS followed by two quick H20 rinses and then mounted on ProLong gold anti-fade reagent with DAPI (Invitrogen-Molecular Probes, Carlsbad, CA, USA). Images were captured with the Nikon Eclipse microscope with Nuance imaging system.

Western Blot

ONH astrocytes were lysed in lysis buffer (M-PER, Thermo Scientific, Rockford, IL) containing Halt protease inhibitor cooktail (1:100; Thermo Scientific, Rockford, IL). The protein samples were run on denaturing 10% polyacrylamide gels and transferred onto PVDF membranes. The membrane was blocked with 10% non-fat dried milk for one hour at room temperature and then incubated with specific primary antibodies at 4 °C overnight on a rotating shaker. The membranes were washed three times with Tris-buffered Saline with 0.1% Tween-20 (TBST) and incubated with corresponding HRP-conjugated secondary antibody for 1 hour. The proteins were then visualized using enhanced chemilluminescence detection reagents (Bio-Rad).

Results

Overexpression of miR-200b-3p downregulates fibronectin expression in optic nerve head astrocytes

We transfected optic nerve head astrocytes with miR-200b-3p at three different concentrations and determined the expression target mRNA fibronectin, tissue transglutaminase 2 (TGM2) and gremlin (Figure 1B). We confirmed that the optimum concentration was 10nM of miR-200b-3p mimic which from the transfection efficiency results show that we sufficiently overexpressed and knocked down expression of miR-200b-3p (Figure 1A). We also analysed protein expression by immunocytochemistry and found that overexpression of miR-200b-3p resulted in a decrease in fibronectin expression, while the inhibitor increased fibronectin expression (Figure 1C).

Effects of miR-200b-3p on TGFβ2 induced-fibronectin expression

We next analysed the effects of miR-200b-3p on fibronectin protein expression (Figure 2*)*. TGFβ2 increased the expression of fibronectin expression in optic nerve head astrocytes. Transfection of miR-200b-3p resulted in downregulation of fibronectin that was induced by TGFβ2 treatment. There was no inhibitory effect with the mir-200b-3p inhibitor, suggesting that miR-200b-3p targets regulates the expression of fibronectin in optic nerve head astrocytes.

Fibronectin expression is elevated in POAG optic nerve head sections

To determine the relevance of miR-200b-3p, we analysed the expression of extracellular matrix proteins in POAG compared to age-matched control optic nerve head tissue sections from human donor eyes. We found the levels of fibronectin were elevated in the POAG optic nerve head (Figure 3).

Discussion

Our results show that fibronectin, an extracellular matrix glycoprotein, is elevated in the glaucomatous optic nerve head. Our lab has previously shown that TGFβ drives the synthesis of extracellular matrix proteins in optic nerve head astrocytes[9]. Here, that overexpression of miR-200b-3p attenuated TGFβ-induced fibronectin in optic nerve head astrocytes.

miRNAs regulate several cellular processes, including extracellular matrix synthesis. In our previous study, we show that TGFβ downregulates the expression of miR-200b-3p in optic nerve head astrocytes (unpublished). We found that inhibition of miR-200b-3p in optic nerve head astrocytes leads to an increase in fibronectin expression; therefore, miRNA expression may be relevant in the context of tissue remodeling in glaucoma. It would be informative to determine the expression of miR-200b-3p in optic nerve head tissue sections from glaucomatous and normal donor eyes as well as expression in optic nerve head astrocytes derived from glaucoma and normal donor optic nerve head explants.

Restoring the expression of miRNAs in optic nerve head cells may be therapeutically relevant to regulate TGFβ2 signaling. Overexpression of miR-200b-3p in optic nerve head astrocytes decreased TGFβ2-induced fibronectin expression. This suggested that miR-200b-3p is a negative regulator of fibrosis. Given that miRNAs target several mRNAs, the identification of other mRNA targets may help elucidate how optic nerve head astrocytes acquire a fibrogenic phenotype in the glaucomatous lamina cribrosa.

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Figures

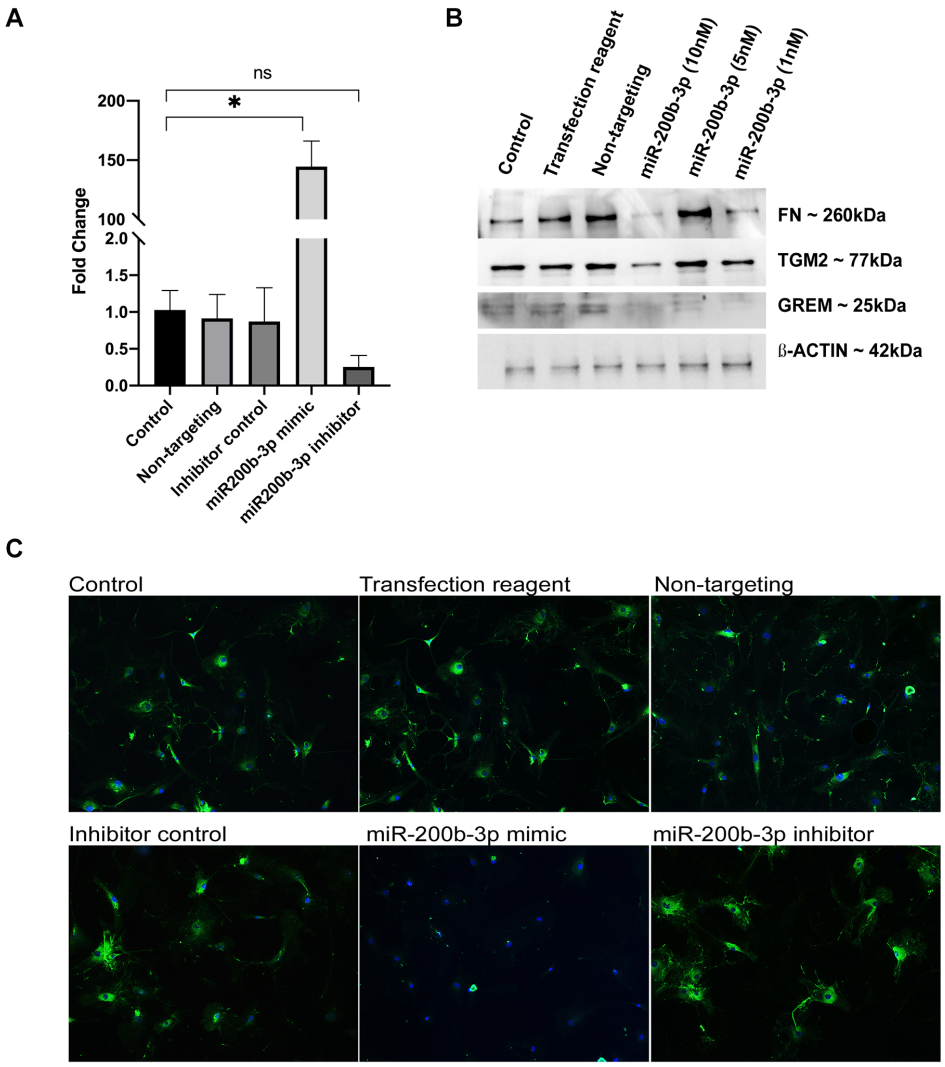

Figure 1. miR-200b-3p regulates fibronectin expression in optic nerve head astrocytes. A) Transfection efficiency showing overexpression and knockdown of miR-200b-3p. B) Dose-response transfection of miR-200b-3p mimic shows 10nm as the optimum concentration. C) miR-200b-3p decreases fibronectin expression in optic nerve head astrocytes (n=2).

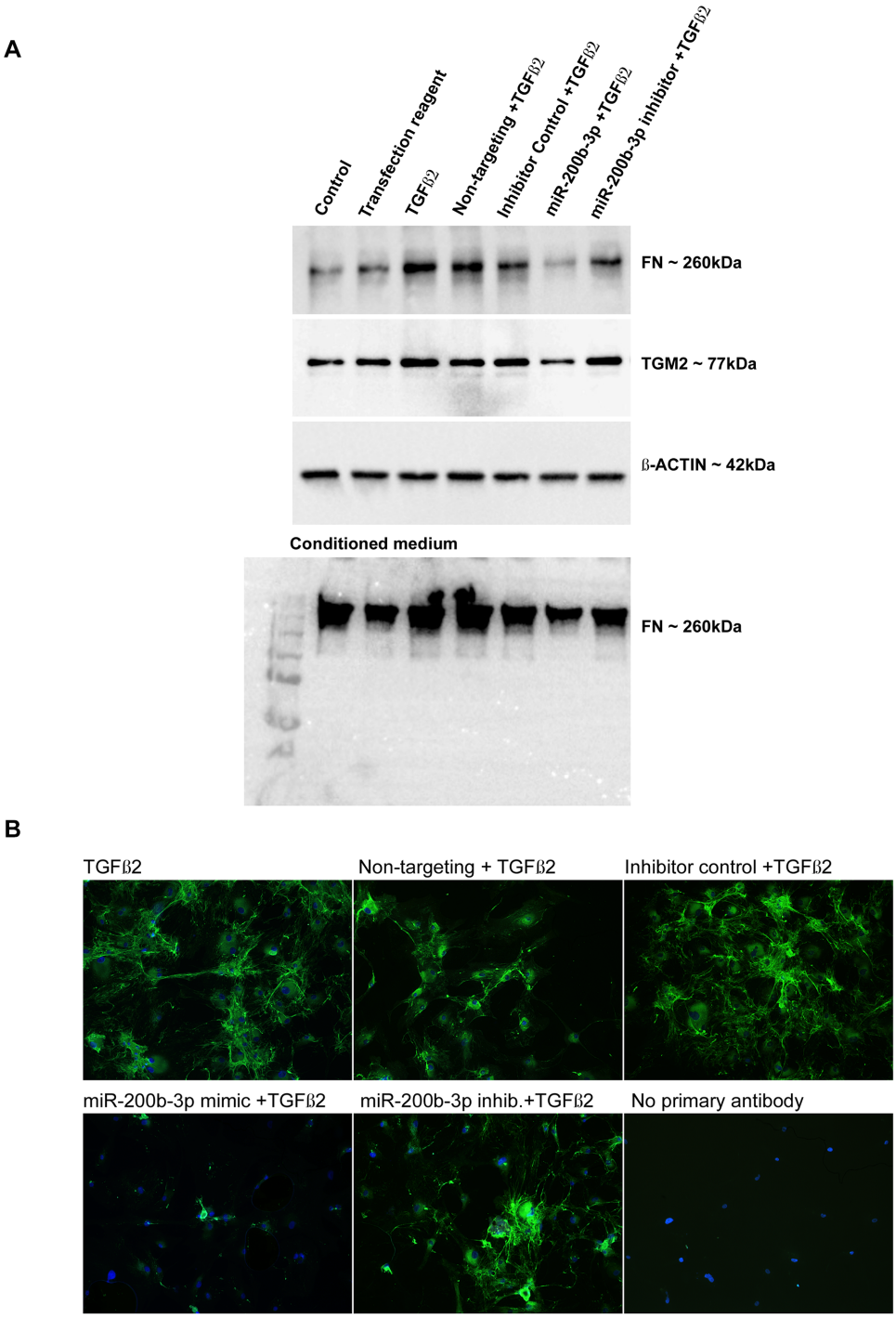

Figure 2. miR-200b-3p decreased TGFβ2-induced fibronectin expression.

A-B) Optic nerve head astrocytes transfected with miR-200b-3p mimic resulted a decrease in TGFβ2-induced fibronectin expression (n=2).

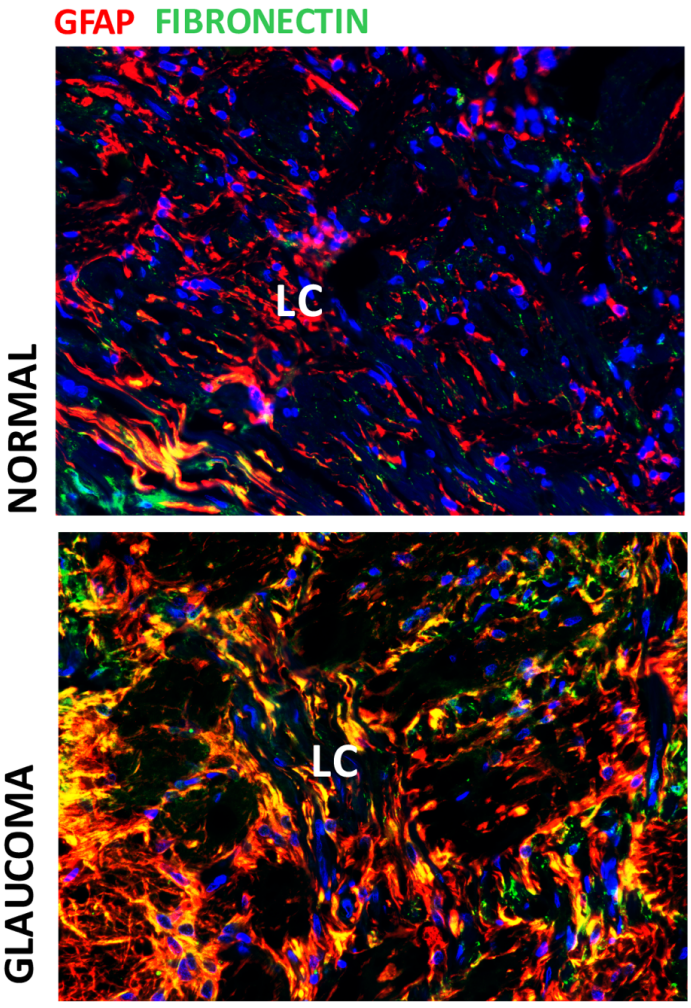

Figure 3. Fibronectin expression in human POAG optic nerve head tissue sections

The levels of fibronectin protein are increased in POAG and co-localise with GFAP positive astrocytes in the lamian cribrosa (n=3).

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

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The lamina cribrosa is a highly organized extracellular matrix structure, which supports the optic nerve head (ONH) and unmyelinated retinal ganglion cell (RGC) axons, and is the primary site of glaucomatous injury[1]. In glaucoma, there is extensive remodeling of the lamina extracellular matrix which contributes to progressive glaucomatous damage[2,3]. The successful culture of cells that populate this region allows us to explore factors that affect the biochemical structure of the lamina cribrosa tissue in glaucoma.

Five different cell types can be cultured from human lamina cribrosa explants: lamina cribrosa cells, scleral fibroblasts, astrocytes, endothelial cells and pericytes. Astrocytes and lamina cribrosa cells have been shown to synthesize extracellular matrix molecules; thus, it is likely that these cells regulate the extracellular matrix of the lamina cribrosa. We have developed a modified method to isolate ONH astrocytes and lamina cribrosa cells from a single explant[4]. Lamina cribrosa cells are broad, flat cells that express alpha-smooth muscle actin (α-SMA)[5,6]. Lamina cribrosa cells were distinguished from scleral fibroblasts by the expression of laminin; fibroblasts do not express basement membrane proteins[6]. ONH astrocytes express the intermediate filament protein, glial fibrillary acidic protein (GFAP). To distinguish pre-laminar astrocytes from lamina cribrosa astrocytes, we confirmed expression of neural cell adhesion molecule (NCAM), indicative of type IB ONH astrocytes present in the lamina cribrosa[7]. We chose to specifically analyze cells in the lamina cribrosa region, since this region is progressively remodeled in glaucoma; however, it is possible to isolate cells from the pre-laminar and myelinated optic nerve regions to determine differences in astrocyte morphology, proteomics and gene expression. A comprehensive profiling study would demonstrate cell-specific expression patterns in different regions of the ONH. It would be interesting to determine the phenotype of ONH cells in different age and race groups, and which may help answer questions about why

there is an increased susceptibility to glaucoma in people over the age of 40[6,8] and in people with African ancestry[9].

ONH astrocytes and lamina cribrosa cells have specific functions. It is important to decipher the cell-specific responses in these cells in glaucoma and in response to glaucomatous conditions. ONH astrocytes and lamina cribrosa cells from POAG donor eyes show differences in expression of growth factors and extracellular matrix genes[10,11]. We show in our preliminary study that there is differential expression of mature miRNAs in POAG lamina cribrosa cells compared to aged matched controls; however, we only have an n=1 for POAG; therefore, it is difficult to draw any definitive conclusions from these results.

TGFβ2 is a cytokine that is elevated in the glaucomatous lamina cribrosa and involved in tissue remodeling. *In-vitro* studies show that TGFβ2 drives the synthesis of extracellular matrix proteins and crosslinking enzymes in ONH astrocytes and lamina cribrosa cells[12-14]. We show that TGFβ2 signaling disrupts the expression of mature miRNAs involved in extracellular matrix synthesis, signal transduction and regulation of inflammatory pathways in these cells. miRNAs upregulated in ONH astrocytes are associated with immune regulation, while miRNAs downregulated in ONH astrocytes and lamina cribrosa cells regulate extracellular matrix synthesis and TGFβ2 signaling. It will be informative to determine if differentially expressed miRNAs in POAG ONH cells cluster with miRNAs dysregulated in ONH cells treated with TGFβ2. If miRNAs are simultaneously expressed, it would indicate that TGFβ2 potentially drives dysregulation of miRNAs in the glaucomatous lamina cribrosa. If miRNA dysregulation is a result of elevated TGFβ2, then we need to determine how TGFβ2 regulates the expression of

miRNAs in lamina cribrosa cells. It is possible that TGFβ2 signaling leads to the recruitment of transcription factors to the promoter of miRNAs, which can affect the transcription of miRNAs (Figure 1)[15]. It is also possible TGFβ2 signaling affects the miRNA processing complex; thus, determining differences in primary, precursor and mature miRNAs would be useful. Designing primers to the hairpin loop, precursor and mature miRNAs would be a way to quantify differences[16,17].

In order to address the question of what occurs first, miRNA or TGFβ2 dysregulation, we need to look at the response to earlier events in glaucomatous optic neuropathy such as elevated intraocular pressure (IOP). Aqueous humor is continuously produced by the ciliary body epithelium to nourish the anterior chamber of the eye, and is drained through the uveoscleral and conventional outflow pathways to maintain IOP within 10 and 21mmHg. The conventional outflow pathway includes the trabecular meshwork and Schlemm's canal and is the primary route of aqueous humor drainage. When aqueous humor outflow pathways are blocked, there is a subsequent increase in resistance to drainage, which can lead to increased pressure in the eye. This pressure is transduced to the ONH and surrounding tissues, exerting stretch and strain which may damage optic nerve head cells and retinal ganglion cell axons (RGC). Studies analysing the effects of the IOP, utilized models to predict stretch and compression of the laminar neural tissue, lamina cribrosa, sclera and pia mater[18]. As levels of IOP increased, the cells within the ONH were subjected to compressive, stretch and shearing deformations, reaching peak strain (15%) at 50mmHg[18]. Based on these predicted studies, further studies have been carried out investigating the gene and protein expression changes in lamina cribrosa cells and

astrocytes exposed to stretch. Lamina cribrosa cells exposed to 15% stretch using the Flexercell system resulted in upregulation of extracellular matrix gene expression including elastin, collagens, lysyl oxidase and TGFβ2[19]. Secretion of TGFβ2 also significantly increased when lamina cribrosa cells were exposed to stretch[19]. Other studies analysed the effects of increasing stretch, 0%, 3% and 12%, on ONH astrocytes and lamina cribrosa cells[20,21]. ONH astrocytes exposed to increasing stretch resulted in an increase in GFAP, indicative of glial activation[21]. In lamina cribrosa cells the level of protein synthesis increased from 3% to 12 % stretch, suggesting lamina cribrosa cells are mechanosensitive and respond to mechanical strain[20]. Activated pathways included the proteins TGFβ, tumor necrosis factor (TNF) and caspase-3 (CASP3)[20]. The degree of IOP change and magnitude of strain on ONH cells may determine the cellular response. To determine if miRNAs are dysregulated in response to mechanical strain, we could analyse miRNA expression in response to stretch at low and high strain conditions. It may be possible that stretch induces dysregulation of miRNAs and leads to increased TGFβ signaling.

miRNAs are essential to the TGFβ signaling pathway and homeostatic regulation of extracellular matrix gene expression[22]. In this study, we further analysed the role of miR-29 in lamina cribrosa cell and miR-200b in ONH astrocytes. We show that miR-29c-3p and miR-200b-3p are downregulated in response to TGFβ2 treatment. It would be interesting to determine the expression over time in response to TGFβ2 in ONH astrocytes and lamina cribrosa cells, since there is some level of cell-cell contact and communication. It is possible that miRNAs could be transferred between cells by exosomes and nanotubes. We aimed to determine to regulatory functions of differentially expressed miRNAs in lamina cribrosa cells and ONH astrocytes treated with TGFβ2. We show that miR-29c-3p is an anti-fibrotic miRNA and downregulates the

expression of extracellular matrix proteins. We also show that miR-29c-3p can target TGFβ2 signaling and decreases extracellular matrix proteins induced by TGFβ2 signaling. miR-29c-3p mRNA targets include collagen types I and IV, however, we do not understand the mechanism by which this occurs. Experiments to elucidate whether miR-29c-3p binds directly to the mRNA of collagens, intracellular SMAD proteins or TGFβ2 ligands would be informative. We also show increased levels of fibronectin in the glaucomatous lamina cribrosa, which co-localizes with GFAP positive astrocytes. Overexpression of miR-200b-3p in ONH astrocytes downregulates the expression of fibronectin; however, we are yet to determine direct targets of miR-200b-3p. It would also be interesting to evaluate the effects of miR-29c-3p in lamina cribrosa cells and miR-200b-3p in ONH astrocytes exposed to stretch. Overexpression of these miRNA in cells exposed to stretch may potentially decrease the secretion of TGFβ2 and the expression of extracellular matrix proteins; thus, protect against tissue remodeling.

In summary, we have used in vitro culture of human lamina cribrosa cells and ONH astrocytes to examine the cell biology and biochemistry of the human lamina cribrosa. Here, we have examined the effects of growth factor signaling on lamina cribrosa cells and astrocytes. TGFβ2 induces extracellular matrix proteins and crosslinking enzymes and contributes to tissue remodeling in the glaucomatous lamina cribrosa. We have suggested that TGFβ2 alters the miRNA expression in the glaucomatous lamina cribrosa to increase extracellular matrix synthesis and tissue remodeling in the lamina cribrosa.

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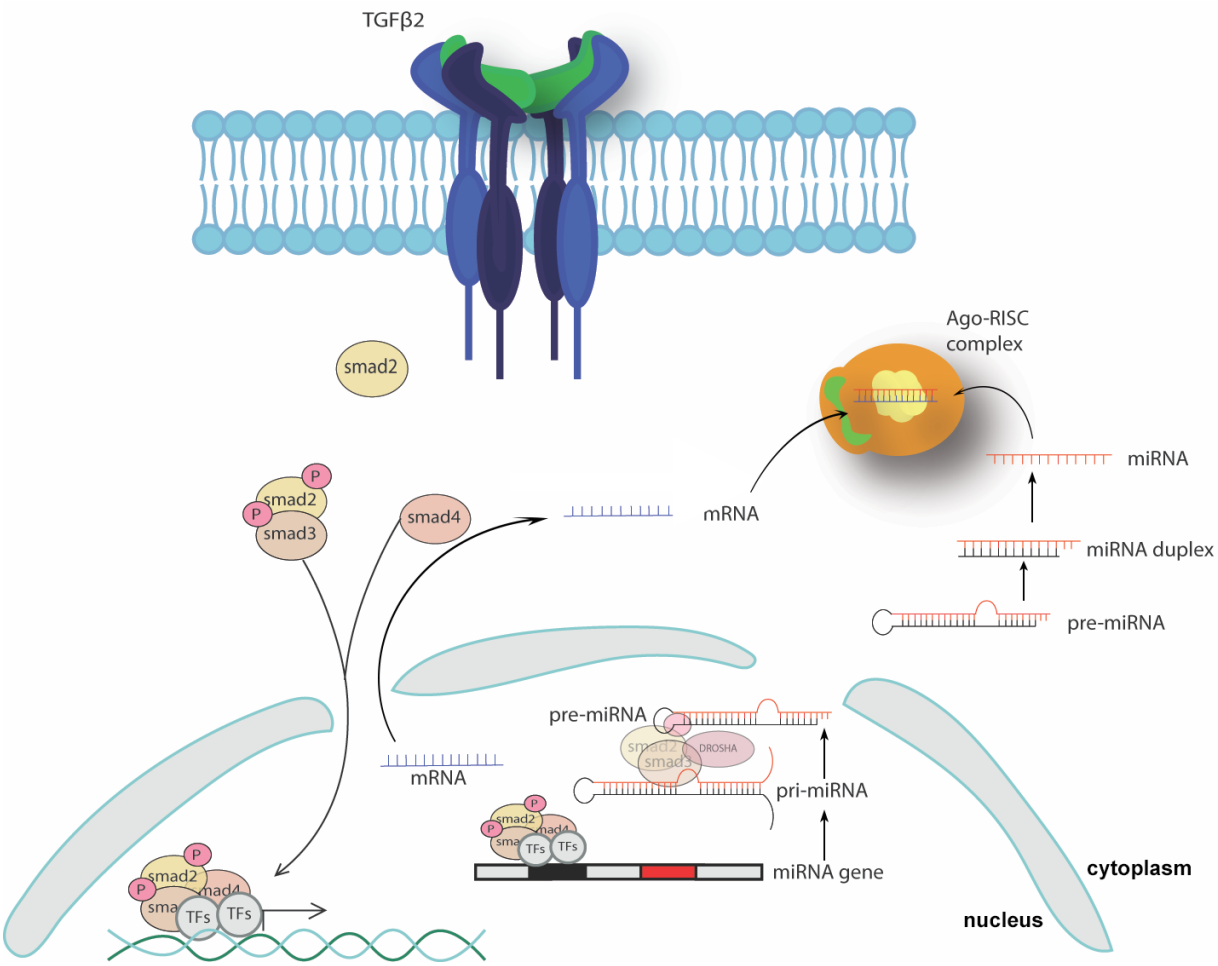
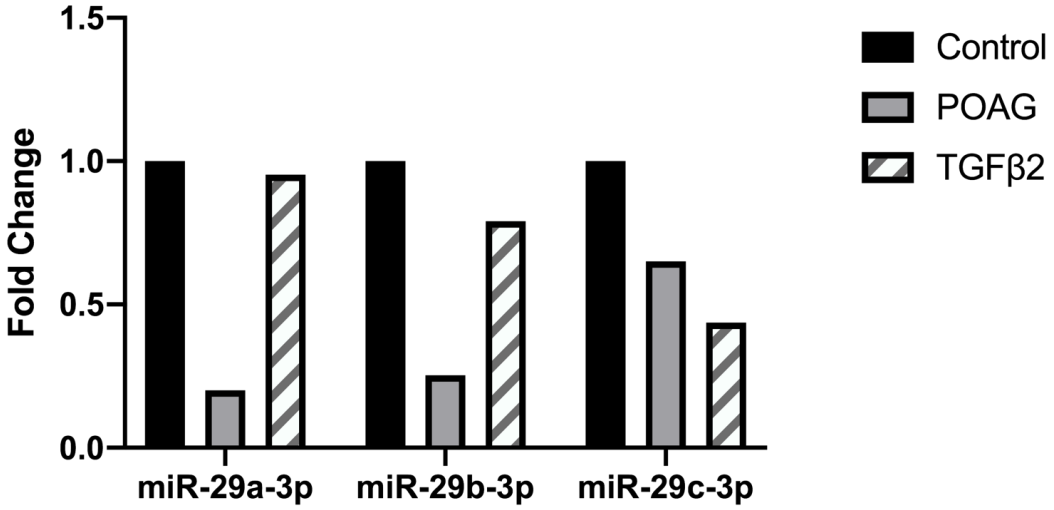


Figure 1. Schematic of how the TGFβ-SMAD signaling pathway may regulate miRNA expression in optic nerve head astrocytes and lamina cribrosa cells.

APPENDIX



Mature ID	POAG	TGFβ2
	Fold Change	Fold Change
hsa-miR-29a-3p	0.2004	0.9532
hsa-miR-29b-3p	0.2532	0.7911
hsa-miR-29c-3p	0.6506	0.437

Figure 1. Comparison of miRNA expression in Normal, POAG, and TGFβ2 treated lamina cribrosa cells from miRNA PCR array. There is downregulation of miR-29b-3p and miR-29c-3p in POAG and TGFβ2 treated lamina cribrosa cells compared to normal lamina cribrosa cells. (POAG, n=1; Normal, n=3; TGFβ2 treated, n=3).

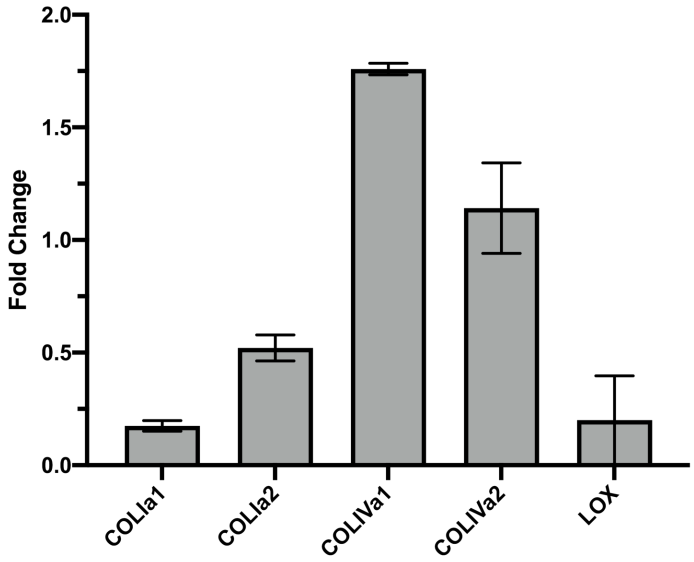


Figure 2. Expression of extracellular matrix proteins in response to TGFβ2 treatment.

Increased expression of collagen type IV in lamina cribrosa cells treated with TGFβ2 (n=2).

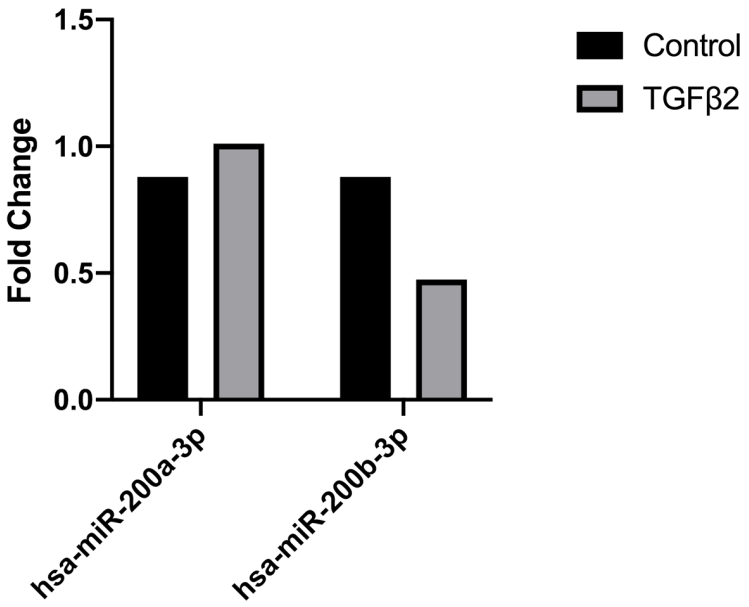


Figure 3. Comparison of miR-200 expression in control and TGFβ2 treated ONH astrocytes from miRNA PCR array. There is downregulation of miR-200b-3p in TGFβ2 treated ONH astrocytes compared to control ONH astrocytes.

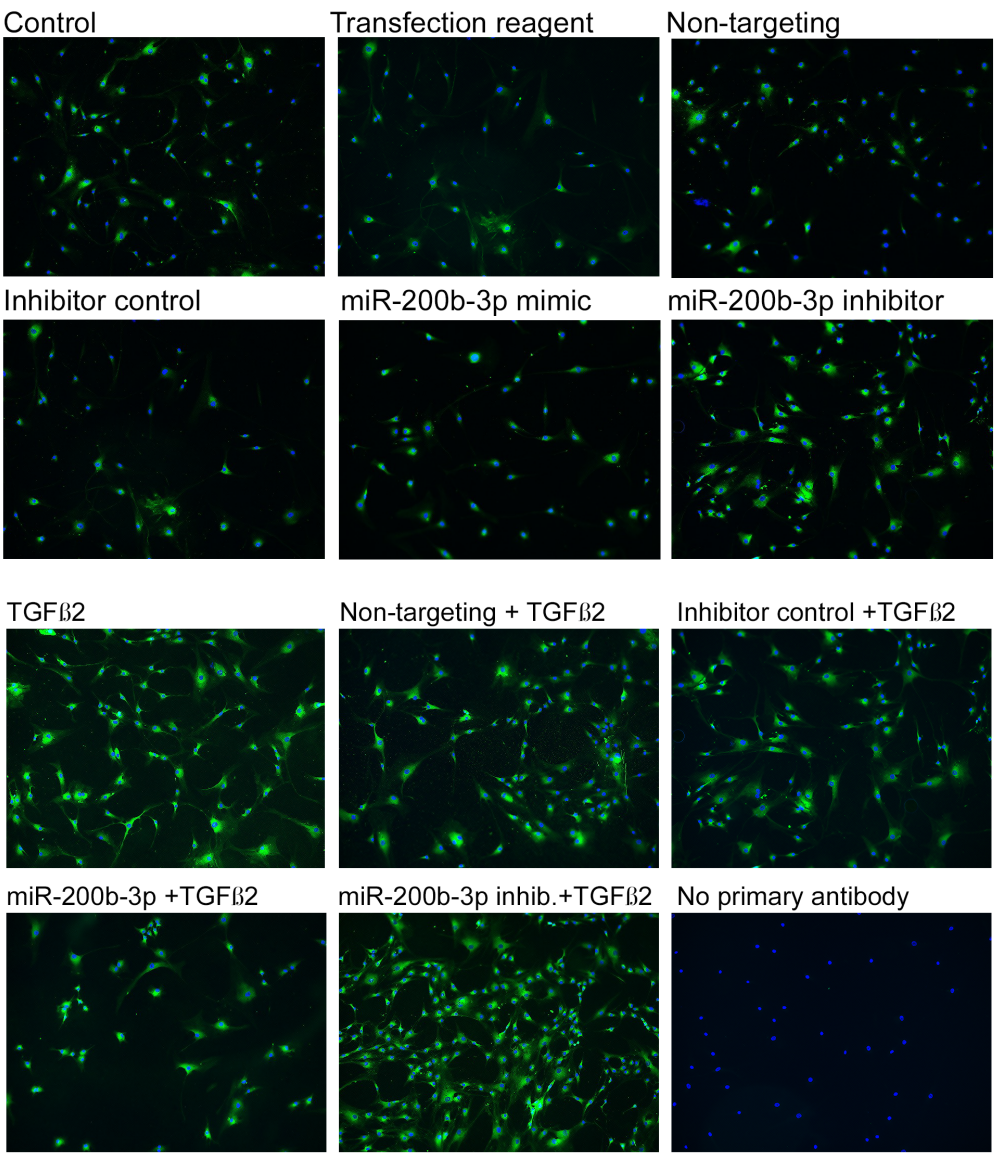


Figure 4. miR-200b-3p regulates collagen IV expression in optic nerve head astrocytes.

miR-200b-3p decreases collagen IV expression in optic nerve head astrocytes (n=2).