# "Understanding the molecular mechanisms of aqueous humor outflow pathway and glaucoma"

*Thesis submitted for the award of*

**DOCTOR OF PHILOSOPHY**

*To*

*Department of Animal Biology*

*University of Hyderabad*


**By**

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**June 2018**

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# CERTIFICATE

This is to certify that the thesis entitled **"Understanding the molecular mechanisms of aqueous humor outflow pathway and glaucoma"** submitted by **Mr. S.Chenna Kesavulu** bearing registration number **12LAPH11** in partial fulfilment of the requirements for award of **Doctor of philosophy** in the **School of Life Sciences** is a bona fide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

A. Published in the following publications:

1) A Novel …adipogenesis. ***Cell Death and Disease.*** ISSN 2041-4889 (**accepted in Jun-2018**).

2) Nutraceuticals…Disease". **"Functional food and human health"**. **(2018). Springer Nature Inc.** (ISBN: 978-981-13-1122-2)

B. Presented in the following conferences:

**1.** Presented in ARVO-INDIA-2017 on "Vision Research-Meeting India's Needs" **(International)**

2. Presented in International Congress of Cell Biology 2018 on "The Dynamic Cell from Molecules and Networks to Form and Function" **(International)**

Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

| Course Code | Name | Credits | Pass/Fail |
|---|---|---|---|
| 1. AS 801 | Seminar 1 | 1 | PASS |
| 2. AS 802 | Research Ethics & Management | 2 | PASS |
| 3. AS 803 | Biostatistics | 2 | PASS |
| 4. AS 804 | Analytical Techniques | 3 | PASS |
| 5. AS 805 | Lab Work | 4 | PASS |

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## DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"Understanding the molecular mechanisms of aqueous humor outflow pathway and glaucoma"** has been carried out by me under the supervision of **Dr. A. Bindu Madhava Reddy,** Department of Animal Biology, School of Life Sciences and this work has not been submitted for any degree or diploma of any other university earlier.

Dated:

**S. Chenna Kesavulu**
**(12LAPH11)**

(Research Scholar)

# Acknowledgements

I would like to express my deepest sense of gratitude to my supervisor ***Dr. A. Bindu Madhava Reddy***, for giving me an opportunity to work under his guidance, constant cooperation, and encouragement throughout my work. I am highly grateful to him for all the efforts he has put in for the successful completion of this thesis.

I thank Head, Dept of Animal Biology, Prof. Anita Jagota, and previous HOD's Prof. Jagan Pongubala Prof. Senthilkumaran, and Prof. Manjula Sritharan for the departmental facilities

I thank the Dean, School of Life Sciences, Prof. K.V.A. Ramaiah and former Dean Prof. M. N. V. Prasad, Prof. P Reddanna, Prof. A.S. Raghavendra, Prof Aparna Dutta Gupta, Prof. R.P. Sharma and Prof Ramanadham for providing the central facilities at the School of Life Sciences

I thank the doctoral committee members Prof. Geeta K. Vemuganti and Prof. Jagan Pongubala for constructive criticism and valuable guidance

I special thanks to Dr. Madhukar Reddy, for providing clinical samples and Dr. Nagaraj for helping during animal experiments.

My special thanks to Dr. Aparna Rachamallu, for her support and suggestions during my course of study.

I thank all my present and past lab mates Suresh, Preethi, Naga Chaitanya, Navya Naidu, Gayathri, for their help and support during my stay in lab

I thank my friends Gangadhar, Shankar, Narayana, Raghu, Jaggu, Maruthi for their support during my critical situations

I thank Bangaraiah, Kumar, Chandram and Prashanth Kumar Yadav for assistance in the laboratory

The financial support from DBT, DST, CSIR, ICMR, UGC, PURSE and DBT-CREBB are highly acknowledged

Financial support through CSIR-JRF and SRF is highly acknowledged

I am extremely grateful to my parents, my Brother and my wife Bindu Bhargavi who has been my real strength, for their unconditional love, support and patience throughout my life.

***S. Chenna Kesavulu***

# Dedicated to my wife Bindu Bhargavi

# Table of Contents

## Abbreviations

| | |
|---|---|
| AH | Aqueous humor |
| AMD | Age-related macular degeneration |
| ATP | Adinosine triphosphate |
| bp | Base pair |
| BSA | Bovine serum albumin |
| cDNA | Complimentary DNA |
| CE | Ciliary Epithelium |
| CLAN | Cross linked actin networks |
| $CO_2$ | Corbon dioxide |
| COL11A1 | Collagen Type XI Alpha 1 Chain, |
| cm | Centimeter |
| CM | Ciliary muscle |
| CYP1B1 | Cytochrome P450 Family 1 Subfamily B Member 1 |
| DAPI | 4,6-diamidino-2-phenylindole |
| DBD | DNA Binding Domain |
| DEPC | Diethyl pyrocarbonate |
| DEX | Dexamethasone |
| DMEM | Dulbecco's modified essential Medium |
| DNA | Deoxy ribo neuclic acid |
| dNTPs | Deoxy nucleotide triphosphates |
| DTT | DL-Dithiothreitol |
| ECM | Extra cellular matrix protein |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immuno sorbent assay |
| EMSA | Electrophoretic mobility shift assays |
| ER | Endoplasmic reticulam |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FGF | Fibroblast growth factor |
| FOXC1 | Forkhead Box C1 |
| GFP | Green fluorescent protein |
| GWAS | Genome-wide association study |
| H&E staining | Hematoxylin and Eosin staining |
| HEK 293T | Human Embryonic Kidney cells |
| HEPES | 2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HTM | Human trabecular meshwork cells |
| IAEC | Institutional Animal Ethics Committee |

| | |
|---|---|
| IOP | Intraocular pressure |
| JCT | Juxtacanalicular Tissue |
| JOAG | Juvenile open angle glaucoma |
| kb | Kilobase pairs |
| kDa | Kilodaltons |
| L-DOPA | L-3,4-dihydroxyphenylalanine |
| LiCl | Lithium chloride |
| LIMK | LIM domain kinase 1 |
| LPS | Lipopolysaccharide |
| LTG | Low tension glaucoma |
| LTBP2 | Latent Transforming Growth Factor Beta Binding Protein 2 |
| mm | Millimeter |
| mM | Millimolar |
| mm Hg | Millimeter of mercury |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger RNA |
| miRNA | Micro RNA |
| MYOC | Myocilin |
| Na+/K+ATPase | sodium-potassium adenosine triphosphatase |
| NAC | N-acetyl cysteine |
| NaCl | Sodium chloride |
| NEB | Nuclear extraction buffer |
| ng | Nanogram |
| nm | Nanometer |
| NM | Normal media |
| NNO1 | Nanophthalmos 1 |
| OD | Optical density |
| Opti-MEM | Optimum minimum essential medium |
| OPTN | Optineurin |
| PACG | Primary angle closure glaucoma |
| PCG | Primary congenital glaucoma |
| PCR | Polymerase chain reaction |
| PCMTD1 | Protein-L-Isoaspartate (D-Aspartate) O-Methyltransferase Domain Containing 1 |
| pg | Picograms |
| pM | Pico moles |
| PMSF | Phenylmethylsulfonyl fluoride |
| POAG | Primary open angle glaucoma |
| RGCs | Retinal ganglion cells |
| RhoA | Ras homolog gene family member A |
| RIPA buffer | Radioimmunoprecipitation assay |
| RNA | Riboneuclic acid |

| | |
|---|---|
| ROCK2 | Rho Associated Coiled-Coil Containing Protein Kinase 2 |
| RT-PCR | Real Time Polymerase chain reaction |
| SC | Schlemm's canal |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| siRNA | Small interfering RNA |
| snRNA | Small nucleolar RNA |
| TAE | Tris base, acetic acid-EDTA |
| TBS | Tris buffer saline |
| TCDD | 2,3,7,8 Tetrachlorodibenzo-p-dioxin |
| TE | Tris EDTA |
| TGF-β2 | Transforming growth factor beta2 |
| TM | Trabecular meshwork cells |
| TMCGS | Trabecular meshwork cell growth factors |
| TMCM | Trabecular meshwork cell medium |
| TMS | 2,3',4,5'-Tetramethoxystilbene |
| TNF-α | Tumor necrosis factor alpha |
| Tris-HCl | Tris hydrochloride |
| UTR | Untranslated region |
| VEGF | Vascular endothelial growth factor |
| WDR36 | WD Repeat Domain 36 |
| WHO | World Health Organization |
| °C | Degrees Celsius |
| µg | Microgram |
| µl | Microliter |
| µM | Micrometer |

# Review of Literature

# Understanding the molecular mechanisms of aqueous humor outflow pathway and glaucoma

## 1. Introduction

Eyes are marvelous organs of sight, which helps us navigate our way through the world. The shape of the eye is spherical and measures 2.5 cm in diameter and is positioned in the orbit of skull and is connected with optic nerves. The anatomical structure of the eye is as shown in figure 1.Vision is the most important one among the senses for humans. Approximately 50% of the sensory receptors in the human body are located in the eyes, and major portion of the cerebral

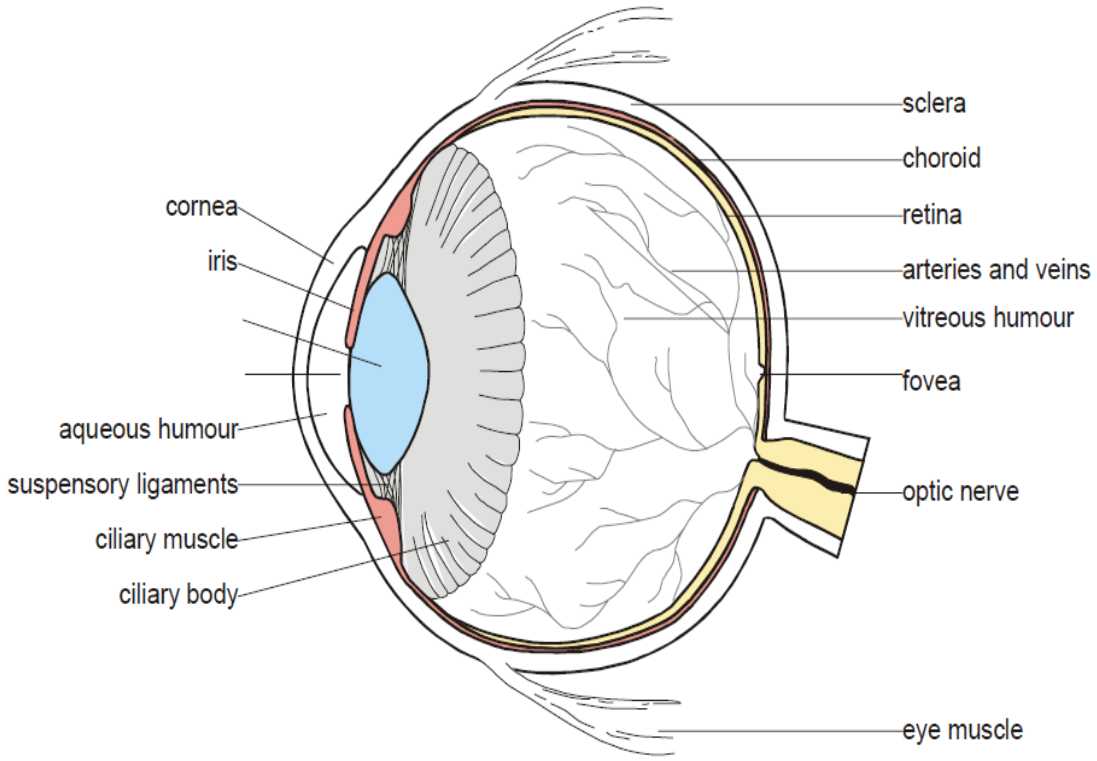



Fig.1. Anatomy of the human eye: Cross section of the human eye |Source. http://www.ekoportal.org/wp content

cortex is devoted to interpreting visual information. The eyes are responsible for detecting visible light, reflected from the objects, ranging from 400 to 700 nanometers in wavelength. They appear as they reflect light, like an object appears white, if it reflects all wavelengths of light and it appears black if it absorbs all wavelengths of light. The fovea is an important part of the eye that contributes to eyesight.

## 1.1 Cross Section of Eye:

### 1.1.1 Eye is made up of three layers

a) Outer layer: Sclera, cornea and conjunctiva
b) Middle layer: ciliary body, choroid and iris
c) Inner layer: retina

**a. Outer layer:**

**1. Sclera:** It is opaque, protective outermost layer consists of fibrous connective tissue. The visible part of the sclera is white in color and contains collagen and elastic fibers [2].

**2. Cornea:** is the thin transparent anterior portion of the eye and is avascular and absorbs oxygen from air [4]. The main function of the cornea is to refract light to focus on to the retina.

**3. Conjunctiva:** A stratified squamous epithelium structure present inside the eyelids and covers the cornea. The function of conjunctiva is to lubricate by producing mucous and tears and protects the cornea.

**b. Middle layer:**

**1. Choroid:** Present between retina and sclera, is highly vascularized, supplies oxygen and nutrients to the retina. Choroid supplies oxygen and nutrition to the outward layer of the retina [5].

**2. Ciliary body:** is located behind the posterior surface of the iris, consists of ciliary muscle which secretes aqueous humor. Ciliary body accommodates lens in position. The function of the ciliary body is to accommodate the lens by contraction or relaxation of muscle based on the light reflection [6].

**3. Iris:** A thin circular structure which controls the pupil. The iris divides the anterior segment into anterior chamber and the posterior chamber. The amount of the pigment decides the color of the iris [7].

**c. Inner layer:**

**Retina:** The retina lines the posterior side of the eye, light-sensitive with millions of cells tightly packed together. Each eye consists of ~ 125 millions of rod cells and ~ 7 millions of cone cells. The fovea centralis is located in the depression of the retinal wall. Fovea centralis contains only cone cells which is sensitive to light and forms sharp images. It has three different types of cells: neuronal cells, photoreceptor cells, and glial cells [7].

### 1.2 Eye lens and chambers

**a) Eye Lens:** It is a transparent and elastic part of the eye present behind the pupil. Due to its elasticity, lens can easily change its shape. As the age progresses the lens becomes hardened due to loss of elasticity, clouded and then becomes challenging to focus, a condition is termed as cataract [7].

**b) Anterior chamber:** Space present between lens and cornea filled with aqueous humor fluid, which comprises of nutrients and respiratory gases that nourishes the lens and cornea. Aqueous humor is hypertonic compared to plasma of mammalians [8].

**c) Vitreous chamber:**

Region behind the lens and in front of the retina filled with transparent gel. Vitreous humor comprises approximately 98 % water, collagen fibrils and hyaluronic acid. The fluid is motionless and visual light passes through and impacts on person's field of vision [7].

### 1.3 Function of Eye:

Cornea, anterior part of the eye, on which light is primarily focused and passes through it. The iris, controls pupil's size depending on light i.e. passed through the cornea. The eye lens is present behind the pupil and light is passes through it, and this lens assists the eye to automatically focus on to the retina. The retina, a light-sensitive area present posterior of the eye converts ocular images into electronic signals. The optic nerve present back of the eye then transmits these electronic signals formed in the retina to the visual cortex of the brain.

### 1.4 Common diseases of the eye:

Various diseases affects different parts of the eye and which may affect the vision. However, most of them are treatable proper diagnosis and treatment should be, should be damage done before any damage.

#### 1.4.1 Dry eye:

Symptoms of dryness in the eye due failing to produce tears consistently which otherwise known as keratoconjunctivitis sicca. Symptoms are burning sensation, ocular dryness, itchy sensation, mucoid discharge, hyperemia and photophobia [9].

### 1.4.2 Meibomian Gland Dysfunction (MGD):

Meibomian glands are known to discharge enough oil into the tears thus keep the eyes moisturized. The dysfunction of which leads to quick evaporation of tears from the eye. Meibomian gland dysfunction is a major problem of dry eye and eyelid disorder (blepharitis) [7].

### 1.4.3 Cataract

Cataract is most common cause of vision loss seen in people with age 40 and above. It is due to clouding of the lens, which decreases vision. It is the primary cause of loss of sight in the world. Clouding may be seen in people taking steroids and suffering with diabetes.[7].

### 1.4.4 Macular degeneration

Macular degeneration also referred as age-related macular degeneration condition leads to loss of vision which affects over 25 million people worldwide. Macular degeneration steadily destroys the macula, part of the eye needed for sharp vision. This disease disturbs central vision, makes it difficult to perform daily activities that require central vision [10, 11]. Risk factors are age, smoking, cardiovascular diseases or high blood pressure, obesity, female, and family history. The age-related macular degeneration is of two types: dry and wet type of age-related macular degeneration. Wet type is more frightening than the dry type of age-related macular degeneration and accounts for up to 90 % of cases for loss of vision in aged people [11]. Loss of vision in age-related macular degeneration occurs due to abnormal function of macula, more specifically photoreceptor cells [12]. There are evidential reports that exposure to sunlight for long period of time may be a causative factor for AMD [13]. As of now there is no permanent cure for AMD.

### 1.4.5 Hypertensive and diabetic retinopathy:

Diabetic retinopathy leads to loss of vision due to damage of retinal blood vessels. Anyone can develop the disease who is suffering with type 1 and type 2 diabetes. One may not develop symptoms apart from mild vision problems initially, eventually it leads to the blindness.

### 1.4.6 Glaucoma

Glaucoma is the term used to refer a group of eye disorders, which leads to the loss of vision. Primary cause of glaucoma is increased intraocular pressure in the anterior chamber of the eye. The fluid pressure in the anterior chamber is known as intraocular pressure (IOP). **Our current study mainly focused on glaucoma pathophysiology.**

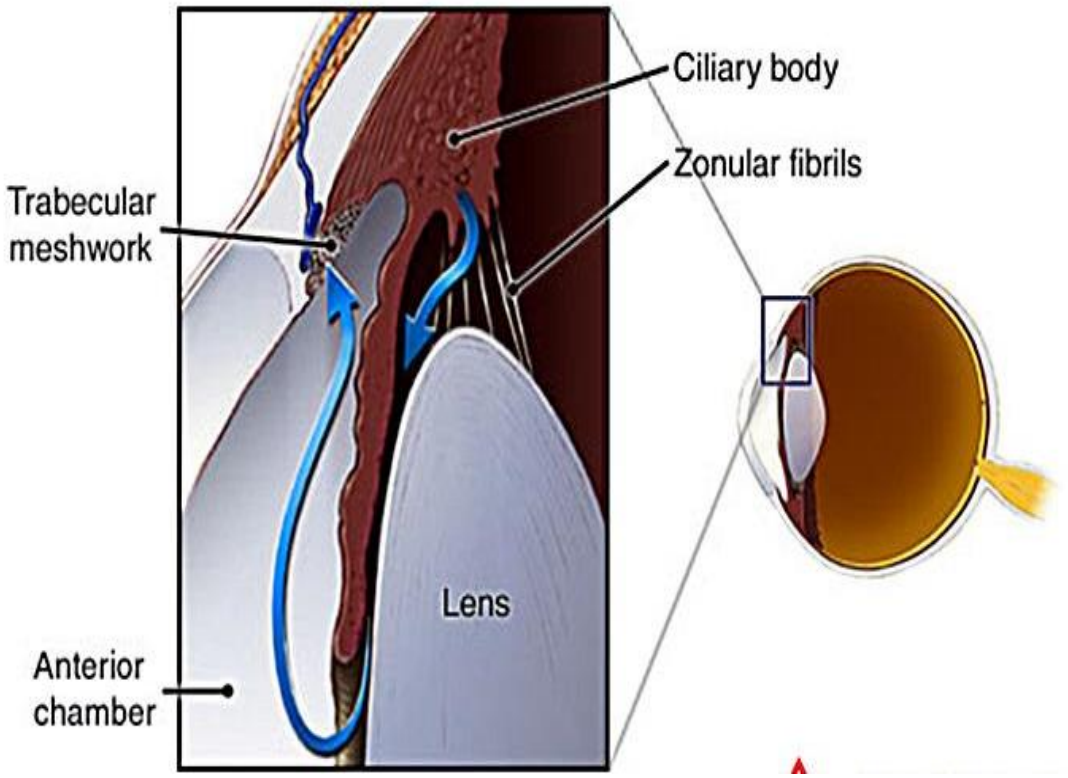



Fig.2 Schematic diagram of aqueous humor production in anterior chamber of the eye| Source: Bright Focus Foundation

## 1.5 Glaucoma

Glaucoma, is the term used to refer a group of eye disorders, is the second most common cause leading to an irreversible loss of sight in the world, [14] in which optic nerves are progressively damaged at the optic disc. The estimated prevalence of glaucoma in 2013 is 64.3 million and may rise to 76 million in 2020 and to 111.8 million in 2040 [15]. Glaucoma is asymptomatic until it is severe. The number of affected people are higher than the number identified [16, 17]. These numbers shows the importance of understanding its underlying pathophysiology, so that it makes easy for better treatment of the disease.

The preliminary cause remains unknown for many types of glaucoma, however reports show that glaucoma is complex in origin, with undisclosed genetic and biological risk factors. Traditionally,

glaucoma is classified based on anterior-segment variations and appearance of the iridocorneal angle.

However, according to modern classification, there are 3 different types of glaucoma, **i) Primary open-angle glaucoma (POAG), ii) primary angle-closure glaucoma (PACG), iii) primary congenital glaucoma (PCG),** as shown in figure 3.

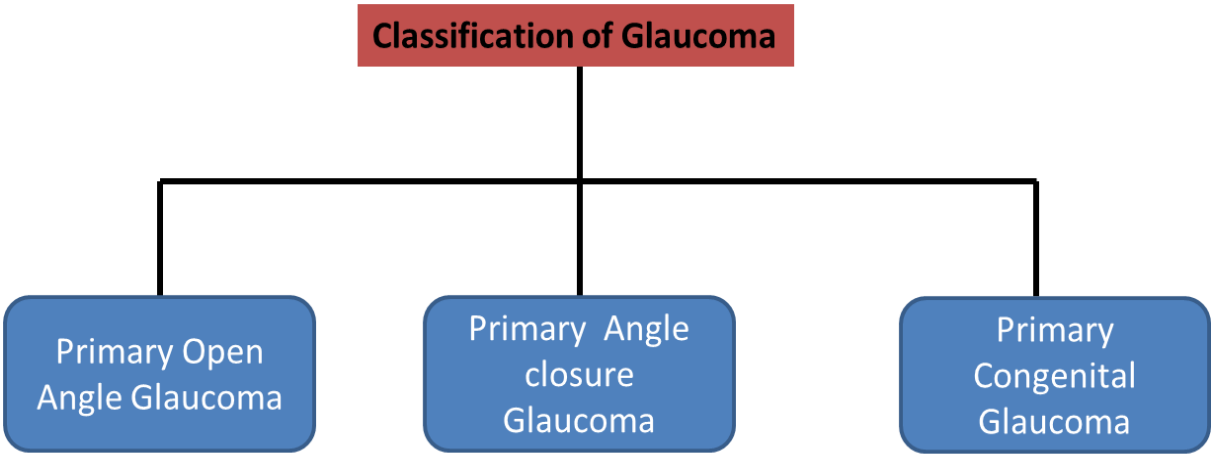



Figure 3. General classification of glaucoma.

### 1.5.1 Primary Open Angle Glaucoma (POAG):

Primary open-angle glaucoma (POAG), in which the angle present between cornea and iris (iridocorneal angle) is open and normal in appearance but aqueous outflow is diminished resulting loss of visual field. Increased intraocular pressure is not necessarily the cause of primary open angle glaucoma since it can also be seen in normal intraocular pressure ( 10 to 21 mm Hg) conditions, however increased intraocular pressure is a key risk factor in glaucoma[18];. Currently, reducing the IOP is the only variable causative factor according to the previous clinical studies [19, 20]. Therefore, treatment options are reducing IOP by means of drugs or surgical [21, 22].

The disease inheritance pattern seems to be multifactorial. So far, genetic linkage and mapping analysis studies shows that more than 20 genetic loci and mutations in WDR36, myocilin, and optineurin have been linked to primary open angle glaucoma. JOAG is rare and categorized with similar clinical features as the adult-onset glaucoma condition like presence of increased IOP, open angle and optic nerve cupping. It can be seen from age 3 to 25 years and extremely high IOPs (frequently >40 mm Hg). [23]. Similar to most forms of glaucomas, the cause is multifactorial, in which some individuals are seen with improper development of the angle and with an increased number of iris processes [24].

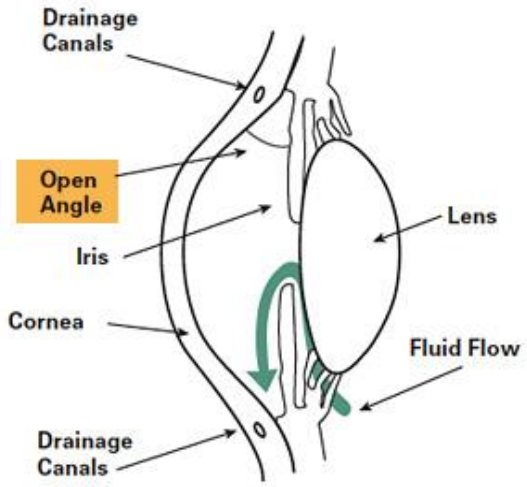



**Fig 4. Schematic diagram to show wide angle in POAG. |Source. The Mayo clinic (www.mayoclinic.com)**

### 1.5.1.1 Genetics of POAG

The disease inheritance pattern is multifactorial. So far, genetic linkage and mapping analysis studies shows that more than 20 genetic loci and candidate WDR36, optineurin, and myocilin have been linked to this phenotype.

**a. Myocilin (MYOC):**

Myocilin was the first gene to be linked with the development of POAG and JOAG. Long term exposure of dexamethasone shown to have, increased production of myocilin protein in TM cells. This was first observation myocilin was identified and seen, associated with the glaucoma pathogenesis [25]. In the year 1993 by linkage analysis, it was initially confined to the GLC1A locus [26] and afterwards related to the MYOC gene in the year 1997,[27] having more than 70 different point mutations in glaucoma patients of various ethnic groups worldwide that accounts to 3 to 5% of mutations were identified in the MYOC gene coding region of POAG cases throughout the world.[28-36].

Myocilin expression was observed in various ocular and nonocular tissues. The highest concentrations of myocilin expression was observed in iris, sclera, and TM [37-39]. It spans ~17kb and comprises 3 exons coding for 504 amino acids [40, 41] with molecular weight of ~57kDa[42].

The reports shows that exon 3 of myocilin harbors most of the mutations related with this phenotype.

Increased expression of myocilin is reported upon increased IOP, dexamethasone treatment and trabecular stress, demonstrating defensive role in the aqueous humor outflow. People have tried to show a relation between mutated myocilin with outflow and glaucoma and found that poor secretion of mutated myocilin protein from cells and accumulate within the endoplasmic reticulum [43, 44]. Apart from this, **the exact function/role of myocilin protein is largely unidentified.**

Mutations in myocilin are intensely linked with POAG and JOAG [45-47]. However, some of these mutations also reported in other forms of glaucoma [45, 48]. Gly399Val mutation reported in adult form of OAG. Whereas the combination of Gly399Val MYOC mutation with Arg368His mutation in CYP1B1 individuals. The severity of the disease also depends on digenic or polygenic interactions & such interaction between several genes had been shown in other forms of diseases like Leber's hereditary optic neuropathy [49].

**b. Optineurin (OPTN)**

Optineurin, another gene associated with OAG initially linked to GLC1E locus on 10p14 [50] and then identified as OPTN as a candidate gene [51]. The studies have reported that polymorphisms were seen in 16.7% of studied NTG cases and missense mutation Glu50Lys is being the most prevalent polymorphism occurred in 13.5% of these families. Further studies have shown that 13.6% of Met98Lys risk-associated variation of affected glaucoma families and 2.1% in controls. Later studies have reported that OPTN mutations are linked with rare form of low tension glaucoma (LTG) and rarely associated with common form of OAG [52-55]. Overall, 1% of OPTN mutations are linked with OAG.

Several nonocular [56] and ocular tissues including TM express optineurin protein [51]. OPTN comprises of 16 exons with 3 different isoforms by alternative splicing, coding for 577 amino acid protein product. AH profiling of numerous species, as well as humans, found OPTN is a secretary protein [51]. **Similar to myocilin, the functional consequences of mutations and its protein function in causing glaucoma is speculative.** OPTN expression has been shown to be increased in the cadaveric human anterior segments by dexamethasone, tumor necrosis factor (TNF)-α and with elevated IOP by perfusion studies [57]. This study shows that secretion of

optineurin may be due to trabecular stress and have a defensive role against glaucoma development.

Overexpression of optineurin in the lens failed to defend against TGF-β1 induced apoptosis in a transgenic mouse model, [58]. Whereas other studies have reported that overexpression of optineurin protects cells from $H_2O_2$ induced cell death whereas E50K mutant failed to show neuroprotective effect [59]. However the exact function of optineurin in the development of glaucoma is not clear.

**c. WDR36**

WDR36 is another gene reported to be associated with POAG. WDR36 gene contains 23-exons and translates a 100-kDa protein. WDR36 mRNA transcripts have been found in ocular and nonocular tissues. The studies reported 4 disease-related mutations in a total of 5% of POAG cases [60]. Further studies have shown less [61, 62] or no link with WDR36 with OAG [63, 64], however, these mutations seems to be associated with escalation in severity of POAG signifying that, WDR36 may be a sensitive gene for POAG [65]. Moreover, WDR36 mutations were not seen in that family where GLC1G locus established linkage with WDR36 gene, and thus raises the question of possibility of association of another gene on the GLC1G locus [66].

The function of WDR36 believed to be involved in ribosomal RNA processing and T-cell activation and proliferation [67] . Loss of function of this gene in Zebrafish shows decreased in eye size and lens anomalies but no signs of glaucoma phenotype [68]. Thus the exact function in the development of the disease needs to be studied carefully.

### 1.5.2 Primary Angle Closure Glaucoma

As per reports, 15 million people were affected worldwide with PACG in 2010 and is predicted to escalate to 21 million by 2020 [14]. Half of the world glaucoma patients are suffering with Angle closure forms. PACG cases are seen mostly in Asia with 86%, nearly 48.0% in China, 23.9% in India and 14.1% in south East Asia [69]. Primary narrow angle cases may be classified into primary angle closure, primary angle closure suspect and primary angle closure glaucoma [70].

**1.5.2.1 Primary Angle Closure or PAC:** The peripheral iris obstructs fluid flow through the normal trabecular meshwork in the absence of any secondary pathology.

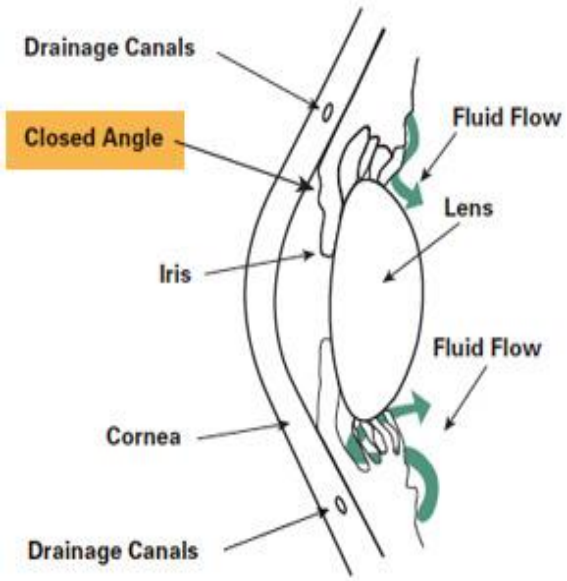



**Fig 5. Schematic diagram to show, blockage of fluid flow in Primary angle closure glaucoma (PACG).|Source. The Mayo clinic (www.mayoclinic.com)**

**1.5.2.2 Primary Angle Closure Glaucoma or PACG:** Fluid flow is obstructed by the trabecular meshwork due to glaucomatous symptoms like damage to the optic nerve head. People were not considered with high IOP without damage to the optic nerves.

This explains that 60-75% of people with high IOP will recover without optic disc damage. While estimating the prevalence of PACG and POAG in South Africa, it was recognized that people with chronic angle closure had IOP as high as 72 mmHg, but were unable to establish a link between symptoms and development of vision loss [71]. Angle closure is more common even in East Asia without any symptoms. Symptoms of angle closure does not alone stipulate the associated mechanism, hence management strategies should not be based on symptoms alone.

**1.5.2.3 Primary angle closure glaucoma suspect (occludable angle):** Described as the presence of high IOP and or peripheral anterior synechiae (PAS) along with presence of disc and field damage. For epidemiological and clinical classification, when more than $270^0$ of the TM cannot be seen through the angle is considered as closed. **Primary angle closure** glaucoma is characterized by damage to the optic disc, corresponding vision loss with occludable angles or signs of PAC. Clinical presentation and identifying the cause is most important for management or treatment of patients with PAC. Treatment options are medical, laser and surgical.

**1.5.2.4 Genetics of PACG:**

The culture and gender-specific susceptibility to PACG shows its relation with genetic basis for the development of pathology in certain populations. Some of the reports strongly shows relation with genes for the development of glaucoma in humans, is a multifactorial disease. [72]. One of the primary cause for the development of PACG is family history with higher chances of heritability [73, 74]. Epidemiological survey in China revealed a six fold increase in risk of PACG

with family history of glaucoma [75]. Moreover, reports shows that anterior chamber size is controlled by genetic factors, signifying its role in the PACG and are also be heritable [73, 74]. Recent studies have shown several genes and genetic loci [27, 51, 60, 76] that may be contributing for POAG, but evidence for linking genes that cause PACG have to be identified. Nanophthalmos is the only human gene identified to be responsible for causing primary angle closure glaucoma [77]. This study has identified nanophthalmos 1 (NNO1) gene on chromosome 11. Mutations in the matrix metalloproteinase-9 (MMP9) gene have been shown to be associated with PACG. MMP9 encodes an enzyme that involves in ECM remodeling. SNP rs2250880 in MMP9 is found to be associated with the disease in southern Chinese population [78]. Another SNP (rs17576) in MMP9 was shown to be associated with PACG in a Taiwanese and a Pakistani patient cohort [79, 80], but rs17576 in MMP9 mutation is not statistically correlating in Singaporean patients [81]. Association studies between PACG and POAG genes in MYOC, OPTN, WDR 36 and CYP1B1 in middle-eastern population failed to identify mutations with risk factor [82]. Association of MYOC to PACG was found in a Quebec population,[83, 84] but not in the Chinese cohort [85]. From the above studies, myocilin is not a major contributor for PACG, but seen its association with POAG. On the other hand, CYP1B1 known to primarily associated with congenital glaucoma, is also positively associated with PACG in Chinese, Indian, and Canadian patients [86, 87].

### 1.5.3 Congenital Glaucoma (PCG):

PCG is a common form of infantile glaucoma. PCG is most common in children and manifests in 80% of cases within the first year itself [88]. The symptoms include corneal edema, epiphora, photophobia, and buphthalmos. PCG affects males (65%) more frequently than females (35%) and 60-80% cases are bilateral [89]. PCG is an autosomal recessive with variable penetrance. PCG is developed due to unusual development of the anterior chamber angle, which results in obstruction of fluid flow [90-92]. Incidence of PCG varies from 1:10,000 to 1:2000 in the developed countries [93] and in the Middle East [94] respectively.

PCG is different from adult form of glaucoma in the optic nerve changes. Optic nerve cupping is fast in newborns [95, 96] and can be reversible if identified early [97]. On the other hand, optic nerve damage is irreversible in glaucomatous adults [95]. PCG is a multifactorial and various genes CYP1B1, LTBP2, MYOC, FOXC1 etc. have been found to be responsible for causing PCG but their molecular mechanism in outflow pathway are speculative and needs to be identified [98].

### 1.5.3.1 Genetics of PCG

Till date, 3 genetic loci GLC3A at 2p21,[99] GLC3B at 1p36,[100] and GLC3C at 14q24.3.116 loci has been linked to PCG, however mutations in CYP1B1 candidate gene, linked to GLC3A locus has been associated to disease phenotype. CYP1B1 belongs to family of cytochrome p450 enzymes and is the first one to be linked to developmental process. [101]. Approximately, 20% to 30% of ethnically mixed populations,[102-105] and 50% to 80% of other more consanguineous groups,[106-109] and nearly 100% of highly consanguineous (in breading) groups [110] were been associated with mutations in CYP1B1 gene. CYP1B1 has 3 exons but only 2 and 3 exons are translated to 543 amino acid protein [111]. CYP1B1 expression is observed in most of the tissues [112] and TM [101]. CYP1B1 is a mono-oxygenase belongs to cytochrome superfamily and dioxin-inducible that can metabolize and activate carcinogenic chemicals [113, 114]. CYP1B1 enzyme involved in the metabolism of drugs and synthesis of steroid hormones and other lipid signaling molecules, differentiation and growth of tissues and thus may be involved in early ocular differentiation [101, 115]. Therefore, it is speculated that loss of CYP1B1 hinders normal development of the anterior chamber.

Moreover, other cytochrome P450 enzymes reported to be involved in producing arachidonic acid metabolites of that can impede a corneal Na+/K+ ATPase involved in corneal transparency, and make cornea more vulnerable to hydration and leads to corneal clouding [116]. CYP1B1 gene knockout mice did not show any developmental defects in the anterior chamber angle and glaucoma [117]. However, CYP1B1−/− knockout mice have shown focal changes in the anterior segment of the eye. The angle structures in CYP1B1−/− knockout mice were normal, only focal areas had some developmental defects and focal area had shown structural changes that resembled with PCG cases. Noticeable iris processes, small/absent Schlemm's canal, peripheral anterior synechiae, and a basal lamina covering the TM were observed in the CYP1B1−/− knockout mice. Interestingly, albino mice with tyrosinase deficiency had shown more severe angle abnormalities than pigmented CYP1B1−/− knockout mice demonstrating that the importance of tyrosinase in the development of the angle by regulating L-Dopa levels. CYP1B1−/− knockout mice supplied with external L-DOPA had shown less angle dysgenesis than controls, which are not supplemented with external L-DOPA. Furthermore, this study shows that other modifying factors like genes and external stimuli may be regulating CYP1B1 to develop PCG phenotype [118].

FOXC1 is another gene reported to be associated with PCG. Mutations in FOXC1 are reported to associate with anterior segment dysgenesis [119]. FOXC1+/− mice exhibited anterior segment abnormalities [120]. From the above studies it is speculated that PCG may arise from alterations in a number of different critical genes necessary for the development of the anterior segment.

## 1.6 Secondary Glaucoma

Secondary glaucoma, where the cause is known, such as diabetes, cataracts, inflammation, eye injury, bleeding in the eye, prolonged use of glucocorticoids and side effects of previous eye surgeries if any may leads to high IOP and glaucoma.

### 1.6.1 Causes of secondary glaucoma

#### 1.6.1.1 Pigment dispersion syndrome

Pigment dispersion syndrome is described as abnormal deposition of pigment in the TM that impedes outflow of aqueous humor. In this disease pigment granules are deposited in the TM that are occasionally rubbed off from iris by the lens. This causes obstruction of fluid outflow from the anterior chamber that leads to increased intraocular pressure. When this increased IOP causes optic nerve damage, is known as pigmentary glaucoma. The condition is mostly seen in adults with mean ages of 25 and 40 years, myopic males having a deep anterior chamber [121].

#### 1.6.1.2. Pseudo-exfoliation glaucoma

In pseudo-exfoliation glaucoma, fine white deposits of a fibrillary material released from the lens by the continuous movement of the iris, deposits in the trabecular meshwork and obstructs aqueous humor outflow. If this condition causes damage to the optic nerve it is called as pseudo-exfoliative glaucoma. It usually seen in any ethnicity but more prevalent in some ethnic groups like Scandinavians and Southern Mediterraneans and usually occurs in patients with mean group of above 65 years. [121].

#### 1.6.1.3. Uveitic glaucoma

Uveitis is inflammation to the uvea. There are different types of uveitis – Anterior uveitis, affects only the front part of the eye. Inflammation in the vitreous called as vitritis and inflammation in the back of the eye retina, choroid layer called as posterior uveitis. Most common cause of uveitis is the use of topical steroids with high intraocular pressure. [121].

#### 1.6.1.4. Neovascular glaucoma

Neovascularization of the iris (rubeosis iridis) and anterior chamber angle is seen in some clinical conditions such as diabetic retinopathy and retinal vein occlusions. In this condition new blood vessels were formed in the anterior chamber angle and initially block the aqueous outflow, which subsequently leads to the development of the glaucoma. [121].

#### 1.6.1.5. Steroid induced glaucoma

Topical, periocular, and intraocular corticosteroids may produce a type of glaucoma called steroid induced glaucoma. Withdrawal of the medication usually reduces the intraocular pressure to base line levels, but permanent damage can occur if the situation goes unrecognized for too long. At times prolonged use of steroids for treating any of the eye diseases may lead to high IOP as a result glaucoma. [121].

### 1.7 Role of Aqueous humor in glaucoma pathophysiology

Several studies have reported that, increased intraocular pressure (IOP) has been found to be a critical contributing factor for glaucoma and, consequently, the decrease in IOP delays or prevents optic nerve axons damage [20, 50, 122-126]. Intraocular pressure is mainly developed due to imbalance between secretion and egression of aqueous humor. The rate of aqueous humor turnover is 2.4 ± 0.6µl/min in the daytime in adults with the age group of 20–83 years. Aqueous humor flow follows circadian rhythm, morning hours fluid flow is more compared to night. Fluid flow in the morning between 8 AM to noon is 2.86 ± 0.73, and from noon to 4 PM is 2.63 ± 0.57, afterwards drops to 1.5µl/min during night times, signifying that it follows a pattern called circadian rhythm in normal human adults [8].

Aqueous humor is a fluid similar to plasma, present in the anterior chamber of the eye and is secreted by the ciliary epithelium (CE) of the ciliary body. AH is secreated from the ciliary epithelium enters into the anterior chamber by passing through the pupil. Then the fluid leaves out of the anterior chamber in pressure dependent manner. Fluid drains out in two different pathways i.e. conventional and unconventional pathway as shown in figure 6 [127]. Aqueous humor nourishes and supplies oxygen to the avascular tissues in the anterior portion of the eye. The pressure generated by the fluid establishes the intraocular pressure, which is important to the anterior chamber to maintain proper shape. AH also helps in transporting ascorbate, in the

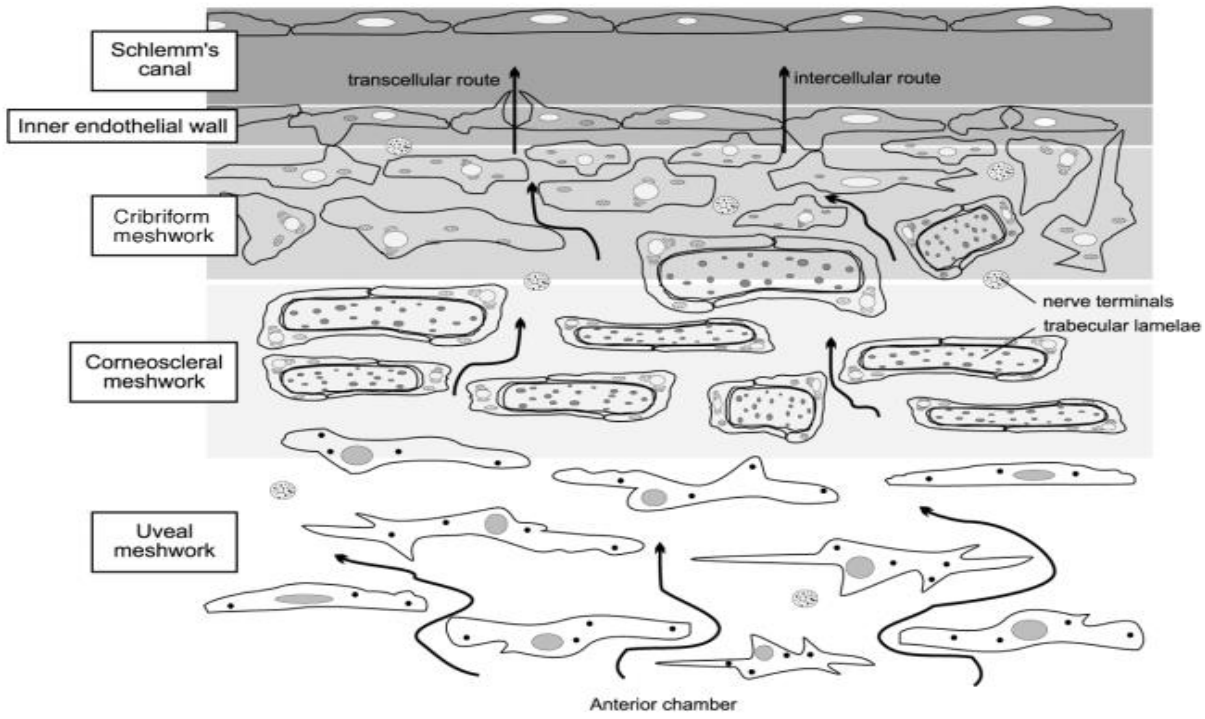



Figure 6. Schematic diagram of the TM shows direction of aqueous humor secretion. The arrows shows the direction fluid flow, from the anterior chamber angle towards Schlemm's canal.| Source:[1]

anterior segment. Immunoglobulins were present in the aqueous humor signifying its role in immune response against invading pathogens [127].

The secretion and draining of aqueous humor are important processes in the eye for its normal function. Average IOP of normal adult eye is of approximately 15mmHg, this pressure developed when fluid flows against resistance of TM [128]. The blockage of AH fluid flow results in increased IOP, which results in the development of the glaucoma pathology. Hence, understanding the molecular mechanisms that control aqueous humor flow is essential for better management of glaucoma [8].

The aqueous humor leaves the eye in two different routes, i) conventional and ii) unconventional pathways. Most of the aqueous humor leaves via the conventional, TM/SC pathway. About 10% drains the eye through unconventional pathway[129]. The trabecular meshwork is a triangular, spongy structure comprises of connective tissue enclosed through endothelium. TM is mainly divided into three different types, juxtacanalicular meshwork, corneoscleral meshwork and uveal meshwork [130]. The front portion towards the anterior chamber is uveal meshwork, which extends from the iris root towards the peripheral cornea. The uveal meshwork comprises of connective tissue, with unequal openings that measuring about 25 to 75μm in size [131].

The middle portion of TM is the corneoscleral meshwork and is comprises of perforated sheets that become smaller nearing Schlemm's canal [131]. The farthest part from the anterior chamber of the trabecular meshwork, is the juxtacanalicular meshwork [132]. Most of the TM resistance developed in the juxtacanalicular meshwork and as a result increased IOP. Elevated IOP is a risk factor of POAG, is directly linked to increase in outflow resistance attributed to juxtacanalicular tissue [133, 134]. Change in structure and organization of trabecular meshwork is seen in glaucomatous eyes. The most distinctive structural changes observed in the trabecular meshwork tissue is the accumulation of extracellular matrix proteins [135-144]. The role of these structural changes in TM of glaucoma eyes are not clearly understood at this time.

The contraction and relaxation of the trabecular meshwork plays a major role in fluid flow from the anterior chamber where contraction of TM reduces fluid outflow which results in elevated IOP, on the other hand relaxation of TM increases fluid flow resulting in decreased IOP [145-149]. Cytoskeletal reorganization plays an important role in TM contraction and relaxation. Increased cross linked actin networks (CLANs) were observed in the TM of glaucomatous eyes. CLANs are polygonal structures and increases the rigidity and stiffness of the trabecular meshwork cells making them more resistant to AH outflow [150-158]. Reports shows that the cytoskeleton of TM cells plays an vital role in inducing aqueous outflow resistance [159]. Studies, on perfusion models have revealed that change in F-actin architecture [159-163] and actin myosin tone [149, 164-168] influences outflow facility. These studies, in combination with other observations of substantial presence of F-actin in TM cells in situ [169-172], and the role of contraction of trabecular meshwork (TM) cells influencing outflow facility [173], provoke further studies in understanding role of actin architecture within the outflow pathway cells.

Treatment of TM cells with dexamethasone induced F-actin reorganization and leading to formation of cross-linked actin networks (CLANs) [152]. Treatment with dexamethasone induces abundant F-actin structures in glaucomatous cultured HTM cells [151], and in human anterior segments perfused outflow structures [150]. F-actin architecture is more disordered in glaucoma outflow tissues compared to normal. Some of these changes may be due to secondary effects of antiglaucoma drugs. These changes affect the outflow tissues in glaucoma but the molecular mechanisms were not clear till date [174].

### 1.8 TGF-β2: role in the pathophysiology of glaucoma

Aqueous humor profiling studies have shown that increased TGF-β2 cytokine levels in the POAG patients, which correlates with increased outflow resistance and high IOP. Approximately 60 - 70% higher concentration of biologically active form of TGF-β2 is observed in aqueous humor of POAG patients compared to the normal cases with similar age group [175-177]. The role of TGF-β2 is found to be pathogenic by *ex- vivo* studies of cultured human, porcine and monkey anterior segments [178-181]. TGF-β2 in these experiments was shown to increase intraocular pressure (IOP) in time dependent manner [181]. In vivo studies by rodent models also showed elevated level of IOP by TGF-β2 [182-186]. TGF-β2 induced secretion and synthesis of ECM in trabecular meshwork cells is shown to be regulated through canonical Smad3 signaling pathway [183, 187, 188]. However, TGF-β2 induced formation of actin stress fibers and contractility use non-canonical RhoGTPase/Rho associated kinase signaling pathways [147, 189-191].

**Above all studies clearly shows that TGF-β2 induces outflow resistance and high IOP through activating both Smad dependent (canonical) and Smad independent (non-canonical) signaling pathways in glaucoma.**

### 1.9 Rho/ROCK signaling pathway in the development of glaucoma pathophysiology

The Rho family (Rho, Rac, and Cdc42) regulates actin cytoskeletal reorganization through activation of downstream effectors [192, 193]. Rho associated kinases (ROCKs) are related in Rho-mediated actin cytoskeletal reorganization, like stress fibers formation and focal adhesions and contraction of smooth muscle cells [194-196]. ROCKs induces actomyosin-based contractility by phosphorylating myosin light chain (MLC) [197]. Cell motility, structural change, cell adhesion and cytokinesis all depend on actin cytoskeletal reorganization [198]. Several extracellular stimuli regulate actin cytoskeletal reorganization. Cofilin regulates the polymerization and depolymerization actin filaments and plays a key role in actin dynamics [199, 200]. Evidence from various studies *in-vitro* and *in-vivo* shows that phosphorylation of cofilin at Ser-3 [201] position by LIMK1 and LIMK2 inactivates it from actin binding and depolymerization in cells [202, 203]. LIMK1 and LIMK2 are serine/threonine kinases [204-208]. Several studies reported that LIM-kinases are activated by Rho family GTPases [202, 203, 209].

### 1.9.1 The functional significance of Rho/ROCK signaling in aqueous humor outflow pathways

Rho and ROCKs expression were found in bovine ciliary muscle (CM) and outflow pathway tissues by *in vitro* and *in vivo* studies [149, 210-213]. RNA levels of ROCK1 and ROCK2 in TM and CM were found using RT-PCR [212]. ROCKs were expressed more abundantly in TM than in CM, both in humans and monkeys [212]. RhoA, ROCK1 and ROCK2 expression were analyzed by immunohistochemical studies in human aqueous outflow tissues (TM, JCT and SC) and found

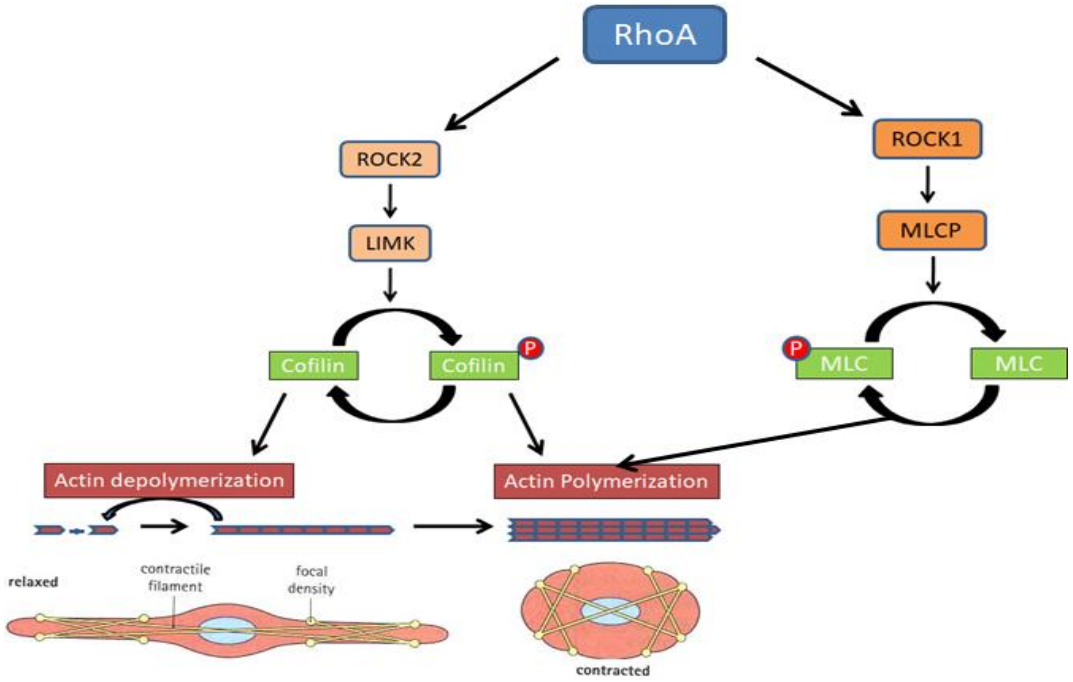



**Fig 7. Model of signaling pathways involving Rho/ROCK in cytoskeleton organization.**

no difference in their expression in glaucoma eyes compared to normal eyes [210]. Therefore, It is hypothesized that anti-glaucoma drugs used to treat glaucoma might be modulating the Rho and ROCK expression in the outflow tissue of glaucomatous eyes. [214].

### 1.9.2 Functional role of Rho/ROCK signaling pathway in IOP regulation

Studies have shown that activation of Rho/ROCK signaling pathway in outflow tissues increases IOP by decreasing aqueous humor flow, however inhibition of Rho/ROCK signaling pathway by different ways shown to decreases IOP by increasing the out flow of aqueous humor. One study reported that expression of RhoAV14 reduced aqueous humor secretion in organ-cultured anterior segments from porcine eyes [215]. Several reports have shown that ROCK inhibitors,

either siRNA or chemical inhibitors ( Y-27632, Y-39983, HA-1077 and H-1152) increase fluid outflow and reduces intraocular pressure in animal models ( rabbits, mouse, rat, monkeys, human and enucleated porcine eyes) [148, 149, 211, 216-224]. Topical application of SNJ-1656 (an ophthalmic solution) effectively reduced intraocular pressure in healthy adult volunteers [219]. **All these studies collectively show that ROCK inhibitors could be good candidates for glaucoma therapy** [212, 218].

### 1.10 Role of miRNAs in the regulation of intraocular pressure

MicroRNAs are small in size ~21 to 23 bps in length. These are of single stranded RNA molecules that bind to the complementary sequence of the target mRNA 3 ' -UTR and leads to the mRNA degradation or translation inhibition [225]. Single miRNA could target number of mRNAs and

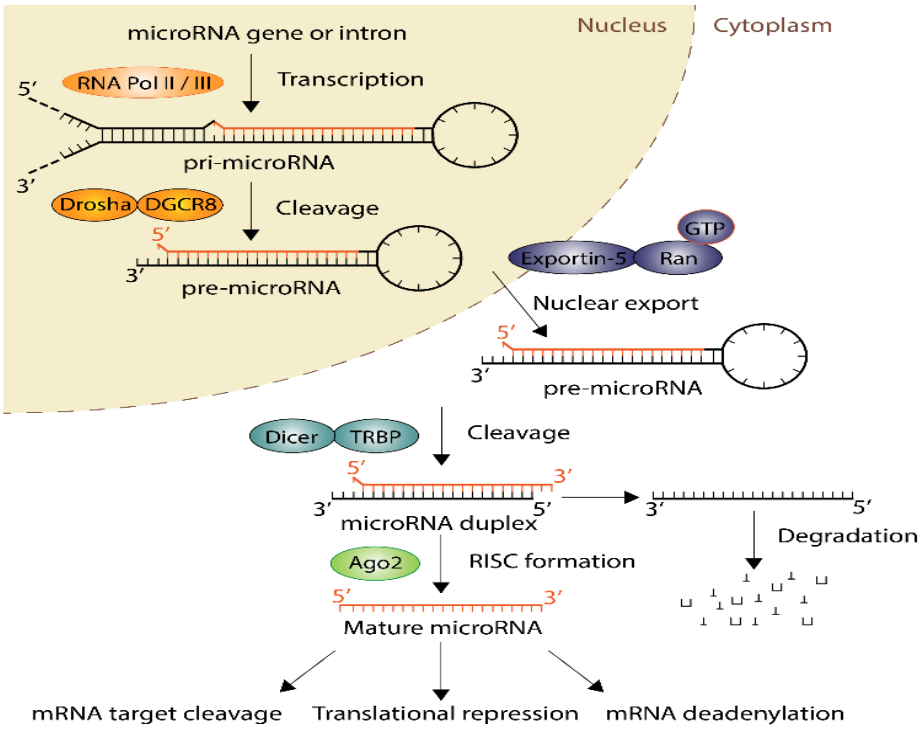



**Fig 8. Diagram showing the miRNA mediated mechanism| Source[3]**

plays significant role in modulating eukaryotic gene expression. [226, 227] miRNA has been shown to have a role in various biological functions such as cell differentiation, proliferation, apoptosis, individual development, and the body's metabolism [228]. Some of the studies have shown link between **miRNAs and its role in maintaining the aqueous humor production, in the**

**outflow tissues, and various functions in retinal ganglion cells (RGC).** These miRNAs may be useful in maintaining the proper shape of the anterior chamber and the pressure of the aqueous humor by regulating target genes related to the anterior chamber organization, such as the trabecular meshwork. **These studies support the hypothesis that miRNAs may regulate key molecules that help in regulation of aqueous humor production and its egression in the anterior chamber of the eye.**

Few studies have shown that miRNAs are associated in maintaining TM physiology of both normal and pathologic TM like contraction of the cell and extracellular matrix synthesis [229]. One of the study had shown that, 181 miRNAs were consistently expressed in the aqueous humor samples of both POAG patients and normal controls. miR-518d and miR-143 were significantly up-regulated and miR-660 was significantly down-regulated in the aqueous humor of POAG patients compared to controls [230]. miRNA profiling using miRNA-Seq in three different anterior segment tissues(cornea, TM and ciliary body) studies identified uniquely expressed miRNAs, signifies miRNA importance in the development of glaucoma [231].

**Despite these recent findings, much is still not known about the expression/function of miRNAs in the development of glaucoma.**

Here we intend to see the relation between TGF-β2 and glaucoma candidate genes in regulating the outflow facility. Our data shows that TGF-β2 and glucocorticoids (dexamethasone and prednisolone) induces FOXC1, OPTN, MYOC and LTBP2 expression and down-regulates CYP1B1 expression in primary HTM cells.

# Objectives

## 2. Objectives:

So far the studies have shown that mutations in some of the genes such as CYP1B1, FOXC1, OPTN, MYOC, LTBP2, WDR36, PITX2 and PAX6 were linked with the development of the glaucoma pathogenesis, but their molecular mechanisms were not clear till date.

Higher concentrations of TGF-β2 levels were found in different forms of glaucoma, signifying its role in the pathogenesis. Similarly prolonged use of glucocorticoids causes secondary glaucoma but the mechanisms inducing high IOP but it's not clear the underlying mechanisms and cause and effect relationship.

There are no glaucoma models to understand the development of the pathophysiology which exactly replicates the pathophysiology in humans. Though studies have reported developing knockout mice of CYP1B1, FOXC1 and OPTN, but did not find any clinical features of glaucoma, which signifies that anterior segment of human eye is complex and there may be possibility of interaction of unknown key molecules with the existing/new candidate genes in developing the pathogenesis.

Thus this doctoral study designed with the following objectives:

- What is the role of TGF-β2 and glucocorticoids in the regulation of IOP and development of glaucoma pathogenesis?
- Role of glaucoma candidate genes in the regulation of IOP and glaucoma development?
- What is the relation between TGF-β2 and glaucoma candidate genes?
- Do glaucoma candidate genes regulate each other? If yes, how could they involve in known signaling pathways in relation with intraocular pressure and disease?

# Materials and Methods

## 3. Materials and Methods

### 3.1 *In silico* miRNA target prediction

miRNA target prediction were performed using open sourced bioinformatics algorithm tools based on sequence complementarity, such as TargetScan7.2 (http://www.targetscan.org)[232], miRanda (http:// www.microrna.org)[233] and miRBase (http://www.mirbase.org/ )[234] , for TGF-β2, ROCK2, CYP1B1, FOXC1, OPTN, LTBP2, MYOC as miR-200 family, miR-590, miR-548c and miR-496 specific targets.

**3.2 Cell culture.** Primary HTM cells (HTM) and its complete culture medium with growth factors (TMCM) were purchased from Sciencell Research Laboratories Inc. and cultured at 37°C and 5% $CO_2$ incubator. Recombinant human TGF-β2 (2ng/ml; Sigma), dexamethasone (300nM/ml; Sigma) and prednisolone (300nM/ml; Sigma) were used to mimic the glaucoma pathology. miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c mimics and anti-miRs against each respective miRNA (Qiagen) were used for transfection in primary HTM cells using Dharmafect duo transfection reagent (Dharmacon). HEK 293T cell lines (ATCC) were used for co-transfection studies. HEK293T cells were cultured in DMEM, supplemented with 10% FBS and 1% Pen/strep (Invitrogen-Gibco Life Technologies) at 5% $CO_2$ and $37^0$ c temperature incubator.

### 3.3 RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was extracted using miRNeasy isolation kit (Qiagen) according to the manufacturer's instructions. RNA yields were measured using Nanodrop 2000 (Thermo scientific). For mRNA analysis complementary DNA (cDNA) was synthesized from 1.0 µg of total RNA using miScript II RT Kit (Qiagen). Real-time PCR was subsequently performed in duplicate with a 1:5 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a CFX96 series PCR machine (BioRad). Data were collected and analyzed by using ddCT method. All mRNA quantification data values were normalized to GAPDH. For miRNA analysis, real time PCR was performed as above; using miRNA prime assays according to the manufacturer's instructions (Qiagen). All miRNA expression data values were normalized to a U6 small nuclear (sn) RNA.

### 3.4 Transfection of synthetic microRNA and inhibitors.

Primary human trabecular meshwork (HTM) cells were seeded at 1.5 x $10^5$ cells per well in 6 well plates and transfected with synthetic miRNA (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-548c and miR-496) mimics (pre-miRs, Qiagen) at a final concentration of 80 nM using Dharmafect duo transfection reagent (Dharmacon, GE Healthcare). Total RNA and protein were collected 48 hours post transfection for analysis. Whereas miRNA inhibitors were transfected at a final concentration of 250 nM (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-548c and miR-496 (Anti-miRs, Qiagen) against each respective miRNA or a negative control inhibitor (250 nM) as above. For experiments where CYP1B1, FOXC1 and OPTN were concurrently knocked down, 100 pM of each siRNA (Qiagen) or a control siRNA (All star negative siCONTROL non-targeting siRNA No. 1, Qiagen) were used.

### 3.5 Immunoblotting.

Extracts were prepared from transfected and treated HTM cells with 1x RIPA lysis buffer (Cell Signaling Technologies) containing protease inhibitor cocktail (Pierce, Rockford, IL) and 50 µg fractionated on 10% SDS polyacrylamide gel. After transfer onto a nitrocellulose membrane, probing was carried out with anti- CYP1B1, anti–OPTN, anti–MYOC, anti-LTBP2, anti-pCofilin, anti–Cofillin, anti-Paxillin, anti- ROCK2, anti-TGF-β2 and anti-GAPDH (Santa Cruz Biotechnology) and anti-FOXC1 (Cell Signaling Technologies) antibodies. Immunocomplexes were detected using the ECL method (GE Healthcare) according to the manufacturer's instructions.

### 3.6 Confocal microscopy and staining for Paxillin and F-actin.

Primary HTM cells plated onto poly-L-lysine ( Sigma) coated coverslips were transfected with miRNA mimics and treated with recombinant human TGF-β2 (2ng/ml; Sigma) as described above, and subjected for staining after 24 hours of post transfection. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and probed with a rabbit- anti-paxillin antibody (1:500; Santa Cruz Biotechnology) followed by goat-anti-rabbit-Alexa 488 conjugated antibodies (1:500; Invitrogen). For F-actin staining, fixed and permeabilized cells were incubated with rhodamine phalloidin (Invitrogen) for 10 min followed by was and mounting along with 4´-

6-Diamidino-2-phenylindole (DAPI; Invitrogen) for visualizing nuclei.. Staining were observed and images were captured using Leica TCS SP8 confocal microscope. Images were analysed with Leica Cell software.

### 3.7 3´UTR reporter analysis.

The 3'UTRs of TGF-β2, ROCK2, CYP1B1, FOXC1 and OPTN were amplified by PCR from HEK393T or K562 genomic DNA and cloned into the pMIR-REPORT luciferase expression vector (ThermoFisher Scientific). Luciferase reporter plasmid (100ng) and pRL-TK control (200ng for normalization; Addgene) were transfected with dharmafect duo (Dharmacon, GE Healthcare) transfection reagent into HEK293T cell lines seeded in 24 well plates ($6x10^4$ cells per well). For co-transfection experiments, 5nM of synthetic miRNAs (pre-miRs, Qiagen) or 30 nM of miRNA inhibitors (Anti-miRs, Qiagen) were added to the above reactions. Cells were collected after 48h of post transfection for dual-luciferase reporter assay (Promega) as described in product protocol. All experiments were performed in triplicates for at least three independent experiments.

### 3.8 Enzyme–Linked Immunosorbent Assay (ELISA)

The amount of TGF-β2 secreted in the samples of conditioned medium of dexamethasone and miRNA transfected primary HTM samples were measured by ELISA kit (R&D systems) which had a sensitivity of 7.0 pg/ml. Briefly, 100 µl of various concentrations of TGF-β2 standards or equal volumes of conditioned medium was added to microtiter ELISA plates coated with human monoclonal antibody. After 2hr of incubation at room temperature, the wells were washed three times with 400 µl of wash buffer and coated with 200 µl of human TGF-β2 conjugate. After 2 hrs of incubation 200 µl of conjugate substrate was added and incubated for another 20 min. The absorbance at 450 nm was measured with a microplate reader (Molecular devices.) All samples of conditioned medium were assayed after acid activation, and all experiments were performed in triplicates.

### 3.9 *In-vivo* dexamethasone treatments.

C57BL/6J mice were purchased from the Liveon Biolabs Private Limited after obtaining Institutional Animal-ethical-clearance. All the procedures and protocols were followed as per the approvals. They were lodged and acclimatized in animal house at the University of Hyderabad.

0.1% dexamethasone phosphate (Sigma) was used for topical administration of mice eye. Sterile phosphate buffered saline (PBS) was used as a control eye drop. 0.1% dexamethasone phosphate were given topically to the right eye of the mice and sterile phosphate buffered saline (PBS) were given to the left as a control eye daily 3 times for up to 3 weeks. Doses were given daily between 9am and 10am; 1pm to 2pm; and 6pm and 7pm. Mice were lightly held for 30 seconds after addition of eye drops to ensure effective penetration in to the eye. At 3 weeks IOP was measured during this throughout 24hrs cycle at every 4hrs interval period. At the end of the study whole eyeball were fixed in 10% formaldehyde (Sigma) for immunohistochemistry.

**3.10 IOP measurements.**

Intraocular pressure (IOP) of each mice was measured using rebound tonometer (TONOLAB, ICARE Instruments), as per the instructions of the manufacturer. In brief, mice were adapted to the room and anesthetized to put them sleep. IOP was measured with a rebound tonometer in between 10 am and 12 pm every week for up to 3 weeks in each group of mice. As each eye responds differently to treatment, we considered each eye measurement as an independent sample. For understanding the circadian rhythm of IOP, IOP was measured in 24hrs cycle at every 4hrs intervals. (12am, 4am, 8am, 12pm, 4pm, and 8pm).

**3.11 Statistical Analysis:**

The data values were expressed as mean ±. SEM statistical analysis was performed using student T-test. The differences between the mean values were analyzed using student T-tests when P- value lesser than 0.05 was considered as statistically significant. For all cell culture studies, a minimum of three independent experiments were carried out.

**3.12 Oligonucleotide sequences.**

All real-time PCR primers of CYP1B1, FOXC1, OPTN, MYOC, LTBP2, TGF-β2, CLOCK, BMAL1, PER1, PER2, CRY1, CRY2, Rev-Erb α and 3'UTRs primers of TGF-β2, ROCK2, CYP1B1, OPTN and FOXC1 were given in the Table 1 & Table 2.

**Table 1. List of qPCR primers used in this study**

| S.No | Name of the gene | Forward primer | Reverse primer |
|---|---|---|---|
| 1 | CYP1B1 | GGATTTGGAGAACGTACCGGCC | GCCAGGACATAGGGCAGGTTG |
| 2 | FOXC1 | ACTCGGTGCGGGAGATGTT | CCTTGATGGGTTCCTTTAGC |
| 3 | OPTN | AGCGGCTCCTCAGAAGATTCC | CGAAGTAATTCTTGGCCCCATC |
| 4 | MYOC | TGCCACCAGGCTCCAGAGAAG | CGCATCCACACACCATACTTGCC |
| 5 | LTBP2 | GCCCCACTGAAGCAGTCCAC | CTGGTCTCCACGCTGTTCTCC |
| 6 | TGF-β2 | CATCTCCAACCCAGCGCTACATC | GTGCAGCAGGGACAGTGTAAGC |
| 7 | BMAL1 | GATTATGTTCTGGAGCACGACGT | GACGAGGCAGCTGAGGTTAC |
| 8 | PER1 | CAACCAGGAATACTACCAGCAGTG | GAACACGTCCCGCTTGCAAC |
| 9 | PER2 | GATGCCTTCAGCGATGCCAAG | GAAGGGGTGGTAGCGGATTTC |
| 10. | CLOCK | CAACACCAACCAAGATCCCGAC | TCTTTCAGATGTTGCATGGCTCC |
| 11 | CRY1 | CTCGTTTGGAAAGGCATTTGG | CGCCATAACAGTTGCCCAT |
| 12 | CRY2 | TGGAAGTAGTGACGGAGAATTC | AGGGCACGCCGTAGGTCT |
| 13 | Rev-Erb α | CCGTCGGAGCATCCAGCAG | GCATCCGCTGCTTCTCTCG |

**Table 2. List of primers used to clone 3'UTR in the study**

| S.No | Name of the gene | Forward primer | Reverse primer |
|---|---|---|---|
| 1 | TGF-β2 | GCAGCTAAAATTCTTGGAAAAGTG | GTTGTTGTTGTCGTTGTTCAC |
| 2 | ROCK2 | CAAAGTCTCAGCCCTGTCTG | GATCTTCGCTCACTGCAACC |
| 3 | CYP1B1 | ACGAGGTGACAAGAGTTGGG | CCTGCTTTGTGTAGTTGACTCT |
| 4 | FOXC1 | CAAATGGCCTTCCCTTCCAG | ACAGGCCACGTAGAGCAG |

**3.13 Electrophoretic mobility shift assays (EMSA):**

Confluent primary HTM cells in 100mm dish were treated and nuclear extracts were collected with nuclear extraction buffer (NEB) containing 20 mM HEPES (pH-7.4), 0.4mM NaCl, 1mM EDTA(pH-8),0.1M EGTA and protease inhibitor cocktail. The 5' end of CYP1B1 promoter sequence (-2622 ATAAAATT**TAAACAACC**AACCAG -2631) along with mutant probes was end labelled with 20 fmol of [γ32p] and unlabeled probe was removed with illustra Microspin-G-50 columns. Different concentrations of nuclear protein (1µg, 5µg, 10µg) of protein was incubated with 10pmol of probe at $37^0$C for 30 min in 1X binding buffer (200mM HEPES (pH 7.4), 40mM DTT, 4mM EDTA (pH-8.0) and 50% glycerol). The nuclear protein complexes were loaded on 6% agarose gel with 6X DNA loading dye. DNA mobility and band shifts were observed

In phosphor imager system (BioRad).

# Results

# 4. Results

## 4.1 TGF-β2 and glucocorticoids regulates the expression of known glaucoma candidate genes in primary HTM cells.

Although elevated levels of TGF-β2 and mutations in glaucoma candidate genes (CYP1B1, MYOC, FOXC1, LTBP2, OPTN etc.) have been linked to cause various forms of glaucoma, the molecular mechanisms involving the genes and TGF-β2 is not clear. Since TGF-β2 is a well-known cytokine and involved in various developmental process by modulating several signaling molecules, for the first time we have tested the relationship between TGF-β2 and glaucoma candidate genes. To investigate the effects of TGF-β2 on glaucoma candidate genes expression, HTM cells were treated with and without TGF-β2 (2ng/ml) and analyzed the expression of glaucoma candidate genes by qRT-PCR and their levels by Western blotting. TGF-β2 induced the expression of MYOC, LTBP2, FOXC1 and OPTN whereas, CYP1B1 mRNA and protein levels were significantly down regulated. These results show that glaucoma candidate genes were altered upon TGF-β2 treatment in HTM cells as shown in figure 9.

a.

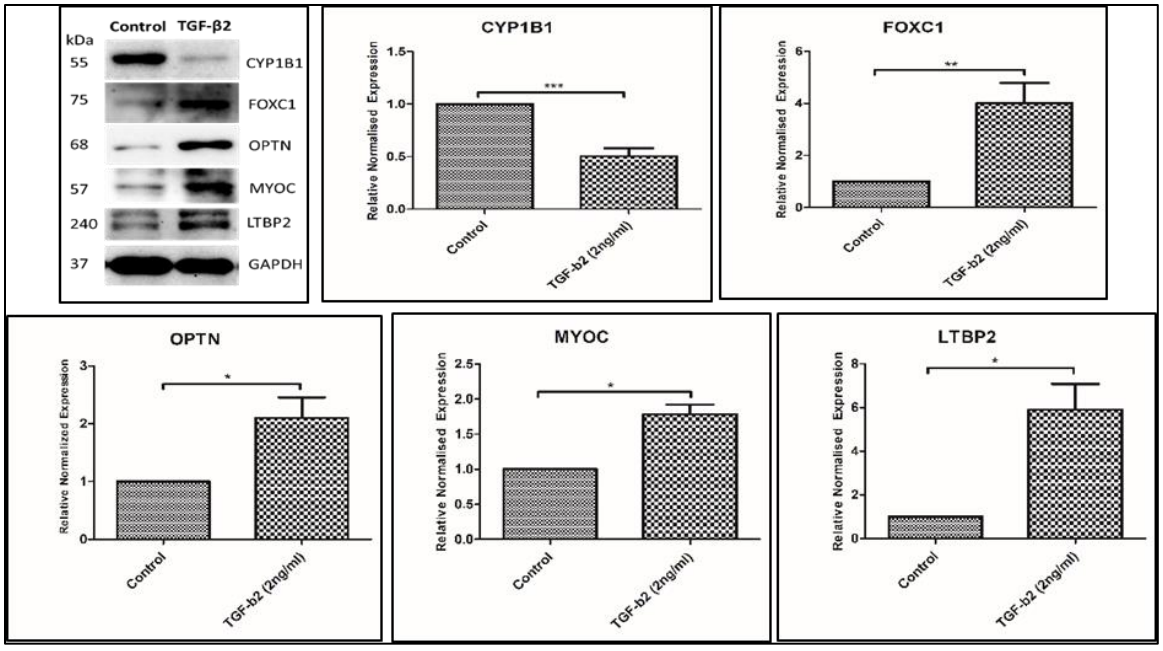



Figure 9. Effect of TGF-β2 treatment on glaucoma candidate genes in primary trabecular meshwork cells. HTM cells were treated with TGF-β2 (2ng/ml) for 48 hrs **a)** Western blot and real time PCR analysis shows that TGF-β2 down-regulated CYP1B1 expression whereas FOXC1, MYOC OPTN, and LTBP2, were up-regulated in primary trabecular meshwork cells. Bars represent standard deviation in three different experiments. Asterisk (*) (**) (***) represents significance at $p<0.5$ $p<0.05$ $p<0.005$ respectively between miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) and control. Standard deviation and significance were calculated by using GraphPad Prism5.

Since prolonged use of glucocorticoids (dexamethasone, prednisolone etc.) causes secondary glaucoma in humans and similar effects were observed in HTM cells treated with glucocorticoids for 5 or more days, thus we have analyzed candidate gene expression upon treatment with dexamethasone (300nM/ml) and prednisolone (300nM/ml). Dexamethasone and prednisolone treatment induced the expression of MYOC, LTBP2, FOXC1 and OPTN whereas, down-regulated CYP1B1 mRNA and protein levels as seen with TGF-β2 treatments as shown in figure 10a.

a.

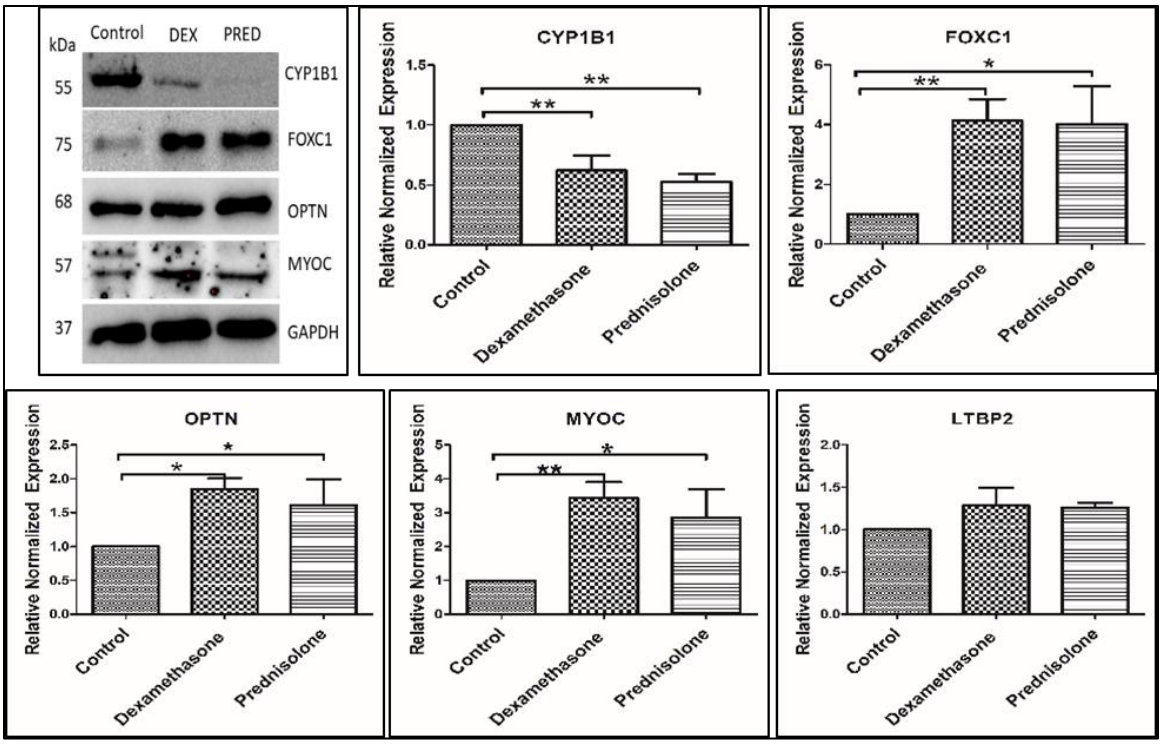



Figure 10. Effect of glucocorticoid treatment on glaucoma candidate genes in primary trabecular meshwork cells. HTM cells were treated with glucocorticoids (Dex 300nm/ml and Pred 300nm/ml) for 7 days. **a)** Western blot and real time PCR analysis shows that glucocorticoids down-regulated CYP1B1 expression whereas FOXC1, MYOC OPTN, and LTBP2, were up-regulated in primary trabecular meshwork cells. Bars represent standard deviation in three different experiments. Asterisk (*) (**) (***) represents significance at $p<0.5$ $p<0.05$ $p<0.005$ respectively between miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) and control. Standard deviation and significance were calculated by using GraphPad Prism5.

Taken together our results for the first time shows that TGF-β2 and glucocorticoids influences the expression of glaucoma candidate genes in trabecular meshwork cells.

**4.2 CYP1B1, FOXC1, OPTN inter regulate each other in primary HTM cells.**

Since our earlier experiments demonstrated TGF-β2 modulates the expression of glaucoma candidate genes and candidate genes belongs to several classes of proteins such as transcription factors (FOXC1, PITX2, PAX6), drug metabolizing enzymes (CYP1B1), stress responsive genes (MYOC), and few others, thus it was curious to see the relationship among the glaucoma candidate genes. To investigate in that line, CYP1B1, FOXC1 and OPTN genes were knocked down individually with respective siRNA's and their levels were analyzed by Western blot and their expression by qRT-PCR. To our surprise, CYP1B1 expression was increased upon knockdown of FOXC1, but no change in FOXC1 protein levels were seen in CYP1B1 knock down cells as shown in figure 11a.

a.

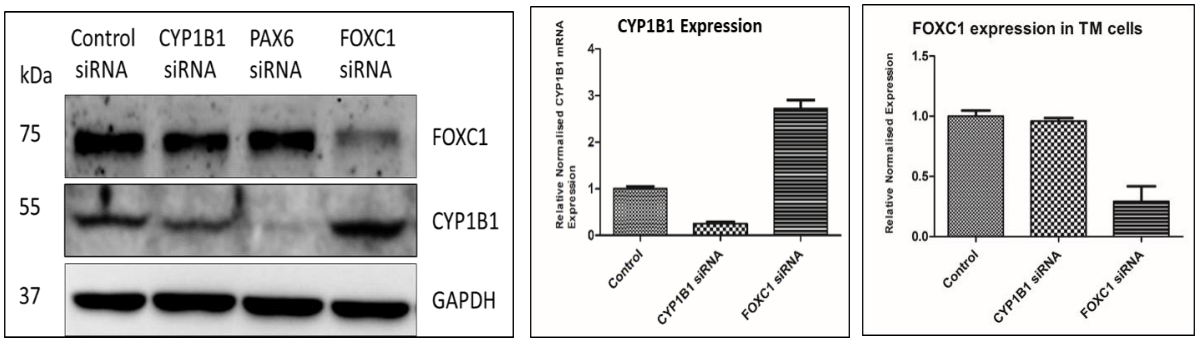



Figure 11a. Relationship among the glaucoma candidate genes. a) Knockdown of FOXC1 and CYP1B1 by respective siRNA and confirmed by Western blot and real time PCR. Up-regulation of CYP1B1 was observed upon knocking down of FOXC1 and no change in expression of FOXC1, upon knockdown of CYP1B1 by siRNA.

These results show that FOXC1 might be regulating the expression of CYP1B1 in trabecular meshwork cells. To further investigate the relationship between CYP1B1and FOXC1, first we analyzed the CYP1B1 promoter for transcription factor binding consensus sequence for FOXC1. Bioinformatics tools couldn't identify one, but using manual analysis for known consensus sequence, we could identify the consensus sequence for FOXC1 (TAAACAACC at position –2622 to –2631) DNA binding site on CYP1B1 promoter (Genbank- U56438.1) as shown in figure 10b. To characterize and confirm the transcriptional binding efficiency, we performed electrophoretic mobility shift assay (EMSA) with synthesized 21-bp oligos of the region containing consensus sequence from CYP1B1 with and without sequence variations as described in the methods. Nuclear fractions extracted from HTM cells were subjected for EMSA. Binding of protein to the probes was observed by corresponding band shifts and super shifts upon subjecting

to the anti-FOXC1 antibody. There was no mobility shift and super shift with controls and mutant probes respectively as shown in figure 12 a, b. Our data shows that TGF-β2 down-regulates the expression of CYP1B1 by modulating the expression of FOXC1, which seems to act as a repressor in HTM cells.

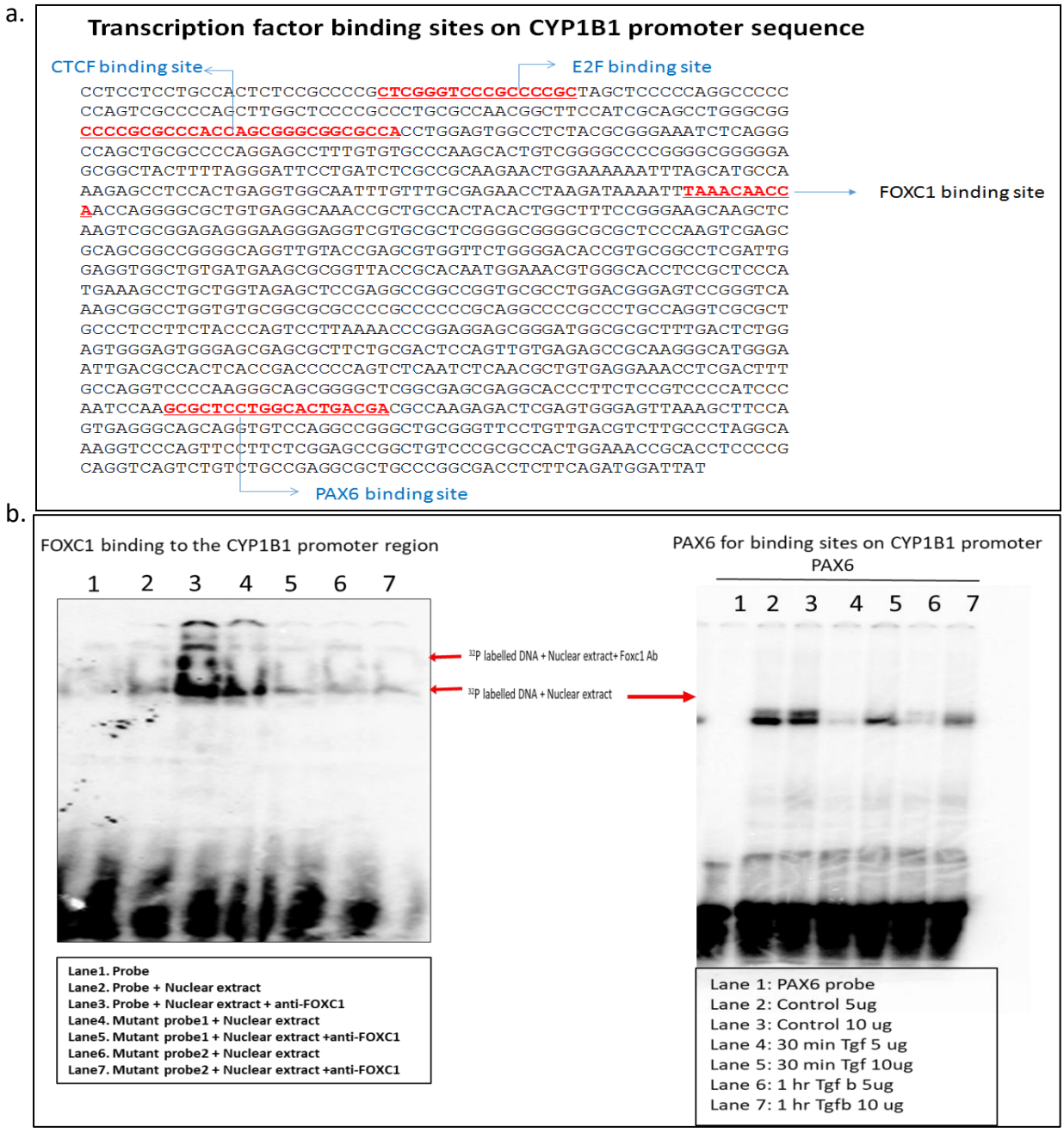



Figure 12. Relationship among the glaucoma candidate genes. a) CYP1B1 promoter analysis for FOXC1 and PAX6 consensus binding sites. b) EMSA experiment showing that FOXC1 and PAX6 binding on to the CYP1B1 promoter sequence respectively. Gel super shift was seen when incubated with FOXC1 specific antibody, confirming its binding on the CYP1B1 promoter sequence.

To investigate the relation between OPTN and CYP1B1 in trabecular meshwork cells, we knocked down OPTN by using siRNA and analyzed CYP1B1 protein levels by western blot and its expression by qRT-PCR. The data shows the reduced levels of CYP1B1 upon knockdown of OPTN, similarly overexpression of OPTN resulted in increased CYP1B1 protein levels, in contrast knockdown of CYP1B1 had no effect on OPTN expression, but overexpression of CYP1B1 had shown increased protein levels of OPTN as shown in figure 13a.

a.

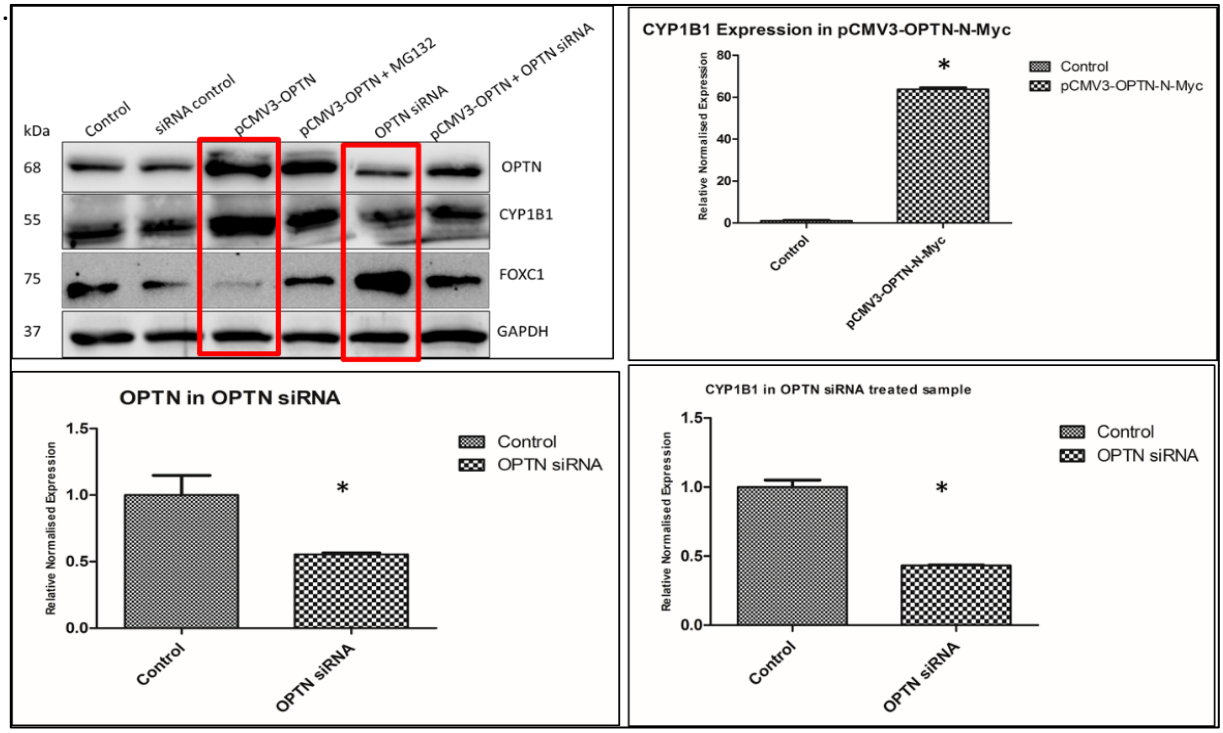
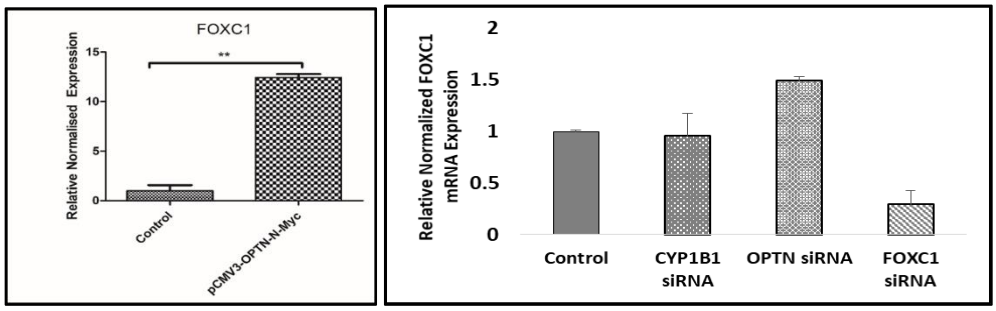



Figure 13. Relationship among the glaucoma candidate genes. a) Western blot and real time PCR results shows that knockdown of OPTN by siRNA lead to the downregulation of CYP1B1 and upon overexpression of wild type OPTN cloned in pCMV3-OPTN-Myc lead to the up-regulation of CYP1B1, shows that CYP1B1 might also be regulated by the OPTN.

We next investigated the relation between OPTN and FOXC1 in trabecular meshwork cells by knock down of OPTN with siRNA and confirmed expression of FOXC1 by Western blot and qRT-PCR analysis. The result shows that OPTN knock down resulted in up-regulation of FOXC1 mRNA and protein levels in HTM cells. Whereas overexpression of OPTN in HTM cells showed significant increase in mRNA levels of FOXC1, but the protein levels were decreased. Thus this data made us to speculate that there could be a possibility of existing regulation at post-translational level. Our further analysis with MG132 (proteasome degradation inhibitor) in

overexpressed OPTN HTM cells resulted in recovery of FOXC1 protein levels compared with and without MG132, confirming the possibility of OPTN induced FOXC1 ubiquitination as shown in figure 14a.

a.




b.

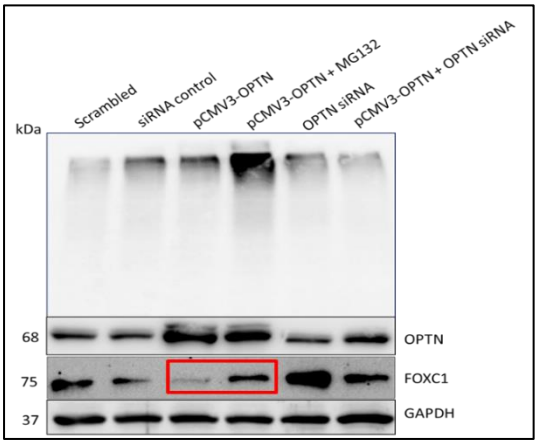



Figure 14. Relationship among the glaucoma candidate genes. a) Shows that increased mRNA levels of FOXC1 in pCMV3-OPTN expression and knock down of OPTN. b) Western blot result shows that knock down of OPTN by respective siRNA lead to the upregulation of FOXC1 and overexpression of wild type pCMV3-OPTN lead to the downregulation of FOXC1 protein levels. Further treatment with MG132 lead to the recovery of FOXC1 protein levels confirming that FOXC1 is regulated by OPTN by ubiquitination process.

Collectively this data shows that, knockdown of CYP1B1/FOXC1 has no effect on OPTN expression, but OPTN regulates expression of CYP1B1 and FOXC1 in HTM cells. However the detailed mechanism needs to be elucidated.

### 4.3 Loss of OPTN induces TGF-β2 and activated Rho/ROCK signaling pathway in primary HTM cells.

The levels of TGFB2 are high in glaucomatous eyes, on the other hand the TGFB2 induced OPTN was seen to regulate the glaucoma candidate gene expression. But their cross talk in the pathophysiology of Glaucoma is yet to be deciphered. It is known that OPTN and TGF-β2 have

unknown functions in pathophysiology of glaucoma. Our earlier experiments suggest that OPTN regulates CYP1B1 and FOXC1, whereas TGF-β2 levels are significant in glaucomatous eyes, and also induces the expression of OPTN. Thus here we thought to investigate the relation between OPTN and TGF-β2 in primary HTM cells. Upon OPTN knock down in HTM cells, up-regulation of TGF-β2 mRNA and protein levels were observed, whereas overexpression of OPTN by pCMV3-OPTN resulted in decreased levels of TGF-β2. The same results were confirmed by ELISA by measuring active form of TGF-β2 in primary trabecular meshwork cells as shown in figure 15 a-d.

Since loss of OPTN induces TGF-β2 expression in primary trabecular meshwork cells, we investigated Rho/ROCK signaling pathway which is known to be a TGF-β2 downstream effectors and have significant role in ECM remodeling in TM tissue and out flow pathway. Interestingly, OPTN knock down up-regulated both ROCK1 and ROCK2 thus confirming the speculation that OPTN knockdown induces TGF-β2, thus activating Rho/ROCK signaling pathway.

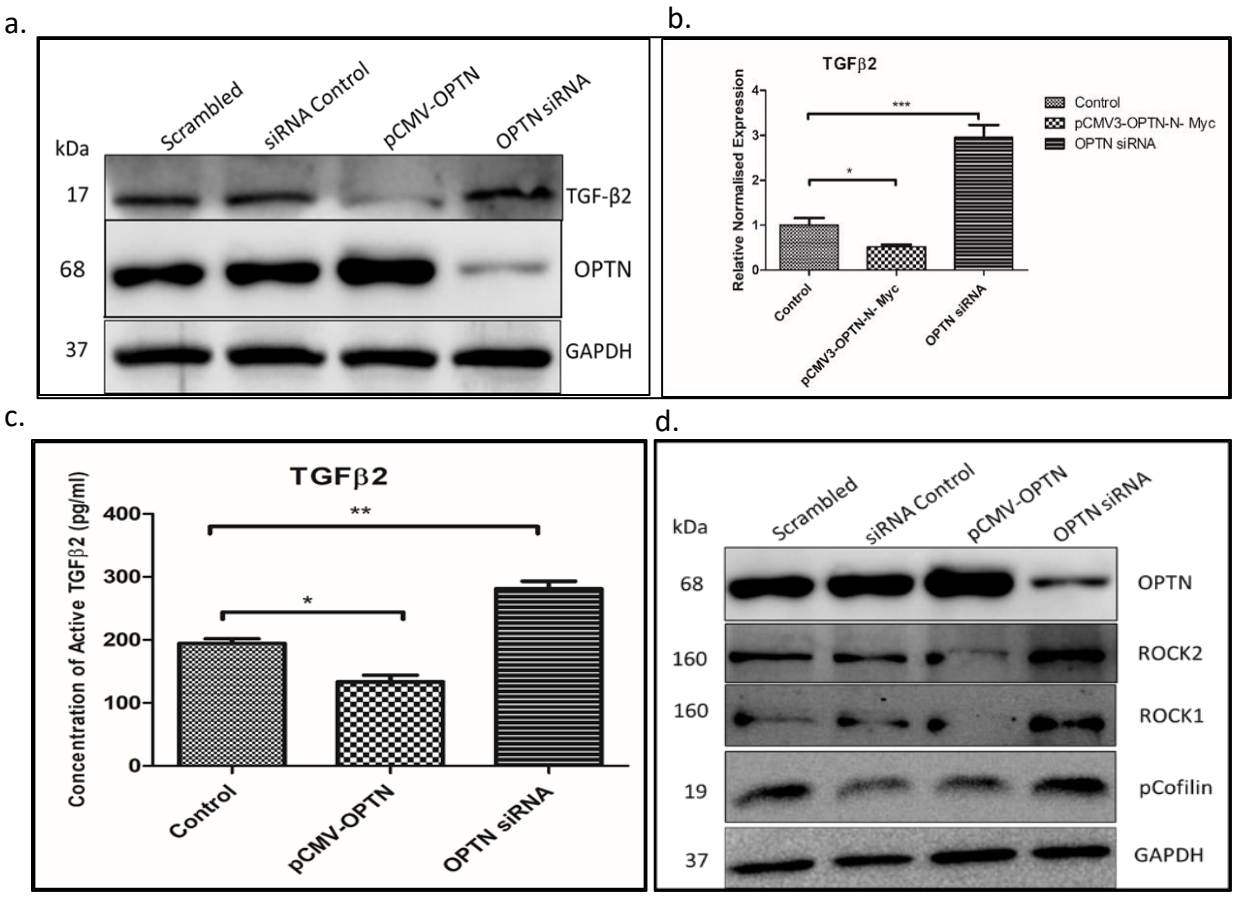


**Figure 15.** Knockdown of OPTN induces TGF-β2 expression in primary HTM cells. a) Knock down of OPTN by siRNA induced of TGF-β2 protein expression and ectopic expression of wild type OPTN cloned in pCMV3-OPTN lead to the downregulation of TGF-β2 protein levels in primary HTM cells. b) Real-time PCR analysis also shown increased mRNA levels of TGF-β2 and decreased mRNA expression in ectopic expression of wild type pCMV3-OPTN in primary HTM cells. c) Quantified active form of the cytokine TGF-β2 levels by ELISA assay in the conditioned medium of the OPTN knockdowned and found increased levels TGF-β2 and decreased in the ectopically expressed wild type OPTN primary HTM cells. d) Western blot analysis showed, activated Rho/ROCK signaling pathway upon knockdown of OPTN was observed in the primary HTM cells. And the opposite effects were observed in ectopically expressed wild type OPTN in primary HTM cells. These results shows that loss of OPTN induces TGF-β2 induced activation of Rho/ROCK signaling pathway. Bars represent standard deviation in three different experiments. Asterisk (*) (**) (***) represents significance at $p<0.5$ $p<0.05$ $p<0.005$ respectively between miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) and control. Standard deviation and significance were calculated by using GraphPad Prism5.

### 4.4 Knockdown of glaucoma candidate genes causes the formation of actin stress fibers and focal adhesions in the primary trabecular mesh work cells.

Harboring functional mutations in CYP1B1, FOXC1, OPTN, LTBP2, MYOC, WDR36 are said to be responsible for causing glaucoma, but their molecular mechanisms in obstructing the outflow pathway in the anterior chamber is not known. From the above experiments we found that CYP1B1, FOXC1 and OPTN are inter-regulating each other and loss of OPTN demonstrated to influence CYP1B1, FOXC1, TGF-β2 and thus Rho/ROCK pathway. Regulation of Rho/ROCK is critical in actin stress fiber formation thus said to increase resistance to the outflow. Here we analyzed the stress fiber formation upon knock down of CYP1B1, FOXC1 and OPTN expression in primary HTM cells, by siRNA based knock down of respective genes and F-actin staining with rhodamine phalloidin and paxillin with anti-paxillin confirms increased actin stress fibers and focal adhesions upon knockdown of CYP1B1, FOXC1 and OPTN gene expression in HTM cells compared to control cells (figure). Similar results were correlated with TMS (Tetramethylsilane), a known chemical inhibitor of CYP1B1, conversely treatment with TCDD (2,3,7,8 Tetrachlorodibenzo-p-dioxin) a known chemical inducer of CYP1B1 resulted in loss of stress fiber as shown in figure 16.

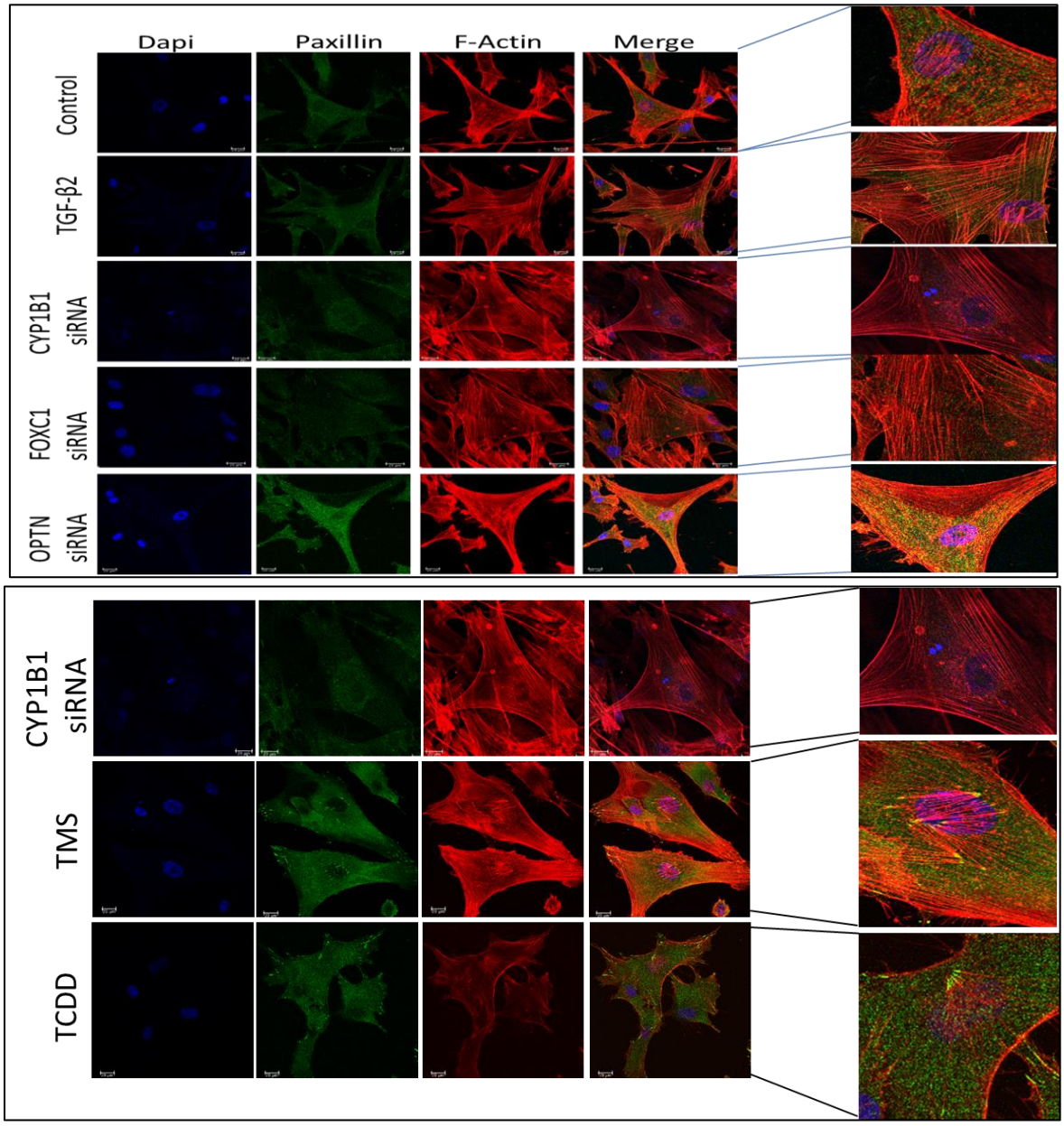



**Figure 16.** Knockdown of glaucoma candidate genes lead to the formation of actin stress fibers and focal adhesions in primary HTM cells. F-Actin was stained with rhodamine phalloidin (Red) and focal adhesion protein paxillin was stained with Anti-Paxillin antibody (Green).

**4.5 miR-200 family, miR-590 and miR-548c down-regulates TGF-β2, ROCK2 and paxillin expression in primary HTM cells.**

Organ-cultured anterior segments from porcine eyes expressing RhoAV14 exhibited significant reduction of aqueous humor outflow [215]. Inhibition of RhoA expression in TM cells with siRNA effectively decreased elevated IOP in mice [235]. Several other studies have shown that, ROCK inhibitors effectively increased outflow facility and/or decreased IOP in living rabbits, mouse, rat, and monkeys, human and enucleated porcine eyes model. From our earlier experiments we found that loss of OPTN induces TGF-β2 which in turn activates Rho/ROCK signaling pathway in primary HTM cells. OPTN also modulates CYP1B1 and FOXC1 expression and finally they all converge to facilitate outflow pathway. Since all have common target on regulating out flow of aqueous humor, we then explored to identify any converging factor for candidate genes/signaling molecules which involved in regulation of out flow.

Interestingly study by Jayaram et.al have shown that normal HTM cells express miR-200a, miR-200b, miR-200c, miR-429, miR-141 but not in ciliary body. Since HTM cells are mainly responsible for the egression of aqueous humor from anterior chamber but not by ciliary body, thus we speculated that these miRNAs may have significant function in outflow tissues. To explore the significance of these miRNAs in HTM and out flow pathway, as an initial experiment, we screened for target genes to miR-200a, miR-200b, miR-200c, miR-429, miR-141 miRNAs using bioinformatics based miRNA target gene prediction tools such as TargetScan (version 7.2, http://www.targetscan.org/)[232], MiRanda (August 2010 Release, http://www.microrna.org/)[236], and miRBase (http://www.mirbase.org/ )[234]. These tools predicted TGF-β2, ROCK2 and glaucoma candidate genes (CYP1B1, FOXC1, and OPTN) are the targets for these miRNAs. Using same tools we then screened for more such miRNAs for list of all genes which predicted another two miRNAs mir-590 and miR-548c shown in the table 3. Further to validate the role of all these predicted miRNAs, the synthetic miRNAs of miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c were ectopically expressed/transfected in primary HTM cells and subjected for analysis by Western blot and qRT-PCR. Expression of TGF-β2, ROCK2 protein levels were down-regulated by miRNAs as shown in (Fig.) whereas expression of anti-miRs of the same miRNAs were abrogated the respective miR's induced down-regulation of TGF-β2, ROCK2. Similar results were observed by ELISA on conditioned medium for active TGF-β2 as shown in figure 17 a-d.

Table 3: Showing list of miRNAs predicted to target glaucoma candidate genes.

| Known Glaucoma Candidate Genes | miRNAs Predicted to Target these genes |
|---|---|
| CYP1B1 | miR-182-5p, miR-200bc-3p/429, miR-27-3p |
| FOXC1 | miR-138-3p, miR-200a/141-3p |
| OPTN | miR-548c-3p, miR-590-3p |
| MYOC | miR-590-3p,miR-486-5p |
| LTBP2 | miR-590-3p, miR-548c-3p |
| WDR36 | miR-590-3p, miR-548c-3p |
| PAX6 | miR-200bc-3p/429, miR-590-3p, miR-548c-3p |
| PITX2 | miR-200a/141-3p, miR-590-5p, miR-548c-3p |
| TGF- β2 | miR-200a/141-3p, miR-590-3p, miR-548c-3p |
| ROCK2 | miR-200bc-3p/429, miR-590-3p, miR-548c-3p, miR-496.1 |

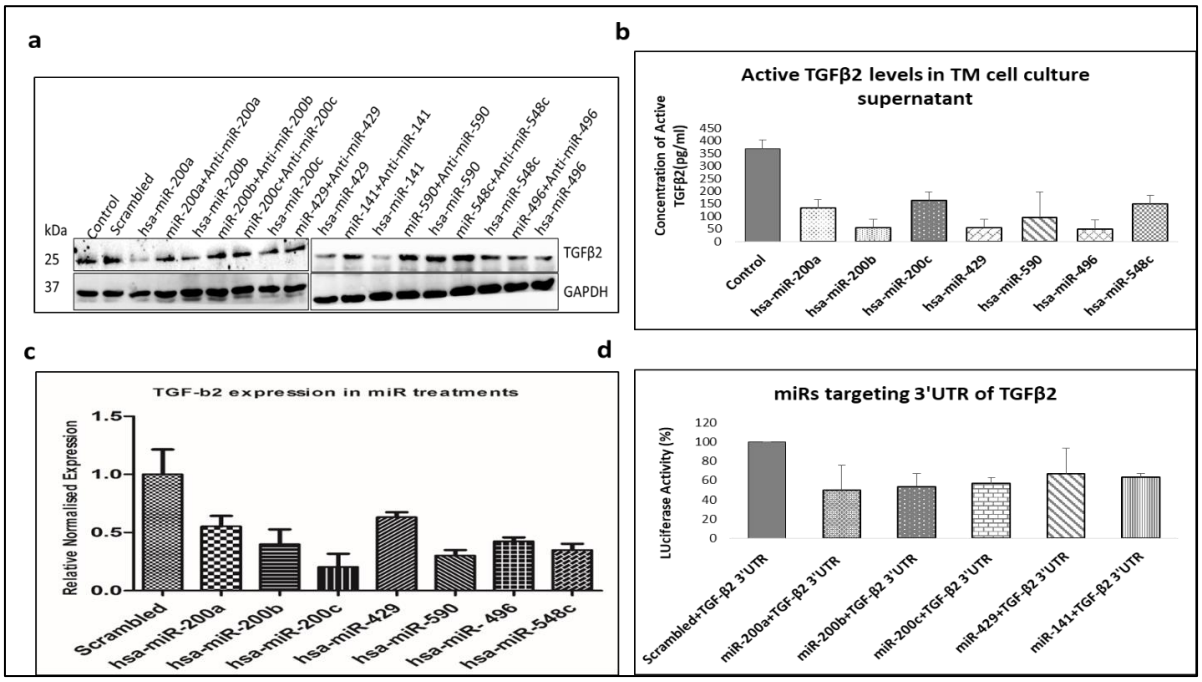


**Figure 17.** Ectopic expression of miRNA-200 family, miR-590, miR-496 and miR-548c down-regulated the expression of TGF-β2 levels in the primary HTM cells. a) Western blot analysis shows that ectopic expression of miRNA-200 family, miR-590, miR-496 and miR-548c mimics inhibited the expression of TGF-β2 and using anti-miRs of respective miRNAs nullified miRNA effects in primary HTM cells. b) By ELISA experiment quantified the active form of TGF-β2 in the condition medium of primary HTM cells by ectopically expressing the miRNA for 48hrs and observed decreased levels compared to control. c) Quantified mRNA levels by using real-time PCR in HTM cell after 48hrs of ectopic expression of miRNAs. d) Luciferase assay was performed to show that TGF-β2 is a direct of miRNA-200 family, miR-590, and miR-496 and miR-548 c. miRNAs were transfected along with TGF-β2 3' UTR cloned in the pMIR-REPORT- luciferase vector and quantified luminescence and observed decreased luminescence in miRNA transfected cells compared to control.

TGF-β2 and ROCK signaling is involved in extracellular matrix remodeling by altering proteins such as focal adhesion molecules such as paxillin. The Western blot analysis of HTM cells treated with miRNA shows that down-regulation of paxillin expression whereas treatment with anti-miR's of respective miRNAs abrogates miRNA's effects thus highlights the involvement of these miRNAs in focal adhesion in HTMs and could be similar mechanism may exists in in vivo.

a.

b.

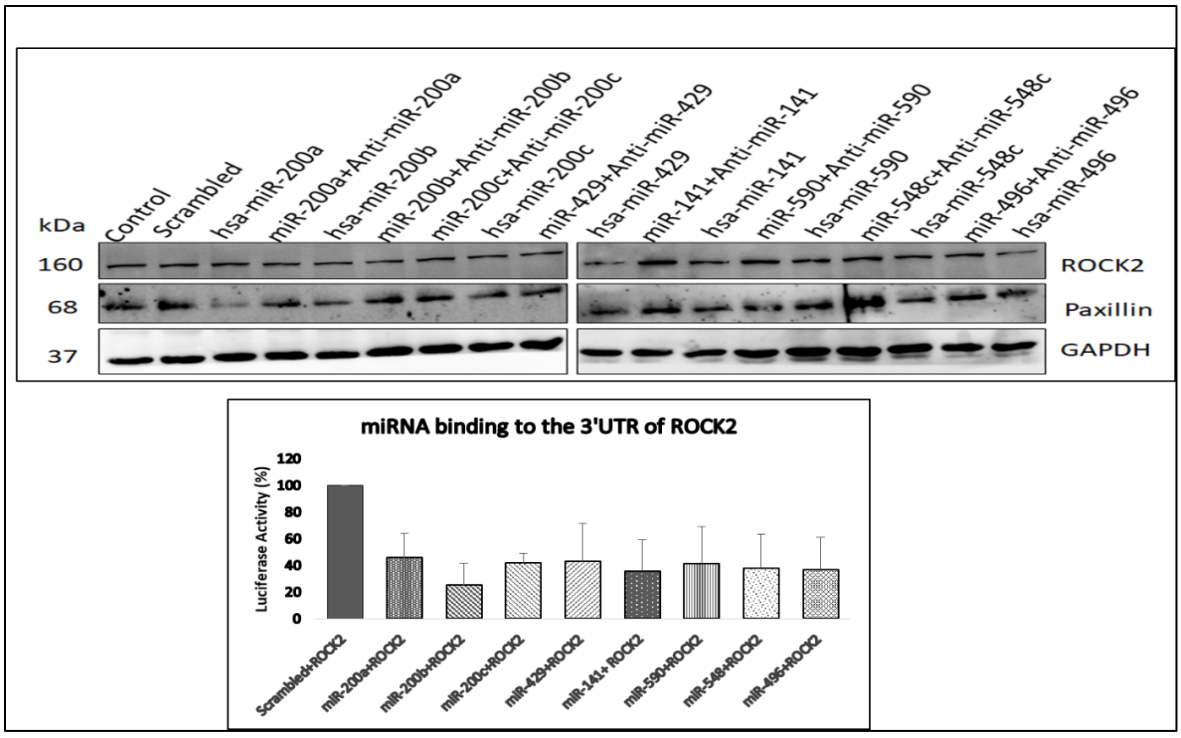



**Figure 18.** Ectopic expression of miRNA-200 family, miR-590, miR-496 and miR-548c down-regulated the expression of TGF-β2 levels in the primary HTM cells. a) Western blot analysis of ROCK2 and paxillin protein expression in primary HTM cells transfected with miRNA-200 family, miR-590, miR-496 and miR-548c mimics and their respective anti-miRs shows that decreased levels of ROCK2 and paxillin protein expression and recovered ROCK2 and paxillin protein levels in primary HTM cells transfected with anti-miRs of respective miRNAs compared with control. b) Luciferase assay was performed to show that ROCK2 is a direct of miRNA-200 family, miR-590, and miR-496 and miR-548 c. miRNAs were transfected along with ROCK2 3' UTR cloned in the pMIR-REPORT- luciferase vector and quantified luminescence after 48hrs and observed decreased luminescence in miRNA transfected cells compared to control. Bars represent standard deviation in three different experiments.

The bioinformatics tools were predicted that TGF-β2 and ROCK2 are the direct targets for these miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c), we then cloned and co-expressed the 3'UTRs of TGF-β2 and ROCK2 along with and without miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) and analyzed for luciferase expression in figure 18b . The results show that miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c directly target TGF-β2 and ROCK2 gene 3'UTRs.

**4.6 miR-200 family, miR-590 and miR-548c inhibited TGF-β2 induced formation of actin stress fibers and focal adhesions by inhibiting Rho/ROCK signaling pathway in primary HTM cells.**

In glaucoma disease models, TGF-β2 is shown to be inducing formation of actin stress fibers and focal adhesions through activation of Rho/ROCK signaling pathway. Formation of actin stress fibers is directly proportional to the increased outflow resistance in glaucoma. Since the above experiments suggests that predicted miRNAs target and down-regulate TGF-β2 and Rho/ROCK pathway, we have tested the expression of ROCK2 and its downstream signaling molecule cofilin

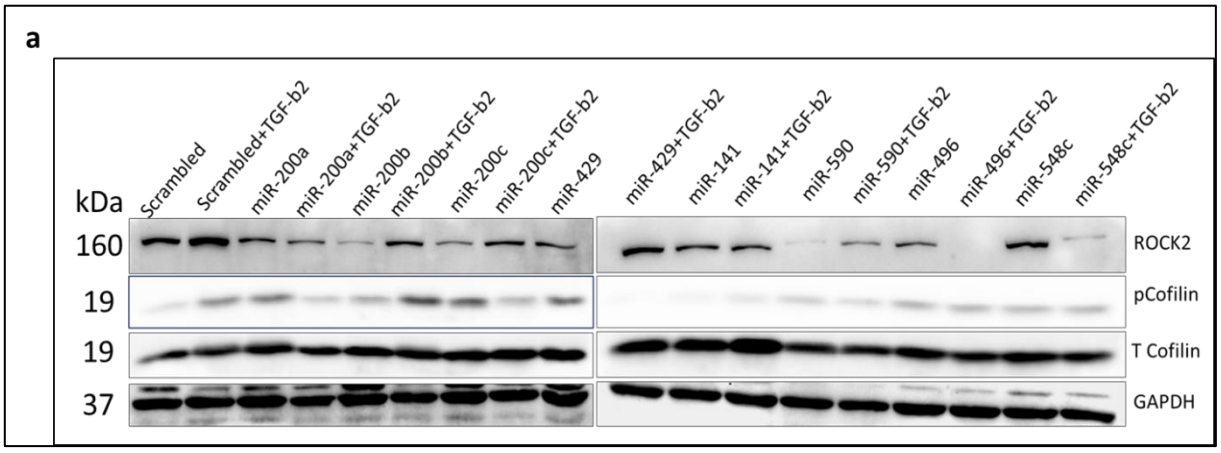



Figure 19. Ectopic expression of miRNA-200 family, miR-590, miR-496 and miR-548c mimics in primary HTM cells nullified TGF-β2 induced downstream signaling effects. A) Western blot analysis showed that downregulation of TGF-β2 induced ROCK2 expression. HTM cells were transfected with miRNA-200 family, miR-590, miR-496 and miR-548c mimics with and without TGF-β2 (2ng/ml) treatment and analysed the expression of ROCK2 and observed downregulation of ROCK2 expression in miRNA-200 family, miR-590, miR-496 and miR-548c mimics transfected cells with and without TGF-β2 (2ng/ml). And also observed decreased cofilin phosphorylation (responsible for F-actin stabilization) in the presence of and it showed that miRNA-200 family, miR-590, miR-496 and miR-548c mimics with and without TGF-β2 (2ng/ml) treatment.

in primary HTM cells transfected with miRNAs and treatment with and without TGF-β2 for 48hrs as shown in figure 19.

Further, we have tested the role of these miRNAs in TGF-β2 induced activation of stress fiber formation and adhesion molecules. Rhodamine phalloidin and paxillin staining on HTM cells treated with and without TGF-β2 and transfected miRNAs revealed loss of TGF-β2 induced disassembled actin stress fibers and paxillin expression by predicted miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c) compared to respective controls and treatments as shown in figure 21. These observations were correlated with effects of Y-27632, a known ROCK inhibitor suggests the mechanism of loss of stress fiber formation by miRNAs could be through modulation of Rho/ROCK pathway as shown in figure 20.

a.

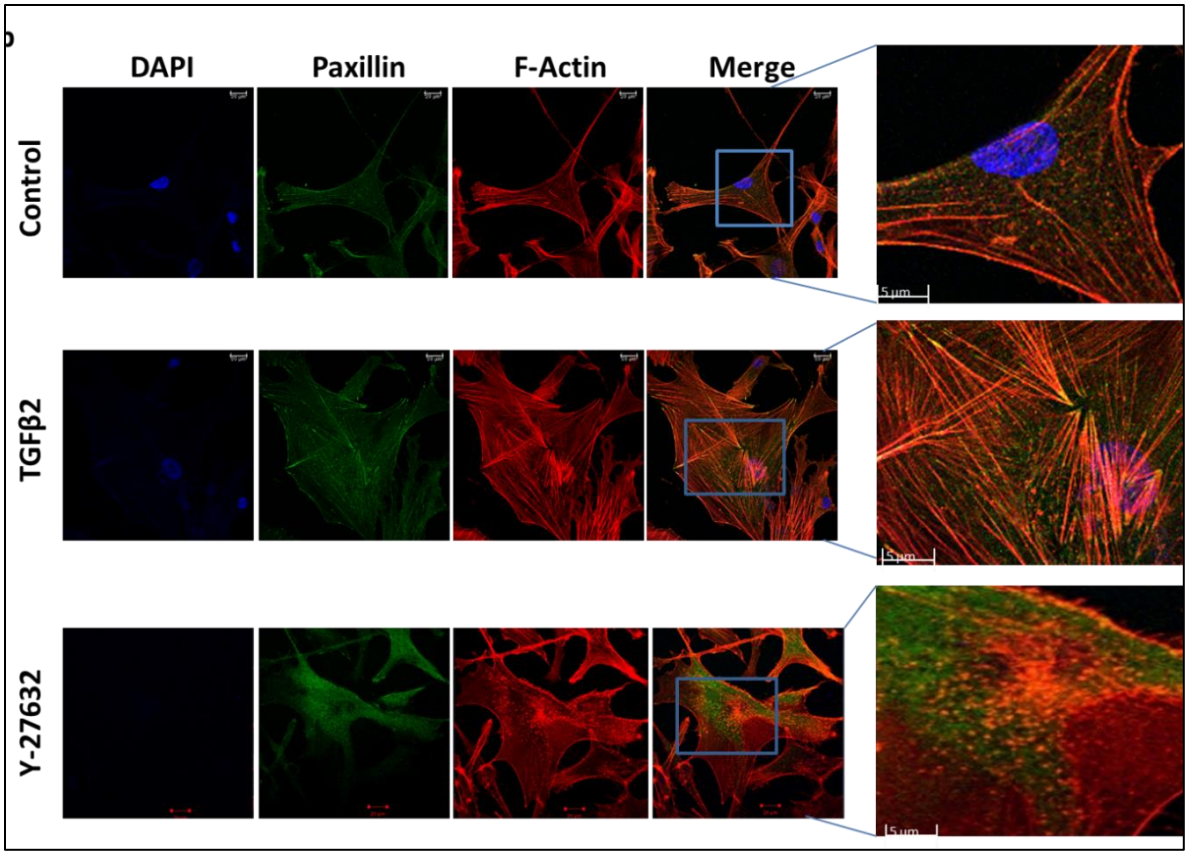
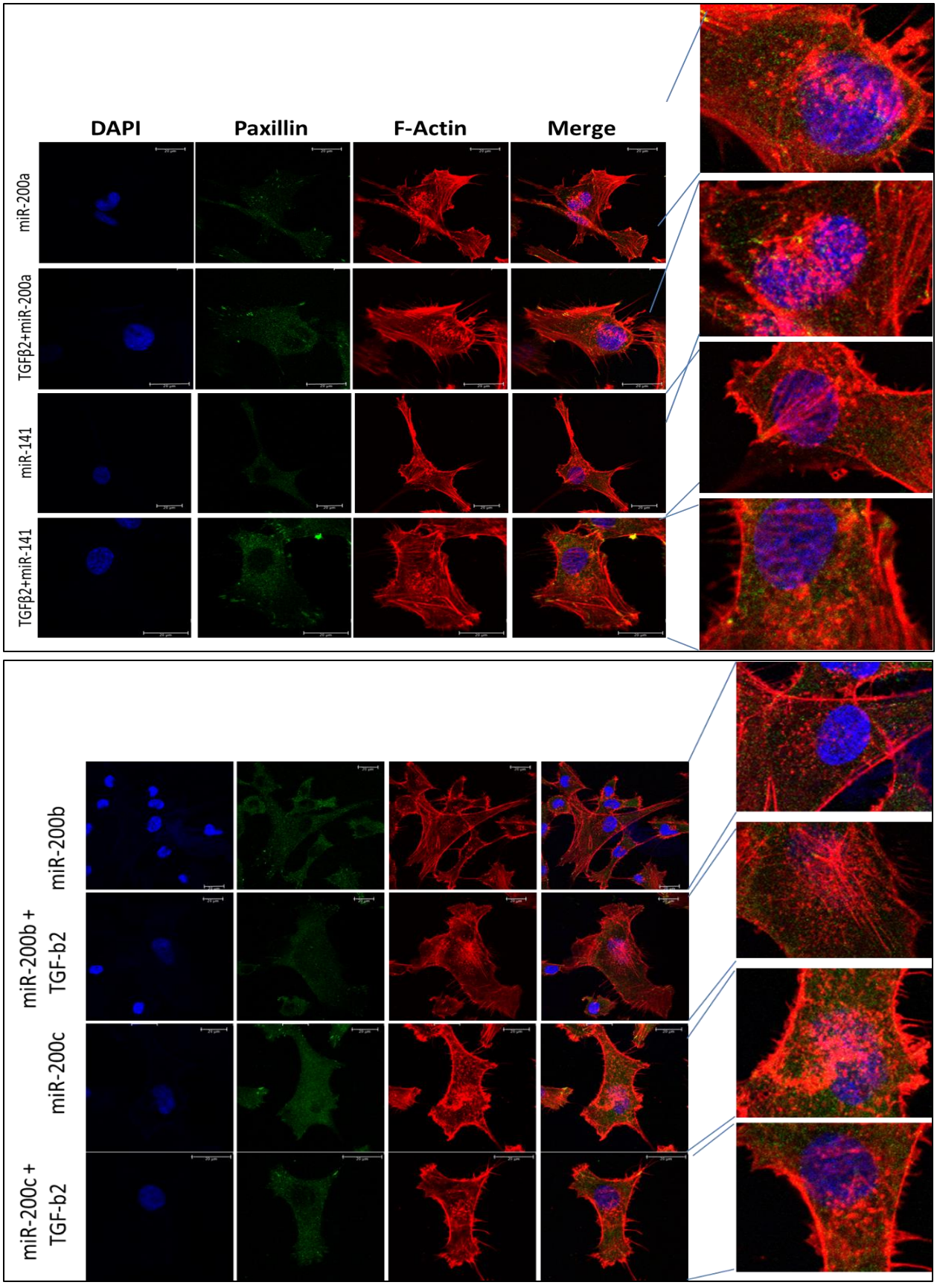



Figure 20. Formation of actin stress fibers in the primary trabecular meshwork cells. a) Confocal microscopy images showed formation of actin stress fiber and increased focal adhesions in the presence of TGF-β2 (2ng/ml) in HTM cells compared to control. And decreased formation of actin stress fiber and focal adhesions in Y-27632 (10μM) treated HTM cells compared to TGF-β2 (2ng/ml) treated primary HTM cells. F-Actin was stained with rhodamine phalloidin (Red) and focal adhesion protein paxillin was stained with Anti-Paxillin antibody (Green).

a.


DAPI
Paxillin
F-Actin
Merge
miR-200a
TGFβ2+miR-200a
miR-141
TGFβ2+miR-141
miR-200b
miR-200b + TGF-b2
miR-200c
miR-200c + TGF-b2

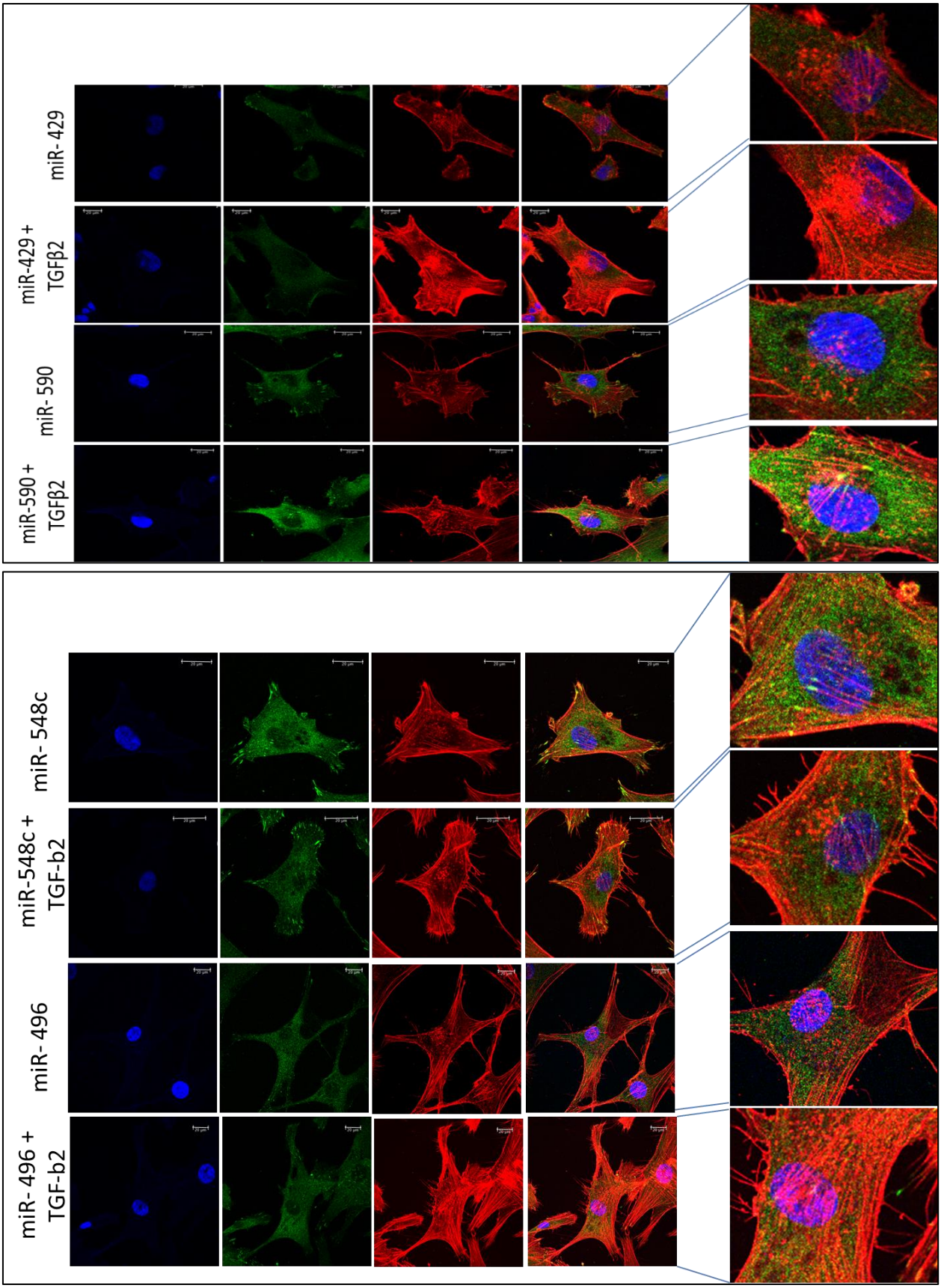

miR- 429
miR-429 + TGFβ2
miR- 590
miR-590 + TGFβ2
miR- 548c
miR-548c + TGF-b2
miR- 496
miR- 496 + TGF-b2

**Figure 21**. Formation of actin stress fibers in the primary trabecular meshwork cells. Further analyzed for formation of actin stress fibers and focal adhesions in HTM cells by transfecting miRNA mimics (miRNA-200 family, miR-590, miR-496 and miR-548c mimics) with and without TGF-β2 (2ng/ml). MiRNAs mimics (miRNA-200 family, miR-590, miR-496 and miR-548c mimics) inhibited the formation of actin stress fibers and focal adhesions even in the presence of TGF-β2 (2ng/ml), showing miRNAs nullified the TGF-β2 induced effects in primary HTM cells.

HTM cells are known to behave like smooth muscle cells in terms of contraction and relaxation. It is clear from earlier anterior chamber perfusion studies that HTM cells treated with ROCK inhibitors known to increase out flow of aqueous humor by relaxing TM cells. Thus the contraction/relaxation of TM cells plays a major role in egression of aqueous humor from anterior chamber. To evaluate the role of these predicted miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c) in contraction and relaxation of HTM cells, Primary HTM cells were transfected with and without miRNAs (scrambled, miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c) for 48hrs and then the cells were embedded in collagen gels for maximum of 24hours. Then cells were detached from the walls and then treated with and without TGF-β2 (2ng/ml) and observed cell contraction for 24hrs and images were taken by using camera after 24hrs. TGF-β2 is known to induce contraction of TM cells and the same was observed whereas miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c treatments abrogated the TGF-β2 induced contraction significantly. These results

a.

b.

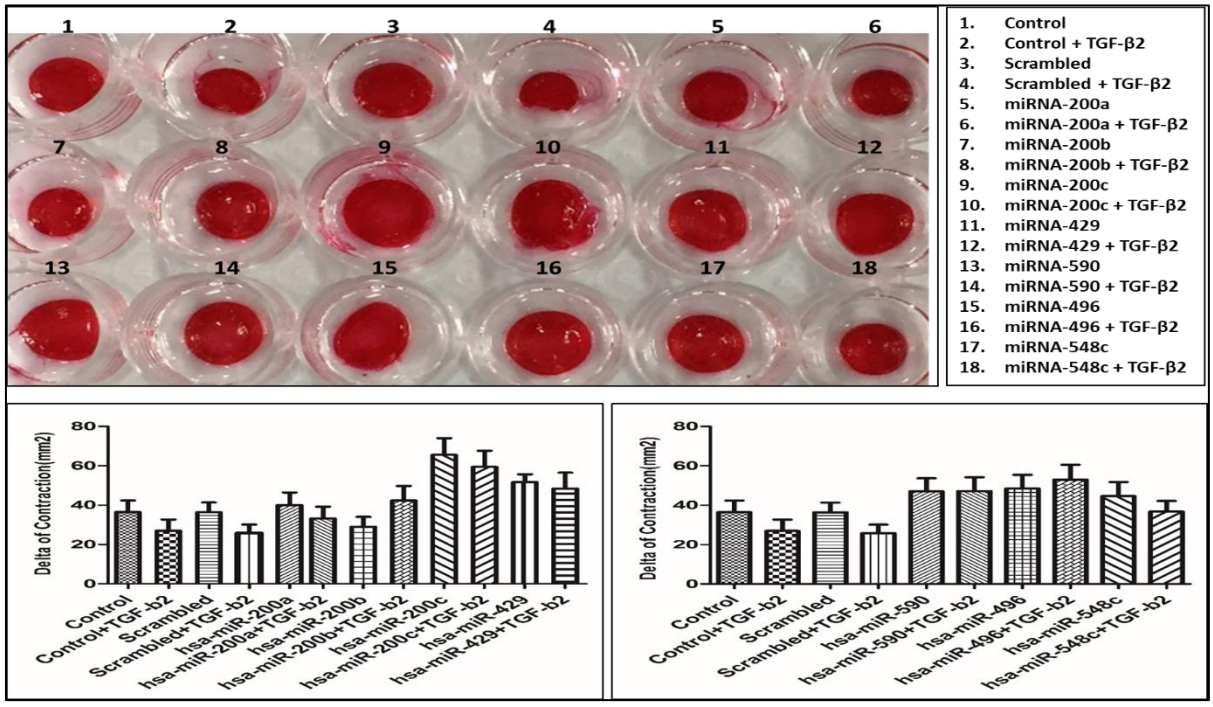


Figure 22. Formation of actin stress fibers in the primary trabecular meshwork cells. c) Representative gel image of HTM cells transfected with miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c mimics) or control and in complete media. Basal level contraction of control was calculated as the difference in area between cells transfected with miRNA control and miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c mimics). miRNAs were ectopically expressed in the HTM cells and after 48hrs, the miRNA transfected HTM cells were embedded in the collagen and then treated with and without TGF-β2 (2ng/ml) and photographed the gel area after for 24 hrs. b) Area of collagen gel size was calculated by using image J software and gel size was represented in the graph. Bars represent standard deviation in three different experiments.

demonstrated further that miRNAs abrogates TGF-β2 induced contraction by modulating ROCK2 pathway as shown in figure 22 a, b.

### 4.7 Glucocorticoids induces TGF-β2 expression in primary HTM cells

Prolonged use of glucocorticoids like dexamethasone and prednisolone etc., are said to be inducing glaucoma phenotype in humans, similarly exposing them to HTM cells for 5-7days resulted in altered expression of ECM proteins that mimics in vivo glaucoma phenotype. Our earlier experiments suggest that TGF-β2 induced molecular effects are similar to glucocorticoids effects in HTMs. We then measured the expression TGF-β2 in prednisolone or dexamethasone treatment by Western blot, qRT-PCR and ELISA. The results show that up-regulation of TGF-β2 in glucocorticoids treatments as shown in the figure 23a, b.

a. b.

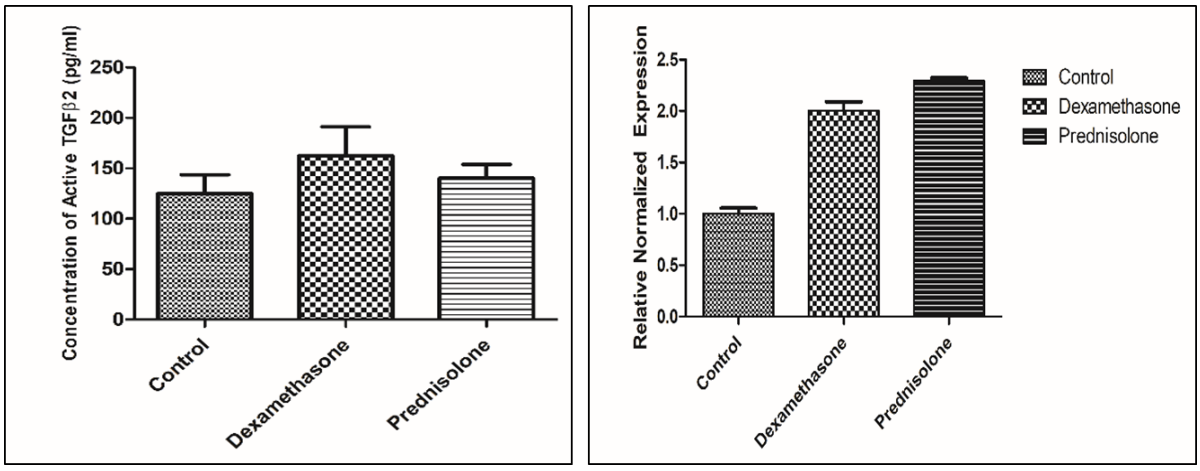



**Figure 23.** Glucocorticoids (Dexamethasone and Prednisolone) induced expression of TGF-β2 in primary HTM cells. a) Primary HTM cells were treated with dexamethasone (300 nM/ml) and prednisolone (300 nM/ml) for 72hrs and quantified TGF-β2 levels in the conditioned medium. b) RNA was isolated and prepared cDNA and quantified mRNA levels of TGF-β2 by using real-time PCR.

To further confirm the effects of TGF-β2 induced downstream signaling pathway, HTM cells were treated with dexamethasone (300nm/ml) or TGF-β2 (2ng/ml) for different time points (0-24hrs) and we observed that phosphorylation of SMAD3, induced ROCK2, paxillin and phospho–cofilin levels in both TGF-β2 or dexamethasone treated cells figure 24a, b. The above experiments suggests that dexamethasone induces the expression of TGF-β2 and formation of actin stress fibers and focal adhesions Figure 25a, b, c. Collectively our data suggests that the glucocorticoids induced alteration of ECM protein expression in HTMs and secondary glaucoma phenotype in in vivo due to overexpression of TGF-β2.

a.

b.

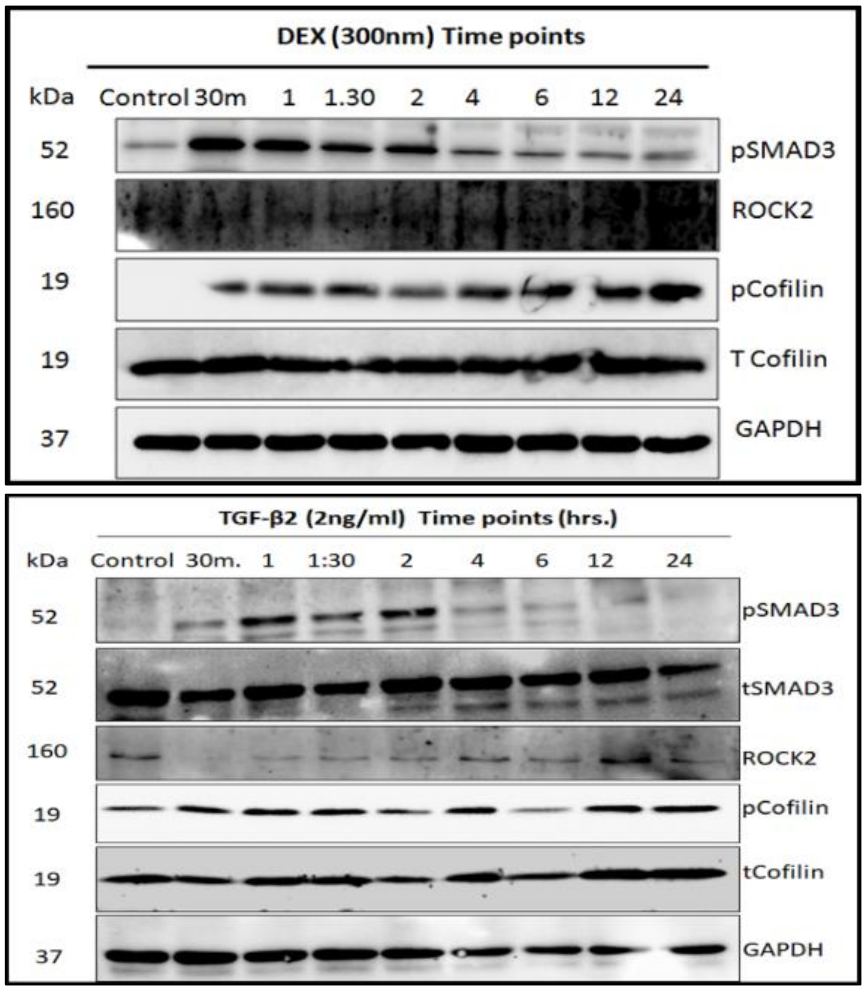



**Figure 24.** Glucocorticoids (Dexamethasone and Prednisolone) induced expression of TGF-β2 in primary HTM cells. a) Primary HTM cells were treated with dexamethasone for different time points (0-24hrs) and analysed for the activation of ROCK signaling pathway by western blot. b) Activation of ROCK signaling pathway was observed by treating HTM cells with TGF-β2 (2ng/ml) for different time points.

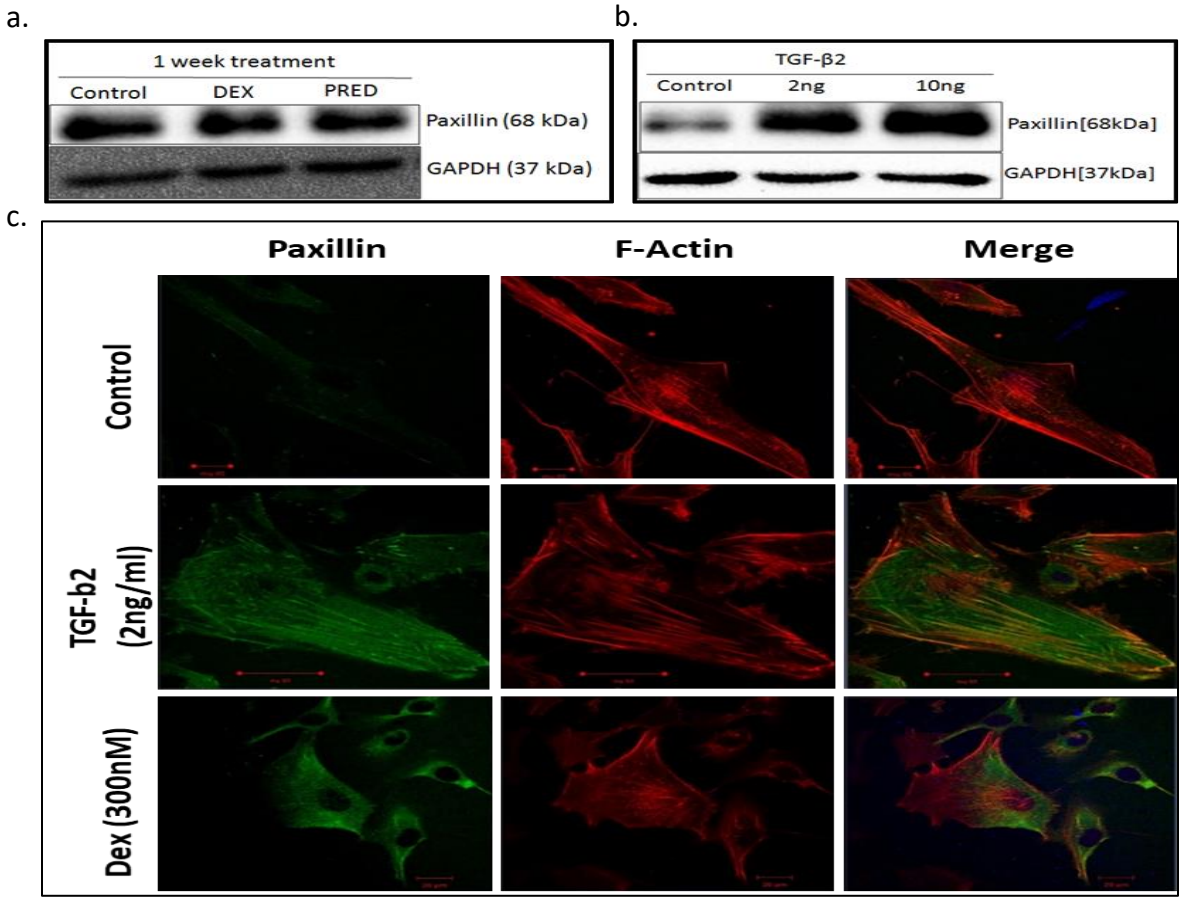


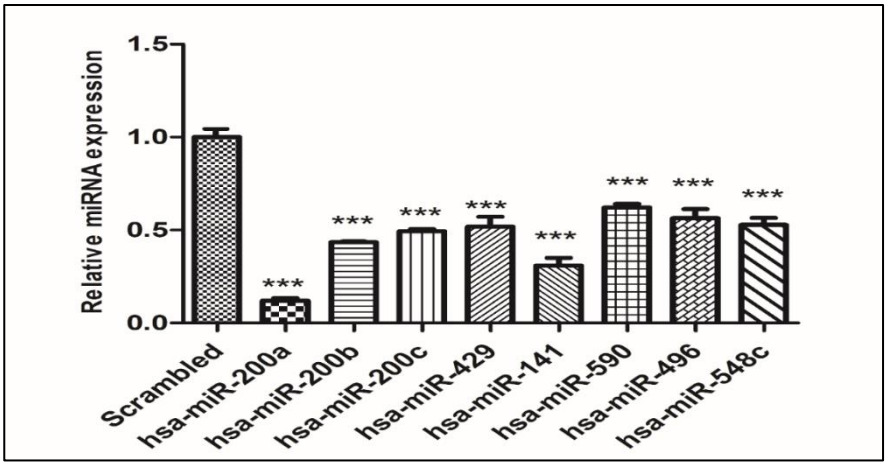

**Figure 25.** Glucocorticoids (Dexamethasone and Prednisolone) induced expression of TGF-β2 in primary HTM cells. a), b) Induced expression of Paxillin (Focal adhesion protein) was observed upon treating with glucocorticoids and TGF-β2 for 24hrs. c) F-Actin was stained with rhodamine phalloidin (Red) and focal adhesion protein paxillin was stained with Anti-Paxillin antibody (Green). Formation of actin stress fibers was seen with dexamethasone and TGF-β2 by confocal microscopy compared to control.

### 4.8 TGF-β2 and dexamethasone down-regulates miR-200 family, miR-590, miR-496 and miR-548c expression in primary HTM cells.

In earlier experiments we have seen that miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c) down-regulates the expression of TGF-β2 and abrogates its downstream effects such as actin depolymerization and relaxation of primary HTM cells. And as TGF-β2 and dexamethasone (DEX) were shown to induce secondary glaucoma thus to establish a cause-effect relationship between miRNA, TGF-β2 and dexamethasone, primary trabecular meshwork (HTM) cells were treated with TGF-β2 (2ng/ml) for 24hrs or dexamethasone

(300nM/ml) for 1 week, and quantified mature miRNA levels. Analysis by qRT-PCR shows decreased expression of miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 miR-496 and miR-548c as shown in figure 26a, b.

a.




b.

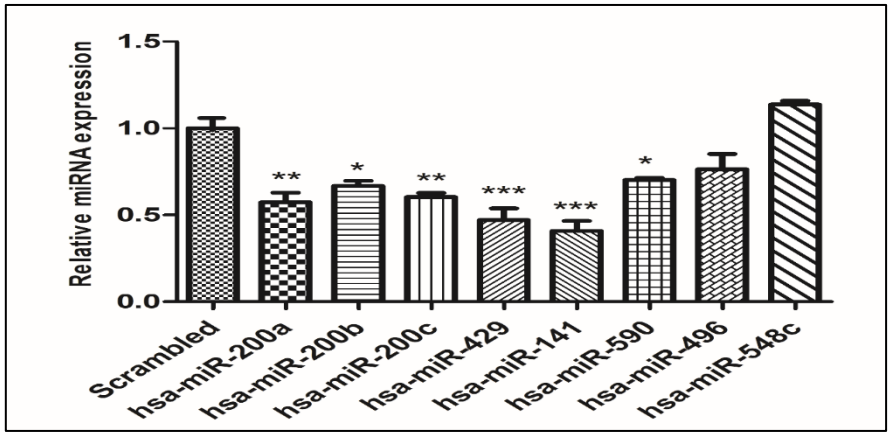



Figure 26. miRNA (miRNA-200 family, miR-590, miR-496 and miR-548c) expression in primary trabecular meshwork cells. a) Primary HTM cells were treated with a) TGF-β2 (2ng/ml) for 24hrs and with b) dexamethasone (300nM/ml) for 7 days, cells were harvested, RNA was isolated and cDNA prepared by using 1µg of RNA and quantified mature miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) by using real-time PCR assay. Bars represent standard deviation in three different experiments. Asterisk (***) represents significance at $p<0.005$ between miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) and control. Standard deviation and significance were calculated by using GraphPad Prism5.

These results highlights the importance of both TGF-β2 and miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c expression in normal functioning of HTM cells. Collectively these results show that tight regulation between TGF-β2 and miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c).

**4.9 Ectopic expression of miR-200 family, miR-590, miR-496 and miR-548c down-regulates the expression of CYP1B1 and FOXC1 in primary HTM cells.**

From the above experiments, we now know that, TGF-β2 induces expression of glaucoma candidate genes (FOXC1, OPTN, MYOC and LTBP2) and down-regulates CYP1B1 expression in primary HTM cells. Conversely, TGF-β2 down-regulate the expression of miRNAs and vice-versa. From our *in-silico* analysis, we found that miRNA (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) targets CYP1B1, FOXC1, OPTN, MYOC and LTBP2 expression apart from TGF-β2 and ROCK2. Thus, here we would like to see the effects of miRNA on expression of glaucoma candidate genes (CYP1B1, FOXC1, OPTN, MYOC and LTBP2). To validate predicted targets, miRNAs are ectopically transfected into primary HTM cells for 48hrs and proteins were analyzed by Western blot for expression of CYP1B1, FOXC1, OPTN, MYOC and LTBP2.as shown in figure 27a. CYP1B1 and FOXC1 genes were down-regulated by the miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c whereas up-regulation of OPTN expression was seen. In proof of concept studies, the expression of candidate genes were rescued upon co-transfection with anti-miRs along with respective miRNAs 27b.

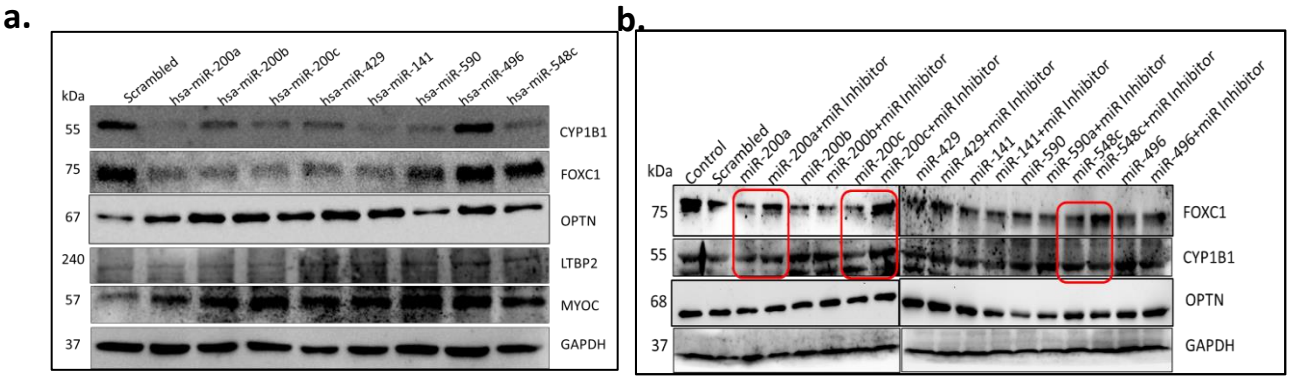



**Figure 27.** Ectopic expression of miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) targeted CYP1B1 and FOXC1 expression in primary HTM cells. a) Primary HTM cells were transfected with miRNA-200 family, miR-590, miR-496 and miR-548c mimics and harvested after 48hrs and analysed for glaucoma candidate genes expression (CYP1B1, FOXC1, OPTN, MYOC AND LTBP2 ) expression by Western blot. b) MiRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) were transfected with and without their respective anti-miRs in primary HTM cells and harvested after 48hrs and analysed for glaucoma candidate genes expression (CYP1B1, FOXC1 and OPTN).

These results were further validated by reporter assays with 3'UTR constructs of CYP1B1 and FOXC1. Collectively these results demonstrated that predicted miRNAs are direct targets for

CYP1B1 and FOXC1 and modulates the expression OPTN along with CYP1B1 and FOXC1 in HTM cells as shown in figure 28a,b.

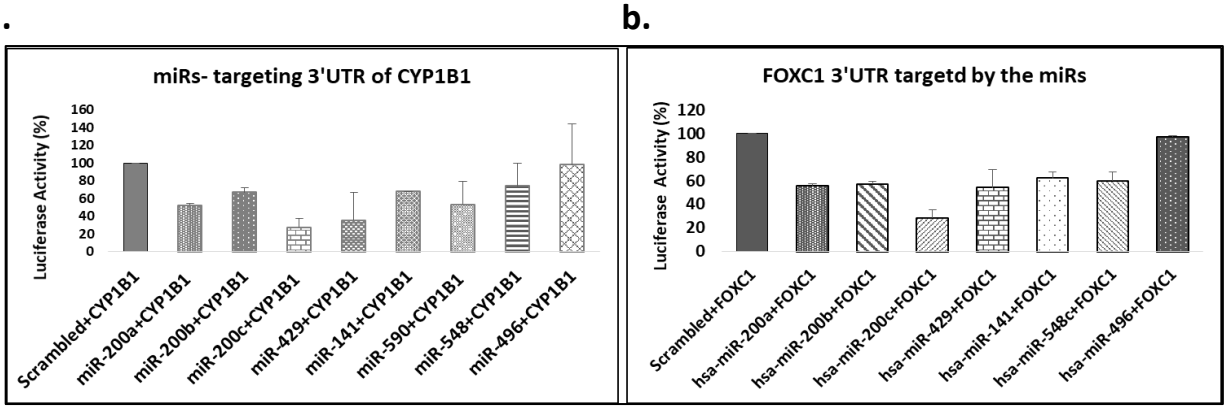



**Figure 28.** Ectopic expression of miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c) targeted CYP1B1 and FOXC1 expression in primary HTM cells. a) miRNAs (miRNA-200 family, miR-590, miR-496 and miR-548c mimics) along with CYP1B1, FOXC1 3'UTR constructs cloned in pMIR-Report vector were transfected in HEK293T cell lines, after 24hrs cells were harvested for dual luciferase assay. And observed decrease in luminescence activity in miRNA-200 family, miR-590, miR-496 and miR-548c transfected along with CYP1B1, FOXC1 3'UTR constructs compared to control. Bars represent standard deviation in three different experiments.

### 4.10 TGF-β2, miRNAs and IOP follow circadian rhythmic pattern in in vitro and murine models.

Several clinical studies demonstrated that normal intraocular pressure (IOP) in humans' as well experimental animal models follow circadian rhythm, where IOP being low during light phase (diurnal) whereas high during dark phase (nocturnal). Expression of TGF-β2 is shown to follow the circadian pattern and over expression of TGF-β2 in normal eyes of mice resulted in altered pattern of rhythm and increased IOP. From our earlier experiments it was demonstrated that TGF-β2, miRNA and glaucoma candidate genes were known to regulate each other at multiple levels, thus it was apparent to check the expression pattern of biological clock genes and further predicted miRNAs and glaucoma candidate genes to elucidate the normal physiological function of circadian rhythm in HTM cells and IOP in in vivo. Overnight serum starved HTM cells were replenished with complete growth medium and collected cell lysates for every 4hrs starting from 0hrs up to 48hrs. qRT-PCR analysis revealed that expression of BMAL1, CLOCK, PER1, PER2, CRY2, CRY1 and Rev-Erb α genes followed 24hrs cycle pattern begins with decreased expression in first 12hrs and recovered in next 12hrs. PER2 and CRY2 also followed similar expression with 12hrs delay whereas Rev-erb α showed with 24hr delay compared to other circadian clock genes

and respective controls. Cyclophilin A was used as a negative control for clock genes. These results show that primary trabecular meshwork (HTM) cells follow a circadian rhythm as shown in figure 29a.

**a.**

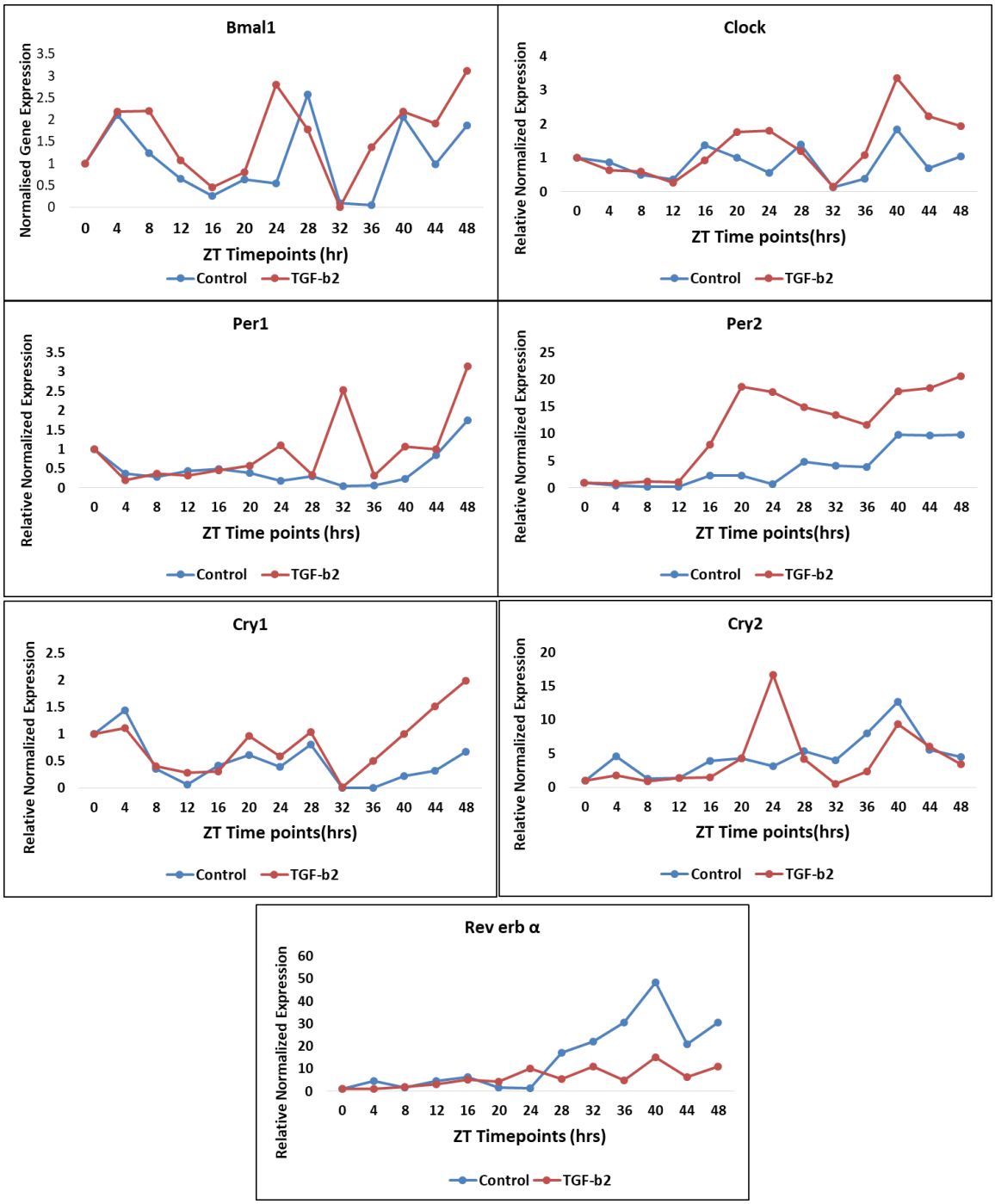



**Figure 29.** Primary HTM cells follows circadian rhythm. a) RNA was isolated from primary HTM cells treated with and without TGF-β2 (2ng/ml) for different time points from 0-48hrs, lysates collected with a span of 4hrs and quantified biological clock genes expression in primary HTM cells.

Since HTM cells follow circadian rhythm, we then checked the expression pattern of TGF-β2, miRNA and glaucoma candidate genes in relation with circadian rhythm. qRT PCR analysis shows that TGF-β2, predicted miRNAs , CYP1B1 and OPTN followed similar pattern like clock genes, whereas treatment with TGF-β2 (2ng/ml) for 0-48hrs disturbed the circadian pattern of gene

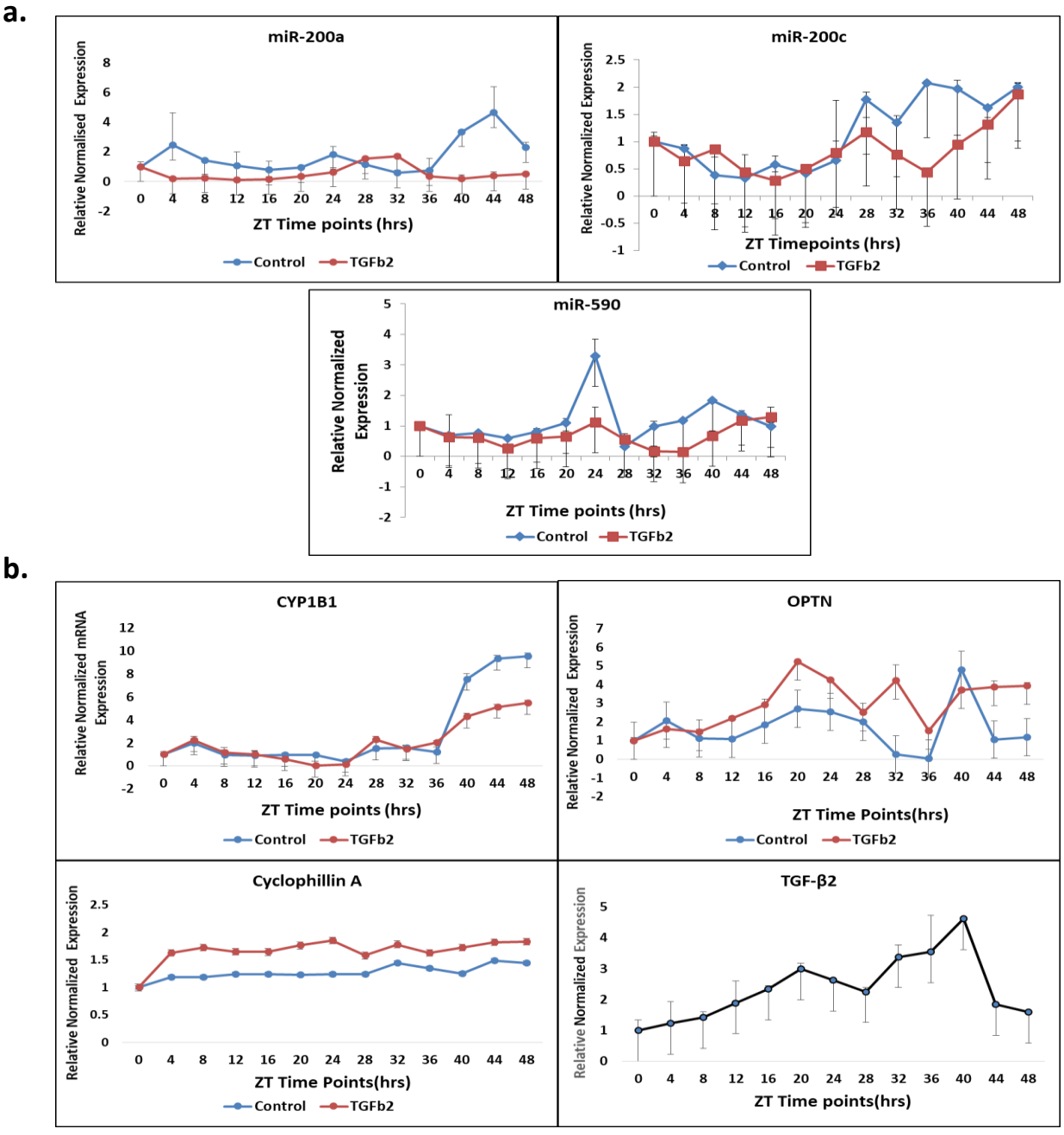



**Figure 30.** Primary HTM cells follows circadian rhythm. a) miRNA-200c, miRNA-200a and miRNA-590 expression in primary HTM cells are following circadian rhythmic pattern. b) Glaucoma candidate gene (CYP1B1, OPTN cyclophilin A and TFG-β2) expression also following circadian rhythm in primary trabecular meshwork cells. And cyclophilin A is used as a control gene as it does not follow any circadian pattern.

expression of miRNAs, CYP1B1, OPTN at every time point compared with control as shown in figure 30a, b.

To check the circadian pattern of IOP and effects of dexamethasone treatments in murine models, one of the eyes of each male C57BL/6 mice were treated ectopically with 0.1% dexamethasone whereas other eye was with vehicle control for 3 weeks and measured IOP using rebound tonometer, Tonolab® at the end of every week end up to 3 weeks of treatment. Intraocular pressure measurements of control eyes at different time points 12AM, 4AM, 8AM, 12 PM, 4PM, 8PM showed the circadian pattern whereas dexamethasone treated eyes were showed disturbed pattern with high IOP compared with controls as shown in figure 31 a.

Collectively these results suggested that regulation of IOP is under the control of circadian rhythm through the modulation of TGF-β2, miRNA and candidate genes however the exact roles of these signaling molecules needs to addressed further.

**a.**

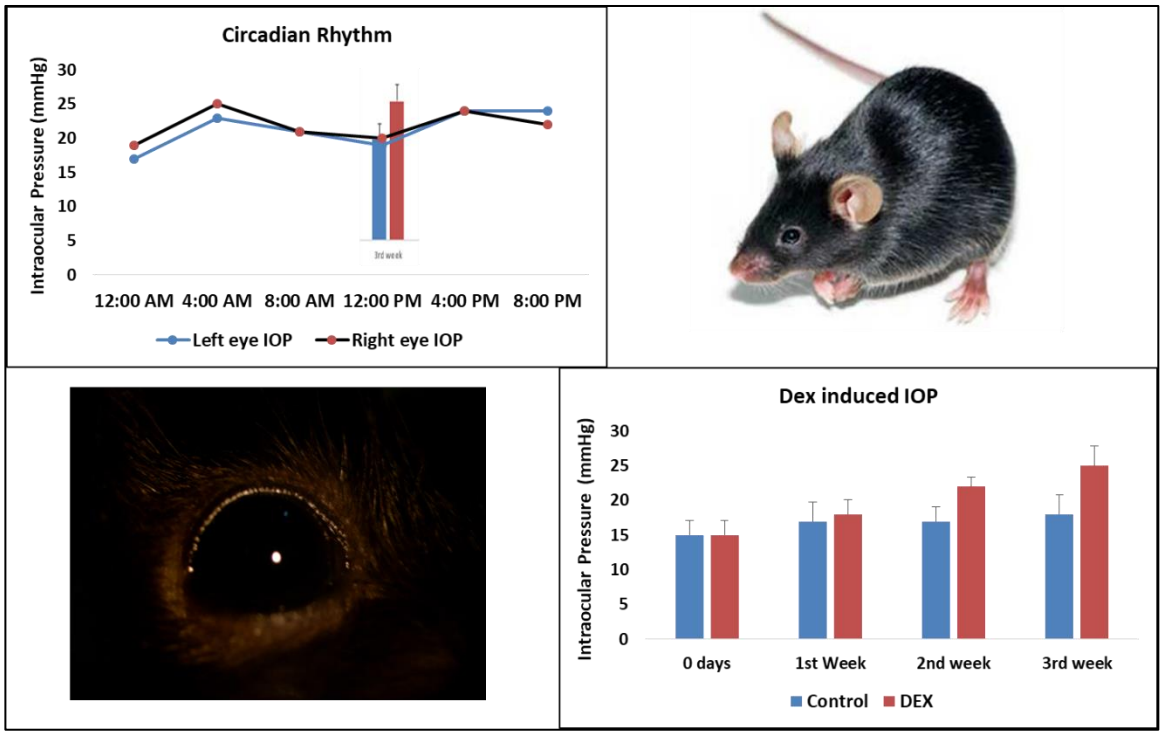



**Figure 31. I**ntraocular pressure in C57BL/6 mice follows circadian rhythm. a) C57BL/6 mice model treated with DEX for 3 weeks and IOP was measured at every week. Significant increase in IOP was seen from second week of DEX treatment compared to control eye. And IOP was measured at different time intervals in 24hrs cycle and observed circadian rhythmic pattern in IOP of normal C57BL/6 mice.

# Discussion

## 5. Discussion

Glaucoma is an irreversible blinding disorder. Several studies have reported that, increased intraocular pressure (IOP) is the causative factor for the development of glaucoma and, therefore, the reduction of intraocular pressure prevents or delays the damage occurring to the optic nerve axons. Various studies have reported that functional mutations in viz; CYP1B1, FOXC1, MYOC, OPTN, WDR36 and LTBP2 have been identified which are segregating with glaucoma pathogenesis, but the molecular mechanisms in the development of the disease is still lacking. Subsequent studies have found that 60-70% of higher concentration of biologically active form of the TGF-β2 in the aqueous humor of various forms of glaucoma compared to age matched controls [73]. TGF-β2 has been implicated in various pathological situations like induction of extracellular matrix proteins [74, 75], and formation of cross linking actin networks in trabecular meshwork cells [76]. Subsequent studies have shown that TGF-β2 found to be associated with tissue stiffness and increase in aqueous outflow resistance seen in glaucomatous TM [70 & Dex ref]. But the association between TGF-β2 and known glaucoma candidate genes were not established till date. So, in this study we explored to demonstrate the pathophysiological significance of TGF-β2 and known glaucoma candidate gene expression in trabecular meshwork cells.

Interestingly, our data shows that TGF-β2 differentially regulates the expression of glaucoma candidate genes. We have also seen the relation of candidate genes with glucocorticoids where dexamethasone and prednisolone induces FOXC1, OPTN, MYOC and LTBP2 expression and down-regulates CYP1B1 expression in primary HTM cells. Further experiments had shown that FOXC1 binds on to the CYP1B1 promoter sequence and negatively regulates its expression. OPTN knockdown studies shows that down-regulation of CYP1B1 and up-regulation of FOXC1, Whereas overexpression of OPTN in HTM cells showed significant increase in mRNA levels of FOXC1, but the protein levels were decreased. Thus this data made us to speculate that there could be a possibility of existing regulation at post-translational level. Our further analysis with MG132 (proteasome degradation inhibitor) in overexpressed OPTN HTM cells resulted in recovery of FOXC1 protein levels compared with and without MG132, confirming the possibility of OPTN induced FOXC1 ubiquitination. Further experiments showed that knockdown of OPTN lead to increased TGF-β2 levels and transient over expression of wild type OPTN lead to the down-regulation of TGF-β2, signifying that OPTN might be regulating CYP1B1 and

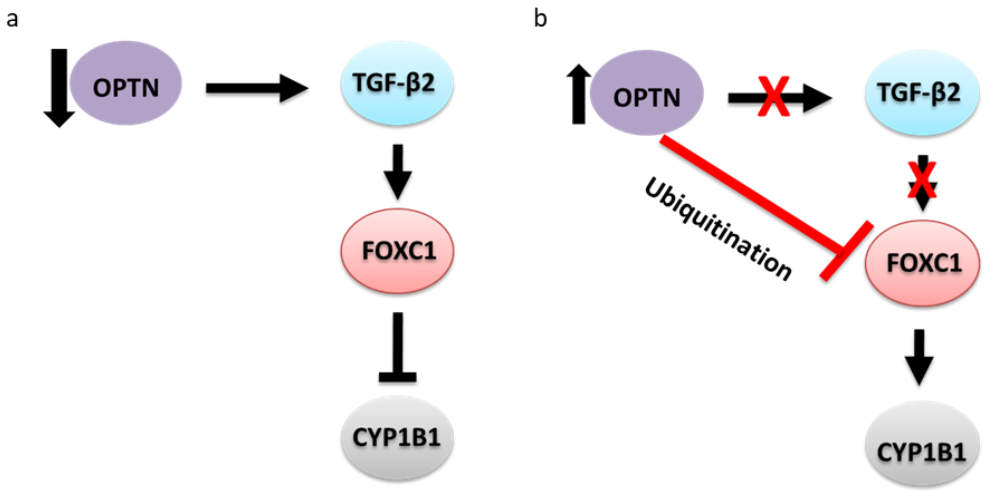



Figure 32. Schematic diagram showing, a) loss of OPTN induces FOXC1, TGF-β2 expression and suppresses CYP1B1 b) Over expression of OPTN inhibits TGF-β2 and down regulates FOXC1 might be through ubiquitination in primary trabecular meshwork (HTM) cells.

FOXC1expression through TGF-β2 regulation in TM cells as shown in the figure 32 a, b. conclusive statement of figure 9- figure-14.

Since TGF-β2 implicated in pathological role in TM cells, we next analyzed TGF-β2 downstream signaling pathways, such as Rho/ROCK signaling pathway, which were shown to play a critical role in AH outflow by modulating actin cytoskeletal reorganization and regulates cell elasticity. Our data shows that knockdown of OPTN lead to the induced expression of both ROCK1 and ROCK2 and phosphorylation of cofilin in TM cells, further confocal microscopy experiments showed the formation of actin stress fibers in the HTM cells, conversely upon overexpression of

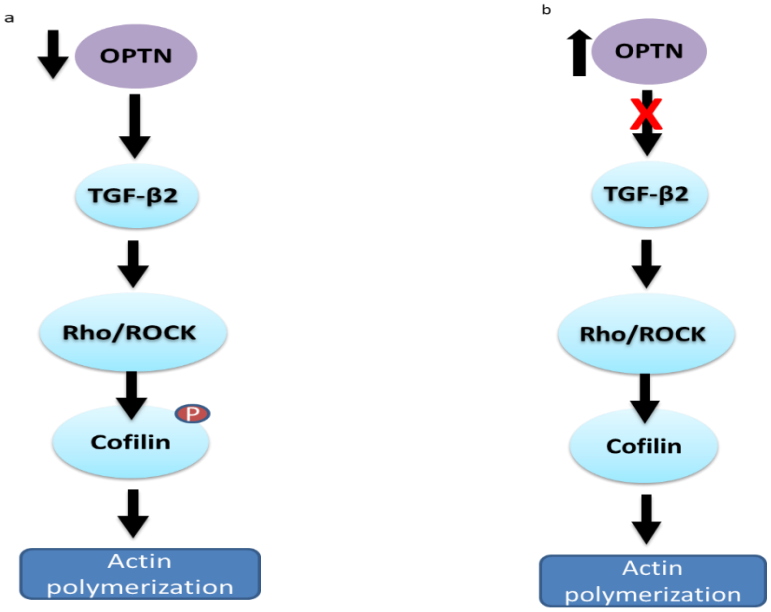



Figure 33. Schematic diagram showing, a) loss of OPTN activates Rho/ROCK signaling pathway by inducing TGF-β2 expression and b) overexpression of OPTN down-regulates TGF-β2, thus abrogates Rho/ROCK signaling pathway in primary human trabecular meshwork (HTM) cells.

wild type OPTN abrogates the Rho/ROCK pathway and actin stress fibers formation. This data shows that loss of OPTN induces Rho/ROCK signaling pathway by inducing TGF-β2 expression in HTM cells as shown in figure 33 a, b. conclusive statement of image-15 & image-16.

It is well documented that activation of Rho/ROCK signaling pathway in the trabecular meshwork cells leads to increased outflow resistance and elevated intraocular pressure (IOP), further our results shows that loss of OPTN leads to the activation of Rho/ROCK signaling pathway by inducing TGF-β2 expression. Organ-cultured anterior segments from porcine eyes expressing RhoAV14 exhibited significant reduction of aqueous humor outflow [215]. Inhibition of RhoA expression in TM cells with siRNA effectively decreased elevated IOP in mice [235]. Several other studies have shown that, ROCK inhibitors effectively increased outflow facility and/or decreased IOP in living rabbits, mouse, rat, and monkeys, human and enucleated porcine eyes model. Now we next analyzed for the inhibitors of Rho/ROCK signaling pathway, like any endogenous molecules that inhibit their activation in TM cells. Some of the studies have shown that miRNA could play a role in maintaining the aqueous humor production in the eye. But their mechanisms were not understood in the regulation of aqueous humor in the eyes. Interestingly there are reports describing the miRNAs that are involved in EMT pathway and ECM remodeling in other pathological conditions such as cancers and diabetes etc, here we looked at the role of these in miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) in targeting the TGF-β2, ROCK2 and glaucoma candidate genes in trabecular meshwork cells since the ECM remodeling is main clinical features in all these instances. From our findings, we found that these miRNA (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) down-regulate the expression of TGF-β2, ROCK2 by binding to its 3'UTR sequences. Further results shows that TGF-β2 and dexamethasone down-regulates miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c expression in HTM cells. These findings made us to think that miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) and TGF-β2 are inter regulated by each other in HTM cells as shown in the figure 34, conclusive statement of image-17 to image-22.

TGF-β2 and dexamethasone have been reported to induce formation of actin stress fibers and focal adhesions and implicated in pathogenic increase in tissue stiffness and aqueous outflow resistance observed in glaucomatous TM. Further to find the role of these miRNAs in regulation of actin polymerization and depolymerization, HTM cells expressing with miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c were treated with and without TGF-β2

have shown reduced formation of actin stress fibers and focal adhesions; imply that miRNAs abolished formation of actin stress fibers in response to TGF-β2 treatments. As mentioned before TGF-β2 induces actin stress fiber formation and focal adhesions by activating Rho/ROCK signaling pathway, activated ROCK kinases ultimately phosphorylates cofilin through LIM kinases, leading to inactivation of cofilin and formation of actin stress fibers. Increased stress fibers have direct effect on cell stiffness and increases aqueous outflow facility. Our results show that miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c inhibit the formation of actin stress fibers and focal adhesions by modifying the expression of TGF-β2 and ROCK2 in HTM cells implying that these miRNAs decrease the IOP by increasing aqueous humor outflow facility. There may be multiple factors/genes influencing contraction or relaxation of trabecular meshwork cells, which is necessary for smooth functioning of aqueous outflow facility. HTM cells expressing miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590 and miR-548c reduced contractile forces in collagen populated HTM cells in the presence of TGF-β2. Which further shows that these miRNAs might be responding to the pressure in the eye and modulates cell morphology.

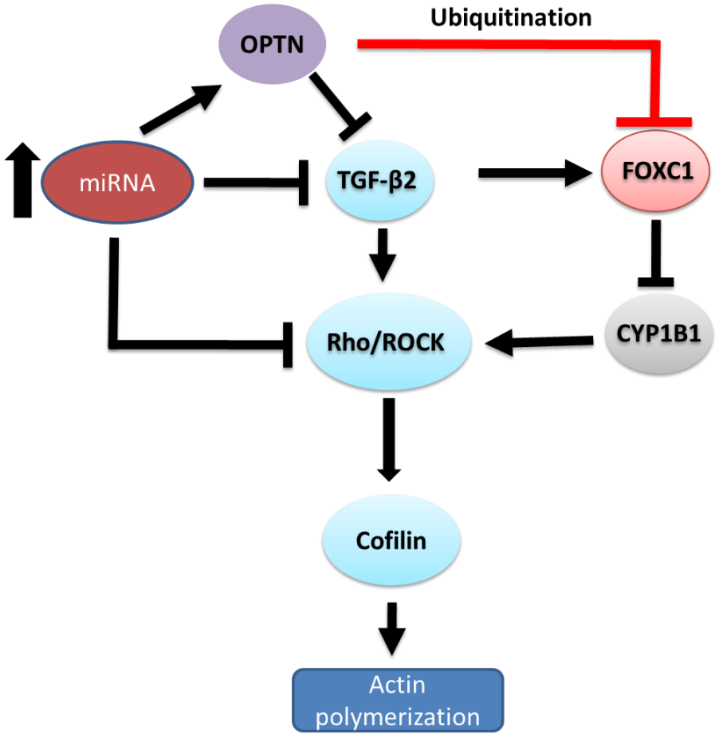



Figure 34. Schematic diagram showing inter-regulation of OPTN, TGF-β2, putative miRNA and Rho/ROCK signaling pathway, which leads to the polymerization and depolymerization of actin filaments that help in cells contraction and relaxation to facilitate outflow of AH.

Though glaucoma candidate genes were found to be responsible for the development of the glaucoma their functional roles were not clear till date, in our study we tried to find their association with disease. As mentioned before the relation between TGF-β2 with FOXC1 OPTN, MYOC and CYP1B1, further we tried to establish relation between predicted miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) and FOXC1 OPTN, MYOC and CYP1B1 and found that miRNAs down-regulate the expression of CYP1B1 and FOXC1and surprisingly we observed up-regulation of OPTN. All these results in combination shows that OPTN, TGF-β2 and miRNAs are inter-regulated by each other as shown in figure 35.

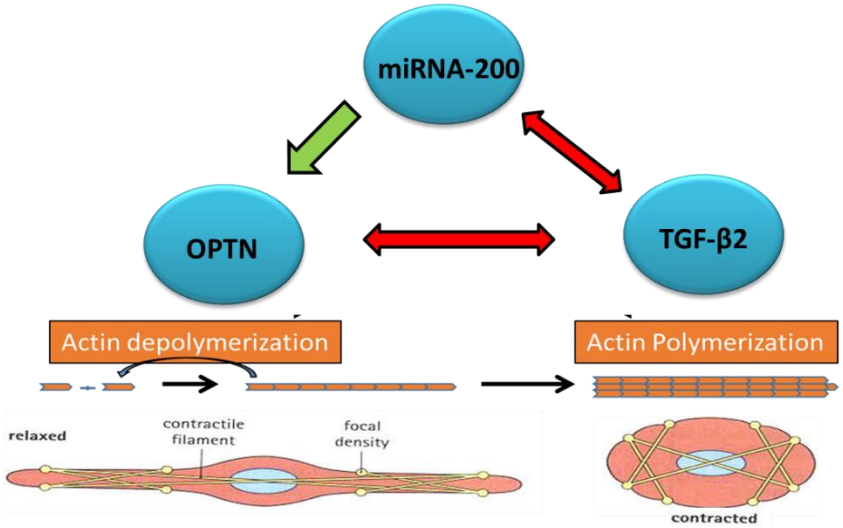



Figure 35. Schematic diagram showing loss of OPTN activates the expression Rho/ROCK signaling pathway by inducing TGF-β2 expression. miRNAs that modulate/inhibit TGF-β2 induced Rho/ROCK signaling pathway by targeting both TGF-β2, ROCK expression as a result leads to the actin depolymerization in primary trabecular meshwork cells.

Aqueous humor production and egression is known to follow a circadian rhythm in humans as well as animals. Intraocular pressure follows a 24hr circadian cycle, but the mechanisms that regulate aqueous humor secretion and egression is not understood well. Here we tried to study the molecular mechanisms that might regulate aqueous secretion and drainage through the anterior chamber. We analyzed expression of OPTN, TGF-β2 and miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c) and found their expression is following circadian rhythm in trabecular meshwork cells. But the molecular mechanisms how they regulate aqueous outflow still needs to be elucidated. From our experiments with C57BL/6 mice, we observed that intraocular pressure follows circadian rhythm in normal mice, and treating with

dexamethasone for 3 weeks induces secondary glaucoma and observed significantly increased intraocular pressure from the 2nd week of the treatment but still the IOP is following the circadian rhythm. Further experiments are in progress to find out the exact mechanisms of IOP regulation in circadian pattern for which our hypothetical model was shown in figure 36.

**Hypothetical model of showing aqueous humor outflow follows circadian rhythm in HTM cells.**

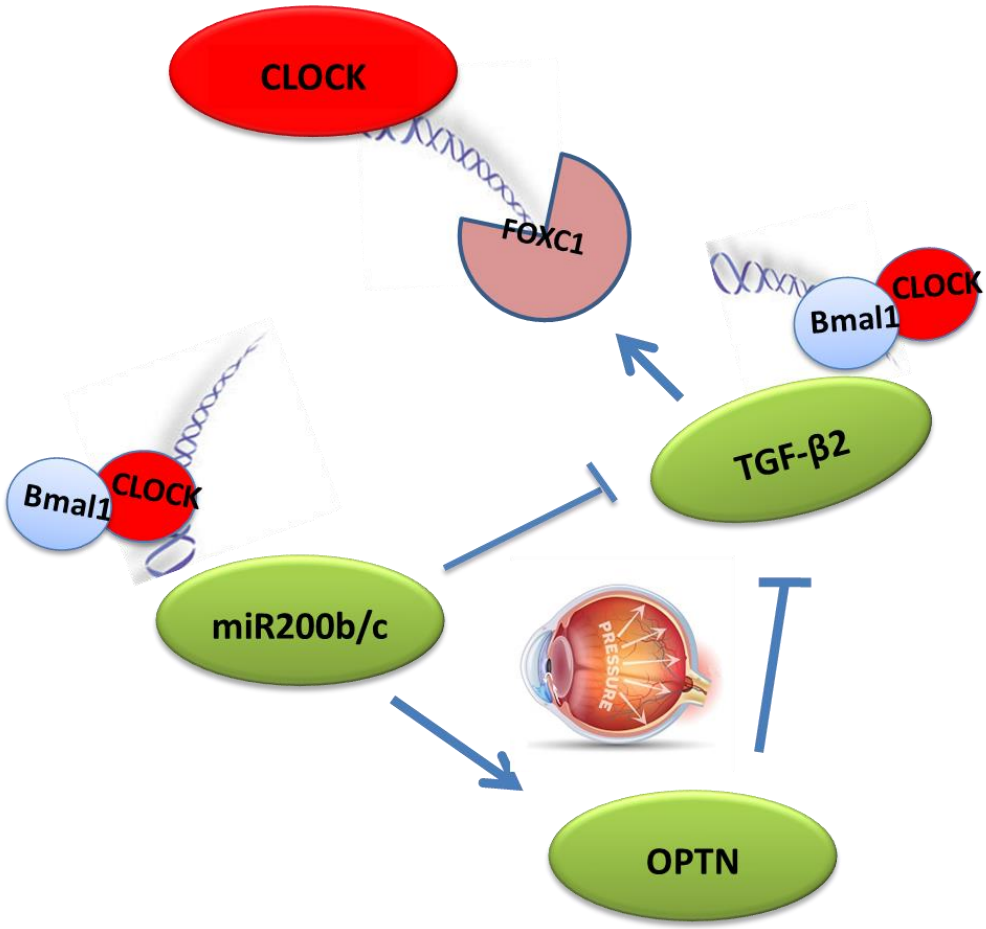



Figure 36. Schematic diagram of hypothetical model showing regulation of aqueous humor outflow facility in circadian rhythm in trabecular meshwork cells. Our data shows that, there is inter-regulation between miRNAs, OPTN and TGF-β2 and biological clock genes, so that that may lead to variation in intraocular pressure in a circadian rhythm *in-vivo* and imbalance in homeostasis leads to pathology due to obstruction of out flow.

# Conclusions

## 6. Conclusions

- *In Silico* identified putative miRNAs regulates the expression of TGF-β2, ROCK2, CYP1B1, FOXC1 and OPTN
- Loss of OPTN activates Rho/ROCK signaling pathway through induction of TGF-β2 in primary HTM cells
- TG-β2 induced ROCK signaling was attenuated by miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c overexpression in primary HTM cells.
- Collagen based contraction assay demonstrated TGF-β2 induced contraction, whereas miRNA attenuates it.
- TGF-β2 and dexamethasone down-regulates putative miRNA expression and overexpression of miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496 and miR-548c down-regulates TGFβ2 expression.
- Primary HTM cells exerts circadian rhythm whereas DEX/TGF-β2 perturbs circadian rhythm in murine model, thus could associates with IOP regulation.

# References

## 7. References


1. Llobet, A., X. Gasull, and A. Gual, *Understanding trabecular meshwork physiology: a key to the control of intraocular pressure?* News Physiol Sci, 2003. **18**: p. 205-9.
2. E, W.C., et al., *Anatomy and physiology of the human eye: effects of mucopolysaccharidoses disease on structure and function – a review.* Clinical & Experimental Ophthalmology, 2010. **38**(s1): p. 2-11.
3. Vrijens, K., V. Bollati, and T.S. Nawrot, *MicroRNAs as potential signatures of environmental exposure or effect: a systematic review.* Environ Health Perspect, 2015. **123**(5): p. 399-411.
4. Refojo, M.F. and F.-L. Leong, *Water-dissolved-oxygen permeability coefficients of hydrogel contact lenses and boundary layer effects.* Journal of Membrane Science, 1978. **4**: p. 415-426.
5. Nickla, D.L. and J. Wallman, *THE MULTIFUNCTIONAL CHOROID.* Progress in retinal and eye research, 2010. **29**(2): p. 144-168.
6. Delamere, N.A., *Ciliary Body and Ciliary Epithelium.* Advances in organ biology, 2005. **10**: p. 127-148.
7. Evelyn Addo, O.A.B.a.R.S., *Ocular Drug Delivery: Advances, Challenges and Applications,*. 2016: Springer International Publishing AG 2016.
8. Goel, M., et al., *Aqueous humor dynamics: a review.* Open Ophthalmol J, 2010. **4**: p. 52-9.
9. CS, F. *Dry eye syndrome*. 2015.
10. Coleman, H.R., et al., *Age-related macular degeneration.* Lancet, 2008. **372**(9652): p. 1835-1845.
11. Jager, R.D., W.F. Mieler, and J.W. Miller, *Age-Related Macular Degeneration.* New England Journal of Medicine, 2008. **358**(24): p. 2606-2617.
12. Beatty, S., et al., *The Role of Oxidative Stress in the Pathogenesis of Age-Related Macular Degeneration.* Survey of Ophthalmology, 2000. **45**(2): p. 115-134.
13. Noell, W.K., et al., *Retinal Damage by Light in Rats.* Investigative Ophthalmology & Visual Science, 1966. **5**(5): p. 450-473.
14. Resnikoff, S., et al., *Global data on visual impairment in the year 2002.* Bull World Health Organ, 2004. **82**(11): p. 844-51.
15. Tham, Y.C., et al., *Global prevalence of glaucoma and projections of glaucoma burden through 2040: a systematic review and meta-analysis.* Ophthalmology, 2014. **121**(11): p. 2081-90.
16. Leite, M.T., L.M. Sakata, and F.A. Medeiros, *Managing glaucoma in developing countries.* Arq Bras Oftalmol, 2011. **74**(2): p. 83-4.
17. Rotchford, A.P., et al., *Temba glaucoma study: a population-based cross-sectional survey in urban South Africa.* Ophthalmology, 2003. **110**(2): p. 376-82.
18. Bahrami, H., *Causal inference in primary open angle glaucoma: specific discussion on intraocular pressure.* Ophthalmic Epidemiol, 2006. **13**(4): p. 283-9.
19. Heijl, A., et al., *Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial.* Arch Ophthalmol, 2002. **120**(10): p. 1268-79.
20. Kass, M.A., et al., *The Ocular Hypertension Treatment Study: a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma.* Arch Ophthalmol, 2002. **120**(6): p. 701-13; discussion 829-30.
21. Alward, W.L., *Medical management of glaucoma.* N Engl J Med, 1998. **339**(18): p. 1298-307.
22. Weinreb, R.N. and P.T. Khaw, *Primary open-angle glaucoma.* Lancet, 2004. **363**(9422): p. 1711-20.
23. Johnson, A.T., et al., *Clinical phenotype of juvenile-onset primary open-angle glaucoma linked to chromosome 1q.* Ophthalmology, 1996. **103**(5): p. 808-14.
24. Tawara, A. and H. Inomata, *Developmental immaturity of the trabecular meshwork in juvenile glaucoma.* Am J Ophthalmol, 1984. **98**(1): p. 82-97.

25. Polansky, J.R., et al., *Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product.* Ophthalmologica, 1997. **211**(3): p. 126-39.
26. Sheffield, V.C., et al., *Genetic linkage of familial open angle glaucoma to chromosome 1q21-q31.* Nat Genet, 1993. **4**(1): p. 47-50.
27. Stone, E.M., et al., *Identification of a gene that causes primary open angle glaucoma.* Science, 1997. **275**(5300): p. 668-70.
28. Allingham, R.R., et al., *Gln368STOP myocilin mutation in families with late-onset primary open-angle glaucoma.* Investigative Ophthalmology & Visual Science, 1998. **39**(12): p. 2288-2295.
29. Angius, A., et al., *Myocilin gln368stop mutation and advanced age as risk factors for late-onset primary open-angle glaucoma.* Archives of Ophthalmology, 2000. **118**(5): p. 674-679.
30. Challa, P., et al., *Prevalence of Myocilin Mutations in Adults with Primary Open-angle Glaucoma in Ghana, West Africa.* Journal of Glaucoma, 2002. **11**(5): p. 416-420.
31. Kennan, A.M., et al., *A novel Asp380Ala mutation in the GLC1A/myocilin gene in a family with juvenile onset primary open angle glaucoma.* Journal of Medical Genetics, 1998. **35**(11): p. 957-960.
32. Mabuchi, F., et al., *A sequence change (Arg158Gln) in the leucine zipper-like motif region of the MYOC/TIGR protein.* Journal Of Human Genetics, 2001. **46**: p. 85.
33. Suzuki, R., Y. Hattori, and K. Okano, *Promoter mutations of myocilin gene in Japanese patients with open angle glaucoma including normal tension glaucoma.* The British Journal of Ophthalmology, 2000. **84**(9): p. 1075-1075.
34. Suzuki, Y., et al., *Mutations in the TIGR gene in familial primary open-angle glaucoma in Japan.* American Journal of Human Genetics, 1997. **61**(5): p. 1202-1204.
35. Taniguchi, F., et al., *The Gly367Arg Mutation in the Myocilin Gene Causes Adult-Onset Primary Open-Angle Glaucoma.* Japanese Journal of Ophthalmology, 2000. **44**(4): p. 445-448.
36. Vasconcellos, J.P., et al., *Novel mutation in the MYOC gene in primary open angle glaucoma patients.* Journal of Medical Genetics, 2000. **37**(4): p. 301-303.
37. Javier, O., E. Julio, and C.-P. Miguel, *Cloning and characterization of subtracted cDNAs from a human ciliary body library encoding TIGR, a protein involved in juvenile open angle glaucoma with homology to myosin and olfactomedin.* FEBS Letters, 1997. **413**(2): p. 349-353.
38. MF Adam, A.B., P Binisti, AP Brezin, F Valtot, A Bechetoille, J-C Dascotte, B Copin, L Gomez, A Chaventre, J-F Bach, and H-J* Garchon,, *Recurrent mutations in a single exon encoding the evolutionarily conserved olfactomedin-homology domain of TIGR in familial open-angle glaucoma.* Human Molecular Genetics, Nov 1997. **6**(12): p. 2091-2097.
39. Tamm, E.R., et al., *Modulation of Myocilin/TIGR Expression in Human Trabecular Meshwork.* Investigative Ophthalmology & Visual Science, 1999. **40**(11): p. 2577-2582.
40. Fingert, J.H., et al., *Characterization and Comparison of the Human and Mouse GLC1A Glaucoma Genes.* Genome Research, 1998. **8**(4): p. 377-384.
41. Kubota, R., et al., *Genomic Organization of the Human Myocilin Gene (MYOC) Responsible for Primary Open Angle Glaucoma (GLC1A).* Biochemical and Biophysical Research Communications, 1998. **242**(2): p. 396-400.
42. Kubota, R., et al., *A Novel Myosin-like Protein (Myocilin) Expressed in the Connecting Cilium of the Photoreceptor: Molecular Cloning, Tissue Expression, and Chromosomal Mapping.* Genomics, 1997. **41**(3): p. 360-369.
43. Caballero, M. and T. Borrás, *Inefficient Processing of an Olfactomedin-Deficient Myocilin Mutant: Potential Physiological Relevance to Glaucoma.* Biochemical and Biophysical Research Communications, 2001. **282**(3): p. 662-670.

44. Caballero, M., L.L.S. Rowlette, and T. Borrás, *Altered secretion of a TIGR/MYOC mutant lacking the olfactomedin domain.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2000. **1502**(3): p. 447-460.
45. Alward, W.M., et al., *Variations in the myocilin gene in patients with open-angle glaucoma.* Archives of Ophthalmology, 2002. **120**(9): p. 1189-1197.
46. Bruttini, M., et al., *Mutations in the myocilin gene in families with primary open-angle glaucoma and juvenile open-angle glaucoma.* Archives of Ophthalmology, 2003. **121**(7): p. 1034-1038.
47. Jansson, M., et al., *Allelic variants in the MYOC/TIGR gene in patients with primary open-angle, exfoliative glaucoma and unaffected controls.* Ophthalmic Genetics, 2003. **24**(2): p. 103-110.
48. Faucher, M., et al., *Founder TIGR/myocilin mutations for glaucoma in the Quebec population*. Vol. 11. 2002. 2077-90.
49. Brown, M.D., et al., *Leber's hereditary optic neuropathy: a model for mitochondrial neurodegenerative diseases.* The FASEB Journal, 1992. **6**(10): p. 2791-2799.
50. *Comparison of glaucomatous progression between untreated patients with normal-tension glaucoma and patients with therapeutically reduced intraocular pressures. Collaborative Normal-Tension Glaucoma Study Group.* Am J Ophthalmol, 1998. **126**(4): p. 487-97.
51. Rezaie, T., et al., *Adult-onset primary open-angle glaucoma caused by mutations in optineurin.* Science, 2002. **295**(5557): p. 1077-9.
52. Alward, W.L.M., et al., *Evaluation of optineurin sequence variations in 1,048 patients with open-angle glaucoma.* American Journal of Ophthalmology, 2003. **136**(5): p. 904-910.
53. Hauser, M.A., et al., *Distribution of Optineurin Sequence Variations in an Ethnically Diverse Population of Low-tension Glaucoma Patients From the United States.* Journal of Glaucoma, 2006. **15**(5): p. 358-363.
54. Leung, Y.F., et al., *Different Optineurin Mutation Pattern in Primary Open-Angle Glaucoma.* Investigative Ophthalmology & Visual Science, 2003. **44**(9): p. 3880-3884.
55. Wiggs, J.L., et al., *Lack of Association of Mutations in Optineurin With Disease in Patients With Adult-onset Primary Open-angle Glaucoma.* Archives of ophthalmology, 2003. **121**(8): p. 1181-1183.
56. Li, Y., J. Kang, and M.S. Horwitz, *Interaction of an Adenovirus E3 14.7-Kilodalton Protein with a Novel Tumor Necrosis Factor Alpha-Inducible Cellular Protein Containing Leucine Zipper Domains.* Molecular and Cellular Biology, 1998. **18**(3): p. 1601-1610.
57. Vittitow, J.L. and T. Borrás, *Expression of optineurin, a glaucoma-linked gene, is influenced by elevated intraocular pressure.* Biochemical and Biophysical Research Communications, 2002. **298**(1): p. 67-74.
58. Kroeber, M., et al., *Transgenic studies on the role of optineurin in the mouse eye.* Experimental Eye Research, 2006. **82**(6): p. 1075-1085.
59. De Marco, N., et al., *Optineurin Increases Cell Survival and Translocates to the Nucleus in a Rab8-dependent Manner upon an Apoptotic Stimulus*. Vol. 281. 2006. 16147-56.
60. Monemi, S., et al., *Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22.1.* Hum Mol Genet, 2005. **14**(6): p. 725-33.
61. Miyazawa, A., et al., *Association between primary open-angle glaucoma and WDR36 DNA sequence variants in Japanese.* Mol Vis, 2007. **13**: p. 1912-9.
62. Pasutto, F., et al., *Profiling of WDR36 Missense Variants in German Patients with Glaucoma.* Investigative Ophthalmology & Visual Science, 2008. **49**(1): p. 270-274.
63. Fingert, J.H., et al., *No association between variations in the WDR36 gene and primary open-angle glaucoma.* Arch Ophthalmol, 2007. **125**(3): p. 434-6.
64. Hewitt, A.W., et al., *A Glaucoma Case-control Study of the WDR36 Gene D658G sequence variant.* Am J Ophthalmol, 2006. **142**(2): p. 324-5.

65. Hauser, M.A., et al., *Distribution of WDR36 DNA sequence variants in patients with primary open-angle glaucoma.* Invest Ophthalmol Vis Sci, 2006. **47**(6): p. 2542-6.
66. Kramer, P.L., et al., *The role of the wdr36 gene on chromosome 5q22.1 in a large family with primary open-angle glaucoma mapped to this region.* Archives of Ophthalmology, 2006. **124**(9): p. 1328-1331.
67. Mao, M., et al., *T lymphocyte activation gene identification by coregulated expression on DNA microarrays.* Genomics, 2004. **83**(6): p. 989-99.
68. Skarie, J.M. and B.A. Link, *The primary open-angle glaucoma gene WDR36 functions in ribosomal RNA processing and interacts with the p53 stress-response pathway.* Hum Mol Genet, 2008. **17**(16): p. 2474-85.
69. Quigley, H.A. and A.T. Broman, *The number of people with glaucoma worldwide in 2010 and 2020.* Br J Ophthalmol, 2006. **90**(3): p. 262-7.
70. Foster, P.J., et al., *The definition and classification of glaucoma in prevalence surveys.* Br J Ophthalmol, 2002. **86**(2): p. 238-42.
71. Salmon, J.F., et al., *The prevalence of primary angle closure glaucoma and open angle glaucoma in Mamre, western Cape, South Africa.* Arch Ophthalmol, 1993. **111**(9): p. 1263-9.
72. Lowe, R.F., *Primary angle-closure glaucoma. Inheritance and environment.* Br J Ophthalmol, 1972. **56**(1): p. 13-20.
73. Alsbirk, P.H., *Anterior chamber depth and primary angle-closure glaucoma. II. A genetic study.* Acta Ophthalmol (Copenh), 1975. **53**(3): p. 436-49.
74. Alsbirk, P.H., *Anterior chamber depth and primary angle-closure glaucoma. I. An epidemiologic study in Greenland Eskimos.* Acta Ophthalmol (Copenh), 1975. **53**(1): p. 89-104.
75. Hu, C.N., *[An epidemiologic study of glaucoma in Shunyi County, Beijing].* Zhonghua Yan Ke Za Zhi, 1989. **25**(2): p. 115-9.
76. Pasutto, F., et al., *Heterozygous NTF4 mutations impairing neurotrophin-4 signaling in patients with primary open-angle glaucoma.* Am J Hum Genet, 2009. **85**(4): p. 447-56.
77. Othman, M.I., et al., *Autosomal dominant nanophthalmos (NNO1) with high hyperopia and angle-closure glaucoma maps to chromosome 11.* Am J Hum Genet, 1998. **63**(5): p. 1411-8.
78. Cong, Y., et al., *Association of the single nucleotide polymorphisms in the extracellular matrix metalloprotease-9 gene with PACG in southern China.* Mol Vis, 2009. **15**: p. 1412-7.
79. Micheal, S., et al., *Polymorphisms in matrix metalloproteinases MMP1 and MMP9 are associated with primary open-angle and angle closure glaucoma in a Pakistani population.* Mol Vis, 2013. **19**: p. 441-7.
80. Wang, I.J., et al., *The association of single nucleotide polymorphisms in the MMP-9 genes with susceptibility to acute primary angle closure glaucoma in Taiwanese patients.* Mol Vis, 2006. **12**: p. 1223-32.
81. Aung, T., et al., *Lack of association between the rs2664538 polymorphism in the MMP-9 gene and primary angle closure glaucoma in Singaporean subjects.* J Glaucoma, 2008. **17**(4): p. 257-8.
82. Abu-Amero, K.K., et al., *Nuclear and mitochondrial analysis of patients with primary angle-closure glaucoma.* Invest Ophthalmol Vis Sci, 2007. **48**(12): p. 5591-6.
83. Faucher, M., et al., *Founder TIGR/myocilin mutations for glaucoma in the Quebec population.* Hum Mol Genet, 2002. **11**(18): p. 2077-90.
84. Vincent, A.L., et al., *Digenic inheritance of early-onset glaucoma: CYP1B1, a potential modifier gene.* Am J Hum Genet, 2002. **70**(2): p. 448-60.
85. Aung, T., et al., *Molecular analysis of the myocilin gene in Chinese subjects with chronic primary-angle closure glaucoma.* Invest Ophthalmol Vis Sci, 2005. **46**(4): p. 1303-6.
86. Chakrabarti, S., et al., *Glaucoma-associated CYP1B1 mutations share similar haplotype backgrounds in POAG and PACG phenotypes.* Invest Ophthalmol Vis Sci, 2007. **48**(12): p. 5439-44.

87. Dai, X., et al., *[Two variants in MYOC and CYP1B1 genes in a Chinese family with primary angle-closure glaucoma].* Zhonghua Yi Xue Yi Chuan Xue Za Zhi, 2008. **25**(5): p. 493-6.
88. Ho, C.L. and D.S. Walton, *Primary congenital glaucoma: 2004 update.* J Pediatr Ophthalmol Strabismus, 2004. **41**(5): p. 271-88; quiz 300-1.
89. Sarfarazi, M. and I. Stoilov, *Molecular genetics of primary congenital glaucoma.* Eye (Lond), 2000. **14 ( Pt 3B)**: p. 422-8.
90. Anderson, D.R., *The development of the trabecular meshwork and its abnormality in primary infantile glaucoma.* Trans Am Ophthalmol Soc, 1981. **79**: p. 458-85.
91. Kupfer, C. and M.I. Kaiser-Kupfer, *Observations on the development of the anterior chamber angle with reference to the pathogenesis of congenital glaucomas.* Am J Ophthalmol, 1979. **88**(3 Pt 1): p. 424-6.
92. Maumenee, A.E., *The pathogenesis of congenital glaucoma: a new theory.* Trans Am Ophthalmol Soc, 1958. **56**: p. 507-70.
93. Francois, J., *Congenital glaucoma and its inheritance.* Ophthalmologica, 1980. **181**(2): p. 61-73.
94. Jay, B. and G. Paterson, *The genetics of simple glaucoma.* Trans Ophthalmol Soc U K, 1970. **90**: p. 161-71.
95. Quigley, H.A., *The pathogenesis of reversible cupping in congenital glaucoma.* Am J Ophthalmol, 1977. **84**(3): p. 358-70.
96. Shaffer, R.N. and J. Hetherington, Jr., *The glaucomatous disc in infants. A suggested hypothesis for disc cupping.* Trans Am Acad Ophthalmol Otolaryngol, 1969. **73**(5): p. 923-35.
97. Iwata, K., et al., *On the reversibility of the glaucomatous disc cupping and the visual field.* Jpn J Clin Ophthalmol, 1977. **31**: p. 759-765.
98. Faiq, M., et al., *Genetic, Biochemical and Clinical Insights into Primary Congenital Glaucoma.* J Curr Glaucoma Pract, 2013. **7**(2): p. 66-84.
99. Sarfarazi, M., et al., *Assignment of a locus (GLC3A) for primary congenital glaucoma (Buphthalmos) to 2p21 and evidence for genetic heterogeneity.* Genomics, 1995. **30**(2): p. 171-7.
100. Akarsu, A.N., et al., *A second locus (GLC3B) for primary congenital glaucoma (Buphthalmos) maps to the 1p36 region.* Hum Mol Genet, 1996. **5**(8): p. 1199-203.
101. Stoilov, I., A.N. Akarsu, and M. Sarfarazi, *Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21.* Hum Mol Genet, 1997. **6**(4): p. 641-7.
102. Kakiuchi-Matsumoto, T., et al., *Cytochrome P450 1B1 gene mutations in Japanese patients with primary congenital glaucoma(1).* Am J Ophthalmol, 2001. **131**(3): p. 345-50.
103. Martin, S.N., et al., *Molecular characterisation of congenital glaucoma in a consanguineous Canadian community: a step towards preventing glaucoma related blindness.* J Med Genet, 2000. **37**(6): p. 422-7.
104. Mashima, Y., et al., *Novel cytochrome P4501B1 (CYP1B1) gene mutations in Japanese patients with primary congenital glaucoma.* Invest Ophthalmol Vis Sci, 2001. **42**(10): p. 2211-6.
105. Ohtake, Y., et al., *Phenotype of cytochrome P4501B1 gene (CYP1B1) mutations in Japanese patients with primary congenital glaucoma.* Br J Ophthalmol, 2003. **87**(3): p. 302-4.
106. Bejjani, B.A., et al., *Mutations in CYP1B1, the gene for cytochrome P4501B1, are the predominant cause of primary congenital glaucoma in Saudi Arabia.* Am J Hum Genet, 1998. **62**(2): p. 325-33.
107. Bejjani, B.A., et al., *Multiple CYP1B1 mutations and incomplete penetrance in an inbred population segregating primary congenital glaucoma suggest frequent de novo events and a dominant modifier locus.* Hum Mol Genet, 2000. **9**(3): p. 367-74.
108. Colomb, E., J. Kaplan, and H.J. Garchon, *Novel cytochrome P450 1B1 (CYP1B1) mutations in patients with primary congenital glaucoma in France.* Hum Mutat, 2003. **22**(6): p. 496.

109. Panicker, S.G., et al., *Identification of novel mutations causing familial primary congenital glaucoma in Indian pedigrees.* Invest Ophthalmol Vis Sci, 2002. **43**(5): p. 1358-66.
110. Chakrabarti, S., et al., *Globally, CYP1B1 mutations in primary congenital glaucoma are strongly structured by geographic and haplotype backgrounds.* Invest Ophthalmol Vis Sci, 2006. **47**(1): p. 43-7.
111. Murray, G.I., et al., *Regulation, function, and tissue-specific expression of cytochrome P450 CYP1B1.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 297-316.
112. Hakkola, J., et al., *Expression of CYP1B1 in human adult and fetal tissues and differential inducibility of CYP1B1 and CYP1A1 by Ah receptor ligands in human placenta and cultured cells.* Carcinogenesis, 1997. **18**(2): p. 391-7.
113. Crespi, C.L., et al., *Development of a human lymphoblastoid cell line constitutively expressing human CYP1B1 cDNA: substrate specificity with model substrates and promutagens.* Mutagenesis, 1997. **12**(2): p. 83-9.
114. Shimada, T., et al., *Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1.* Cancer Res, 1996. **56**(13): p. 2979-84.
115. Nebert, D.W., *Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions.* Mol Endocrinol, 1991. **5**(9): p. 1203-14.
116. Schwartzman, M.L., et al., *12(R)-hydroxyicosatetraenoic acid: a cytochrome-P450-dependent arachidonate metabolite that inhibits Na+,K+-ATPase in the cornea.* Proc Natl Acad Sci U S A, 1987. **84**(22): p. 8125-9.
117. Buters, J.T., et al., *Cytochrome P450 CYP1B1 determines susceptibility to 7, 12-dimethylbenz[a]anthracene-induced lymphomas.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 1977-82.
118. Libby, R.T., et al., *Modification of ocular defects in mouse developmental glaucoma models by tyrosinase.* Science, 2003. **299**(5612): p. 1578-81.
119. Nishimura, D.Y., et al., *The forkhead transcription factor gene FKHL7 is responsible for glaucoma phenotypes which map to 6p25.* Nat Genet, 1998. **19**(2): p. 140-7.
120. Hong, H.K., J.H. Lass, and A. Chakravarti, *Pleiotropic skeletal and ocular phenotypes of the mouse mutation congenital hydrocephalus (ch/Mf1) arise from a winged helix/forkhead transcriptionfactor gene.* Hum Mol Genet, 1999. **8**(4): p. 625-37.
121. *Secondary Glaucomas*. 2017: International Glaucoma Association.
122. *The effectiveness of intraocular pressure reduction in the treatment of normal-tension glaucoma. Collaborative Normal-Tension Glaucoma Study Group.* Am J Ophthalmol, 1998. **126**(4): p. 498-505.
123. *The Advanced Glaucoma Intervention Study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration.The AGIS Investigators.* Am J Ophthalmol, 2000. **130**(4): p. 429-40.
124. Anderson, D.R., *Collaborative normal tension glaucoma study.* Curr Opin Ophthalmol, 2003. **14**(2): p. 86-90.
125. Gordon, M.O., et al., *The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma.* Arch Ophthalmol, 2002. **120**(6): p. 714-20; discussion 829-30.
126. Leske, M.C., et al., *Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial.* Arch Ophthalmol, 2003. **121**(1): p. 48-56.
127. Mohammad Shahidullah, Waleed Hassan Al-Malki, and N.A. Delamere, *Mechanism of Aqueous Humor Secretion, Its Regulation and Relevance to Glaucoma*. 2011, Glaucoma - Basic and Clinical Concepts: InTech.

128. Millar C and K. PL., *Aqueous humor: secretion and dynamics*. 1995, Philadelphia: Lippincott-Raven; : Duane's foundations of clinical ophthalmology. .
129. Abu-Hassan, D.W., T.S. Acott, and M.J. Kelley, *The Trabecular Meshwork: A Basic Review of Form and Function.* J Ocul Biol, 2014. **2**(1).
130. EM., V.B., *Clinical Atlas of Glaucoma.* . 1986, Philadelphia.
131. Flocks, M., *The anatomy of the trabecular meshwork as seen in tangential section.* AMA Arch Ophthalmol, 1956. **56**(5): p. 708-18.
132. Fine, B.S., *OBSERVATIONS ON THE DRAINAGE ANGLE IN MAN AND RHESUS MONKEY: A CONCEPT OF THE PATHOGENESIS OF CHRONIC SIMPLE GLAUCOMA. A LIGHT AND ELECTRON MICROSCOPIC STUDY.* Invest Ophthalmol, 1964. **3**: p. 609-46.
133. Acott, T.S. and M.J. Kelley, *Extracellular matrix in the trabecular meshwork.* Exp Eye Res, 2008. **86**(4): p. 543-61.
134. Johnson, M., *'What controls aqueous humour outflow resistance?'.* Exp Eye Res, 2006. **82**(4): p. 545-57.
135. Lutjen-Drecoll, E., *Importance of trabecular meshwork changes in the pathogenesis of primary open-angle glaucoma.* J Glaucoma, 2000. **9**(6): p. 417-8.
136. Lutjen-Drecoll, E., *Morphological changes in glaucomatous eyes and the role of TGFbeta2 for the pathogenesis of the disease.* Exp Eye Res, 2005. **81**(1): p. 1-4.
137. Lutjen-Drecoll, E. and J. Rohen, *Functional Morphology of the Trabecular Meshwork*. 2001, J.B. Lippincott Company; Philadelphia: Duane's Foundations of Clinical Ophthalmology.
138. Lutjen-Drecoll, E., et al., *Quantitative analysis of 'plaque material' in the inner- and outer wall of Schlemm's canal in normal- and glaucomatous eyes.* Exp Eye Res, 1986. **42**(5): p. 443-55.
139. Lütjen-Drecoll, E., E. Tamm, and G. Krieglstein, *Differences in the amount of `plaque-material' in the outflow system of eyes with chronic simple and exfoliation glaucoma*. 1987.
140. Rohen, J. and E. Lutjen-Drecoll, *Biology of the trabecular meshwork*. 1st ed. ed. 1982, Stuttgard; Schattauer FK, Verlap: Basic Aspects of Glaucoma Research.
141. Rohen, J.W., *Why is intraocular pressure elevated in chronic simple glaucoma? Anatomical considerations.* Ophthalmology, 1983. **90**(7): p. 758-65.
142. Rohen, J.W., et al., *Ultrastructure of the trabecular meshwork in untreated cases of primary open-angle glaucoma (POAG).* Exp Eye Res, 1993. **56**(6): p. 683-92.
143. Rohen, J.W., D.O. Schachtschabel, and R. Wehrmann, *Structural changes of human and monkey trabecular meshwork following in vitro cultivation.* Graefes Arch Clin Exp Ophthalmol, 1982. **218**(5): p. 225-32.
144. Roos, H., et al., *Markers of cartilage matrix metabolism in human joint fluid and serum: the effect of exercise.* Osteoarthritis Cartilage, 1995. **3**(1): p. 7-14.
145. Kaufman, P. and T. Mittag, *Medical therapy of glaucoma*. 1994, Mosby; St Louis: Textbook of Ophthalmology.
146. Kaufman, P.L., *Enhancing trabecular outflow by disrupting the actin cytoskeleton, increasing uveoscleral outflow with prostaglandins, and understanding the pathophysiology of presbyopia interrogating Mother Nature: asking why, asking how, recognizing the signs, following the trail.* Exp Eye Res, 2008. **86**(1): p. 3-17.
147. Pattabiraman, P.P. and P.V. Rao, *Mechanistic basis of Rho GTPase-induced extracellular matrix synthesis in trabecular meshwork cells.* Am J Physiol Cell Physiol, 2010. **298**(3): p. C749-63.
148. Rao, P.V., et al., *Regulation of myosin light chain phosphorylation in the trabecular meshwork: role in aqueous humour outflow facility.* Exp Eye Res, 2005. **80**(2): p. 197-206.
149. Rao, P.V., et al., *Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632.* Invest Ophthalmol Vis Sci, 2001. **42**(5): p. 1029-37.

150. Clark, A.F., et al., *Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue.* Cell Motil Cytoskeleton, 2005. **60**(2): p. 83-95.
151. Clark, A.F., et al., *Cytoskeletal changes in cultured human glaucoma trabecular meshwork cells.* J Glaucoma, 1995. **4**(3): p. 183-8.
152. Clark, A.F., et al., *Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells.* Invest Ophthalmol Vis Sci, 1994. **35**(1): p. 281-94.
153. Faralli, J.A., et al., *Functional properties of fibronectin in the trabecular meshwork.* Exp Eye Res, 2009. **88**(4): p. 689-93.
154. Filla, M.S., et al., *Dexamethasone-associated cross-linked actin network formation in human trabecular meshwork cells involves beta3 integrin signaling.* Invest Ophthalmol Vis Sci, 2011. **52**(6): p. 2952-9.
155. Filla, M.S., et al., *Beta1 and beta3 integrins cooperate to induce syndecan-4-containing cross-linked actin networks in human trabecular meshwork cells.* Invest Ophthalmol Vis Sci, 2006. **47**(5): p. 1956-67.
156. Liu, X., et al., *The effect of C3 transgene expression on actin and cellular adhesions in cultured human trabecular meshwork cells and on outflow facility in organ cultured monkey eyes.* Mol Vis, 2005. **11**: p. 1112-21.
157. O'Reilly, S., et al., *Inducers of cross-linked actin networks in trabecular meshwork cells.* Invest Ophthalmol Vis Sci, 2011. **52**(10): p. 7316-24.
158. Tan, J.C., D.M. Peters, and P.L. Kaufman, *Recent developments in understanding the pathophysiology of elevated intraocular pressure.* Curr Opin Ophthalmol, 2006. **17**(2): p. 168-74.
159. Tian, B., et al., *Cytoskeletal involvement in the regulation of aqueous humor outflow.* Invest Ophthalmol Vis Sci, 2000. **41**(3): p. 619-23.
160. Peterson, J.A., et al., *Latrunculin-A increases outflow facility in the monkey.* Invest Ophthalmol Vis Sci, 1999. **40**(5): p. 931-41.
161. Peterson, J.A., et al., *Effect of latrunculin-B on outflow facility in monkeys.* Exp Eye Res, 2000. **70**(3): p. 307-13.
162. Peterson, J.A., et al., *Latrunculins' effects on intraocular pressure, aqueous humor flow, and corneal endothelium.* Invest Ophthalmol Vis Sci, 2000. **41**(7): p. 1749-58.
163. Sabanay, I., et al., *H-7 effects on the structure and fluid conductance of monkey trabecular meshwork.* Arch Ophthalmol, 2000. **118**(7): p. 955-62.
164. Epstein, D.L., L.L. Rowlette, and B.C. Roberts, *Acto-myosin drug effects and aqueous outflow function.* Invest Ophthalmol Vis Sci, 1999. **40**(1): p. 74-81.
165. Honjo, M., et al., *A myosin light chain kinase inhibitor, ML-9, lowers the intraocular pressure in rabbit eyes.* Exp Eye Res, 2002. **75**(2): p. 135-42.
166. Khurana, R.N., et al., *The role of protein kinase C in modulation of aqueous humor outflow facility.* Exp Eye Res, 2003. **76**(1): p. 39-47.
167. Rao, P.V., et al., *Expression of dominant negative Rho-binding domain of Rho-kinase in organ cultured human eye anterior segments increases aqueous humor outflow.* Mol Vis, 2005. **11**: p. 288-97.
168. Vittitow, J.L., et al., *Gene transfer of dominant-negative RhoA increases outflow facility in perfused human anterior segment cultures.* Mol Vis, 2002. **8**: p. 32-44.
169. de Kater, A.W., A. Shahsafaei, and D.L. Epstein, *Localization of smooth muscle and nonmuscle actin isoforms in the human aqueous outflow pathway.* Invest Ophthalmol Vis Sci, 1992. **33**(2): p. 424-9.
170. Ethier, C.R., A.T. Read, and D. Chan, *Biomechanics of Schlemm's canal endothelial cells: influence on F-actin architecture.* Biophys J, 2004. **87**(4): p. 2828-37.

171. Flu¨gel, C., et al., *Age-related loss of a-smooth muscle actin in normal and glaucomatous human trabecular meshwork of different age groups.* J. Glaucoma 2002. **1**: p. 165–173.
172. Gipson, I.K. and R.A. Anderson, *Actin filaments in cells of human trabecular meshwork and Schlemm's canal.* Invest Ophthalmol Vis Sci, 1979. **18**(6): p. 547-61.
173. Wiederholt, M., H. Thieme, and F. Stumpff, *The regulation of trabecular meshwork and ciliary muscle contractility.* Prog Retin Eye Res, 2000. **19**(3): p. 271-95.
174. Read, A.T., D.W. Chan, and C.R. Ethier, *Actin structure in the outflow tract of normal and glaucomatous eyes.* Exp Eye Res, 2006. **82**(6): p. 974-85.
175. Inatani, M., et al., *Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes.* Graefes Arch Clin Exp Ophthalmol, 2001. **239**(2): p. 109-13.
176. Min, S.H., et al., *Transforming growth factor-beta levels in human aqueous humor of glaucomatous, diabetic and uveitic eyes.* Korean J Ophthalmol, 2006. **20**(3): p. 162-5.
177. Picht, G., et al., *Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development.* Graefes Arch Clin Exp Ophthalmol, 2001. **239**(3): p. 199-207.
178. Bachmann, B., et al., *Ultrastructural and biochemical evaluation of the porcine anterior chamber perfusion model.* Invest Ophthalmol Vis Sci, 2006. **47**(5): p. 2011-20.
179. Bhattacharya, S.K., et al., *Cochlin expression in anterior segment organ culture models after TGFbeta2 treatment.* Invest Ophthalmol Vis Sci, 2009. **50**(2): p. 551-9.
180. Fleenor, D.L., et al., *TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure.* Invest Ophthalmol Vis Sci, 2006. **47**(1): p. 226-34.
181. Gottanka, J., et al., *Effects of TGF-beta2 in perfused human eyes.* Invest Ophthalmol Vis Sci, 2004. **45**(1): p. 153-8.
182. Hill, L.J., et al., *Decorin Reduces Intraocular Pressure and Retinal Ganglion Cell Loss in Rodents Through Fibrolysis of the Scarred Trabecular Meshwork.* Invest Ophthalmol Vis Sci, 2015. **56**(6): p. 3743-57.
183. McDowell, C.M., et al., *Smad3 is necessary for transforming growth factor-beta2 induced ocular hypertension in mice.* Exp Eye Res, 2013. **116**: p. 419-23.
184. Robertson, J.V., et al., *Ocular gene transfer of active TGF-beta induces changes in anterior segment morphology and elevated IOP in rats.* Invest Ophthalmol Vis Sci, 2010. **51**(1): p. 308-18.
185. Shepard, A.R., et al., *Adenoviral gene transfer of active human transforming growth factor-{beta}2 elevates intraocular pressure and reduces outflow facility in rodent eyes.* Invest Ophthalmol Vis Sci, 2010. **51**(4): p. 2067-76.
186. Swaminathan, S.S., et al., *TGF-beta2-mediated ocular hypertension is attenuated in SPARC-null mice.* Invest Ophthalmol Vis Sci, 2014. **55**(7): p. 4084-97.
187. Fuchshofer, R., et al., *Gene expression profiling of TGFbeta2- and/or BMP7-treated trabecular meshwork cells: Identification of Smad7 as a critical inhibitor of TGF-beta2 signaling.* Exp Eye Res, 2009. **88**(6): p. 1020-32.
188. Tovar-Vidales, T., A.F. Clark, and R.J. Wordinger, *Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells.* Exp Eye Res, 2011. **93**(4): p. 442-51.
189. Han, H., et al., *Elasticity-dependent modulation of TGF-beta responses in human trabecular meshwork cells.* Invest Ophthalmol Vis Sci, 2011. **52**(6): p. 2889-96.
190. Pattabiraman, P.P., R. Maddala, and P.V. Rao, *Regulation of plasticity and fibrogenic activity of trabecular meshwork cells by Rho GTPase signaling.* J Cell Physiol, 2014. **229**(7): p. 927-42.
191. Von Zee, C.L., K.A. Langert, and E.B. Stubbs, Jr., *Transforming growth factor-beta2 induces synthesis and secretion of endothelin-1 in human trabecular meshwork cells.* Invest Ophthalmol Vis Sci, 2012. **53**(9): p. 5279-86.

192. Hall, A., *Rho GTPases and the actin cytoskeleton.* Science, 1998. **279**(5350): p. 509-14.
193. Narumiya, S., T. Ishizaki, and N. Watanabe, *Rho effectors and reorganization of actin cytoskeleton.* FEBS Lett, 1997. **410**(1): p. 68-72.
194. Amano, M., et al., *Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase.* Science, 1997. **275**(5304): p. 1308-11.
195. Leung, T., et al., *The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton.* Mol Cell Biol, 1996. **16**(10): p. 5313-27.
196. Uehata, M., et al., *Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension.* Nature, 1997. **389**(6654): p. 990-4.
197. Kimura, K., et al., *Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase).* Science, 1996. **273**(5272): p. 245-8.
198. Welch, M.D., et al., *Actin dynamics in vivo.* Curr Opin Cell Biol, 1997. **9**(1): p. 54-61.
199. Bamburg, J.R., A. McGough, and S. Ono, *Putting a new twist on actin: ADF/cofilins modulate actin dynamics.* Trends Cell Biol, 1999. **9**(9): p. 364-70.
200. Carlier, M.F. and D. Pantaloni, *Control of actin dynamics in cell motility.* J Mol Biol, 1997. **269**(4): p. 459-67.
201. Agnew, B.J., L.S. Minamide, and J.R. Bamburg, *Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site.* J Biol Chem, 1995. **270**(29): p. 17582-7.
202. Arber, S., et al., *Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase.* Nature, 1998. **393**(6687): p. 805-9.
203. Yang, N., et al., *Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization.* Nature, 1998. **393**(6687): p. 809-12.
204. Bernard, O., et al., *Kiz-1, a protein with LIM zinc finger and kinase domains, is expressed mainly in neurons.* Cell Growth Differ, 1994. **5**(11): p. 1159-71.
205. Mizuno, K., et al., *Identification of a human cDNA encoding a novel protein kinase with two repeats of the LIM/double zinc finger motif.* Oncogene, 1994. **9**(6): p. 1605-12.
206. Nunoue, K., et al., *LIMK-1 and LIMK-2, two members of a LIM motif-containing protein kinase family.* Oncogene, 1995. **11**(4): p. 701-10.
207. Ohashi, K., et al., *Molecular cloning of a chicken lung cDNA encoding a novel protein kinase with N-terminal two LIM/double zinc finger motifs.* J Biochem, 1994. **116**(3): p. 636-42.
208. Okano, I., et al., *Identification and characterization of a novel family of serine/threonine kinases containing two N-terminal LIM motifs.* J Biol Chem, 1995. **270**(52): p. 31321-30.
209. Maekawa, M., et al., *Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase.* Science, 1999. **285**(5429): p. 895-8.
210. Goldhagen, B., et al., *Elevated levels of RhoA in the optic nerve head of human eyes with glaucoma.* J Glaucoma, 2012. **21**(8): p. 530-8.
211. Honjo, M., et al., *Effects of protein kinase inhibitor, HA1077, on intraocular pressure and outflow facility in rabbit eyes.* Arch Ophthalmol, 2001. **119**(8): p. 1171-8.
212. Nakajima, E., et al., *Contribution of ROCK in contraction of trabecular meshwork: proposed mechanism for regulating aqueous outflow in monkey and human eyes.* J Pharm Sci, 2005. **94**(4): p. 701-8.
213. Thieme, H., et al., *Mediation of calcium-independent contraction in trabecular meshwork through protein kinase C and rho-A.* Invest Ophthalmol Vis Sci, 2000. **41**(13): p. 4240-6.
214. Wang, J., X. Liu, and Y. Zhong, *Rho/Rho-associated kinase pathway in glaucoma (Review).* Int J Oncol, 2013. **43**(5): p. 1357-67.

215. Zhang, M., R. Maddala, and P.V. Rao, *Novel molecular insights into RhoA GTPase-induced resistance to aqueous humor outflow through the trabecular meshwork.* Am J Physiol Cell Physiol, 2008. **295**(5): p. C1057-70.
216. Fukunaga, T., et al., *The effect of the Rho-associated protein kinase inhibitor, HA-1077, in the rabbit ocular hypertension model induced by water loading.* Curr Eye Res, 2009. **34**(1): p. 42-7.
217. Honjo, M., et al., *Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility.* Invest Ophthalmol Vis Sci, 2001. **42**(1): p. 137-44.
218. Nishio, M., et al., *The effect of the H-1152P, a potent Rho-associated coiled coil-formed protein kinase inhibitor, in rabbit normal and ocular hypertensive eyes.* Curr Eye Res, 2009. **34**(4): p. 282-6.
219. Tanihara, H., et al., *Intraocular pressure-lowering effects and safety of topical administration of a selective ROCK inhibitor, SNJ-1656, in healthy volunteers.* Arch Ophthalmol, 2008. **126**(3): p. 309-15.
220. Tian, B. and P.L. Kaufman, *Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys.* Exp Eye Res, 2005. **80**(2): p. 215-25.
221. Tokushige, H., et al., *Effects of topical administration of y-39983, a selective rho-associated protein kinase inhibitor, on ocular tissues in rabbits and monkeys.* Invest Ophthalmol Vis Sci, 2007. **48**(7): p. 3216-22.
222. Waki, M., et al., *Reduction of intraocular pressure by topical administration of an inhibitor of the Rho-associated protein kinase.* Curr Eye Res, 2001. **22**(6): p. 470-4.
223. Whitlock, N.A., et al., *Decreased intraocular pressure in mice following either pharmacological or genetic inhibition of ROCK.* J Ocul Pharmacol Ther, 2009. **25**(3): p. 187-94.
224. Yu, M., et al., *H-1152 effects on intraocular pressure and trabecular meshwork morphology of rat eyes.* J Ocul Pharmacol Ther, 2008. **24**(4): p. 373-9.
225. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Res, 2009. **19**(1): p. 92-105.
226. Ambros, V., *The functions of animal microRNAs.* Nature, 2004. **431**(7006): p. 350-5.
227. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
228. Sayed, D. and M. Abdellatif, *MicroRNAs in development and disease.* Physiol Rev, 2011. **91**(3): p. 827-87.
229. Gonzalez, P., et al., *Role of microRNAs in the trabecular meshwork.* J Ocul Pharmacol Ther, 2014. **30**(2-3): p. 128-37.
230. Jayaram, H., et al., *Comparison of MicroRNA Expression in Aqueous Humor of Normal and Primary Open-Angle Glaucoma Patients Using PCR Arrays: A Pilot Study.* Invest Ophthalmol Vis Sci, 2017. **58**(7): p. 2884-2890.
231. Drewry, M., et al., *miRNA Profile in Three Different Normal Human Ocular Tissues by miRNA-Seq.* Invest Ophthalmol Vis Sci, 2016. **57**(8): p. 3731-9.
232. Agarwal, V., et al., *Predicting effective microRNA target sites in mammalian mRNAs.* Elife, 2015. **4**.
233. John, B., et al., *Human MicroRNA targets.* PLoS Biol, 2004. **2**(11): p. e363.
234. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**(Database issue): p. D140-4.
235. Liu, Q., et al., *siRNA silencing of gene expression in trabecular meshwork: RhoA siRNA reduces IOP in mice.* Curr Mol Med, 2012. **12**(8): p. 1015-27.
236. Betel, D., et al., *Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites.* Genome Biol, 2010. **11**(8): p. R90.

References

# Publications

1) Suresh C; **Chennakesavulu S**; Meher Gayatri; Reddy AB*. "A Novel Phosphorylation by AMP-activated Kinase regulates RUNX2 from Ubiquitination in Osteogenesis over Adipogenesis." ***Cell Death and Disease*** (**accepted in jun-2018**).

2) Devi A, **Chennakesavulu S**, Suresh C, Reddy AB*. Chapter-17. "Nutraceuticals in Health and Disease". **"Functional food and human health"**. **(2018) Springer Nature Inc.** (Book chapter ISBN: 978-981-13-1122-2)

3) **"TGF-β2- OPTN- Bmal1 axis regulated by miR-200 cluster controls aqueous humor outflow pathway and intraocular pressure".** (In Preparation)

# Understanding the molecular mechanisms of aqueous humor outflow pathway and glaucoma

*by* S. Chenna Kesavulu

**Submission date:** 25-Jun-2018 04:40PM (UTC+0530)
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