https://doi.org/10.33472/AFJBS.6.14.2024.8613-8631

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

UNDERSTANDING THE MOLECULAR MECHANISMS OF AQUEOUS HUMOR OUTFLOW PATHWAY AND GLAUCOMA

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Article Info

volume 6, Issue 14, 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.14.2024.8613-8631

ABSTRACT

Glaucoma is a leading cause of irreversible blindness globally, primarily resulting from the progressive degeneration of the optic nerve. Central to the pathophysiology of glaucoma is the dysregulation of aqueous humor outflow, which leads to elevated intraocular pressure (IOP), a critical risk factor for the disease. The aqueous humor outflow pathway involves a complex interplay between the trabecular meshwork (TM), Schlemm's canal (SC), and the uveoscleral pathway. This review provides a comprehensive analysis of the molecular mechanisms governing aqueous humor dynamics and their implications in glaucoma. We explore the roles of extracellular matrix (ECM) remodeling, cytoskeletal alterations, and cellular signaling pathways in the TM, emphasizing the impact of mechanobiological forces and oxidative stress. Additionally, we discuss the contribution of genetic factors and novel molecular targets in the regulation of IOP. Understanding these mechanisms is essential for developing innovative therapeutic strategies aimed at enhancing aqueous humor outflow and preventing glaucomatous damage. Future research directions are proposed to further elucidate the molecular underpinnings of aqueous humor outflow and identify potential biomarkers for early diagnosis and targeted treatment of glaucoma.

Keywords: Aqueous Humor Outflow, Intraocular Pressure, Glaucoma, Schlemm's Canal, Cytoskeletal Alterations, Cellular Signaling Pathways, Mechanobiology, Oxidative Stress

INTRODUCTION

Eyes are remarkable organs that enable us to see and navigate the world around us. Each eye is spherical, with a diameter of about 2.5 cm, and is situated within the skull's orbit, connected to the brain via the optic nerves. The anatomical structure of the eye is illustrated in Figure 1.

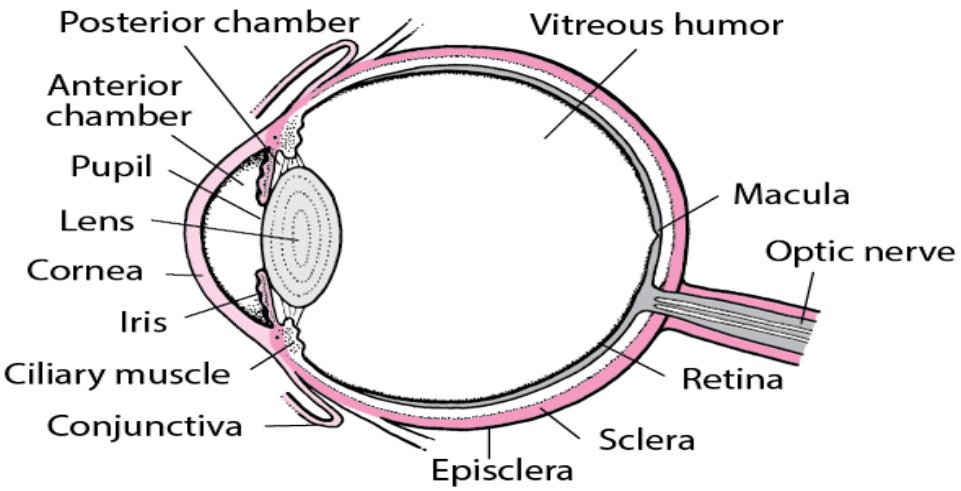


Fig 1: Anatomy of the human eye: Cross section of the human eye

Vision is the most vital sense for humans, with approximately 50% of the body's sensory receptors located in the eyes, and a significant portion of the cerebral cortex dedicated to processing visual information. The eyes detect visible light, which is reflected from objects, with wavelengths ranging from 400 to 700 nanometers. Objects appear in different colors based on the light they reflect; for instance, an object appears white if it reflects all wavelengths of light, and black if it absorbs all wavelengths. The fovea is a crucial part of the eye that plays a significant role in vision.

Cross Section of Eye

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: The sclera is the opaque, protective outer layer of the eye, composed of fibrous connective tissue. The visible portion is white and contains collagen and elastic fibers .[2]
2. Cornea**:** The cornea is the thin, transparent front part of the eye. It is avascular and absorbs oxygen from the air. [4]. The primary function of the cornea is to refract light to focus it onto the retina.
3. Conjunctiva: This is a stratified squamous epithelium that lines the inside of the eyelids and covers the cornea. Its functions include lubricating the eye by producing mucus and tears, as well as protecting the cornea.

b. Middle layer

1. Choroid: Located between the retina and the sclera, the choroid is highly vascularized and provides oxygen and nutrients to the retina, particularly to its outer layer. [5]
2. Ciliary Body: Positioned behind the posterior surface of the iris, the ciliary body contains ciliary muscles that secrete aqueous humor. It helps to hold the lens in place and adjusts its position by contracting or relaxing in response to light conditions. [6]
3. Iris: The iris is a thin, circular structure that regulates the size of the pupil. It divides the anterior segment of the eye into the anterior and posterior chambers. The amount of pigment present in the iris determines its color. [7]

c. Inner layer

Retina: The retina lines the back of the eye and is light-sensitive, containing millions of closely packed cells. Each eye has approximately 125 million rod cells and about 7 million cone cells.

The fovea centralis, located in a depression in the retinal wall, contains only cone cells, which are sensitive to light and responsible for sharp image formation. The retina comprises three types of cells: neuronal cells, photoreceptor cells, and glial cells. [7]

Eye lens and chambers

a) Eye Lens: The eye lens is a transparent and elastic structure located behind the pupil. Its elasticity allows it to easily change shape for focusing. With age, the lens can become hardened due to a loss of elasticity, leading to clouding and difficulties in focusing, a condition known as cataract. [7]

b) Anterior Chamber: This is the space between the lens and the cornea, filled with aqueous humor. This fluid contains nutrients and respiratory gases that nourish both the lens and cornea. The aqueous humor is hypertonic compared to the plasma of mammals. [8]
c) Vitreous Chamber: This region is located behind the lens and in front of the retina, filled with a transparent gel known as vitreous humor. Comprising about 98% water, along with collagen fibrils and hyaluronic acid, this gel is motionless, allowing light to pass through and impact the person's field of vision. [7]

Function of Eye
The cornea, located at the front of the eye, is where light is primarily focused as it passes through. The iris regulates the size of the pupil based on the amount of light that enters through the cornea. Behind the pupil is the eye lens, which further focuses the light onto the retina. The retina, situated at the back of the eye, is light-sensitive and converts visual images into electrical signals. These signals are then transmitted by the optic nerve to the visual cortex of the brain for processing.

Common diseases of the eye
Various diseases can impact different parts of the eye, potentially affecting vision. Fortunately, many of these conditions are treatable, but proper diagnosis and timely treatment are essential to prevent further damage.

Dry eye
Symptoms of dry eye, also known as keratoconjunctivitis sicca, occur due to inadequate tear production. Common symptoms include a burning sensation, dryness in the eyes, itching, mucoid discharge, redness (hyperemia), and sensitivity to light (photophobia) [9]

Meibomian Gland Dysfunction (MGD)
Meibomian glands secrete oil into the tears, helping to keep the eyes moisturized. When these glands malfunction, it can result in the rapid evaporation of tears. Meibomian gland dysfunction is a significant contributor to dry eye and eyelid disorders, such as blepharitis. [7].

Cataract
Cataract is the most common cause of vision loss in individuals aged 40 and older, resulting from clouding of the lens, which impairs vision. It is the leading cause of blindness worldwide. Clouding may occur in people who use steroids or have diabetes.[7].

Macular degeneration
Macular degeneration, also known as age-related macular degeneration (AMD), is a condition that results in the loss of vision and affects over 25 million people worldwide. It progressively damages the macula, which is crucial for sharp central vision. This disease disrupts the ability to perform daily activities that rely on central vision [10, 11]. Risk factors include age, smoking, cardiovascular diseases or high blood pressure, obesity, being female, and having a family history of AMD.
There are two main types of AMD: dry AMD and wet AMD. Wet AMD is more severe and accounts for up to 90% of cases of vision loss in older individuals [11]. Vision loss in AMD occurs due to abnormal function of the macula, particularly the photoreceptor cells [12]. Long-term exposure to sunlight is also suggested as a potential contributing factor to AMD [13]. Currently, there is no permanent cure for AMD available.

Hypertensive and diabetic retinopathy
Diabetic retinopathy causes vision loss due to damage to the retinal blood vessels. It can affect anyone with type 1 or type 2 diabetes. Initially, individuals may experience mild vision issues without significant symptoms, but the condition can progress to blindness over time.

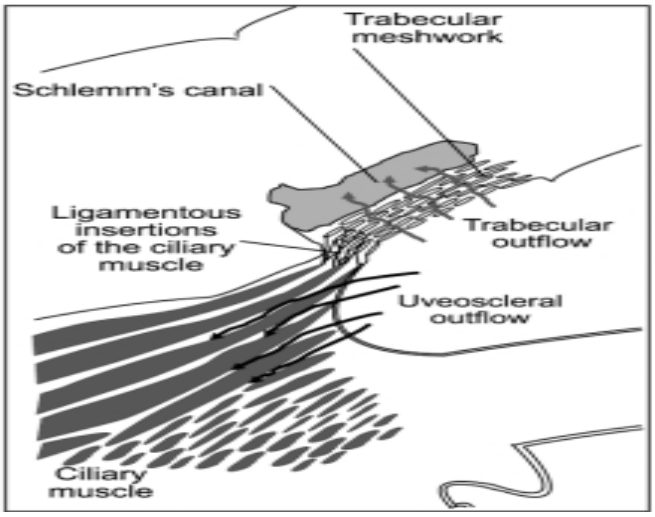


Fig 2: Schematic diagram of aqueous humor production in anterior chamber of the eye

Glaucoma

Glaucoma refers to a group of eye disorders and is the second most common cause of irreversible vision loss globally, characterized by progressive damage to the optic nerves at the optic disc. In 2013, the estimated prevalence of glaucoma was 64.3 million, projected to rise to 76 million by 2020 and 111.8 million by 2040 [15]. The condition is often asymptomatic until it reaches a severe stage, resulting in more people affected than diagnosed [16, 17]. These figures highlight the need for a better understanding of its underlying pathophysiology to improve treatment options. While the exact cause remains unknown for many types of glaucoma, it is understood to be complex, involving undisclosed genetic and biological risk factors.

Traditionally, glaucoma has been classified based on variations in the anterior segment and the appearance of the iridocorneal angle. However, modern classification recognizes three main types of glaucoma: i) Primary Open-Angle Glaucoma (POAG), ii) Primary Angle-Closure Glaucoma (PACG), and iii) Primary Congenital Glaucoma (PCG), as illustrated in Figure 3.

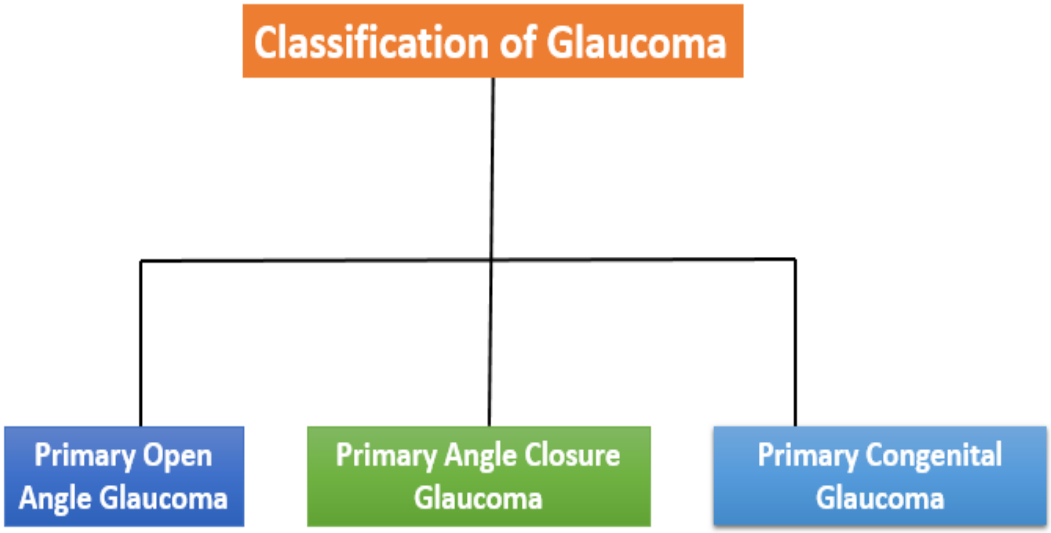


Figure 3. General classification of glaucoma.

Primary Open Angle Glaucoma (POAG)

Primary open-angle glaucoma (POAG) is characterized by a normal-looking iridocorneal angle between the cornea and iris, yet there is reduced aqueous outflow, leading to loss of visual field. While increased intraocular pressure (IOP) is a significant risk factor for glaucoma, POAG can also occur with normal IOP levels (10 to 21 mm Hg). Recent clinical

studies indicate that the only modifiable causative factor is the reduction of IOP. Consequently, treatment options primarily involve lowering IOP through medications or surgical interventions.

The inheritance pattern of the disease is multifactorial. Genetic linkage and mapping studies have identified more than 20 genetic loci associated with primary open-angle glaucoma, including mutations in WDR36, myocilin, and optineurin. Juvenile open-angle glaucoma (JOAG) is rare and shares clinical characteristics with adult-onset glaucoma, such as elevated intraocular pressure (IOP), an open angle, and optic nerve cupping. JOAG typically presents between the ages of 3 and 25, often with very high IOPs exceeding 40 mm Hg. Similar to other forms of glaucoma, its etiology is multifactorial, with some individuals showing improper angle development and an increased number of iris processes.

Genetics of POAG

The inheritance pattern of the disease is multifactorial. Genetic linkage and mapping studies have identified more than 20 genetic loci linked to this phenotype, including candidate genes like WDR36, optineurin, and myocilin.

a. Myocilin (MYOC):

Myocilin was the first gene identified in connection with the development of primary open-angle glaucoma (POAG) and juvenile open-angle glaucoma (JOAG). Long-term exposure to dexamethasone has been shown to increase the production of myocilin protein in trabecular meshwork (TM) cells.

This was the initial observation linking myocilin to the pathogenesis of glaucoma[25]. In 1993, linkage analysis initially localized myocilin to the GLC1A locus [26] subsequently, in 1997, it was linked to the MYOC gene, which has been associated with over 70 different point mutations in glaucoma patients from various ethnic groups worldwide. These mutations account for 3 to 5% of the mutations identified in the MYOC gene coding region in cases of POAG globally..[28-36].

Myocilin expression has been detected in both ocular and non-ocular tissues. The highest levels of myocilin expression have been observed in the iris, sclera, and trabecular meshwork (TM). [37-39]. The MYOC gene spans approximately 17 kb and consists of 3 exons, coding for a protein of 504 amino acids with a molecular weight of about 57 kDa.[42].

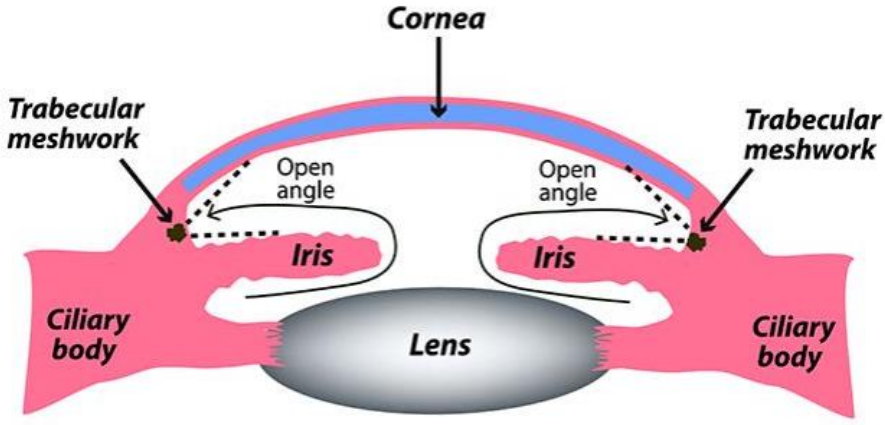


Fig 4. Schematic diagram to show wide angle in POAG

Reports indicate that exon 3 of myocilin contains the majority of mutations associated with this phenotype.

Increased expression of myocilin has been reported in response to elevated intraocular pressure (IOP), dexamethasone treatment, and trabecular stress, suggesting a protective role in aqueous humor outflow. Researchers have attempted to establish a connection between mutated myocilin and glaucoma, finding that mutated myocilin proteins exhibit poor secretion from cells and tend to accumulate within the endoplasmic reticulum. [43, 44]. Aside from this, the precise function and role of myocilin protein remain largely unknown.

Mutations in myocilin are strongly associated with both primary open-angle glaucoma (POAG) and juvenile open-angle glaucoma (JOAG). [45-47]. However, some of these mutations have also been reported in other forms of glaucoma. The Gly399Val mutation is noted in adult open-angle glaucoma, while the combination of the Gly399Val MYOC mutation with the Arg368His mutation has been observed in individuals with CYP1B1 mutations. The severity of the disease may also be influenced by digenic or polygenic interactions, similar to interactions seen in other conditions, such as Leber's hereditary optic neuropathy. [49].

b. Optineurin (OPTN)

Optineurin, another gene associated with open-angle glaucoma (OAG), was initially linked to the GLC1E locus on chromosome 10p14, [50] it was subsequently identified as the OPTN 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 H202 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.

Primary Angle Closure Glaucoma

As per reports, 15 million people were affected worldwide with PACG in 2010 and is predictedto 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].

Fluid flow is hindered by the trabecular meshwork due to glaucomatous conditions such as damage to the optic nerve head. Individuals were not classified as having high intraocular pressure (IOP) unless there was damage to the optic nerves.

This indicates that 60-75% of individuals with high intraocular pressure (IOP) will recover without damage to the optic disc. When estimating the prevalence of primary angle-closure glaucoma (PACG) and primary open-angle glaucoma (POAG) in South Africa, it was noted that individuals with chronic angle closure could have IOP levels as high as 72 mmHg, but there was no clear association established between symptoms and the onset of vision loss. [71]. Angle closure is prevalent even in East Asia, often occurring without noticeable symptoms. Symptoms alone do not fully indicate the underlying mechanism of angle closure, highlighting the importance that management strategies should not rely solely on symptomatic presentation.

Congenital Glaucoma (PCG)

Primary congenital glaucoma (PCG) is a prevalent type of glaucoma in infants. It typically manifests in children, with approximately 80% of cases appearing within the first year of life [88]. Symptoms of primary congenital glaucoma (PCG) include corneal edema, epiphora (excessive tearing), photophobia (sensitivity to light), and buphthalmos (enlargement of the eyeball). PCG affects males more frequently (65%) than females (35%), and between 60% to 80% of cases are bilateral (affecting both eyes) [89]. Primary congenital glaucoma (PCG) is inherited in an autosomal recessive manner with variable penetrance. It develops due to abnormal development of the anterior chamber angle, leading to obstruction of fluid flow within the eye. [90-92]. The incidence of primary congenital glaucoma (PCG) varies widely, ranging from 1 in 10,000 to 1 in 2,000 in developed countries and in the Middle East [93]. [94] respectively.

Primary congenital glaucoma (PCG) differs from the adult form of glaucoma in terms of optic nerve changes. In newborns with PCG, optic nerve cupping progresses rapidly. [95, 96] and optic nerve cupping in primary congenital glaucoma (PCG) can be reversible if identified early and appropriate treatment is initiated promptly. [97]. On the other hand, optic nerve damage in adults with glaucoma is generally irreversible.[95]. Primary congenital glaucoma (PCG) is multifactorial, and several genes such as CYP1B1, LTBP2, MYOC, and FOXC1 have been identified as responsible for causing PCG. However, the molecular mechanisms through which these genes affect the outflow pathway remain speculative and require further identification and study. [98].

Objectives

This doctoral study aims to:

1. Investigate the roles of TGF-β2 and glucocorticoids in regulating intraocular pressure (IOP) and contributing to the development of glaucoma.
2. Explore how candidate genes associated with glaucoma influence IOP regulation and contribute to the disease's development.
3. Determine any relationship between TGF-β2 and glaucoma candidate genes in the pathogenesis of glaucoma.
4. Investigate whether glaucoma candidate genes interact with each other and how these interactions may be involved in known signaling pathways related to intraocular pressure and disease progression.

MATERIALS AND METHODS

In silico miRNA target prediction

miRNA target prediction was conducted using freely available bioinformatics algorithms that rely on sequence complementarity. These tools include TargetScan7.2 (http://www.targetscan.org) [232], miRanda (http://www.microrna.org) [233], and miRBase (http://www.mirbase.org/) [234]. They were utilized to identify potential targets for miR-200 family members, miR-590, miR-548c, and miR-496 among TGF-β2, ROCK2, CYP1B1, FOXC1, OPTN, LTBP2, and MYOC.

Cell culture

Primary human trabecular meshwork cells (HTM) and their complete culture medium with growth factors (TMCM) were obtained from Sciencell Research Laboratories Inc. and cultured in a 37°C, 5% CO2 incubator. To simulate glaucoma pathology, recombinant human TGF-β2 (2 ng/ml; Sigma), dexamethasone (300 nM/ml; Sigma), and prednisolone (300 nM/ml; Sigma) were used. For transfection experiments in primary HTM cells, miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-496, and miR-548c mimics, as well as anti-miRs targeting each respective miRNA (Qiagen), were utilized with Dharmafect duo transfection reagent (Dharmacon). Co-transfection studies were conducted using HEK 293T cell lines (ATCC), which were cultured in DMEM supplemented with 10% FBS and 1% Pen/strep (Invitrogen-Gibco Life Technologies) at 37°C in a 5% CO2 incubator.

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

Total RNA was extracted using the miRNeasy isolation kit (Qiagen) following the manufacturer's instructions. RNA yields were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific). For mRNA analysis, complementary DNA (cDNA) was synthesized from 1.0 μg of total RNA using the miScript II RT Kit (Qiagen). Real-time PCR was conducted 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 using the ddCt method, with all mRNA quantification values normalized to GAPDH.

For miRNA analysis, real-time PCR was performed similarly using miRNA primer assays according to the manufacturer's instructions (Qiagen). All miRNA expression values were normalized to U6 small nuclear (sn) RNA.

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 mimics (miR-200a, miR-200b, miR-200c, miR-429, miR-141, miR-590, miR-548c, and miR-496) 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.

For experiments using miRNA inhibitors, they 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) or a negative control inhibitor (250 nM), using Anti-miRs (Qiagen) against each respective miRNA.

In experiments where CYP1B1, FOXC1, and OPTN were simultaneously knocked down, 100 pM of each siRNA (Qiagen) or a control siRNA (All star negative siCONTROL non-targeting siRNA No. 1, Qiagen) were utilized.

Immunoblotting

Cell extracts from transfected and treated HTM cells were prepared using 1x RIPA lysis buffer (Cell Signaling Technologies) supplemented with protease inhibitor cocktail (Pierce, Rockford, IL), and 50 µg of protein was fractionated on a 10% SDS polyacrylamide gel. Following gel electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was then probed with antibodies against CYP1B1, OPTN, MYOC, LTBP2, phosphorylated Cofilin (pCofilin), total Cofilin, Paxillin, ROCK2, TGF-β2, and GAPDH (Santa Cruz Biotechnology), as well as FOXC1 (Cell Signaling Technologies). Immunocomplexes were visualized using the ECL method (GE Healthcare) following the manufacturer's instructions.

Confocal microscopy and staining for Paxillin and F-actin

Primary HTM cells plated on poly-L-lysine (Sigma) coated coverslips were transfected with miRNA mimics and treated with recombinant human TGF-β2 (2 ng/ml; Sigma) as previously described. After 24 hours post-transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then incubated with a rabbit anti-paxillin antibody (1:500; Santa Cruz Biotechnology). Subsequently, cells were treated with 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 minutes, followed by washing and mounting with DAPI (Invitrogen) to visualize nuclei.

Stained cells were observed and images were captured using a Leica TCS SP8 confocal microscope. Image analysis was performed using Leica Cell software.

´UTR reporter analysis

The 3'UTRs of TGF-β2, ROCK2, CYP1B1, FOXC1, and OPTN were amplified by PCR from HEK293T or K562 genomic DNA and cloned into the pMIR-REPORT luciferase expression vector (ThermoFisher Scientific). For luciferase reporter assays, 100 ng of the luciferase reporter plasmid and 200 ng of pRL-TK control vector (Addgene) for normalization were co-transfected into HEK293T cells seeded in 24-well plates (6 x 10^4 cells per well) using Dharmafect duo transfection reagent (Dharmacon, GE Healthcare).

In co-transfection experiments, 5 nM of synthetic miRNAs (pre-miRs, Qiagen) or 30 nM of miRNA inhibitors (Anti-miRs, Qiagen) were included in the transfection mixtures. Cells were harvested 48 hours post-transfection, and dual-luciferase reporter assays (Promega) were performed according to the manufacturer's protocol. Each experiment was conducted in triplicate and repeated independently at least three times.

Enzyme–Linked Immunosorbent Assay (ELISA)

The amount of TGF-β2 secreted in conditioned medium samples from dexamethasone and miRNA-transfected primary HTM cells was quantified using an ELISA kit from R&D Systems, which has a sensitivity of 7.0 pg/ml. Briefly, 100 µl of various concentrations of TGF-β2 standards or equal volumes of conditioned medium were added to microtiter ELISA plates coated with a human monoclonal antibody. After 2 hours of incubation at room temperature, the wells were washed three times with 400 µl of wash buffer and then incubated with 200 µl of human TGF-β2 conjugate for another 2 hours. Subsequently, 200 µl of substrate for the conjugate was added and incubated for an additional 20 minutes. The absorbance at 450 nm was measured using a microplate reader (Molecular Devices). All samples of conditioned medium were assayed after acid activation, and each experiment was performed in triplicate.

In-vivo dexamethasone treatments

C57BL/6J mice were procured from Liveon Biolabs Private Limited after obtaining Institutional Animal Ethics Committee clearance. All procedures were conducted in accordance with approved protocols. The mice were housed and acclimatized in the animal facility at the University of Hyderabad.

For topical administration, 0.1% dexamethasone phosphate (Sigma) was applied to the right eye of the mice, while sterile phosphate-buffered saline (PBS) served as a control for the left eye. The administration was performed three times daily for up to 3 weeks. Doses were given at 9-10 am, 1-2 pm, and 6-7 pm each day. Mice were gently restrained for 30 seconds after applying eye drops to ensure effective penetration into the eye.

Throughout the 3-week period, intraocular pressure (IOP) was measured every 4 hours over a 24-hour cycle. At the end of the study, whole eyeballs were fixed in 10% formaldehyde (Sigma) for immunohistochemistry analysis.

IOP measurements

Intraocular pressure (IOP) in each mouse was measured using a rebound tonometer (TONOLAB, ICARE Instruments) following the manufacturer's instructions. Mice were acclimatized to the room environment and anesthetized to induce sleep. IOP measurements were taken between 10 am and 12 pm every week for up to 3 weeks in each group of mice. Each eye's measurement was considered an independent sample due to potential variations in response to treatment.

To study the circadian rhythm of IOP, measurements were taken at every 4-hour interval over a 24-hour cycle: specifically at 12 am, 4 am, 8 am, 12 pm, 4 pm, and 8 pm.

Statistical Analysis

The data were presented as mean ± SEM (standard error of the mean), and statistical analysis was conducted using the Student's t-test. Differences between mean values were considered statistically significant when the p-value was less than 0.05. For all cell culture studies, a minimum of three independent experiments were performed to ensure robustness and reliability of the findings.

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

Electrophoretic mobility shift assays (EMSA)

Confluent primary HTM cells in a 100 mm dish were treated, and nuclear extracts were collected using nuclear extraction buffer (NEB) containing 20 mM HEPES (pH 7.4), 0.4 mM NaCl, 1 mM EDTA (pH 8.0), 0.1 M EGTA, and a protease inhibitor cocktail.

The 5' end of the CYP1B1 promoter sequence (-2622 ATAAAATTTAAACAACCAACCAG -2631) along with mutant probes was labeled with 20 fmol of [γ-32P] and unlabeled probe was removed using Illustra Microspin-G-50 columns.

Different concentrations of nuclear protein (1 µg, 5 µg, 10 µg) were incubated with 10 pmol of probe at 37°C for 30 minutes in 1X binding buffer (200 mM HEPES pH 7.4, 40 mM DTT, 4 mM EDTA pH 8.0, and 50% glycerol).

The nuclear protein complexes were then loaded onto a 6% agarose gel with 6X DNA loading dye. DNA mobility and band shifts were visualized using a phosphor imager system (BioRad).

RESULTS

Glaucoma, a leading cause of irreversible blindness worldwide, is often associated with elevated levels of TGF-β2 (Transforming Growth Factor Beta 2) and mutations in key genes like CYP1B1, MYOC, FOXC1, LTBP2, and OPTN. Despite these associations, the molecular mechanisms linking TGF-β2 with these glaucoma candidate genes remain

unclear. In this study, we investigated how TGF-β2 influences gene expression in primary human trabecular meshwork (HTM) cells, crucial for regulating intraocular pressure. HTM cells were treated with TGF-β2 at a concentration of 2ng/ml, and changes in gene expression were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) and protein levels assessed by Western blotting.

Our findings reveal a significant regulatory role of TGF-β2 in modulating the expression profiles of glaucoma candidate genes. Specifically, TGF-β2 stimulation led to a notable increase in the expression of MYOC, LTBP2, FOXC1, and OPTN genes within HTM cells. These genes are known to play critical roles in maintaining trabecular meshwork function and have been implicated in various forms of glaucoma pathogenesis. Conversely, the expression of CYP1B1, another important glaucoma-related gene, was markedly downregulated at both the mRNA and protein levels following TGF-β2 treatment.

The observed changes suggest a complex interplay between TGF-β2 signaling and the regulation of these glaucoma candidate genes in HTM cells. This study provides novel insights into potential molecular mechanisms underlying glaucoma development and progression, highlighting TGF-β2 as a key regulatory factor in influencing gene expression patterns critical for trabecular meshwork homeostasis. Further research into these pathways could pave the way for targeted therapeutic strategies aimed at modulating TGF-β2 activity to mitigate glaucoma-related pathology effectively.

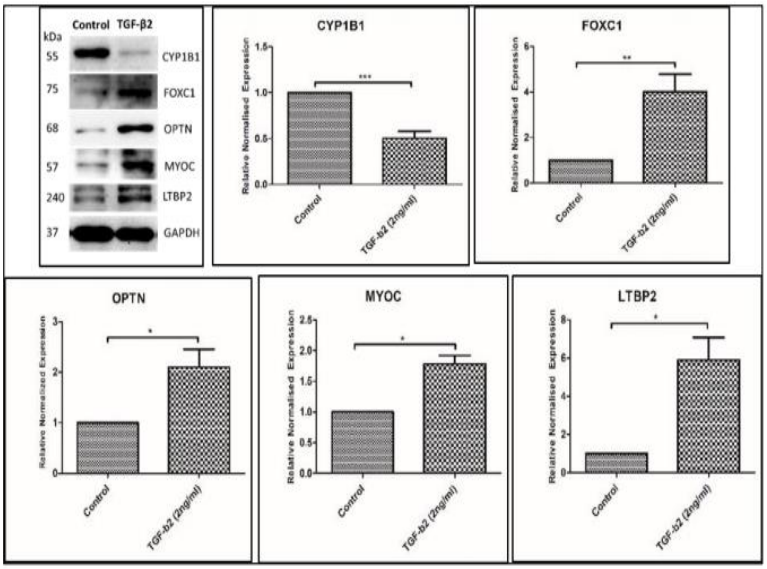


Figure 5. depicts the impact of TGF-β2 treatment on glaucoma candidate genes in primary trabecular meshwork (HTM) cells. HTM cells were exposed to TGF-β2 at a concentration of 2ng/ml for 48 hours. The results from Western blotting and real-time PCR analyses indicate that TGF-β2 treatment resulted in the downregulation of CYP1B1 expression. Conversely, genes such as FOXC1, MYOC, OPTN, and LTBP2 showed increased expression levels in response to TGF-β2 stimulation within HTM cells. The bars on the graphs represent standard deviation across three independent experiments. Significance levels (*, **, *) denote p-values less than 0.05, 0.01, and 0.005, respectively, comparing miRNAs (miRNA-200 family, miR-590, miR-496, and miR-548c) against the control. Statistical calculations were performed using GraphPad Prism5 software.**

Given the prolonged use of glucocorticoids like dexamethasone and prednisolone is known to cause secondary glaucoma in humans, similar effects were examined in HTM cells treated with these drugs for 5 days or more. In this study, the expression of glaucoma candidate genes was analyzed following treatment with dexamethasone (300nM/ml) and prednisolone (300nM/ml). Results showed that both dexamethasone and prednisolone induced the expression of MYOC, LTBP2, FOXC1, and OPTN genes. Conversely, similar to TGF-β2 treatments, these glucocorticoids downregulated the expression of CYP1B1 at both mRNA and protein levels, as illustrated in Figure 10a. These findings suggest a shared regulatory effect of glucocorticoids and TGF-β2 on the expression profiles of these glaucoma-associated genes in HTM cells.

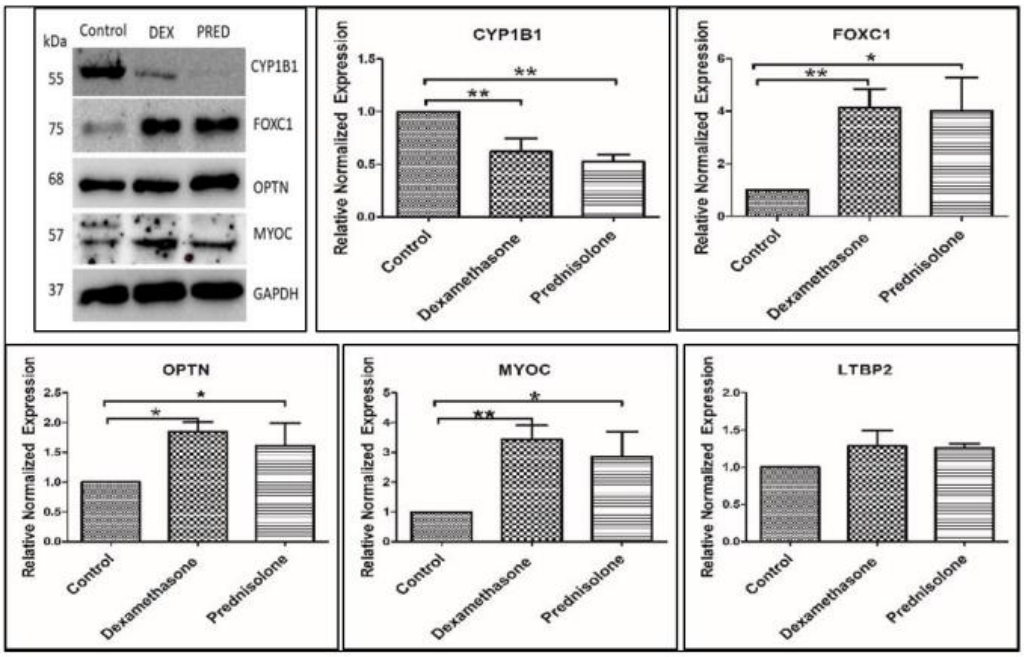


Figure 6. illustrates the impact of glucocorticoid treatment on glaucoma candidate genes in primary trabecular meshwork (HTM) cells. HTM cells were treated with two types of glucocorticoids, dexamethasone (Dex) at 300nM/ml and prednisolone (Pred) at 300nM/ml, for a duration of 7 days. The results from Western blotting and real-time PCR analyses indicate that both Dex and Pred treatments led to the downregulation of CYP1B1 expression. In contrast, genes such as FOXC1, MYOC, OPTN, and LTBP2 showed increased expression levels in response to glucocorticoid stimulation within HTM cells. The bars on the graphs represent standard deviation across three independent experiments. Significance levels (*, **, *) denote p-values less than 0.5, 0.05, and 0.005, respectively, comparing miRNAs (miRNA-200 family, miR-590, miR-496, and miR-548c) against the control. Statistical calculations were performed using GraphPad Prism5 software. These findings highlight a consistent pattern where glucocorticoid treatment influences the expression profiles of glaucoma-associated genes in HTM cells, mirroring some effects observed with TGF-β2 treatment.**

In summary, our findings reveal for the first time that both TGF-β2 and glucocorticoids play significant roles in modulating the expression of glaucoma candidate genes in trabecular meshwork cells. TGF-β2, a well-known cytokine involved in various developmental processes, and glucocorticoids, including dexamethasone and prednisolone, which are clinically linked to secondary glaucoma, were shown to induce distinct changes in gene expression profiles. Specifically, TGF-β2 and glucocorticoid treatments consistently upregulated the expression of genes such as FOXC1, MYOC, OPTN, and LTBP2, while concurrently downregulating CYP1B1 expression levels.

These findings underscore the complex regulatory mechanisms involved in glaucoma pathogenesis, where cytokine signaling and steroid treatments can dynamically alter the molecular landscape of trabecular meshwork cells. Understanding these interactions provides valuable insights into potential therapeutic strategies aimed at mitigating glaucoma-related pathology by targeting specific gene expression pathways influenced by TGF-β2 and glucocorticoids.

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

In our previous experiments, we established that TGF-β2 influences the expression of glaucoma candidate genes, which encompass diverse classes such as transcription factors (FOXC1, PITX2, PAX6), drug metabolizing enzymes (CYP1B1), stress-responsive genes (MYOC), and others. Given this broad spectrum, we were intrigued by the interrelationships among these genes. To explore this further, we individually knocked down CYP1B1, FOXC1, and OPTN genes using specific siRNAs, and assessed their levels using Western blotting and their expression via qRT-PCR. Surprisingly, when FOXC1 was knocked down, the expression of CYP1B1 actually increased. However, no corresponding change in FOXC1 protein levels was observed when CYP1B1 was knocked down, as depicted in Figure 11a. These results suggest a complex regulatory network among glaucoma candidate genes, where knocking down one gene can unexpectedly influence the expression of another. This finding underscores the intricate and potentially interconnected roles these genes play in the molecular pathways involved in glaucoma pathogenesis.

Further exploration of these interactions could yield valuable insights into the underlying mechanisms contributing to glaucoma development and progression. Understanding these relationships may also offer new avenues for targeted therapeutic interventions aimed at manipulating specific gene interactions to mitigate glaucoma-related pathology effectively.

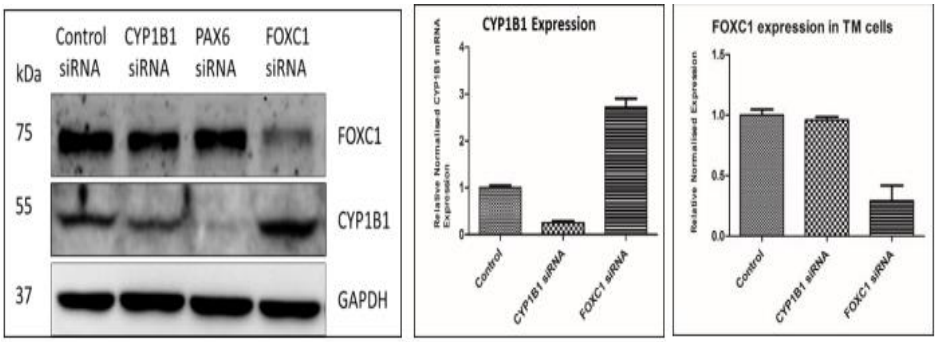
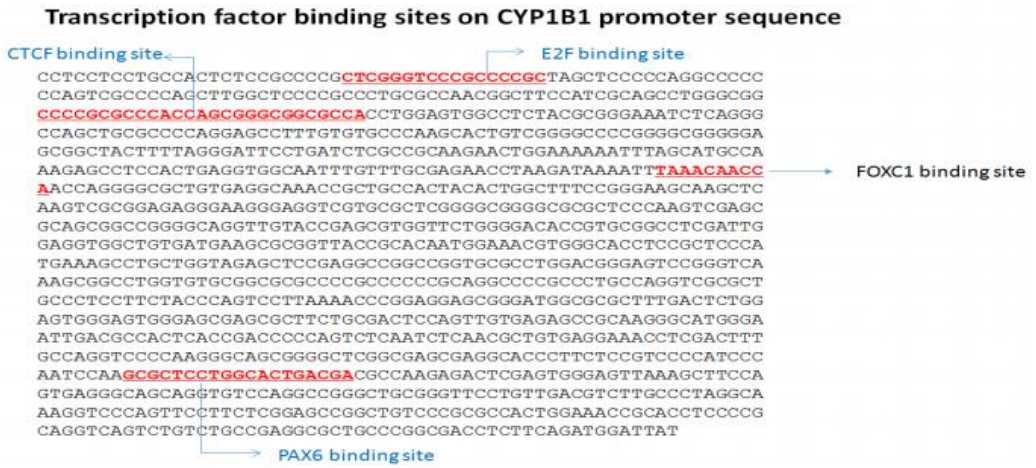


Figure 7. demonstrates the interaction among glaucoma candidate genes through siRNA knockdown experiments. Specifically, the study targeted FOXC1 and CYP1B1 genes using siRNAs and confirmed their knockdown efficiency through Western blot and qRT-PCR analyses. Interestingly, knocking down FOXC1 resulted in an unexpected increase in CYP1B1 expression levels. Conversely, knocking down CYP1B1 did not lead to significant changes in FOXC1 expression at the protein level, despite similar experimental assessments. These findings underscore a complex regulatory network among glaucoma-related genes, suggesting potential interconnected roles and signaling pathways that warrant further investigation.

These findings suggest that FOXC1 potentially regulates the expression of CYP1B1 in trabecular meshwork cells. To explore this relationship further, we initially examined the CYP1B1 promoter for the presence of transcription factor binding sites specific to FOXC1. While bioinformatics tools did not identify such a consensus sequence, manual analysis based on known motifs revealed a potential FOXC1 binding site (TAAACAACC) located at positions -2622 to -2631 on the CYP1B1 promoter (Genbank- U56438.1), as depicted in Figure 10b.

To characterize and confirm the transcriptional binding activity, we conducted electrophoretic mobility shift assays (EMSA) using synthesized 21-bp oligos derived from the CYP1B1 promoter region containing the identified FOXC1 consensus sequence, with and without sequence variations as described in the methods. Nuclear fractions extracted from HTM cells were utilized in the EMSA experiments. The binding of proteins to these probes was observed through band shifts and super shifts when anti-FOXC1 antibodies were introduced. In contrast, no mobility shift or super shift was observed with control probes or mutant variants, respectively, as illustrated in Figure 12a and 12b.

These results support the hypothesis that TGF-β2 influences the downregulation of CYP1B1 expression by modulating FOXC1 expression, which appears to act as a repressor in HTM cells. This study highlights the intricate regulatory mechanisms involving FOXC1 and CYP1B1 in trabecular meshwork cells, suggesting potential avenues for further exploration into their roles in glaucoma pathogenesis.



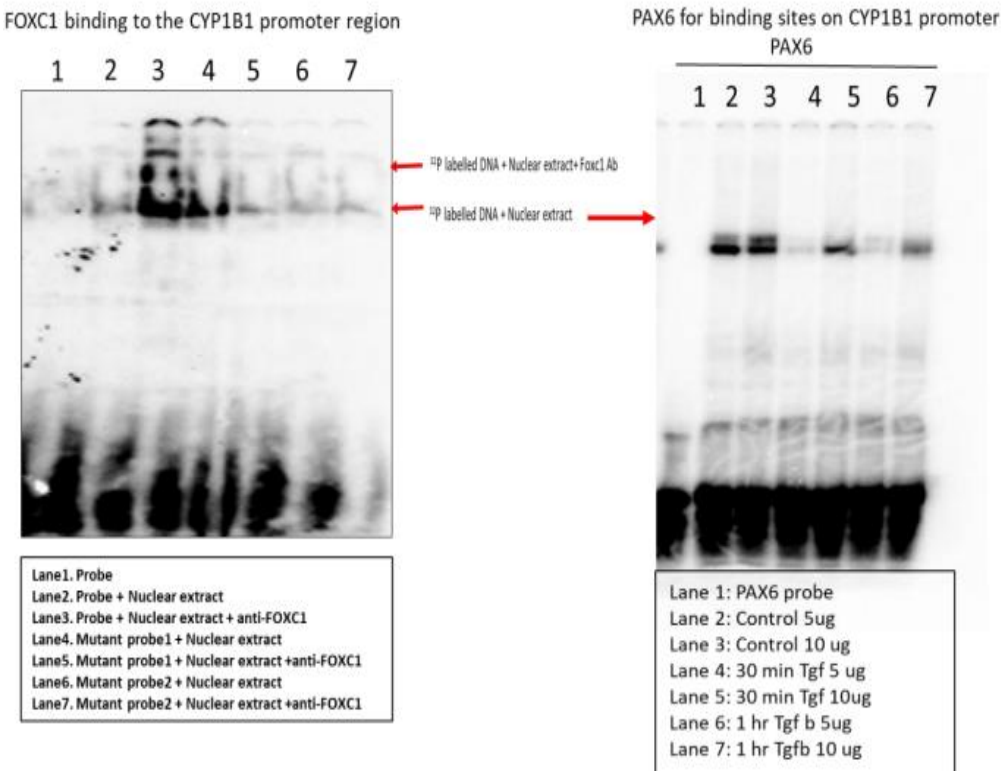


Figure 8. illustrates the relationship among glaucoma candidate genes through promoter analysis and EMSA experiments. Panel a shows the analysis of the CYP1B1 promoter for consensus binding sites for FOXC1 and PAX6. Panel b presents the EMSA experiment results, demonstrating that FOXC1 and PAX6 bind to the CYP1B1 promoter sequence. The gel super shift observed upon incubation with a FOXC1-specific antibody confirms FOXC1's binding to the CYP1B1 promoter sequence.

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

Levels of TGF-β2 are elevated in glaucomatous eyes, and TGF-β2-induced OPTN is known to regulate the expression of glaucoma candidate genes. However, the interplay between TGF-β2 and OPTN in the pathophysiology of glaucoma remains unclear. While both OPTN and TGF-β2 have roles in glaucoma, their specific functions and interactions have not been fully elucidated. Our previous experiments indicated that OPTN regulates CYP1B1 and FOXC1, with TGF-β2 levels being significant in glaucomatous eyes and capable of inducing OPTN expression. This prompted us to investigate the relationship between OPTN and TGF-β2 in primary human trabecular meshwork (HTM) cells.

Upon knocking down OPTN in HTM cells, we observed an upregulation of TGF-β2 mRNA and protein levels. Conversely, overexpression of OPTN via pCMV3-OPTN plasmid resulted in decreased TGF-β2 levels. These findings were corroborated by ELISA, which measured the active form of TGF-β2 in primary trabecular meshwork cells, as shown in Figure 15a-d. These results indicate that the loss of OPTN leads to an increase in TGF-β2 expression in primary trabecular meshwork cells.

Given that OPTN knockdown induces TGF-β2 expression, we further investigated the Rho/ROCK signaling pathway, a known downstream effector of TGF-β2 with a significant role in extracellular matrix (ECM) remodeling and the outflow pathway in trabecular meshwork tissue. Interestingly, knocking down OPTN upregulated both ROCK1 and ROCK2, confirming that OPTN knockdown induces TGF-β2 and activates the Rho/ROCK signaling pathway. These findings highlight a potential mechanistic link between OPTN and TGF-β2 in the pathogenesis of glaucoma, with implications for understanding ECM remodeling and intraocular pressure regulation.

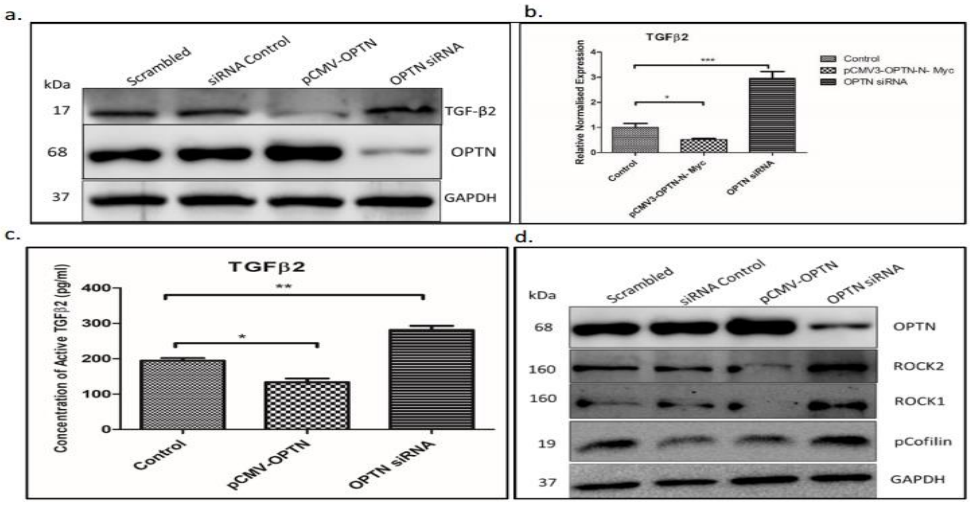


Figure 9. Knockdown of OPTN induces TGF-β2 expression in primary HTM cells.a) Knockdown of OPTN by siRNA led to an increase in TGF-β2 protein expression, while ectopic expression of wild-type OPTN cloned in pCMV3-OPTN resulted in the downregulation of TGF-β2 protein levels in primary HTM cells.b) Real-time PCR analysis showed increased mRNA levels of TGF-β2 with OPTN knockdown and decreased mRNA expression with ectopic expression of wild-type pCMV3-OPTN in primary HTM cells.c) ELISA assay quantified the active form of TGF-β2 in the conditioned medium of OPTN-knockdown cells, revealing increased levels of TGF-β2, whereas decreased levels were observed in cells with ectopic expression of wild-type OPTN.d) Western blot analysis indicated activation of the Rho/ROCK signaling pathway upon OPTN knockdown in primary HTM cells. Conversely, the opposite effects were observed with ectopic expression of wild-type OPTN in primary HTM cells. These results demonstrate that loss of OPTN induces TGF-β2, leading to activation of the Rho/ROCK signaling pathway. Bars represent standard deviation across three different experiments. Significance levels (*, **, *) denote p-values less than 0.5, 0.05, and 0.005, respectively, comparing miRNAs (miRNA-200 family, miR-590, miR-496, and miR-548c) against the control. Standard deviation and significance were calculated using GraphPad Prism5.**

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

Functional mutations in genes such as CYP1B1, FOXC1, OPTN, LTBP2, MYOC, and WDR36 are believed to contribute to the development of glaucoma, yet the molecular mechanisms by which they obstruct the outflow pathway in the anterior chamber remain unclear. Our experiments revealed that CYP1B1, FOXC1, and OPTN regulate each other, with the loss of OPTN influencing CYP1B1, FOXC1, TGF-β2, and the Rho/ROCK pathway. Regulation of the Rho/ROCK pathway is crucial for actin stress fiber formation, which increases resistance to aqueous humor outflow.

To further investigate this, we analyzed stress fiber formation following siRNA-based knockdown of CYP1B1, FOXC1, and OPTN in primary human trabecular meshwork (HTM) cells. F-actin staining with rhodamine phalloidin and paxillin staining with anti-paxillin antibodies confirmed increased actin stress fibers and focal adhesions in knockdown cells compared to controls, as illustrated in the figure.

Additionally, similar results were observed using tetramethylsilane (TMS), a known chemical inhibitor of CYP1B1, which increased stress fibers. Conversely, treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a known chemical inducer of CYP1B1, resulted in a reduction of stress fibers, as shown in Figure 16. These findings suggest that the regulation of these genes and pathways plays a significant role in the cellular mechanisms contributing to glaucoma.

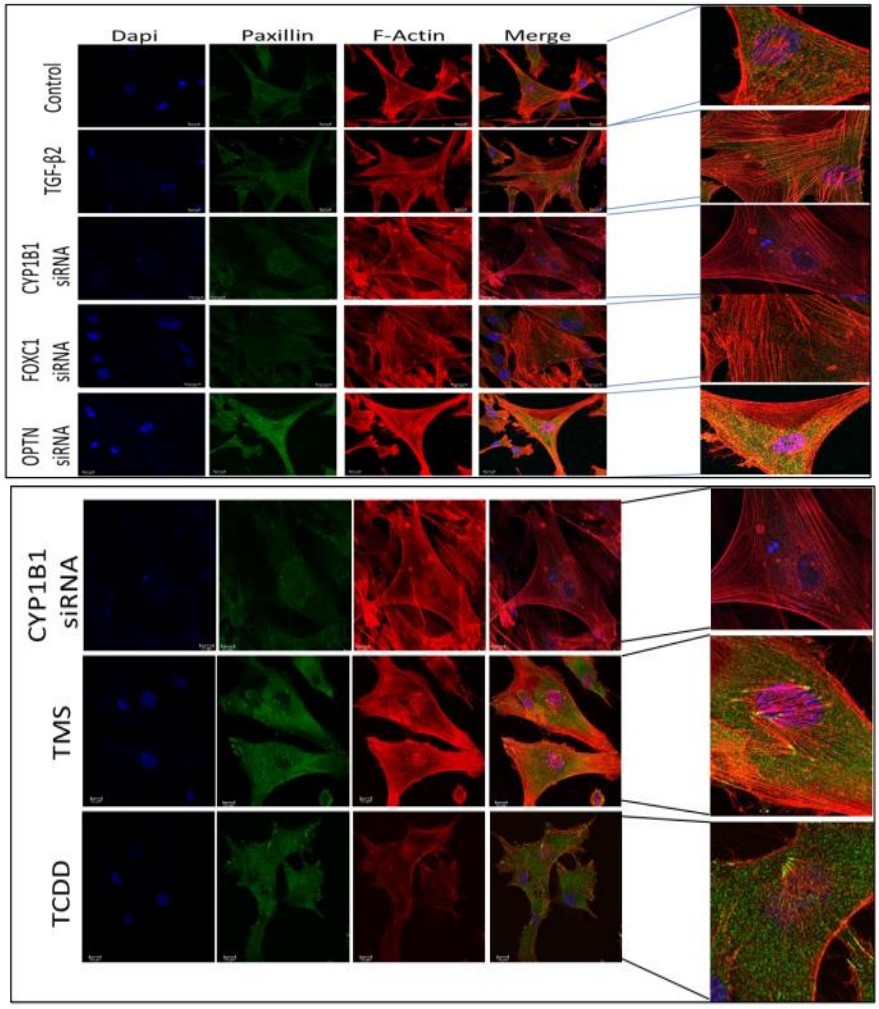


Figure 10. Knockdown of glaucoma candidate genes leads to the formation of actin stress fibers and focal adhesions in primary HTM cells. F-actin was stained with rhodamine phalloidin (Red), and the focal adhesion protein paxillin was stained with an anti-paxillin antibody (Green).

DISCUSSION

Glaucoma is a leading cause of irreversible blindness, primarily due to increased intraocular pressure (IOP) which damages the optic nerve. Genetic mutations in genes such as CYP1B1, FOXC1, MYOC, OPTN, WDR36, and LTBP2 are linked to glaucoma, but their exact roles in disease progression are not fully understood. Elevated levels of TGF-β2 in the aqueous humor have been observed in glaucoma patients, suggesting its involvement in disease pathology through mechanisms like extracellular matrix (ECM) protein induction and actin network formation in trabecular meshwork (TM) cells. This study aimed to elucidate the interplay between TGF-β2 and glaucoma candidate genes in TM cells.

The findings reveal that TGF-β2 differentially regulates glaucoma candidate genes. Glucocorticoids such as dexamethasone and prednisolone were shown to induce FOXC1, OPTN, MYOC, and LTBP2 expression while down-regulating CYP1B1. Further investigation indicated that FOXC1 negatively regulates CYP1B1 by binding to its promoter sequence. Knockdown of OPTN led to increased levels of TGF-β2, which in turn activates the Rho/ROCK signaling pathway, crucial for actin stress fiber formation and TM cell elasticity. The study demonstrated that OPTN loss induces TGF-β2 expression, thereby influencing the Rho/ROCK pathway and potentially increasing outflow resistance in the eye.

Additionally, the study explored the role of miRNAs in modulating glaucoma-related pathways. miRNAs such as miR-200 family, miR-590, miR-496, and miR-548c were found to down-regulate TGF-β2 and ROCK2 expression, suggesting a regulatory feedback loop. TGF-β2 and dexamethasone treatments reduced these miRNAs, promoting actin stress fiber formation and focal adhesions, which contribute to increased outflow resistance and IOP. Overexpression of these miRNAs in TM cells reduced stress fiber formation and increased outflow facility, indicating their potential in lowering IOP and modulating TM cell morphology. These findings highlight the complex regulatory networks involving

TGF-β2, glaucoma candidate genes, and miRNAs in TM cells, providing insights into the molecular mechanisms underlying glaucoma.

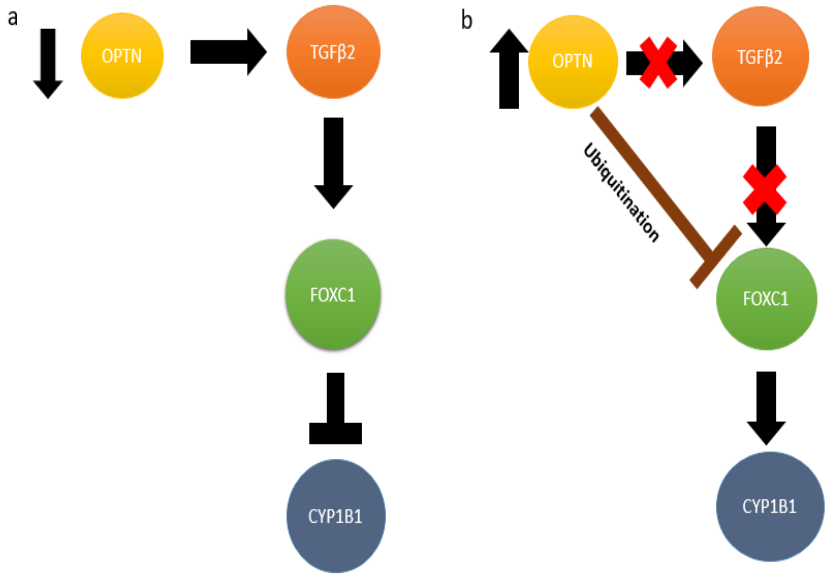


Figure 11. Schematic diagram illustrating:a) Loss of OPTN induces the expression of FOXC1 and TGF-β2 while suppressing CYP1B1 in primary trabecular meshwork (HTM) cells.b) Overexpression of OPTN inhibits TGF-β2 and down-regulates FOXC1, potentially through ubiquitination mechanisms in primary HTM cells.

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