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Abstract: The trabecular meshwork (TM) is a complex tissue that regulates aqueous humor outflow from the eye. Dysfunction of the TM is a major contributor to the pathogenesis of open-angle glaucoma, a leading cause of irreversible blindness worldwide. The TM is a porous structure composed of trabecular meshwork cells (TMC) within a multi-layered extracellular matrix (ECM). Although dysregulation of the outflow throughout the TM represents the first step in the disease process, the underlying mechanisms of TM degeneration associate cell loss and accumulation of ECM, but remain incompletely understood, and drugs targeting the TM are limited. Therefore, experimental models of glaucomatous trabeculopathy are necessary for preclinical screening, to advance research on this disease's pathophysiology, and to develop new therapeutic strategies targeting the TM. Traditional animal models have been used extensively, albeit with inherent limitations, including ethical concerns and limited translatability to humans. Consequently, there has been an increasing focus on developing alternative in vitro models to study the TM. Recent advancements in three-dimensional cell culture and tissue engineering are still in their early stages and do not yet fully reflect the complexity of the outflow pathway. However, they have shown promise in reducing reliance on animal experimentation in certain aspects of glaucoma research. *This review provides an overview of the existing alternative models for studying TM and their potential for advancing research on the pathophysiology of open-angle glaucoma and developing new therapeutic strategies.*

Keywords: glaucoma model, trabecular meshwork, 3D culture, in vitro model, tissue engineering, outflow

## Introduction

Glaucoma is a blinding optic neuropathy affecting over 70 million people worldwide [1]. Its most important risk factor is elevated intraocular pressure (IOP). The trabecular meshwork (TM) plays a key role in the pathophysiology of glaucoma. This filter is located within the iridocorneal angle and constitutes the main outflow pathway for the aqueous humor. It is a fenestrated triangle-form structure in which trabecular meshwork cells (TMC) populate a multi-layered extracellular matrix. The TM tissue is not a rigid structure, but a highly dynamic, avascular filtration system that has a multitude of roles, including filtering aqueous humor of waste material, sensing and regulating IOP through the mechanical stretch, and altering ECM composition and deposition [2–4].The TM contributes to the regulation of IOP by regulating the outflow of aqueous humor from the eye's anterior chamber, primarily through the juxtacanalicular tissue and the endothelium of Schlemm's canal (SC) [2,5]. The juxtacanalicular tissue, located adjacent to the inner wall of SC, plays a crucial role in the regulation of outflow resistance. It consists of specialized cells and extracellular matrix (ECM) components that modulate the flow of aqueous humor [6]. Dysfunction of the TM and the inner wall of SC is the cause of IOP elevation and represents the *primum movens* of primary open-angle glaucoma (POAG) but the precise mechanisms at the origin are still unclear. It is known that abnormal TMC function and excess of ECM deposit contribute to TM stiffening in POAG[7]. Alterations in the structure and composition of the TM ECM generate aqueous humor outflow resistance. Cell-ECM interactions within the TM and SC play a role in regulating outflow resistance. Cellular responses to ECM components and their remodeling can influence the contractility and overall functionality of the tissue[6,8,9]. The endothelial cells lining the inner wall of SC exhibit contractile properties. Contraction and relaxation of these cells regulate the diameter of the canal and thereby influence outflow resistance[10,11]. Tight junctions between endothelial cells of SC also contribute to the formation of a barrier that controls the paracellular movement of aqueous humor[12]. A second 'unconventional' outflow

pathways exists in the human eye, but it only accounts for less than 10% of the total outflow, and thus does not significantly contribute to the regulation of IOP in the normal eye[13].

The development of robust models of physiologic and pathologic TM is necessary to study the different mechanisms at the origin of this deregulation but also to develop and test treatments targeting TM, which are currently rare. Several experimental models have been described with advantages and disadvantages, from animal models or perfusion-cultured anterior segments to cell culture models.

To study the pathophysiology of the TM or its changes under the effects of treatments, all models that artificially increase the IOP by blocking or sclerosing the outflow pathways, for example using laser photocoagulation, episcleral vein cauterization, injection of microbeads or hyaluronic acid into the anterior chamber or hypertonic saline injection into the episcleral veins are not appropriate as they do not mimic the natural course of glaucomatous dysfunction to the aqueous outflow pathway[14]. Indeed, a distinction must be made between animal models of ocular hypertension made to study RGC loss and those specifically developed for the study of TM.

Models of glaucoma exist in mice, rats, dogs, cats, and primates[15]. Each has its challenges, including the availability of genetic resources and difficulty of genetic manipulation, ethical considerations, cost, and maintenance. Translational research in glaucoma is faced with numerous challenges. One significant factor is the divergence in eye anatomy between animals and humans. Furthermore, the etiology of the disease differs between glaucoma animal models and human patients. Additionally, there are notable disparities in study design and statistical analysis methods employed in preclinical and clinical investigations. Moreover, significant differences exist in terms of dosage, scale, timing of intervention, methodologies,

endpoints, age groups studied, and the presence or absence of IOP-lowering treatment, which further complicates the comparison between preclinical and clinical studies in glaucoma[16]. The animal whose TM anatomy is the closest to humans is the primate, but their use is limited because of their expensive price, the inaccessibility of transgenic strains and the rise of ethical concern. Most preclinical studies in the field of glaucoma used mice as their outflow tract anatomy is comparable to humans. It is described as follows: TM, juxtacanalicular tissue, Schlemm canal, collector channels, and episcleral veins[15]. However, the main anatomical difference is that the human TM structure consists of 9 to 18 trabecular beams whereas in the mouse TM, there are only 2 to 5 layers of lamellae. This difference in the structure of the animal TM as well as the regulation of aqueous humor excretion constitutes a limitation to use of animal models. The results obtained with animal models are therefore not always transposable to humans although the majority of IOP lowering glaucoma medications in human are also effective in mouse eyes[16]. Moreover, the lack of cooperation of animals and the use of anesthesia to perform experimentation affects the results. The IOP values vary by animal breed, method of sedation, and measure. In general, excessive restraint, inadequate positioning, or lack of experience in the use of equipment can increase IOP. Last but not least, they are questioned from an ethical point of view. Recently, a consensus recommendation for mouse models to study the aqueous humor outflow was published in 2022 to set standard practice in this field [17]. The most useful models are genetic models that allow OHT by transduction of the TM with glaucoma-related genes[18] (e.g., *MYOC*[19,20], *TGFβ*[21–23], *GREM1*[24], *CTGF*[25], *DKK1*[26], *SFRP1*[27], *CD44*[28]). To meet the expectations of this consensus recommendation animal models used to study outflow physiology must[17]:

- have an open iridocorneal angle
- present decreased outflow facility
- present elevated IOP

- include morphologic descriptions of the conventional outflow tract (TM, Schlemm's canal, collector channels, and intrascleral and episcleral regions) preferably by electronic microscopy to be able to analyze the organization of the ECM
- and assessment of TMC numbers.

The Myoc$^{Y437H}$ mouse, responsible for a juvenile glaucoma, is the best-known and most used[20]. The genetically modified mice express high levels of mutant myocilin in the TM and sclera resulting in a decline of AH outflow facility and a secondary increase of IOP. Morphological changes can be observed in the TM: decreased intertrabecular spaces and a progressive loss of TMC. However, the level of IOP can vary based on the genetic background of the mouse strain selected for inducing the MYOCY437H mutation[29].

In recent years, progress in three-dimensional cell cultures and tissue engineering fabrication has offered promising approaches to reduce the use of animal models, partly encouraged by the three Rs rule (Reduce the number of animals used; Replace the living animal with alternative experimental techniques; and Refine the techniques to minimize animal suffering) [30,31]. While conventional 2D cell culture models represent an attractive alternative to animal models for analyzing the TM and allow for more accurate assessment at the cellular scale, they are limited by the absence of differentiation, polarization, or relationship with an extracellular matrix, making them insufficient in reflecting the actual porous architecture of the TM [32,33]. However, this deficient architecture is precisely the cause of its dysfunction [34]. *Ex vivo* models such as organ perfusion chambers, whole tissue explants, and decellularized tissues are commonly used natural sources; however, their limited availability restricts their use in perfusion studies and drug testing[35,36]. This is why a three-dimensional (3D) cellular model of the TM would be an interesting tool to advance research on this pathology by considering the biomechanics, which is a key element in the pathophysiology of glaucoma[4]. 3D cell culture would enable the recreation of the

microenvironment encountered *in vivo* and provide cells with an environment allowing them to interact with each other[33]. This, in turn, would lead to a better understanding of the physiological functioning of the TM, its behavior under conditions of stress or toxicity, and the effect of treatments[37]. Additionally, *in vitro* models provide a more precise analysis of cell behavior and molecular mechanisms involved in the pathology than do animal experiments[38]. This approach allows to investigate specific biological phenomena in isolated cells, eliminating potential confounding factors present in whole organisms. However, it is crucial to acknowledge that while in vitro systems provide controlled environments, they cannot fully replicate the complex conditions and interactions that occur within a living organism. Technological advances in TM *in vitro* models can help fill the gap in considering the mechanistic modifications involved in glaucoma, such as changes in porosity resulting from alterations in morphology and the mechanical properties of the interaction between TMC and ECM.

In this literature review, we will provide an overview of existing alternative TM models to animal experimentation. We will detail the different cell types used, culture modes, and means to obtain a pathological model. Finally, we will focus on the potential applications of these different models.

## ***Available cell types***

*Primary cell cultures:*

TM is composed of three regions, from the anterior chamber to the Schlemm canal: the uveal meshwork, the corneoscleral meshwork, and the juxtacanalicular tissue, the location of greatest resistance to AH outflow. TMC have a different organization in these three regions,

but it is complex to isolate cells from only one of these three regions. Thus, TMC cultures are generally a mixture of cells from all three regions [32].

Cultures of primary human TMC (HTMC) are sampled from donor eye tissue commonly from a corneal rim discarded from corneal transplant[39]. Whole globe or anterior segment from normal subjects, developing fetuses, or patients with glaucoma can also be purchased.

Cultures of TMC from patients with glaucoma are more difficult due to the accelerated loss of these cells. Nevertheless, they retain their glaucomatous characteristics after culture[40]. TMC change their morphology after 6 to 8 passages, thus it is recommended to use TMC from human eyes before the 7th passages. Methods used to validate that cells are TMC is required for publications including at least responsiveness of myocilin expression by cells to dexamethasone [32].TMC cultures can also come from animal eyes with the limitations that this brings.

*Immortalized human TM cells*

Immortalized TMC lines can be generated with TMC transfected with an original defective mutant of the SV40 virus[41,42]. However, during the immortalization process, some properties of the TMC can be lost, for example the myocilin expression. It is recommended that cell line findings be replicated with non-immortalized TMC[32].

*Induced pluripotent stem cell-derived trabecular meshwork (iPSC-TM)*

The reproducibility of primary cell cultures is a challenge and immortalized cell lines are considered poorly relevant to TMC physiology and disease patterns. The experimental transplantation of iPSC-derived TM (iPSC-TM) highlighted the huge therapeutic potential of these new human cell models, offering perspectives for toxicological or therapeutic evaluations[43,44]. Moreover, the pathogenesis of the glaucomatous disease is explained by a

TMC loss greater than the physiological age-related cell loss. This loss has been suggested to affect the ability of the human TM to regulate aqueous humor outflow and to lead to IOP elevation. In addition to providing a source of reproducible and valuable cells for the constitution of an *in vitro* model iPSC-TM cells are promising autologous cell sources that can be used to regenerate the declining TMC population and function of IOP regulation[45,46].

*TM progenitor cells*

A population of progenitor cells has been identified in Schwalbe's Ring, which is located at the junction between the corneal endothelium and the anterior portion of the TM[47]. These progenitors can be isolated and expanded, and studies have shown that they have the ability to differentiate into both keratocytes and TMC. Zhang et al. developed an optimized method to expand multipotent progenitors from human TMC in a two-dimensional (2D) culture followed by three-dimensional (3D) culture in Matrigel using a modified embryonic stem cell medium[48]. The expanded cells expressed TM markers, embryonic stem cell (ESC) markers, and neural crest (NC) markers. Although some markers were lost after passage, the cells regained the markers when seeded on 3D Matrigel via activation of the canonical BMP signaling[48]. These cells can be used in an *in vitro* model system to help better understand how TM is affected in glaucoma and whether TM progenitor cells may have potential therapeutic applications for glaucoma.

***Generation of Pathological Models:***

As discussed earlier, obtaining cells from the glaucomatous TM is a challenging task for researchers, which is why different molecules are used to induce a diseased phenotype.

Applying mechanical stress to the TM can induce changes similar to those observed in glaucoma. This can be done by stretching or compressing the tissue using a variety of devices. In a previous study, Schlunck et al. demonstrated that the stiffness of the ECM could alter the structure of the cytoskeleton of trabecular cells as well as the profiles of certain protein expressions [8].

Various chemicals can be used to induce glaucomatous changes in the TM, such as transforming growth factor β2 (TGF-β2), and have been shown to contribute to the changes in the ECM of the TM.

TGF-β2 is a profibrotic cytokine known to be elevated in the aqueous humor of patients with glaucoma[49,50]. It has been used in many studies as a model of pathological TM [41,42,51–53]. Studies have revealed that TGF-β2 can increase intraocular pressure (IOP) by promoting the synthesis of certain ECM components by trabecular cells (collagens, fibronectin) through epithelial-mesenchymal transition in TMC [54,55]. Furthermore, TGF-β2 can increase cell rigidity by triggering the formation of Cross-linked Actin Networks (CLANs) via the Rho-GTPase pathway[53,56].

Hydrogen peroxide (H2O2), another molecule used to induce a glaucomatous phenotype, is a chemical compound with powerful oxidizing properties and has been shown to promote cellular senescence, rearrange cytoskeletal structure, and increase proinflammatory mediators such as IL-6, IL-8, and endothelial–leukocyte adhesion molecule 1 (ELAM-1) in the TM[57].

Endothelin-1 (ET-1) is another biomarker found in the aqueous humor of patients with POAG[58,59]. It has been shown that ET-1 can induce TMC contraction in culture and that it can affect the outflow facility[60,61]. Wang et al. showed that in a cultured HTMC model, treatment with ET-1 increased the expressions of fibronectin and collagen IV, and that in an ex-vivo model, IOP increased after ET-1 administration[62]. Zhou et al. also used ET-1 in a whole eye

perfusion system and found a decreased outflow after ET-1 exposition and successfully tested several pretreatments to reverse this effect[63].

Benzalkonium chloride (BAK) *in vitro* induced apoptosis, oxidative stress, and also an IOP increase, with reduction of aqueous outflow *in vivo*. BAK enhances all characteristics of TM degeneration typical of glaucoma and causes degeneration in acute experimental conditions, potentially mimicking TM degeneration[64]. In an *in vitro* 3D TM model, Bouchemi et al. showed that BAK disorganized the TMC and decreased their number resulting in an enlargement of spaces between cells [65].

***Available 3D models:***

*3D scaffolds culture*

The first successful scaffold was a micro-fabricated SU-8 porous structure, where TMC were cultured to study steroid-induced glaucoma. Scaffolds with pores of approximately 20 micrometers in thickness, which were seeded with primary HTM cells, were able to imitate some of the normal tissue functions *in vivo*. This included being able to induce or reverse glaucomatous conditions using medication [66,52,67]. A follow-up study showed that applying a hyaluronic acid-containing hydrogel coating to the SU-8 scaffold improved cell proliferation. Over time, various technologies and materials have been explored, including traditional polymeric filters, SU-8 membranes, electrospun nanofibers, and other methods. These methods offer precise control over morphological characteristics such as porosity and beam thickness in both 2D and 3D environments. However, the stiffness of the scaffold cannot be directly controlled using most of these methods. Despite these limitations, 3D cultures have the potential to create an in vivo-like microenvironment for HTM cell growth. Tissue engineering aims to produce functional biomimetic replicas of tissues of interest, but only a

limited number of studies have been reported on bioengineered 3D HTM *in vitro* models. These models partially mimic normal tissue function and provide a platform for drug testing and evaluating the effectiveness of different treatment options. Wlodarczyk-Biegun et al. studied the biofabrication technique of melt electrowriting (MEW), a marriage between electrospinning and 3D printing, as a means of producing fibrillar and porous scaffolds with thermoplastic polymers that replicate the multilayer and gradient structure of the natural HTM [68]. HTMC cultured on these scaffolds maintained the phenotype of native HTMC and infiltrated the scaffolds. However, some may argue that these models are more comparable to a 2D cell culture system rather than a true 3D model, as they cannot fully replicate the 3D cell-ECM interface apart from the ECM secreted by the HTM cells grown on top of the synthetic polymer scaffold.

*Hydrogel- based TM scaffolds*

Recent research has focused on using hydrogels as scaffolds to study the behavior of HTMC in response to environmental changes and disease conditions[69,70]. Hydrogels are networks of crosslinked, hydrophilic polymers used to recapitulate the 3D architecture of organ systems in tissue engineered models. These materials are so useful in cell culture because they provide a biocompatible, degradable, hydrated microenvironment that mimics the cell–ECM interactions of natural tissues. Hydrogel scaffolds offer higher control over the morphology, stiffness, and 3D environment compared to photolithography and electrospinning, while also maintaining structural integrity.

Ideally, 3D cell culture matrices can reproduce the features of the ECM to closely resemble the *in vivo* environment. The interaction between cells and ECM is essential for a range of biophysical and biochemical functions, such as the transportation of signaling molecules, nutrients, and waste metabolites, as well as mechanical integrity. Thus, these

matrices need to reflect the specific ECM characteristics of each tissue for a given application. Moreover, the mechanical properties of 3D matrices are also significant, as they can directly influence cell adhesion, thereby affecting both the shape and response of cells[71]. The utilization of degradable scaffolds presents an opportunity for more molecular research, as opposed to permanent ones. Hydrogels can be created using various synthetic or natural components. In tissue engineering, natural polymers are the most commonly used approach to developing hydrogels[72]. Collagen, fibrin, and elastin, which are components of the ECM of the TM, have been used as attachment factors for HTMC to study specific functions and interactions [65]. 3D Corning® Matrigel® Matrix (Corning Life Sciences, Tewksbury, MA, United States) contents several proteins found in extracellular matrix (ECM) such as laminin, collagen IV, heparin sulfate proteoglycan, and entactin/nidogen[73,74]. Several studies demonstrated that unlike cells cultured on traditional 2D planar surfaces, cells in 3D scaffolds are more physiologically relevant concerning in vivo characteristics exhibited by in-vivo surrogates[75] (figure 1). Vernazza et al. conducted a study to compare the response of HTMC in 2D and 3D *in vitro* models following chronic stress exposure. Their results revealed that 3D TMC cultured on Matrigel exhibited a higher sensitivity to the production of intracellular reactive oxygen species induced by hydrogen peroxide treatment compared to 2D cultures. Furthermore, the 3D models demonstrated a more precise regulation of apoptosis triggers and cell adaptation mechanisms than the 2D models [33]. Another scaffold-based approach by Osmond and colleagues utilized HTMC cultured on various collagen scaffolds containing different glycosaminoglycans (GAGs) and different pore architectures to better understand how HTMC respond to changes in their extracellular environment. The cellular response was assessed by quantifying cellular proliferation and the expression of fibronectin, an important extracellular matrix (ECM) protein. Fibronectin plays a crucial role in organizing ECM proteins both among themselves and with trabecular cells, thereby contributing to the

resistance of outflow [76–78]. The pore architecture of the scaffolds was altered using the freeze-casting technique to make both large and small pores that are aligned or with a non-aligned random structure. The composition of the scaffolds was altered with the addition of chondroitin sulfate and/or hyaluronic acid. It was found that HTMC grown on large pore scaffolds proliferate more than those grown on small pores. There was an increase in the fibronectin expression with the incorporation of GAGs, and its morphology was changed by the underlying pore architecture. These works offer a better understanding of how human TMC behave in response to alterations in their microenvironment and highlight the importance of the mimicry of the 3D strucutre[79–81]. However, the study did not explore how the constructs would react under conditions that induce glaucoma. Furthermore, if the accumulation of extracellular matrix (ECM) proteins is a characteristic feature of the pathogenic process in glaucoma, it is important to highlight that cell proliferation is not[77,82]. Therefore, it is crucial to determine whether cells can survive under normal conditions on these new substrates. However, it should be noted that the ability to proliferate does not necessarily indicate an appropriate glaucoma model.

3D bioprinting can produce a variety of architectural patterns on a wide array of biomaterials. Li and colleagues developed a hydrogel using a tissue-engineering approach for HTM. The hydrogel consisted of ECM biopolymers and normal HTMC obtained from a donor. By mixing HTMC with collagen type I, hyaluronic acid (HA), and elastin-like polypeptide (ELP) - each containing photoactive functional groups - researchers were able to create HTM hydrogels in various sizes and shapes. Short UV cross-linking, mediated by photo-initiators, was used to solidify the hydrogels. To induce glaucomatous changes, dexamethasone (DEX) was administered, and the therapeutic effects of the ROCK inhibitor Y27632 were evaluated[83].

To create an *in vitro* 3D TM scaffold for potential use as a tissue scaffold in glaucoma patients after trabeculectomy, Waduthanthri et al. developed a hydrogel peptide called

MAX8B which partly mimics the motif of cellular integrins and enables interactions with ECM components [84]. The scaffold material demonstrated the ability to undergo shear-thinning and exhibited biocompatibility, facilitating appropriate growth and proliferation of TMC in tightly packed cell monolayers resembling typical TMC morphology. Moreover, the MAX8B scaffold was utilized in an in vitro perfusion system to investigate the impact of Dexamethasone on the outflow facility of the trabecular meshwork proving the effectiveness of this three-dimensional (3D) model as a platform for drug testing[84].

*Spheroids*

Although 3D culture techniques have gained popularity for their ability to better mimic *in vivo* environments, there are some limitations when it comes to replicating physiological and pathological conditions of human TM. This is because the use of scaffolds in 3D cultures is not reflective of the absence of such structures in human TM. However, 3D spheroid cell cultures have recently emerged as a promising alternative to conventional 2D cell cultures, particularly as *in vivo* models for various diseases. These spheroids allow for more intercellular interactions in a 3D space, potentially resulting in protein networks that resemble those found in real tissues. This makes it possible for 3D spheroids to replicate biological features associated with real tissues.

The spheroid model of TM refers to a 3D culture system that mimics the structural and functional properties of the TM in the eye. HTMC have been cultured as spheroids *in vitro* to study their role in glaucoma. These spheroids have been shown to exhibit features of the TM *in vivo*, such as the presence of ECM components and cytoskeletal proteins. These spheroids have been shown to respond to mechanical stress and exhibit physiological responses similar to those observed *in vivo*. These spheroids have been shown to be structurally and functionally similar to the TM *in vivo* and have been used to study the effects of various drugs

on TMC behavior. 3D HTM spheroids became significantly and differently smaller and stiffer in response to TGF-β2 or dexamethasone stimulation[41,85]. Watanabe et al. successfully obtained 3D HTM spheroids and found that TGFβ2 significantly induced the down-sizing and stiffness of 3D spheroids from human orbital fibroblasts, and those effects were substantially inhibited by a ROCK-inhibitor.[42,86]

*Outflow studies*

Perfusion studies of outflow in HTMC began in the late 1980s and have since evolved to include a range of techniques and models. One of the earliest studies involved the use of filters to culture HTMC and measure hydraulic conductivity using a pressure/flow circuit[87]. This study led to further investigations into the biomechanics of HTMC. The perfusion system developed by Yubing Xie's group enabled continuous pressure monitoring at different flow rates to investigate the effects of drugs such as Lat-B, ROCK inhibitors, or TGFβ2[52,66,67]. As previously mentioned, 3D culture models of TMC are superior to 2D models due to the ability to enable cell-cell and cell-ECM interactions. However, these 3D models fail to reproduce the dynamic continuous supply of nutrients, oxygen, and removal of metabolic waste products. Recent advances propose models that combine the benefits of 3D culture with milli-fluidic techniques to improve the physiological relevance of the culture and address the issues related to cell responses under static culture conditions. Microfluidic systems allow for the creation of a 3D microenvironment that mimics the *in vivo* conditions of the TM, including the presence of shear stress and fluid flow. Recently, the MAX8 peptide-hydrogel scaffold and a 3D Matrigel® model have been tested in perfusion chambers to evaluate their use as artificial TM scaffolds[39,84].

In their closed-circuit *in vitro* model developed by Tirendi et al., 3D-HTMCs cultured in Matrigel were provided with a continuous medium supply. This was achieved by

connecting single-flow bioreactor culture chambers to a peristaltic pump. The milli-fluidic technology as well as the 3D culture model mimicked cell responses found *in vivo* as a result of the increase in outflow resistance [57]. This type of model can be used to investigate the effects of various factors on TM function, such as mechanical stress and changes in ECM composition.

***Ex vivo models***

Ex vivo models, specifically perfusion studies, utilizing animal eyes such as pigs, cows, and primates, have been instrumental in advancing our understanding of glaucoma [88–91]. These models offer valuable insights into the dynamics of aqueous humor outflow and provide a platform to investigate the effects of various experimental interventions on the disease. By perfusing the enucleated eyes with a controlled flow of fluid, researchers can mimic physiological conditions and measure parameters such as intraocular pressure and outflow facility. These models have helped elucidate the mechanisms underlying glaucoma and evaluate potential treatments[63,89]. For example, Zhou et al. developed a platform to simultaneously evaluate outflow facility and its time- and dose-dependent responses to treatments of 20 eyes. They used whole porcine and bovine eyes to develop a perfusion system and studied the regulation of outflow facility by endothelin-1, nitric oxide donor, and sphigosine1-phosphate [63].

However, it is important to acknowledge the limitations of ex vivo models. They do not fully replicate the complex in vivo environment of the eye, including interactions with surrounding tissues and systemic factors. Additionally, the use of animal eyes may introduce species-specific differences that may not fully reflect human physiology. Given these limitations and the fact that they do not represent an alternative to the reliance on animal experimentation, we will primarily focus on human models.

The human anterior segment perfusion culture model is a valuable tool for studying the TM and aqueous humor outflow in glaucoma[89,92,93]. *Ex vivo* models possess several significant benefits compared to other models, including their ability to maintain the structure of pathways and their capacity to facilitate analysis in nearly ideal physiological conditions[94]. Outflow facility measurements can be performed *ex vivo* or *in vivo*, with *ex vivo* measurements offering a simpler approach by avoiding confounding factors that are difficult to control. However, *in vivo* measurements are more representative of real-life conditions.

Bahler et al. used perfusion organ culture of human anterior segments to study the effect of prostaglandin on the trabecular outflow. Since this human anterior segment culture model lacks a choroid or functional ciliary body, the uveoscleral pathway is absent. This simplification facilitates the analysis by directly assessing the sclera's impact on outflow facility[93].

Peng et al. have created an *ex vivo* model of human corneal rim for perfusion culture experiments as an alternative to the human anterior segment perfusion culture model. This model can be used to study the TM and aqueous humor outflow in glaucoma while improving cost and availability. The corneal rims were affixed to 3D-printed perfusion culture plates and perfused in constant flow mode. Pressure changes were recorded using a computerized system. TM stiffness of corneal rims treated with dexamethasone was significantly higher than in the control group[95]. Additionally, the model allows histology or immunohistochemistry of the TM to investigate biomechanical changes or treatments.

Baudouin et al. examined TM specimens using immunohistology and reverse transcriptase–polymerase chain reaction. Trabecular specimens of glaucomatous patients showed extremely low densities of trabecular cells and the presence of cells expressing fractalkine and fractalkine receptor and their respective mRNAs[64]. These explants methods have the advantages to retain tissue architecture and cellular interactions closer to in vivo conditions as

opposed to traditional cell culture methods. They are suitable for studying tissue responses and drug effects at the cellular level[96]. The low cell count of TMC in TM explants from glaucomatous patients can be circumvented by using TM from healthy donors and exposing them to TGF-β2. The addition of TGF-β2 to healthy TM permits reproduction of the changes in TM cell cytoskeletal organization and ECM compaction, while providing sufficient material for a transcriptomic study[2,82,97].

## Discussion

This article discusses the importance of developing models of TM, a structure within the eye that plays a crucial role in regulating IOP, to study the pathophysiology of glaucoma. The TM is a dynamic filtration system that helps regulate IOP by controlling the outflow of aqueous humor.

Developing new 3D in vitro models is crucial to studying TM pathophysiology in glaucoma. They mimic the physiological microenvironment of the TM, providing a more physiologically relevant model than traditional 2D cell culture methods[98].

One of the key advantages of these 3D models is that they reduce the need for animal studies, which can be costly, time-consuming, and ethically challenging[15,30]. *In vitro* models can be used as a complementary tool to animal studies, as they can provide useful data on mechanisms and drug efficacy before moving to animal models or clinical trials.

While *in vitro* models offer several advantages, they also have limitations that need to be considered. One of the main challenges is that *in vitro* models may not fully recapitulate the complexity of the TM *in vivo*, such as interactions with other tissues and the influence of systemic factors. To address this limitation, researchers often use a multiplicity of models to collect data for a particular question. For example, to study the modification of ECM, a natural hydrogel medium that closely resembles the components of TM ECM is more

interesting than a synthetic one. As it provides a more physiologically relevant environment that can better mimic the ECM interactions in the TM. Similarly, a microfluidic bioreactor can be used to study the effect of sheer stress or biomechanical impact on TMC[66,99]. This type of model allows researchers to control the flow of fluids and apply mechanical forces to the cells, providing more accurate simulations of the TM microenvironment. A comparison of innovative 3D TM models and measured outcomes is presented in supplementary table 1.

Furthermore, biomimetic 3D in vitro models, in addition to enhancing our understanding of TM tissue biology and outflow pathology, have the potential to be used therapeutically for restoring compromised TM function[100]. Promising research has demonstrated the effectiveness of stem cell therapy in repairing TM tissue and preserving vision in glaucoma patients [46]. Moreover, the presence of TM progenitor cells capable of differentiating into functional TM cells further supports the potential for tissue repair[101,102]. Advanced biofabrication techniques allow for the creation of scaffolds that closely mimic the native ECM and provide cues for stem or progenitor cell differentiation, replicating cellular responses observed in vivo[103]. By incorporating biomaterials alongside TM progenitor cells, the development of a delivery system for effective stem cell therapy can be facilitated.

In conclusion, the use of multiple models that can better replicate the different aspects of the TM *in vivo* can provide more robust and accurate data. By using a combination of *in vitro*, *ex vivo*, and *in vivo* models, researchers can gain a more comprehensive understanding of glaucoma pathology and develop better treatments for this disease.

However, it is important to consider the limitations of non-animal. The progress made in the alternative models presented in this study does not imply that we can completely eliminate the need for animal experimentation at present. In vivo experiments enable a substantial prediction of the effect of hypotonic treatment on IOP, even if the organization of their outflow system is not totally similar to that of humans[16]. These alternative models are

still in their early stages and may not fully replicate the complexity of the TM or its interactions within the eye. They may not provide the same comprehensive data as animal models, particularly in terms of assessing IOP, estimating natural flow rate, accessing the outflow facility, evaluating cellularity, tissue integrity, and capturing natural expression profiles as it would be in a living in vivo system. Additionally, organ culture has a significant limitation whereby the regulation of IOP relies solely on external manipulative regulations, lacking intrinsic regulation in enucleated eyes. Nonetheless, despite these current limitations, the progress made in developing these alternative models is encouraging. While they may not completely replace the need for animal models, they do hold the potential to significantly reduce their utilization, provided of course that the trabecular cells used are not derived from animals.

Overall, this progress in *in vitro* and *ex vivo* models offers a promising tool for studying the TM in glaucoma and reducing the need for animal studies. While it has limitations, it provides a more physiologically relevant model than traditional 2D cell culture methods, and its potential applications in drug discovery and testing make it a valuable addition to glaucoma research.

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**Figure 1: Confocal microscopy image of the 3D cultured pHTMCs in Matrigel.** The pHTMC is organized in a mesh conformation with interconnections and the formation of intercellular spaces. Actin fibers are stained in red by phalloidin, membranes with DiO (green), and nuclei with DAPI (blue). Magnification 200X.