BIOENGINEERED, STEM CELL DERIVED
OCULAR OUTFLOW TISSUE

By

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Glaucoma is one of the leading causes of irreversible blindness in the world. Despite decades of research, intraocular pressure (IOP) is the only known treatable risk factor. IOP is affected by the timely removal of aqueous humor through the conventional outflow track, which is made up of the trabecular meshwork and adjacent Schlemm’s canal. Dysfunction in these tissues due to aging, oxidative stress, metabolic or pathological changes lead to increased flow resistance, elevated IOP, and ultimately glaucoma.

Recent advances in ocular regenerative therapy have the potential to rescue glaucomatous tissue function and restore its delicate microenvironment. The possibility of using stem cell-derived trabecular meshwork and Schlemm’s canal cells to recreate a functional outflow tissue are explored in this thesis. Previously, our lab developed a well-defined, micro-porous substrate that promotes in vivo-like physiology and outflow function in primary trabecular meshwork and Schlemm’s canal cell cultures. Using these primary cell cultures as controls, we have created 3D stem cell-derived outflow tissues, evaluated and compared their morphology, expression, outflow facility, and drug responsiveness. To explore the importance of the dynamic microenvironment in outflow function, we developed a dual-flow microfluidic chamber that mimics the basal-to-apical and shear flow of aqueous humor through the conventional outflow track. Overall, this dissertation demonstrates the promising application of stem cells in future glaucoma drug screening and treatment.
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>A2P</td>
<td>L-Ascorbic acid 2-phosphate</td>
</tr>
<tr>
<td>ADSC</td>
<td>Adipose-derived stem cell</td>
</tr>
<tr>
<td>AH</td>
<td>Aqueous humor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BtA</td>
<td>Basal-to-apical</td>
</tr>
<tr>
<td>CD-31 (PECAM1)</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>CLAN</td>
<td>Cross-linked actin network</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CollIV</td>
<td>Collagen type IV</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenate</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>HSC</td>
<td>Human schlemm’s canal</td>
</tr>
<tr>
<td>HTM</td>
<td>Human trabecular meshwork</td>
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<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
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<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>iPSC-TM</td>
<td>iPSC-derived trabecular meshwork</td>
</tr>
<tr>
<td>JCT</td>
<td>Juxtacanalicular connective tissue</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Media</td>
</tr>
<tr>
<td>MYOC</td>
<td>Myocilin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OCT 3/4</td>
<td>Octamer-binding transcription factor 3/4</td>
</tr>
<tr>
<td>ONH</td>
<td>Optic nerve head</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>POAG</td>
<td>Primary open-angle glaucoma</td>
</tr>
<tr>
<td>PROX1</td>
<td>Prospero homeobox protein 1</td>
</tr>
<tr>
<td>q-PCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cell</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SU-8</td>
<td>SU-8 negative photoresist</td>
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<tr>
<td></td>
<td>Description</td>
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<tr>
<td>-----</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>Transforming growth factor-beta 2</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
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1.1 Introduction

Glaucoma is a debilitating neurodegenerative eye disease that has lead to visual impairment in over 64 million people worldwide (Tamm et al., 2014). Due to its tendency to remain asymptomatic until advanced stage, glaucoma is the primary cause of irreversible blindness (Weinreb et al., 2016). Patients living with advanced glaucoma not only suffer physically and emotionally, but are also met with the financial burden of disease management (Carreras et al., 2017). Currently, there is no cure for glaucoma, and the only known, modifiable risk factor is intraocular pressure (IOP). Typical management plan involves medication(s) and/or surgery to reduce IOP, and regular clinic visits to monitor treatment efficacy and disease progression (Chae et al., 2013).

In the United States alone, the annual economic burden of glaucoma-related disorders is $5.5 billion, and will continue to grow with the increase in elderly population (Frick et al., 2007). Moreover, a recent meta-analysis estimates that the global prevalence of glaucoma will exceed 111 million in 2040, with Africa and Asia affected more than the rest of the world (Fig. 1.1; Weinreb et al., 2016; Tham et al., 2014). Therefore, there is an urgency to develop a more permanent and cost-effective treatment strategy for glaucoma through a better understanding of its pathophysiology.
Primary Open Angle Glaucoma

Primary open angle glaucoma (POAG) is the most common type of glaucoma, responsible for 90% of all reported cases. Among the few well-recognized risk factors for POAG, such as age, race, gender, and family history, elevated IOP is the only modifiable risk factor during disease development. In fact, several large population-based studies in the past have confirmed that the reduction of IOP reduces the progression of POAG in patients with or without elevated IOP (Heijl et al., 2002).
In human, IOP is determined by the continuous generation of aqueous humor (AH) by the ciliary processes and its elimination through the conventional outflow pathway, located in the anterior chamber angle between the cornea and iris (Gabelt et al., 2005; Carreon et al., 2017). The trabecular meshwork, together with Schlemm’s canal, collector channels, and aqueous veins, constitute this major outflow pathway that removes up to 90% of AH (Dautriche et al., 2014). Any dysfunction in the trabecular meshwork and Schlemm’s canal, such as internal blockage or age-related cell death, could lead to increased resistance to AH outflow (Weinreb et al., 2016). In POAG, such imbalance between AH production and outflow leads to increased pressure gradient across the eye, which can be especially devastating to optic nerve health.

Although the pathophysiology is not well understood, POAG is characterized by progressive degeneration of the retinal ganglion cells (RGCs) in the optic nerve (Fig.1.2; Weinreb et al., 2014). Since RGCs are postmitotic neurons that do not regenerate, any vision loss sustained from their death become irreversible (Ju et al., 2008). On the clinical level, its identifiable features include thinning of the neuroretinal rim and the retinal nerve finger layer, and optic nerve cupping (Burgoyne et al., 2015).

The optic nerve is a bundle of roughly one-million RGC axons, which are crucial for transmitting visual information from the photoreceptors to the brain. Elevated IOP causes mechanical stress and strain on the RGCs, and if left untreated, could interrupt the transport of essential neurotrophic factors, degradation of the axons, death of RGCs, and ultimately loss of visual field (Nguyen et al., 2015).
Figure 1.2. Pathophysiology of glaucoma. (A) Optic nerve head (ONH) in a healthy eye, with axons of retinal ganglion cells constituting the retinal nerve finger layer of the inner retina and converging at the ONH. (C) ONH in glaucomatous eyes, displaying optic disc cupping, characterized by progressive neuroretinal rim narrowing. Biomechanical deformation of the ONH often leads to progressive and irreversible loss of RGC axons and cell bodies. Reprinted by permission from Springer Nature: Primary open-angle glaucoma, Weinreb et al., Copyright © 2016

1.3 Conventional Outflow Pathway

The conventional outflow pathway, located at the intersection between the cornea and ciliary body, is the primary system that regulates AH removal and IOP (Fig. 1.3; Weinreb et al., 2016, Tamm et al., 2009). AH is a transparent fluid secreted by the epithelium of the ciliary body. It circulates around the lens, through the pupil, and into the
anterior chamber to provides nourishment (i.e. amino acids and antioxidants) and immune protection for the avascular ocular tissues (Braunger et al., 2015). To maintain a constant ocular fluid volume and IOP, the removal rate must balance the rate of AH production, at roughly 2.5 μL/min (Gabelt et al., 2005).

As AH leaves the eye through the conventional outflow pathway, it first encounters the trabecular meshwork, which is responsible for filtration and resistance generation. The human trabecular meshwork (HTM) tissue consists of three distinct regions: (i) the inner uveal meshwork consists of 1~3 layers of collagen and elastin lamellae with large irregular intertrabecular spaces; (ii) the middle corneoscleral meshwork is composed of 8~15 layers of perforated collagen and elastin plates; and (iii) the deepest juxtacanalicular connective tissue (JCT) is a loose connective tissue with trabecular meshwork cells embedded in a fibrillar extracellular matrix (ECM), composed of interwoven elastin, collagen and fibronectin fibers (Johnson et al., 2006; Tamm et al., 2009).

The uveal and corneoscleral trabecular meshwork cells are highly phagocytic toward any cellular debris and reactive oxygen species (ROS) in the AH (Stamer et al., 2017). Their main function as a self-cleaning filter ensures a “clear” outflow path toward the resistance generating JCT region. Trabecular meshwork cells in the JCT are contractile and capable of continual remodeling of its surrounding ECM, altering the tissue “permeability” in response to IOP fluctuations (Llobet et al., 2013).
Figure 1.3. Aqueous humor production and flow. Aqueous humor is produced by the ciliary body and flows from the posterior chamber, around the lens, into the anterior chamber of the eye.

Adjacent to the JCT is the inner wall region of the human Schlemm's canal (HSC), which provide the final barrier to AH drainage (Aspelund et al., 2014; Ramos et al., 2007). The Schlemm’s canal is a ring-shaped vessel lined with a single non-fenestrated layer of spindle-shaped endothelial cells, with their long axis aligned and parallel to the direction of flow (Ramos et al., 2007). The inner wall Schlemm’s canal cells experience a remarkably dynamic microenvironment, due to both the basal-to-apical and shear flow of AH (Ethier et al., 2004; Fautsch et al., 2006; Ashpole et al., 2014). The basal-to-apical flow, in particular, generates a massive transcellular mechanical load, leading to the formation of a fluid-filled extracellular cavity or “giant vacuole” that fuse with the cell membrane (Alvarado et al., 2004; VanderWyst et al., 2011). These pressure-dependent protrusions into the canal lumen are often accompanied by transcellular and/or
paracellular pores, which allow AH drainage without compromising the blood-aqueous barrier (Braakman et al., 2016).

The inner wall Schlemm’s canal cells share a discontinuous basement membrane with the JCT region of trabecular meshwork cells. By themselves, neither structure appears to generate enough resistance to maintain physiological IOP (Johnson et al., 2006). However, evidence suggests that the trabecular meshwork and Schlemm’s canal cells work together to synergistically regulate resistance likely via a hydrodynamic effect known as “funneling” (Stamer et al., 2012). It is suspected that AH flowing through he JCT preferentially converge or “funnel” toward inner wall regions with discrete pore sites, in order to pass through the Schlemm’s canal. This effect could potentially explain the nonuniform and segmental flow of AH around the circumference of the outflow pathway, a well-known phenomenon that creates regions of high and low conductance (Carreon et al., 2017). Taking segmental flow into consideration, the funneling model reduces the area of JCT that is actively involved in filtration, thereby significantly increase its effective hydraulic resistance (Stamer et al., 2012; Overby et al., 2009). This suggests that the bulk of outflow resistance is actually generated in the JCT, while its magnitude is modulated by the pores and vacuoles of the inner wall endothelium of Schlemm’s canal (Johnson et al., 2006).

The external wall of Schlemm’s canal is sparsely lined with entrances to the collector channels, which traverse radially onto the scleral surface and connect to the aqueous vein. Finally, AH is transported through the aqueous vein to the episcleral and conjunctival veins that return blood to the heart.
1.4 Pathogenesis of POAG

Despite decades of scrutiny, researchers have yet reached a consensus regarding the pathological mechanism underlying elevated IOP at the outflow tissue, which is the only risk factor for primary open-angle glaucoma. Although the mechanisms underlying glaucoma are not well understood, it has been established that glaucoma does not result from a single pathological mechanism, but rather a combination of pathways influenced by genes, age, and environment. The JCT region of the trabecular meshwork together with the inner wall of Schlemm’s canal is known to be the major site of aqueous outflow resistance. In glaucomatous patients, several physiological changes, including loss of trabecular meshwork cells, ECM remodeling, tissue stiffness, and decreased Schlemm’s canal porosity, have been observed and linked to increased outflow resistance. Understanding the development and mechanism of these changes could shed light on the pathogenesis of POAG.

1.4.1 Decreased Cellularity

The trabecular meshwork has been under significant scrutiny over the past decade as the primary site of dysfunction. This is because trabecular meshwork cells reside in a biologically demanding environment, constantly exposed to a combination of oxidative, metabolic, and mechanical stress. Naturally, trabecular meshwork cells adapt to these biomechanical challenges through its extensive cytoskeleton, complex cell-cell and cell-matrix attachments, and almost macrophage-like phagocytic ability (Stamer et al., 2017). However, accumulation of oxidative stress, cellular debris over a lifetime, along with other age-related metabolic changes, could compromise critical trabecular meshwork
functions and lead to cell death (Weinreb et al., 2014; Stamer et al., 2012; Gabelt et al., 2005; Tan et al., 2006). In fact, statistics over the past 60 years suggest that trabecular meshwork cellularity decreases with age, and in particular, it is significantly lower in POAG eyes than in age-matched healthy eyes (Alvarado et al., 1981; Grierson et al., 1987; Liton et al., 2005). Partial repopulation of cell-depleted outflow tissue with cultured trabecular meshwork cells has been shown to restore some key functions, such as responding to pressure spikes by reducing outflow resistance (Abu-Hassan et al., 2015). Luckily, the outflow pathway is relatively insensitive to normal immune rejection, so cell transplantation could become a promising alternative treatment for POAG.

1.4.2 Altered ECM remodeling

Due to the continuous flow of AH, the outflow pathway ECM is relatively dynamic, undergoing pressure-induced turnover and remodeling. The ECM is comprised of fibrillar and non-fibrillar collagen, elastin-containing microfibrils, glycosaminoglycan, and proteoglycans (Acott and Kelley, 2008). Structural and physiological changes in cells directly affect ECM production and degradation, which leads to changes in mechanical property and outflow resistance. Mechanical stretching or distortion of the ECM and attached JCT and Schlemm’s canal inner wall endothelium have been shown to initiate a cascade of events involving matrix metalloproteinase activity (Acott and Kelley, 2008; Bradley et al., 2001). The activation of these enzymes is necessary for ECM turnover and outflow facility regulation, and their inhibition could temporarily decrease outflow (Swaminathan et al., 2013). Gene expression analysis comparing glaucomatous and normal eyes has found an increase in the total protein levels of matrix metalloproteinase,
yet reduced activity, suggesting that the degradation mechanism is overwhelmed (De Groef et al., 2013).

Another ECM-related alteration found in glaucomatous eyes is the elevated level of TGF-β2 in the AH of glaucomatous eyes (Swaminathan et al., 2013). This is often accompanied by increased ECM deposition in the trabecular meshwork and decreased AH outflow. In addition, protein aggregates known as sheath-derived plaques have also been found in the JCT region of glaucomatous trabecular meshwork (Tektas et al., 2009). While the formation of these plaques is not known, it contains many ECM proteins, including elastin, collagen, and proteoglycans. Since either increasing ECM production or decreasing ECM degradation will lead to elevated IOP, compounds targeting these mechanisms are currently undergoing clinical trials.

1.4.3 Increased Tissue Stiffness

Stiffness is a measure of the tendency of a material to resist deformation when it is loaded. Under the context of outflow tissue, stiffness has two components, (i) the intrinsic stiffness of the ECM, determined by the constituents of the ECM as well as their three-dimensional nanoscale organization, and (ii) a dynamic component that is the stretching of the JCT and inner wall of SC, induced by elevated IOP (Last et al., 2011). In glaucomatous outflow tissue, ECM stiffness initiates a feedback loop that increases stiffness in trabecular meshwork cells and reduced matrix degradation. Changes in Schlemm’s canal cell contractility and basement membrane stiffness significantly impede giant vacuole and pore formation and lead to increased outflow resistance and IOP elevation (Stamer et al., 2015). These observations suggest that it is the presence of
glaucoma and not simply an aging of the tissue that is responsible for the increase in the elastic modulus. Understanding the underlying mechanism in IOP modulation could lead to the development of effective targets for new pharmacologic agents.

1.4.4 Modulation of Schlemm’s Canal Pores

The conventional pathway for AH outflow has a hydraulic conductivity that is surprisingly 2-5 orders of magnitude greater than that of non-fenestrated endothelial barriers. It was suggested that such high conductivity results from micron-sized pores within the Schlemm’s canal endothelium. It was later confirmed through SEM and TEM that indeed such pores exist and its density decreased in POAG. Although the mechanism of pore formation is not clear, some suspect that the mechanical stress induced by the basal-to-apical pressure gradient, as evidenced by the giant vacuole. This lead to studies on the mechanical properties, more specifically the elastic modulus of the Schlemm’s canal endothelium as the stiffness of the cells could lead to fewer pores. Using magnetic pulling cytometry, Zhou et al. 2012 investigated the responsiveness of Schlemm’s canal mechanical properties to pharmacological agents. By treating Schlemm’s canal with agents of known effects, they were able to show that Schlemm’s canal is highly contractile and responsive to pharmacological intervention; cell stiffness increased by up to 200 percent with thrombin and decreased by up to 80 percent with Latrunculin A. More importantly, cell stiffness correlates with outflow resistance, supporting the hypothesis that mechanical properties of Schlemm’s canal contribute to AH outflow possibly through pore formation modulation.
1.5 POAG Management

Currently available and approved treatments for glaucoma include various combination of pharmacologic, laser-based, and surgical procedures aimed at lowering IOP to prevent further optic nerve damage (Chamling et al., 2016; Fan and Wiggs 2010). Topical medications are the primary line of treatment applied directly to the ocular surface to reducing AH production or promote outflow. Based on the stage of disease, the rate of progression and life expectancy of the patient, one or more types of therapeutic agents are used to reduce IOP. Some of the most popular agents include prostaglandin analogues, cholinergic agonists, β-blockers and carbonic anhydrase inhibitors (Table 1). Limitations related to existing medications including harmful side effects, a significant portion of non-responders, low adherence, and need for combination therapy to achieve target IOP (Vicente et al., 2017).

Recent understanding in mechanisms leading to outflow dysfunction has illuminated novel strategies to target this primary pathological site. Several new pharmacological agents, such as Rho-kinase (ROCK) inhibitors and nitric oxide donors, directly influence the trabecular meshwork and Schlemm’s canal (Kopczynski and Epstein, 2014) and have shown encouraging results in the clinic (Hoy, 2018). Clinical trials are critical for determining drug efficacy, dosage, safety profiles, target populations, and the effects of combined therapy. This costly, multiphase process requires significant investment in infrastructure and sufficient number of suitable, compliant volunteers. A comprehensive study of major clinical trials database revealed 158 registered cases of drug-based interventions targeting POAG during the last 20 years (Vicente et al., 2017).
Of these drugs, IOP modulation was the most common mechanism of action (87.2%, n = 138), while combined IOP modulation and neuroprotection were tested in 10.1% (n = 16) of these trials. Various prostaglandin analogues (62.7%, n = 99), ROCK inhibitors (9.5%, n = 15), carbonic-anhydrase inhibitors (8.9%, n = 14), and β-blockers (4.4%, n = 7) were the focus of these studies.

Table 1. Available IOP-lowering medications for POAG (Zhang et al., 2012 with modifications)

<table>
<thead>
<tr>
<th>Target</th>
<th>Class</th>
<th>Mechanism of action</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous humour outflow</td>
<td>Prostaglandin analogus</td>
<td>Increase in outflow of aqueous humour, primarily through the remodeling of the <strong>uveoscleral outflow tract</strong></td>
<td>Highly effective and well tolerated: generally used as first-line therapy</td>
</tr>
<tr>
<td></td>
<td>Cholinergic agonists</td>
<td>Increase trabecular meshwork outflow through <strong>ciliary muscle contraction</strong></td>
<td>Highly effective but cause dim vision and discomfort</td>
</tr>
<tr>
<td>Aqueous humour formation</td>
<td>β-adrenergic receptor blockers</td>
<td>Decrease inflow by regulating <strong>aqueous humour formation</strong> in the ciliary processes</td>
<td>Occasional systemic effects include fatigue and bradycardia</td>
</tr>
<tr>
<td></td>
<td>α-adrenergic receptor agonists</td>
<td>Decrease inflow by inactivating adenyl cyclase in ciliary processes; may also increase uveoscleral outflow</td>
<td>TBD</td>
</tr>
<tr>
<td></td>
<td>Carbonic anhydrase inhibitors</td>
<td>Decrease <strong>aqueous humour formation</strong> by inhibiting carbonic anhydride and HCO3 production in the ciliary epithelium</td>
<td>TBD</td>
</tr>
</tbody>
</table>

Currently, all commercially available and clinical approaches to POAG management act by modifying aqueous dynamics, inhibiting AH production through the ciliary process and/or by increasing aqueous outflow through the trabecular and/or
uveoscleal outflow pathways (Toris et al., 2010). While these treatments can be effective in managing disease progression, they neither target the underlying cause of resistance, nor restore vision that has already been lost. Since POAG tends to remain asymptomatic until the cascade of IOP-related damages leading to vision loss has occurred, it is imperative to seek alternative approaches in rescuing damaged outflow functions.

### 1.6 Potential of Stem Cell in POAG

Stem cell has the therapeutic potential to replace damaged or diseased tissues and provide trophic support to maintain homeostasis. Adult mammalian tissues often possess adult stem cells and progenitor cells that provide a reservoir of replacements throughout an organism’s life. These endogenous stem cells have been expanded \textit{in vitro} and exhibit effective tissue regeneration when introduced to pathologic tissues \textit{in vivo}.

Approximately 35 years ago, an unusual population of the trabecular meshwork was accidentally discovered in the non-filtering region located beneath the Schwalbe’s line (Kelly et al., 2009). Treatment of this region with laser trabeculoplasty unexpectedly increased HTM cell division by 4-fold, and caused them to migrate out of the insert region to repopulate laser burn site within a week (Acott et al., 1989). Followup studies in human and monkey explants also reported increasing cell division and migration from this region either with or without laser stimulation (Grierson et al., 1983; Alexander and Grierson, 1989; Alexander et al., 1989; Dueker et al., 1990). This unique replicative ability was also found in some primary HTM cells, which exhibit different morphology, ultrastructure, growth patterns, and gene expression than normal HTM (Challa et al., 2003). Together, these findings strongly suggest the existence of endogenous trabecular
meshwork cells with stem-like properties, later termed as novel cells (Du et al., 2012). Several markers were identified to help differentiate between normal and novel trabecular meshwork cells using fluorescence-activated cell sorting, free-floating neurosphere, and clonal culture. These isolated novel cells are multipotent and able to home to the trabecular meshwork region and differentiate into HTM cells \textit{in vitro} and \textit{in vivo} (Du et al., 2012; Du et al., 2013; Du et al., 2016). Aging or glaucomatous HTM cells showed senescence phenotype (e.g., ROS accumulation, pathobiological changes) and were susceptible to ROS-induced damage (Chhunchha et al., 2017; Saccà et al., 2007). Stem cell-based therapy that targets HTM could provide an alternative avenue to glaucoma treatment, by restoring outflow tissue function in patients who have already lost vision (Chamling et al., 2016; Pearson and Martin, 2015; Yu et al., 2011).

However, isolation of this unique group is no easy task, since the stem-like trabecular meshwork makes up only 2-5\% of the entire trabecular meshwork cell population. Therefore, stem cell-based restoration of trabecular meshwork function in glaucomatous eyes would be limited by the availability of endogenous trabecular meshwork-like stem cells. A more reliable approach might be to derive TM-like cells from induced pluripotent stem cells (iPSCs) that have been genetically reprogrammed from the patient’s own dermal fibroblasts or other tissues. Mouse iPS cells can be differentiated by co-culturing with HTM cells for up to 21 days (Ding et al., 2014). These iPSC-TM cells closely resembled cultured HTM cells in morphology and protein expression. Additionally, transplantation of these iPSC-TM cells restored IOP homeostatic function in a human anterior segment model \textit{ex vivo} and in glaucoma mouse.
models in vivo (Du et al., 2016; Abu-Hassan et al. 2015; Zhu et al., 2016; Zhu et al., 2017). Several labs have already begun development of a stem cell-based replacement therapy for rebuilding the trabecular meshwork tissue and its regulatory functions. However, preclinical and clinical studies of stem cell-derived trabecular meshwork are needed to demonstrate the safety and functional restoration of IOP in vivo and efficacy of glaucoma treatment.

Currently, there has not been any known attempts in the field to differentiate Schlemm’s canal cells from stem cells. HSC cells are critical for understanding outflow physiology and glaucoma etiology. However, the primary HSC cells from donor frequently used in research are difficult to isolate. Therefore, the development of an alternative source of HSC cells could potentially expedite understanding in HSC biology. The embryological origin of HSC is controversial, sharing both vascular and lymphatic-like vessel characteristics (Dautriche et al., 2015).

The development of HSC is postnatal and can be classified into four stages: Schlemm’s canal progenitor cell-fate specification, lateral sprouting, lumenization, and separation from venous vasculature. In order to form mature Schlemm’s canal, the progenitor cells subsequently express vascular endothelial growth factor receptor (VEGFR) 2, tunica interna endothelial cell kinase 2, prospero Homeobox 1 (PROX1) and VEGFR-3. The lymphatic transcription factor, PROX1, and VEGF-C are required for proper Schlemm’s canal development. VEGF-C (VEGFc+/LacZ) heterozygous mice exhibited delayed budding of Schlemm’s canal endothelial cells from the venous system and retarded tubular fusion.
Similar to lymphatic cells, Schlemm’s canal cells experience flow from a basal to apical direction. Shear stress plays a critical role in maintaining HSC integrity, function, and PROX1 expression. Reduction of AH, presumably the loss of biomechanical cue from shear stress, resulted in endothelial-mesenchymal transition and loss of the lymphatic identity. While these studies were conducted in mice, expression of PROX1 by Schlemm’s canal endothelial cells in humans, zebrafish, and mice indicates that the lymphatic-like identity of the Schlemm’s canal is conserved in vertebrate evolution (Aspelund et al., 2014), and suggests that similar developmental pathways are likely to occur in humans, albeit prenatally rather than postnatally. Understanding the exact molecular footprint of Schlemm’s canal differentiation and organogenesis from stem cells will facilitate the establishment of platforms for manipulating Schlemm’s canal progenitor cells to address the scarcity of Schlemm’s canal cells available for research. Furthermore, it will advance our understanding of human Schlemm’s canal inner wall cell biology and physiology and its role in regulating IOP and glaucoma.

1.7 Construction of Stem Cell-Derived Outflow Tissue

Bioengineering approaches can benefit stem cell research by providing scalable and cost-efficient stem cell isolation, purification, expansion, differentiation, as well as a 3D engineered tissue constructs. Advanced biomaterials with tunable mechanical properties, micro-/nanopatterned topography, or gradients of biomacromolecules such as growth factors, have great potential to induce ocular tissue regeneration using stem cells. Advancement in 3D printing has the potential to allow fabrication and assembly of a complex and functional eye model (Barbara et al., 2014; Kador et al., 2016). Such a
platform would allow evaluation of therapeutics effectiveness and interaction at a multiple-tissue level.

Bioengineering trabecular meshwork has been attempted by growing primary cells on biomaterials. Primary HTM cells have been cultured on natural polymers, such as Matrigel (Bouchemi et al., 2017) and collagen (Osmond et al., 2017) to mimic the 3D morphology of HTM. Recently, our lab has demonstrated the feasibility of recreating the trabecular meshwork outflow function in vitro by growing primary HTM cells on synthetic, microfabricated, well-defined porous SU-8 scaffolds. These scaffolds have 12-\(\mu\)m pores to mimic the ECM of the in vivo microenvironment. The bioengineered 3D HTM on SU-8 scaffolds exhibited the in vivo-like phenotype and drug responsiveness to outflow modulation as expected (Torrejon et al., 2016a; Torrejon et al., 2016b; Torrejon et al., 2013). Furthermore, co-cultured 3D HTM/HSC constructs were created to better mimic the conventional outflow pathway (Dautriche et al., 2015). Compared to expensive and labor-intensive animal studies, our bioengineered HTM and/or primary HSC cells on scaffold provided a reliable, accessible, and scalable in vitro model to study disease pathogenesis and facilitate drug discovery (Torrejon et al., 2016a; 2016b; Dautriche et al., 2015). It laid a solid foundation for future preclinical and clinical testing of novel glaucoma drugs. However, there is an unmet need for stem cell-derived outflow tissue for understanding outflow physiology/pathology and high-throughput drug screening.

To evaluate whether stem cell-derived outflow tissue components could be used for understanding glaucoma pathogenesis and screening for potential therapeutic agents, the following studies have been performed as detailed in chapter 2-4, respectively.
Study 1: Construction and evaluation of 3D induced pluripotent stem cell-derived trabecular meshwork cells (iPSC-TM) on well-defined, microporous SU-8 scaffolds (Chapter 2)

Study 2: Differentiation of human adipose-derived stem cells (ADSCs) into HSC-like cells through biochemical and mechanical cues (Chapter 3)

Study 3: Design and fabrication of a dual-flow fluidic chamber for evaluation of biomechanical effects on the outflow tissue (Chapter 4)
1.7 References


Swaminathan, S. S., & Oh, D. J. understanding trabecular meshwork Outflow.


CHAPTER 2.

INDUCED PLURIPOTENT STEM CELL- DERIVED TRABECULAR MESHWORK AS A BIOENGINEERED DRUG TESTING PLATFORM

2.1 Introduction

Primary open-angle glaucoma (POAG) is a group of optic neuropathies characterized by progressive degeneration of retinal ganglion cells and their axons (Weinreb et al., 2004; Almasieh et al., 2012) caused by abnormally high intraocular pressure (IOP) (Weinreb et al., 2014; Stamer et al., 2012). IOP is the only modifiable risk factor of glaucoma. It is determined by the steady state balance between the rate of aqueous humor production by the ciliary process (inflow) and the rate of aqueous humor removal (outflow) (Gabelt et al., 2005; Carreon et al., 2017).

In humans, the majority of the aqueous humor (up to 90%) is eliminated through the conventional outflow pathway (Dautriche et al., 2014). The trabecular meshwork (TM) and Schlemm's canal make up this pressure-sensitive outflow pathway, dampening IOP fluctuation by adjusting the resistance to aqueous humor outflow in inverse proportion to IOP (Tamm et al., 2009). In particular, TM cells have the ability to remodel their extracellular matrix (ECM) and cytoskeleton in response to pressure-induced mechanical stress. Additionally, TM cells remove debris from the aqueous humor through phagocytosis to avoid local accumulation. However, accumulation of oxidative stress and
cellular debris over a lifetime, along with other age-related metabolic changes can lead to
cell death and potentially compromise TM functions (Weinreb et al., 2014; Stamer et al.,
2012; Gabelt et al., 2005; Tan et al., 2006). Work performed over the past 60 years
suggests that TM cellularity decreases with age. TM cellularity is also significantly lower
in eyes of patients with primary open angle glaucoma (POAG) than in eyes from age-
matched healthy controls (Alvarado et al., 1981; Grierson et al., 1987; Liton et al., 2005).
Therefore, glaucoma is a disease associated with aging and age-induced TM loss may
play a critical (and causative) role in increasing outflow tissue resistance, which could
lead to POAG (Gabelt and Kaufman, 2005). Current treatments for glaucoma rely on
pharmacologic or surgical reduction of IOP. Although generally effective in slowing
disease progression, most if not all of these treatments fail to replenish damaged TM cells
or repair outflow function (Barton et al., 2013). This lack of therapies that target the site
of pathology in glaucoma underscores the need to develop such targeted therapeutics that
would act on the TM. Furthermore, they highlight the value of in vitro models that can
recapitulate the TM morphology and function and can be used for understanding of
outflow physiology and screening of pharmacological agents for glaucoma.

Our lab has demonstrated the feasibility of simulating the TM outflow function in
vitro by growing primary human TM (HTM) cells on micropatterned, porous SU-8
scaffolds, which allows them to maintain their in vivo phenotypes and functional
responses to pharmacological agents (Torrejon et al., 2013). Compared to expensive and
labor-intensive animal studies, our bioengineered outflow pathway constructs provide a
reliable, accessible, and scalable in vitro model to study disease pathogenesis and
facilitate drug discovery (Torrejon et al., 2016a; 2016b; Dautriche et al., 2015). However, there is an unmet need of reproducible and scalable cell sources that can be used in the *in vitro* outflow pathway model.

TM progenitor cells represent a promising source of endogenous cells that can be used for HTM regeneration. These multipotent TM cells that occupy the non-filtering region of the TM can migrate towards the injury site after laser trabeculoplasty (Du et al., 2012; 2013; 2016; Samples et al., 1989). A number of protocols have been developed to isolate (Gonzalez et al., 2006; Kelley et al., 2009; Yun et al., 2016), expand and differentiate them to express an HTM phenotype (Samples et al., 1989; Yu et al., 2011; Ding et al., 2014). Unfortunately, since only 2-5% of the total cell population in this region is stem-like, it is hard to imagine that they can be used for autologous cell replacement therapy (Kelley et al., 2009). A more realistic approach might be to differentiate TM-like cells from autologous induced pluripotent stem cells (iPSCs). Such iPSCs could potentially be created by reprogramming the patient’s own dermal fibroblasts or cells from other tissues. These iPSC-derived TM cells have shown to have the capacity to restore the IOP homeostatic function in a human anterior segment organ culture model and in glaucoma mouse models *in vivo* (Du et al., 2016; Abu-Hassan et al. 2015; Zhu et al., 2016; Zhu et al., 2017). Hence, development of a stem cell-based TM replacement therapy seems like a logical long-term solution for restoring the TM cellularity and receiving its regulatory functions.

In this study, we evaluated whether human iPSC-derived TM cells can form functional, *in vivo*-like HTM tissue when cultured on SU-8 scaffolds. Morphology,
characteristic HTM protein expression, phagocytic activity, and outflow facility were evaluated. In addition, we evaluated whether exposure to dexamethasone alters cell phenotype and physiology in a manner similar to that of primary HTM cells in vitro and in vivo.

2.2 Materials and Methods

2.2.1 SU-8 Scaffold Fabrication

The microporous, free-standing SU-8 scaffolds (Fig. 2.1) used for cell culture were microfabricated as described previously (Torrejon et al., 2013). Briefly, a release layer was first spin-coated onto the silicon wafer and baked between 120–150°C. Next, SU-8 2010 (MicroChem Corp., Newton, MA) was spin-coated onto the release layer to <5 µm, baked at 95°C, then cooled to room temperature. The photoresist was exposed to UV-light (140 mJ/cm2) through a custom-designed mask with the desired pattern, baked at 95°C, and developed in PGMEA (MicroChem Corp.) Finally, the scaffolds were removed from the release layer, washed with isopropyl alcohol, and air dried.

2.2.2 Primary Human Trabecular Meshwork Cell Culture

Primary HTM cells were isolated from donor tissue rings discarded after penetrating keratoplasty. Isolation of these cells was performed under an IRB exempt protocol approved by the SUNY Downstate IRB. Isolation and culture conditions were as previously described (Torrejon et al., 2013). All HTM cell strains were characterized for expression of αβ-crystalline and α-smooth muscle actin (α-SMA) before experiments.
HTM cells were initially expanded in 75 cm² cell culture flasks coated with 1% gelatin and fed every 48 hours with 10% FBS (Atlas Biologicals, Fort Collins, CO) in Improved MEM (Corning Cellgro, Manassas, VA) with 1% gentamicin. All studies were conducted using cells before the 5th passage.

Figure 2.1. Schematics of the fabrication process for micropatened, porous SU-8 scaffolds via photolithography. First, (A) silicon wafer substrate is cleaned and treated with Omnicoat as the release layer, followed by (B) spin-coating of SU-8 2010. Then, (C) the wafer is exposed to a patterned mask. (D) Since SU-8 is a negative photoresist, unexposed, and therefore uncrosslinked SU-8 will be removed during the development phase. (E) Optical and (F) scanning electron microscopy of the freestanding SU-8 scaffold with 12 μm pores. Scale bar = 20 μm

2.2.3 iPSC-TM Cell Culture

Human iPSC-derive TM cells were first passaged onto feeder-free tissue culture plates coated with Matrigel (BD Biosciences, San Jose, CA, USA) and expanded in
mTeSR1 (StemCell Technologies, Vancouver, BC, Canada) as colonies. Cells were then cultured on extracellular matrix (ECM) derived from A549 cells in a culture medium containing for neural crest cell differentiation for 10 days. The neural crest cells were then induced to differentiate into TM cells by culturing on ECM derived from primary TM cells in a culture medium containing TM cell-derived conditioning medium for 10-14 days (manuscript in preparation). Cultures were fed every 48 hours with fresh media containing DMEM/F12 (Sigma), 10% FBS (Atlas Biologicals), conditioning medium and 100 µM ascorbic acid 2-phosphate (Sigma).

2.2.4 Cell Culture on SU-8 Scaffolds and Dexamethasone Treatment

Prior to use in cell culture, SU-8 scaffolds were mounted on aluminum tape rings, UV sterilized, coated with 1% gelatin to promote cell attachment. trabecular meshwork cells were seeded at a density of 50,000 cells/scaffold and cultured in the appropriate medium for 14 days. Samples were treated with Dexamethasone (100 nM, Sigma Aldrich, ST. Louis, MO) for 7 days to induce myocilin expression in both HTM and iPSC-TM cells.

2.2.5 Optical and Scanning Electron Microscopy

Cell growth was monitored on a daily basis using the Nikon TS100-F inverted microscopy (Micro Video Instrument, Avon, MA). Cell morphology was characterized via the LEO 1550 field emission scanning electron microscope (SEM; Leo Electron Microscopy Ltd, Cambridge, UK). To prepare for SEM, samples were fixed with 2% glutaraldehyde solution in 0.1M phosphate buffer and 0.1M sucrose for 2 hours at room
temperature. Then, samples were chemically dehydrated in a graded ethanol and slowly infiltrated with a graded hexamethyldisilazane (HMDS)-ethanol. Critically dried samples were mounted on SEM stubs using carbon tape and were sputter-coated with iridium to avoid charging effects. Images were collected at 3kV gun voltage and working distance of 5 mm. Cell alignment was quantified using the ImageJ plugin, OrientationJ Analysis and Distribution, which creates histogram of cell orientation based on the SEM images. The standard deviation of the histogram was calculated to represent the alignment index, where high alignment index indicates random orientation and low alignment index indicates similar orientation.

### 2.2.6 Immunocytochemistry

Samples were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked using 5% bovine serum. Samples were subsequently incubated with the appropriate primary antibodies: rabbit anti-myocilin, mouse anti-α-smooth muscle actin (α-SMA), rabbit anti-fibronectin, and rabbit anti-OCT3/4 (Abcam, Cambridge, MA). Secondary antibody, mouse anti-rabbit Alexa Fluor 647 was used to detect myocilin, fibronectin and OCT3/4, goat anti-mouse Alexa Fluor 594 was used to detect α-SMA (1:200, Invitrogen, Grand Island, NY). These samples were further counter-stained with 4’,6-diamidino-2-phenylindole (DAPI) to reveal cell nuclei. Laser scanning confocal microscopy was performed using a Leica SP5 confocal microscope, and images were acquired at 20X or 63X magnifications using an oil-immersion objective. Confocal images were processed using Leica LasAF software, and all confocal images within a
given experiment were imaged and captured using the same laser intensity and gain settings in order to be able to compare intensities across samples.

### 2.2.7 Western Blot

Cellular proteins were extracted with ice-cold radioimmunoprecipitation assay (RIPA) buffer, containing protease inhibitors (Complete Protease Inhibitor; Roche, Manheim, Germany). Proteins were quantified by bicinchoninic acid assay (Thermo Fischer Scientific). 20 µg of proteins from each sample were separated by SDS polyacrylamide gel electrophoresis on a 12% gel in MOPS running buffer (ThermoFisher Scientific), transferred onto a nitrocellulose membrane and probed with primary antibodies against myocilin, OCT3/4, and β-actin (Abcam). After incubation in HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Invitrogen), bound antibodies were detected using FluorChem E (Protein Simple). Protein expression was analyzed by densitometry using ImageJ, and normalized to the house-keeping β-actin.

### 2.2.8 Phagocytosis Assay

Primary HTM and human iPSC-derived TM cells were seeded on SU-8 scaffolds and incubated with 488-conjugated dextran beads (50 ng/ml, Sigma) for 0, 15, 30, 45, 60, 90, 120, and 180 minutes. After incubation, each scaffold was washed with Trypan Blue to deactivate extracellular fluorescence and washed with PBS three times. Phagocytosis was quantified by measuring the fluorescent intensity using a plate reader (Tecan, Mennedorf, Switzerland). Each data point represents the average of four replicates from
two experiments. Selected samples were fixed with 4% paraformaldehyde and stained with DAPI for visualizing the ingested particles using confocal microscopy.

2.2.9 Cytoskeleton Stain and Nuclear Size Analysis

F-actin cytoskeleton and CLAN formation were stained using phalloidin (Life Technology) and co-stained with DAPI to reveal cell nuclei. Nuclei size was measured using ImageJ. Ten images from five control samples and eight images from four dexamethasone-treated samples were used for the quantification.

2.2.10 Perfusion Assay

A perfusion apparatus was used as previously described (Torrejon et al., 2013). Samples were securely placed in the perfusion chamber and perfused at various rates for 5 hours per flow rate (2, 4, 8, and 16 µL/min). Samples were perfused in an apical-to-basal direction with perfusion medium consisting of Dulbecco's modified Eagle's medium (DMEM; Cellgro) with 0.1% gentamicin. Pressure was continuously monitored and recorded. After perfusion, samples were fixed and stained for SEM or confocal imaging. The “outflow facility” of the bioengineered 3D TM models was calculated from the inverse of the slope of the pressure versus flow graph, per unit surface area. Six sample replicates per condition were studied under perfusion.

2.2.11 Statistical Analysis

All experimental data, unless otherwise specified, were expressed as means ± standard error of the mean from at least three independent experiments. Comparisons of
means of three or more groups were performed using two-way ANOVA followed by post-hoc testing. p values of <0.05 were considered statistically significant.

Figure 2.2. Schematics of the flow system constructed perfusion studies.
2.3 Results

2.3.1 Human iPSC-TM cells grown on SU-8 scaffold exhibited primary HTM-like morphology

Primary HTM cells form long processes to attach to each other and their surrounding ECM (Grierson and Lee, 1974; Grierson et al., 1978; Lutjen-Drecoll, 1999). When grown on the plastic surface of cell culture flasks, HTM cells tended to be randomly oriented and form a single layer (Fig. 2.3A). When cultured on SU-8 scaffolds, HTM cells not only formed multiple cell layers, but also became more aligned and elongated (Fig. 2.3B and C).

![Figure 2.3. Primary HTM and iPSC-TM cell morphology on conventional 2D culture flask and 3D SU-8 scaffolds.](image)

(A and E) Optical images of HTM and iPSC-TM cells grown on 2D culture flask. Scale bar = 10 µm. SEM images of (B-C) HTM and (F-G) iPSC-TM cells cultured on SU-8 scaffolds at different magnifications, along with a zoomed in view of the decellularized extracellular matrix (D and H). (B, F) Scale bar = 50 µm. (C, G) Scale bar = 10 µm. (D, H) Scale bar = 2 µm.

Similar transformation was observed in human iPSC-derived TM cells when comparing morphology of cells cultured on a 2D plastic surface (Fig. 2.3E) versus on 3D SU-8 scaffolds (Fig. 2.3F and G). Human iPSC-derived TM cells showed full coverage of
the SU-8 scaffold with elongated and aligned cell bodies. After 14 days, decellularized scaffolds showed a thin deposition of ECM over the 12-µm pores of the SU-8 scaffold from both HTM and iPSC-derived TM cells on SEM imaging (Fig. 2.3D and H), demonstrating the ECM deposition capacity of iPSC-derived TM cells on 3D porous scaffolds.

Figure 2.4. Confocal images showed expression of HTM markers of myocilin and α-SMA in 3D cultured trabecular meshwork cells. (A-D) iPSC-TM cells grown on glass coverslips. (E-H) iPSC-TM cells grown on SU-8 scaffolds. (I-L) Primary HTM grown on SU-8 scaffolds as the control. (M) ImageJ quantification of fluorescence intensity. Scale bar = 100 µm.
Figure 2.5. Confocal images showed expression of HTM markers of fibronectin and f-actin in 3D cultured trabecular meshwork cells. (A-D) iPSC-TM cells grown on glass coverslips. (E-H) iPSC-TM cells grown on SU-8 scaffolds. (I-L) Primary HTM grown on SU-8 scaffolds as the control. (M) ImageJ quantification of fluorescence intensity. Scale bar = 50 µm.

2.3.2 iPSC-TM on SU-8 scaffold exhibited characteristic HTM protein markers

The ability of human iPSC-TM cells to express characteristic HTM proteins including myocilin, α-SMA, and ECM protein fibronectin was determined using immunocytochemistry and confocal microscopy. DAPI-stained nuclei were used to reveal
total cell population and phalloidin-stained F-actin was used to show cytoskeleton organization (Fig. 2.5).

Immunofluorescent analysis demonstrated that after 14 days in culture only a small fraction of iPSC-derived TM cells expressed myocilin when cultured on glass (Fig. 2.4B), while a significantly larger percentage of iPSC-derived TM cells cultured on the porous SU-8 scaffold expressed myocilin (Fig. 2.4F), with expression levels reaching those seen in primary HTM (Fig. 2.4J). α-SMA expression for all cells under the culture conditions was similar. Fibronectin-based ECM-cell connections appear to be very important for the adhesion of Schlemm’s canal endothelial cells to the fibrillar ECM of the HTM. Primary HTM and iPSC-derived TM cells grown on the SU-8 scaffold expressed slightly higher level of fibronectin than those grown on glass coverslips (Fig. 2.5). However, ImageJ quantification analysis of the difference did not show any statistical significance (Fig. 2.5M).

**2.3.3 Dexamethasone induced changes to cell morphology of 3D iPSC-TM**

To further validate the feasibility of using 3D iPSC-derived TM as a model for drug testing, human iPSC-derived TM cells grown on SU-8 scaffolds were treated with dexamethasone, which is known to change HTM cell morphology, ECM deposition, myocilin expression, and outflow resistance. Dexamethasone-treated primary HTM and iPSC-derived TM cells grown on glass coverslips (Fig. 2.6A) and on the SU-8 scaffold showed a disturbed morphology. In addition, instead of a smooth, full coverage of cells grown on the SU-8, there is significant spacing between cells on the scaffold when cells were exposed to dexamethasone (compare Fig. 2.6B-C to Fig. 2.3B-C).
Figure 2.6. Cell morphology of primary HTM and iPSC-TM cells after treatment with dexamethasone. (A and E) Optical images of primary HTM and iPSC-TM cells grown on 2D plastic surface. Scale bar = 10 µm. SEM images of (B and C) HTM and (F and G) iPSC-TM cells cultured on gelatin-coated SU-8 scaffolds at different magnifications, along with a zoomed in view of the decellularized ECM (D and H). (B, F) Scale bar = 50 µm. (C, G) Scale bar = 10 µm. (D, H) Scale bar = 2 µm.

Dexamethasone-treated iPSC-derived TM showed shrinkage in cell volume, as well as decreased cell coverage on glass coverslips (compare Fig. 2.6E to 2.3E) and on the SU-8 scaffold (compare Fig. 2.6F-G to Fig. 2.3F-G). Both dexamethasone-treated HTM and iPSC-derived TM showed thicker, cord-like ECM deposition when compared to the non-treated controls (Fig. 2.6D, H).

Figure 2.7. Quantification of nuclei size after DEX-treatment in HTM and iPSC-TM cells. **p < 0.001, ***p < 0.0005.
The effects of dexamethasone treatment on nuclei size and the actin skeletal network were evaluated using confocal microscopy and quantified using ImageJ. The nuclei in both dexamethasone-treated HTM and iPSC-derived TM cells were significantly enlarged when compared to the controls (Fig. 2.7), similar to previous reports in *ex vivo* dexamethasone-induced glaucoma models (Clark et al., 1995). Dexamethasone treatment also induced F-actin reorganization and crosslinked actin...
network (CLAN) formation in both HTM and iPSC-derived TM cells, although the percentage of CLAN forming cells is significantly higher in the primary HTM culture (41% in HTM versus 18% in iPSC-TM; Fig. 2.8). This confirms that 3D iPSC-derived TM cells are responsive to dexamethasone in terms of changes in cell morphology, nuclei size, and cytoskeleton re-arrangement, although the extent of this response may be slightly lower than that seen by primary HTM cells.

**2.3.4 Phagocytosis Activity of 3D iPSC-derived TM cells in Response to Dexamethasone Treatment**

Phagocytosis is an important function of TM cells that is not observed in iPSCs. Here we used FITC-conjugated dextran beads to evaluate the ability of 3D iPSC-derived TM cells to carry out phagocytosis. We measured fluorescence every 30 minutes for up to 3 hours after adding dextran beads. The 3D cell cultures with HTM cells exhibited high phagocytic activity with maximal fluorescence readings achieved 2 hours after addition of dextran beads (Fig. 2.9C). Treatment with dexamethasone drastically decreases the phagocytic ability of primary HTM cells, as demonstrated by the significant decrease in fluorescence intensity in comparison to control after 2 hours (Fig. 2.9A-C).

In comparison, 3D iPSC-derived TM cells displayed lower fluorescence throughout the entire incubation period. Maximum fluorescence intensity at hour 3 is roughly one third that of the primary HTM cells at the same time point (Fig. 2.9D), suggesting that either a portion of the iPSC-derived TM cells are not phagocytic or that the overall population has lower phagocytic capacity than primary HTM cells. Dexamethasone-treated iPSC-derived TM displayed similar levels of phagocytic activity
as dexamethasone-treated HTM cells (Fig. 2.9D-F), although the differences between control and dexamethasone-treated iPSC-derived TM cells are not significant at the 3-hour time point.

![Confocal images of (A-B) HTM and (D-E) iPSC-TM that have ingested the fluorescent beads (green); nuclei are stained with DAPI (blue). (C,F) Quantification of phagocytic activity by measuring fluorescence intensity. Scale bar = 20 µm. *p < 0.01, **p < 0.001, ***p < 0.0005.]

**Figure 2.9. Effect of dexamethasone on phagocytosis in HTM and iPSC-TM after 3 hours of incubation with 488-conjugated dextran beads.** Confocal images of (A-B) HTM and (D-E) iPSC-TM that have ingested the fluorescent beads (green); nuclei are stained with DAPI (blue). (C,F) Quantification of phagocytic activity by measuring fluorescence intensity. Scale bar = 20 µm. *p < 0.01, **p < 0.001, ***p < 0.0005.

### 2.3.5 Dexamethasone induced changes to characteristic HTM protein expression

Immunoblotting demonstrated a marked increase of myocilin in both primary HTM and iPSC-derived TM cells grown on SU-8 scaffolds after dexamethasone treatment (Fig. 2.11). Dexamethasone-induced increase in myocilin expression is characteristic of TM cells, but not observed in iPSCs. In addition, there was a decrease in stem cell marker, OCT3/4 expression after dexamethasone treatment.
Figure 2.10. Confocal analysis of protein expression in IPSC-TM with and without DEX treatment. Characteristic trabecular meshwork markers (B and F) myocilin and (C and G) α-SMA, as well as stem cell marker (J and N) OCT3/4 and (K and O) f-actin were evaluated. Scale bar = 50 µm.

Immunocytochemistry also confirmed the dexamethasone-induced increase in myocilin expression in iPSC-derived TM cells grown on SU-8 scaffolds (Fig. 2.10B and F). The dexamethasone-induced ECM deposition in 3D iPSC-derived TM constructs was further confirmed by immunocytochemistry analysis, which clearly showed enhanced
fibronectin and collagen IV expression (Fig. 2.12). Deposition thickness, as measured from confocal z-stack cross-sections, revealed that dexamethasone treatment increased fibronectin and collagen IV deposition by 32.2% and 62.6%, respectively (Fig. 8E).

Figure 2.11. Western blot analysis of myocilin and Oct3/4 expression in HTM and iPSC-TM before and after DEX treatment. (A) Western blot images. (B) Densitometry of western blot normalized to β-actin. *p < 0.01, ***p < 0.0005, ****p < 0.0001.

Figure 2.12. Confocal analysis of extracellular matrix proteins deposition by 3D iPSC-TM. Deposition of (A and C) fibronectin and (B and D) collagen IV with and without DEX treatment. (E) Quantification of deposition thickness from confocal cross-section via ImageJ. Scale bar = 50 µm. **p < 0.001, ***p < 0.0005.
2.3.6 Outflow Studies with or without Dexamethasone Treatment

Finally, we examined whether the 3D iPSC-derived TM constructs can regulate fluid outflow in a “physiologic manner” in response to dexamethasone treatment. We used a perfusion apparatus to measure the transmembrane pressure and compared results to those obtained by using primary HTM cells with or without dexamethasone treatment in the same constructs. Dexamethasone is known to cause an increase in flow resistance, and therefore, decrease outflow facility in vitro, ex vivo and in vivo. Dexamethasone-treated 3D primary HTMs have a significantly lower outflow facility than untreated control samples (Fig. 2.13). Similarly, dexamethasone-treated 3D iPSC-derived TM displayed a decreased outflow facility as well (Fig. 9C).

![Graphs showing outflow studies](image)

**Figure 2.13. Effect of dexamethasone on outflow facility of HTM and iPSC-TM.** (A and C) Relationship between perfusion flow rate and transmembrane pressure in (A) HTM and (C) iPSC-TM cells. (B and D) Outflow facility of (B) HTM and (D) iPSC-TM calculated from the inverse slope of the flow rate versus pressure graph. *p < 0.01, **p < 0.001.
2.4 Discussion

In this study, we demonstrate the feasibility of using human stem cells to recreate a 3D iPSC-derived TM model that recapitulates the HTM morphology, gene and protein expression, and outflow function. The in vivo microenvironment around HTM contains a compliant, 3D network of ECM proteins that provides critical topographic cues and influence cell phenotype and function (Russell et al., 2008; Gasiorowski et al., 2009). Conventional 2D culture plastics lack this complex surface support and have been reported to down-regulate myocilin expression in TM cells (Tamm et al., 1999). Polyacrylamide gels more closely mimic the compliance of the in vivo ECM, hence leading to fewer stress fibers and spreading of the HTM cell bodies than HTM cells plated onto stiff plastic (Schlunck et al., 2008). While soft gels are useful for studying the biomechanics of HTM, they are unsuitable for functional studies of outflow facility. The Transwell membrane has been used as a cell substrate insert for perfusion systems, however, due to its non-uniform pore size and density, it exhibits flow resistance much higher than that of in vivo TM. Previously, we have shown that microfabricated SU-8 scaffolds with well-defined micropores could mimic the TM microenvironment and maintain primary HTM phenotype in vitro (Torrejon et al., 2013). In this study, we examine whether iPSC-derived TM cells could proliferate, sustain HTM-like marker expression and certain HTM functions when maintained on the SU-8 scaffolds.

Initial observation of cell morphology on flat surfaces versus microporous scaffolds revealed that like primary HTM cells, iPSC-derived TM cells grown on SU-8 scaffolds altered their alignment (Fig. 2.3F-G) and deposited a thin layer of ECM
covering the porous scaffold (Fig. 2.3H). This morphologic response in HTM to micro-/nano-scale features has been observed by other labs as well (Russell et al., 2008). In addition to morphology, expression of characteristic HTM proteins, such as myocilin by iPSC-derived HTM cells parallels that of primary HTM cells. Primary HTM cells down-regulate myocilin expression when grown in 2D culture (Tamm et al., 1999). This phenomenon was also observed in iPSC-derived TM cells where myocilin expression is expressed in abundance when the cells are grown on SU-8 scaffolds (Fig. 2.4F) while only sparingly when grown on 2D flat surface (Fig. 2.4B).

Dexamethasone has been shown to cause increases in nuclear size, CLAN formation, and myocilin expression, while decreasing in phagocytosis in TM cells (Zhu et al., 2016), which all lead to decreased ECM turnover and outflow facility. Human iPSC-derived TM cells treated with dexamethasone also showed drastic changes in morphology (Fig. 2.5F-G), most notably shrinkage in cell body size, enlarged nuclei (Fig. 2.7), and CLAN formation (Fig. 2.8).

Phagocytosis is an integrin-mediated, multistep process employed by TM cells to clear away ECM, cellular debris and other materials that might be restricting aqueous humor outflow (Peotter et al., 2016). Analysis of phagocytic activity after treatment with dexamethasone showed a decrease in ingested fluorescent bead intensity in primary HTM cells, as that has been previously reported in TM cells isolated from both POAG and glucocorticoid-induced glaucoma patients (Zhang et al., 2006), and is related to formation of CLAN (Gasiorowski et al., 2009). Dexamethasone-treated iPSC-derived TM cells showed similar phagocytosis activity to its HTM counterpart, however, due to lower
baseline activity, the difference between control and treated iPSC-derived TM cells is not significant at the 3-hour time point.

To further understand potential differences between primary HTM and iPSC-derived TM cells we looked at the expression and regulation of myocilin. Myocilin is a secreted glaucoma-associated protein that interacts with intracellular and ECM proteins in the TM microenvironment. In HTM cells, myocilin overexpression can be induced by dexamethasone, and mutations in myocilin are linked to elevated intraocular pressure and glaucoma (Borrás et al., 2014). Both confocal and western blot analysis showed increased myocilin expression in dexamethasone-treated HTM and iPSC-derived TM cells (Fig. 2.10-11). Myocilin has also been shown to interact and co-localize with fibronectin and collagen type IV, both major ECM components of the juxta-canalicular trabecular (JCT) region (Ueda et al., 2002). Interestingly, quantification of confocal cross-sections showed a significant increase in deposition thickness of these ECM proteins after dexamethasone-treatment of both primary and iPSC-derived TM cells (Fig. 2.12).

A major advantage of the SU-8 scaffold is its porosity which enables the measurement of outflow facility through perfusion studies. Dexamethasone-induced changes described previously translated to a significant increase in outflow resistance in both HTM and iPSC-derived TM populated 3D constructs. Thus it appears that the 3D iPSC-derived TM cultures resemble 3D HTM cultures in their physiologic response to flow despite some difference in other functions. The establishment of this 3D iPSC-derived HTM model highlights the utility of human iPSCs as reproducible and scalable
cell sources in this in vitro HTM model that can be used for understanding of outflow physiology and screening of pharmacologic agents that act on the TM.

2.5 Conclusion

In this study, we have demonstrated that human iPSC-derived TM cells can form functional HTM-like tissue when cultured on microfabricated SU-8 scaffolds. The 3D iPSC-derived TM exhibits at least partially HTM-like cell morphology, ECM deposition, marker expression, phagocytosis, and drug responsiveness to dexamethasone treatment. It offers a promising stem cell-based platform for glaucoma drug screening and further understanding of outflow pathway physiology.
2.6 References


CHAPTER 3.

BIOENGINEERING STEM CELL-DERIVED SCHLEMM’S CANAL-LIKE CELLS FOR IN VITRO GLAUCOMA DRUG SCREENING

3.1 Introduction

Primary open angle glaucoma is a complex neurodegenerative disease often associated with elevated intraocular pressure (IOP) (Weinreb et al., 2016). As the leading cause of blindness and visual impairment, it affects more than 70 million individuals worldwide (Kapetanakis et al., 2016). However, while most of the currently available glaucoma therapies target aqueous humor production or the uvea-scleral outflow pathway, they do not address the primary conventional outflow pathway, which is normally responsible for 70-90% of aqueous humor drainage into the systemic circulation (Goel et al., 2010; Weinreb et al., 2014). Dysfunction and blockage in this primary pathway can lead to increased outflow resistance, elevated IOP, and eventually glaucoma. Although pathogenetic mechanisms that lead to IOP elevation are still unclear, previous studies have identified the inner wall region of Schlemm’s canal as the primary site of outflow resistance (Grant et al., 1963; Mäepea et al., 1989; Ethier et al., 2002; Johnson et al., 2006).

The inner wall region of Schlemm’s canal is composed of the inner wall endothelium of Schlemm’s canal, its discontinuous basement membrane, and the adjacent juxtacanalicular connective tissue of the trabecular meshwork (Johnson et al., 2006).
Schlemm’s canal is a ring-shaped, often non-continuous vessel that encircles the ocular anterior chamber at the iridocorneal angle. Its lumen is lined with a single non-fenestrated layer of spindle-shaped endothelial cells, with their long axis aligned and parallel to the direction of flow (Ramos et al., 2007).

As the final barrier to aqueous humor drainage, the inner wall Schlemm’s canal cells experience a remarkably dynamic microenvironment, due to both the basal-to-apical and shear flow of aqueous humor (Ethier et al., 2004; Fautsch et al., 2006; Ashpole et al., 2014). The basal-to-apical flow, in particular, generates a massive transcellular mechanical load, leading to the creation of fluid-filled intracellular cavities or “giant vacuoles” that fuse with the cell membrane (Alvarado et al., 2004; VanderWyst et al., 2011). These pressure-dependent protrusions into the canal lumen are often accompanied by transcellular and/or paracellular pores, which allow aqueous humor drainage without compromising the blood-aqueous barrier (Braakman et al., 2016). In glaucomatous eyes, changes in Schlemm’s canal cell contractility and basement membrane stiffness significantly impede giant vacuole and pore formation and lead to increased outflow resistance and IOP elevation (Stamer et al., 2015). Understanding the underlying mechanism and role of Schlemm’s canal cells in IOP modulation could lead to the development of effective targets for new pharmacologic agents (Stack et al., 2018).

Isolated primary human Schlemm’s canal (HSC) cells provide a valuable in vitro cellular model to study their role in outflow physiology and pathology and in discovering new therapeutic targets (Stamer et al., 1998). In fact, HSC isolated from healthy and glaucomatous eyes have measurable differences in gene expression and cytoskeletal
stiffness that affect pore density and outflow resistance (Overby et al., 2014). New IOP-lowering agents that include actin depolymerizers and Rho kinase inhibitors target and regulate cell stiffness affecting HSC cells (Rao et al., 2001; Inoue et al., 2013). Unfortunately, HSC cell scarcity and technical difficulty in their isolation create a bottleneck for effective and affordable high-throughput drug screening using HSC cells. Stem cell differentiation is a promising approach to create an alternative source that is physiologically and functionally similar to HSC cells for in vitro cell-based drug screening.

Adipose tissue-derived stem cells (ADSCs) are adult stem cells with the ability to proliferate, self-renew and differentiate (Fraser et al., 2006). They can be easily isolated from adipose tissue, which can be obtained in large quantity through liposuction, a commonly performed, low-risk surgical procedure (Housman et al., 2002). To date, ADSCs have been successfully differentiated into adipocytes (Rodriguez et al., 2004; Huang et al., 2009), osteoblasts (Knippenberg et al., 2005, 2006), chondrocytes (Erickson et al., 2002; Awad et al., 2004), myocytes (Rangappa et al., 2003; Planat-Benard et al., 2004), smooth muscle cells (Rodriguez et al., 2006), neurons (Safford et al., 2002; Ashjian et al., 2003), vascular endothelium (Fischer et al., 2009; dela Paz et al., 2012; Colazzo et al., 2014) and lymphatic endothelium-like cells (Yang et al., 2015; Deng et al., 2017) under lineage-specific culture conditions. In particular, the capacity of ADSCs to differentiate into vascular and lymphatic endothelium-like cells makes them a promising candidate for HSC cell differentiation since HSC cells exhibit a combination of vascular and lymphatic phenotypes.
Schlemm’s canal barrier integrity and functions depend on the induction and continual expression of PROX1, a master regulator of the lymphatic system, and VEGFR3, the surface receptor for vascular endothelial growth factors (VEGFs) (Aspelund et al., 2014; Kizhatil et al., 2014; Karpinich et al., 2014). Short-term treatment using VEGF-C has been shown to induce expression of both vascular endothelial markers (CD31) and lymphatic markers (PROX1) in ADSCs, but not VEGFR-3 (Yan et al., 2011). Fluid shear stress, on the other hand, is known to activate VEGFR-3 expression in both blood and lymphatic endothelial cells (Baeyens et al., 2015). In addition to growth factor and fluid flow, cellular substrate stiffness, composition, and porosity could also alter the three-dimensional (3D) microenvironment needed for differentiation (Engler et al. 2006; Baker et al., 2012). Previously, we have shown that human trabecular meshwork (HTM) cultured on microporous SU8 scaffolds maintained its in vivo phenotype, along with the ability to secrete extracellular matrix (ECM), regulate outflow facility and respond to IOP-altering agents (Torrejon et al., 2013, 2016; Dautriche et al., 2015), making it the ideal cellular substrate to support and guide ADSC differentiation into Schlemm’s canal-like cells.

In this study, we tested the hypothesis that ADSCs can be differentiated into HSC-like cells that not only express both vascular and lymphatic markers, but also maintain comparable outflow resistance. VEGF-C, shear stress, and co-culture with primary HTM cells were used to promote HSC-like differentiation by mimicking the biological, mechanical and cellular microenvironment in vivo. Schlemm’s canal phenotypes were subsequently evaluated by examining the cell morphology using optical and scanning
electron microscopy (SEM), gene/protein expression of the HSC marker (e.g., PROX1) using immunocytochemistry, immunoblotting and qPCR analysis, and functional assessment of outflow facility using perfusion studies.

Figure 3.1. Schematics of differentiation strategy to induce ADSCs into SC-like cells using a combination of VEGF-C, shear stress and co-culture with HTM cells on the SU-8 scaffold.
3.2 Materials and Methods

3.2.1 Collection, Isolation, and Culture of Human ADSCs

Human ADSCs were isolated and cultured as described previously (Du et al., 2010). Briefly, human subcutaneous adipose tissue was obtained from patients undergoing elective lipoaspiration surgery with informed consent under a protocol approved by the Institutional Review Board (IRB) of the University of Pittsburgh, consistent with the principles of the Declaration of Helsinki. ADSCs were initially expanded in 75 cm$^2$ cell culture flasks and fed every 48 hours with 10% FBS (HyClone; Fisher Scientific, Pittsburg, PA) in DMEM/F21 (Gibco, Grand Island, NY) with 100 nM Dexamethasone. Cells were maintained at 37°C in a humidified atmosphere with 5% CO$_2$ until 80–90% confluence, and subcultured using 0.05% trypsin/0.5 mM EDTA (Gibco, Grand Island, NY).

3.2.2 Primary Human Schlemm’s Canal Cell Culture

Primary HSC cells were provided by Dr. Stamer at Duke University. The protocol for HSC cell extraction from postmortem human eyes is described in detail elsewhere (Stamer et al., 1998). Extracted cells were shipped overnight in T-25 flasks filled with DMEM/Low glucose (Life Technologies; Carlsbad, CA) medium with 10% FBS (Atlas Biologicals, Fort Collins, CO) and 1% penicillin/streptomycin. Upon arrival, the culture medium was replaced with fresh medium and flasks were stored in the incubator at 37°C and 5% CO$_2$. Medium was changed every other day. Cells were trypsinized and passaged.
at 1:3 ratio when 80% confluent. All studies were conducted using cells before the 6th passage.

### 3.2.3 Primary Human Trabecular Meshwork Cell Culture

Primary HTM cells were isolated from donor tissue rings discarded after penetrating keratoplasty. Isolation of these cells was performed under an IRB-exempt protocol approved by the SUNY Downstate IRB. Isolation and culture conditions were as previously described (Torrejon et al., 2013). All HTM cell strains were characterized for expression of $\alpha$B-crystalline and $\alpha$-smooth muscle actin ($\alpha$SMA) before experiments. HTM cells were initially expanded in 75 cm$^2$ cell culture flasks coated with 1% gelatin and fed every 48 hours with 10% FBS (Atlas Biologicals, Fort Collins, CO) in Improved MEM (Corning Cellgro, Manassas, VA) with 1% gentamicin. All studies were conducted using cells before the 5th passage.

### 3.2.4 Differentiation of Human ADSCs

The protocol for endothelial differentiation of human ADSCs was modified from a previous study (Dardkid et al., 2005). Briefly, human ADSCs were passaged onto 6-well plates and cultured for 3 days under static conditions in the ADSC medium described above. When culture was 50% confluent, the ADSC medium was exchanged with EGM-2MV (Endothelial Cell Growth Medium BulletKit™; Lonza, Walkersville, MD) supplemented with 50 ng/ml of VEGF-A (Sigma-Aldrich, St Louis, MO). Then the plate was secured onto an orbital shaker (Scilogex, Rocky Hill, CT) and maintained at 37°C, 5% CO$_2$. Plates were rotated at 210 cycles/min producing roughly 12 dynes at the
periphery of the wells, representing the average shear noted within the human common femoral artery (Dardik et al. 2005, Reneman et al., 2006). Shear stress was applied for 11 days, and the medium was changed every 2 days. Experiments were performed in triplicate and repeated three times.

The protocol for Schlemm’s canal-like differentiation also began with passaging human ADSCs onto a 6-well plate with a glass coverslip and culturing for 3 days under static conditions. When culture reached 50% confluence, the ADSC medium was supplemented with 50 ng/ml VEGF-C (Sigma-Aldrich) and subjected to the same amount of shear stress for 11 days. All other cell culture parameters were kept the same as endothelial differentiation. Vehicle-treated ADSCs were used as the control. Experiments were performed in triplicate and repeated three times.

**Figure 3.2. Differentiation timeline of ADSCs into HSC-like cells through VEGF-C and shear stress treatments.**

### 3.2.5 Microfabrication of SU-8 Scaffolds

The microporous, free-standing SU-8 scaffolds used for cell culture were microfabricated as described previously (Torrejon et al., 2013). Briefly, a release layer was first spin-coated onto the silicon wafer and baked between 120–150°C. Next, SU-8
2010 negative-tone photoresist (MicroChem Corp., Newton, MA) was spin-coated onto the release layer to a thickness <5 µm, baked at 95°C, then cooled to room temperature. The photoresist was exposed to UV-light (140 mJ/cm²) through a custom-designed mask with the desired pattern, baked at 95°C, and developed in PGMEA (MicroChem Corp.) Finally, the scaffolds were removed from the release layer, rinsed with isopropyl alcohol, and air dried.

3.2.6 Co-culture with HTM cells on SU-8 Scaffolds

Prior to use in cell culture, SU-8 scaffolds were mounted on aluminum tape rings, UV sterilized, coated with 1% gelatin to promote cell attachment. Primary HTM cells were seeded at a density of 50,000 cells/scaffold and cultured in HTM medium for 7 days. For co-culture, the scaffold was flipped upside-down, seeded with either human ADSC or HSC cells, and cultured for an additional 7-11 days in HTM medium or until confluency.

3.2.7 Optical and Scanning Electron Microscopy

Cell growth was monitored on a daily basis using a Nikon TS100-F inverted microscope (Micro Video Instrument, Avon, MA). Cell morphology was characterized via a LEO 1550 field emission scanning electron microscope (SEM; Leo Electron Microscopy Ltd, Cambridge, UK). To prepare for SEM, samples were fixed with 2% glutaraldehyde solution in 0.1 M phosphate buffer and 0.1 M sucrose for 2 hours at room temperature. Then, samples were chemically dehydrated in a graded ethanol and slowly infiltrated with a graded hexamethyldisilazane (HMDS)-ethanol. Critically dried samples
were mounted on SEM stubs using carbon tape and were sputter-coated with iridium to eliminate charging effects. Images were collected at 3 kV gun voltage and working distance of 5 mm. Cell alignment was quantified using the ImageJ plugin, OrientationJ Analysis and Distribution, which creates histogram of cell orientation based on the SEM images. The standard deviation of the histogram was calculated to represent the alignment index, where high alignment index indicates random orientation and low alignment index indicates similar orientation.

3.2.8 Immunocytochemistry Analysis

Samples were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked using 5% bovine serum. Samples were subsequently incubated with the appropriate primary antibodies: rabbit anti-CD31, goat anti-VEGFR2, rabbit anti-PROX1, mouse anti-Vimentin, rabbit anti-myocilin, mouse anti-αSMA, rabbit anti-OCT3/4 (Abcam, Cambridge, MA). Secondary antibodies: mouse anti-rabbit Alexa Fluor 488 was used to detect CD31, mouse anti-goat Alexa Fluor 647 was used to detect VEGFR2, mouse anti-rabbit Alexa Fluor 647 was used to detect PROX1, myocilin and OCT3/4, and goat anti-mouse Alexa Fluor 594 was used to detect vimentin and αSMA (1:200, Invitrogen, Grand Island, NY). These samples were further counter-stained with 4,6-diamidino-2-phenylindole (DAPI) to reveal cell nuclei. Laser scanning confocal microscopy was performed using a Leica SP5 confocal microscope, and images were acquired at 20x or 63X magnifications using an oil-immersion objective. Confocal images were processed using Leica LasAF software, and all confocal images within a
given experiment were imaged and captured using the same laser intensity and gain settings in order to be able to compare intensities across samples.

### 3.2.9 Quantitative real-time PCR Analysis

Total RNA was extracted using RNeasy Mini Kit as per the manufacturer's instructions (Qiagen, Hilden, Germany). RNA was quantified using a NanoDrop ND1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). PCR probes and primers were generated using Integrated DNA Technologies probe/primer design software (http://www.idtdna.com/pages/products/geneexpression/primetime-qpcr-assays-and-primers). Specific primer sequences are listed in Table 1. qRT-PCR was carried out as directed using StepOnePlusTM Real Time PCR system (Applied Biosystems, Foster City, CA). Samples were amplified using a SYBR® green I PCR master mix. Reactions were analyzed in triplicate and expression levels were normalized to the housekeeping gene GAPDH. Relative quantitative data analysis was performed using the comparative Ct, with GAPDH as the endogenous reference. Experiments were run in triplicate and the average values are presented as mean ± standard error of the mean.

**Table 3.1. qPCR primer sequences used in this study.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROX1</td>
<td>5-TAC GCA CGT CAA GCC ATC AA-3</td>
<td>5-CAG GAA TCT CTC TGG AAC CTC A-3</td>
</tr>
<tr>
<td>VEGFR3</td>
<td>5-GCA CCG AGG TCA TTG TGC-3</td>
<td>5-CCT CCA GTC ACG GCA C-3</td>
</tr>
<tr>
<td>CD31</td>
<td>5-ATT GCT CTG GTC ACT TCT CC-3</td>
<td>5-CAG GCC CAT TGT TCC C-3</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>5-GGA AGC TCC TGA AGA TCT GT-3</td>
<td>5-GAG GAT ATT TCG TGC CGC-3</td>
</tr>
<tr>
<td>OCT3/4</td>
<td>5-TGA GTA GTC CCT TCG CAA GC-3</td>
<td>5-TTA GCC AGG TCC GAG GAT CA-3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5GAT TCC ACC CAT GGC AAA TTC-3</td>
<td>5-GTC ATG CCT TCC ACG ATA C-3</td>
</tr>
</tbody>
</table>
3.2.10 Protein Extraction and Western Blot Analysis

Cellular proteins were extracted with ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Complete Protease Inhibitor; Roche, Manheim, Germany). Proteins were quantified by bicinchoninic acid assay (Thermo Fischer Scientific). 20 µg of proteins from each sample were separated by SDS polyacrylamide gel electrophoresis on a 12% gel in MOPS running buffer (ThermoFisher Scientific), transferred onto a nitrocellulose membrane, and probed with primary antibodies against PROX1, CD31, OCT3/4, and β-actin (Abcam). After incubation in HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Invitrogen), bound antibodies were detected using FluorChem E (Protein Simple). Protein expression was analyzed by densitometry using ImageJ, and normalized to the house-keeping β-actin.

3.2.11 Perfusion Studies

A perfusion apparatus was used as previously described (Torrejon et al., 2013). Samples were securely placed in the perfusion chamber and perfused at various rates for 5 hrs per flow rate (2, 4, 8, and 16 mL/min). Samples were perfused in an apical-to-basal direction with perfusion medium consisting of DMEM (Cellgro) with 0.1% gentamicin in the absence or presence of 100 nM dexamethasone. Pressure was continuously monitored and recorded. After perfusion, samples were fixed and stained for SEM or confocal imaging. The “outflow facility” of the bioengineered 3D HTM and co-culture models was calculated from the inverse of the slope of the pressure versus flow graph, per unit surface area. Six sample replicates per condition were studied under perfusion.
3.2.12 Statistical Analysis

All experimental data, unless otherwise specified, were expressed as means ± SEM from at least three independent experiments. Multiple comparisons were performed using two-way ANOVA. p-values of <0.05 were taken to indicate statistical significance.
3.3 Results

3.3.1 Initial Endothelial Differentiation of Human ADSC

To demonstrate their differentiation potential toward endothelial-like cells, human ADSCs were seeded and grown in ADSC medium for three days, then cultured in EGM-2V medium supplemented with VEGF-A in the presence or absence of shear stress for 11 more days. After 3-day treatment, cells started to show morphological changes (Fig. 3.3A-D). After 11-day treatment, significant morphological changes were observed in the treated ADSCs (Fig. 3.3F-H) versus the vehicle-treated ADSC control (Fig. 3.3E).

![Figure 3.3. Optical images of endothelial differentiation of ADSCs induced by (A-D) 3-day and (E-H) 11-day treatment with VEGF-A and shear stress. (A, E) Vehicle-treated ADSCs as the control. (B, F) ADSCs treated with VEGF-A. (C, G) ADSCs exposed to shear stress. (D, H) ADSCs treated with VEGF-A under shear flow. White arrows indicate cord-like formation after differentiating ADSCs induced by shear stress in the absence (G) or presence of VEGF-A (H). Scale bar = 100 µm.](image)

While the morphology in all treated samples became more elongated and directional, ADSCs exposed to both VEGF-A and shear stress formed robust cord-like structures throughout the culture (Fig. 3.3H). ADSCs that underwent shear stress alone
also formed some cords after 11-day treatment (Fig. 3.3G), but not as robustly when compared to the combined treatment.

Figure 3.4. Expression of endothelial markers in differentiated ADSCs induced by 11-day treatment with vehicle, VEGF-A, and shear stress. Immunocytochemistry reveals a significant increase in expression of endothelial markers of CD31 and VEGFR2 after co-treatment with VEGF-A and shear stress. (A-D) CD31. (E-H) VEGFR2. Scale bar = 100 µm.

These changes in cell morphology were echoed in gene and protein expression analysis as shown in Fig. 3.4. ADSCs exposed to 11-day treatment with VEGF-A and shear stress significantly increased expression of endothelial markers CD31 and VEGFR2.
at both protein and gene level (Fig. 3.5A-B; CD31: 5-fold; VEGFR2: 17-fold).

Interestingly, CD31 and VEGFR2 were minimally expressed in ADSCs treated with VEGF-A alone, as detected by both qPCR and immunocytochemistry analysis. The decreased level of pluripotency marker OCT3/4 gene expression as shown in Fig. 3.5C indicates that ADSCs treated with VEGF-A underwent differentiation.

**Figure 3.5.** qPCR analysis of endothelial markers expressions in differentiated ADSCs induced by 11-day treatment of VEGF-A and shear stress. Significant increase in expression of endothelial markers (A) CD31 and (B) VEGFR2, and decreased expression of stem cell marker (C) OCT3/4 as a result of these stimulations. *p < 0.01, **p < 0.001, ***p < 0.0005, ****p < 0.0005, and ns: not significant.

### 3.3.2 Differentiation of ADSCs towards Schlemm’s canal-like cells

Given the multipotent nature of human ADSCs, we further investigated their ability to become Schlemm’s canal-like cells by inducing lymphatic endothelial differentiation using VEGF-C in the presence or absence of shear stress. VEGF-C was previously successfully used in differentiating ADSCs into lymphatic-like endothelial cells (Yan et al., 2011; Yang et al., 2015). As shown in Fig. 3.6, the morphologic transition of ADSCs from a fibroblast-like appearance (Fig. 3.6A and B) to a typical endothelial cell-specific cobblestone-like appearance seen in HSC cells (Fig. 3.6I and J)
could be clearly observed in ADSCs exposed to 11-day treatment with VEGF-C (Fig. 3.6C and D), shear stress (Fig. 3.6E and F) or both (Fig. 3.6G-H).

**Figure 3.6. Differentiation of ADSCs into Schlemm’s canal-like cells induced by VEGF-C and shear stress for 11 days.** (A-J) SEM images of ADSCs showed dramatic changes in cell morphology and orientation after 11-day treatment. (A and B) Vehicle-treated ADSCs. (C, D) ADSCs treated with VEGF-C. (E, F) ADSCs exposed to shear stress. (G, H) ADSCs treated with VEGF-C under shear flow. (I, J) Primary HSC cells as the positive control (A, C, E, G, I) Scale bar = 100 µm. (B, D, F, H, J) Scale bar = 20 µm. (K) Quantitative analysis of the degree of cell orientation based on the alignment index. A high alignment index, like that of the vehicle-treated ADSC control, represents high randomness in cell orientation. HSCs and differentiated ADSCs are much more oriented than vehicle-treated ADSCs. *p < 0.01, ***p < 0.0005, and ns: not significant.

ImageJ was used to quantify the degree of cell orientation using alignment index. While vehicle-treated ADSCs were randomly organized, ADSCs exposed to both VEGF-C and shear stress showed low level alignment index, indicating directionality and higher degree of cell alignment/orientation, comparable to that of primary HSCs (Fig. 3.6K).
Figure 3.7. Expression of characteristic HSC markers demonstrated that ADSCs exhibited HSC-like differentiation after 11-day treatment with VEGF-C and shear stress. (A-D) qPCR analysis of gene expression of HSC markers of (A) PROX1, (B) VEGFR3, (C) CD31, and (D) VEGFR2. *p < 0.01, **p < 0.001, ***p < 0.0005, and ns: not significant.

HSC exhibits a combination of blood, vascular, and lymphatic markers (Kizhatil et al., 2014). To verify HSC-like differentiation of human ADSCs, we evaluated the gene expression using qPCR analysis of both lymphatic markers (PROX1 and VEGFR3) and vascular markers (CD31 and VEGFR2), along with stem cell marker Oct3/4 after 11-day treatment with VEGF-C and shear stress. As shown in Fig. 3.7A-D, in comparison to vehicle-treated ADSCs, the mRNA expression level of PROX1 and VEGFR3 was...
increased by 4.61-fold and 1.23-fold in VEGF-C treated ADSCs, respectively; 3.11-fold and 1.75-fold for ADSCs under shear stress, respectively; and 7.11-fold and 3.99-fold for ADSCs exposed to both shear stress and VEGF-C treatment.

Western blot analysis revealed a noticeable increase in the level of PROX1 after 11-day co-treatment with VEGF-C and shear stress (Fig. 3.8). Expression of OCT3/4, a stem cell pluripotency marker, decreased in the treated groups when compared to the vehicle-treated ADSC control, confirming that these induced ADSCs are undergoing differentiation (Fig. 3.8C).

Figure 3.8. Western blot analysis of HSC marker in ADSCs after 11-day treatment with VEGF-C and shear stress. (A) Representative immunoblot and densitometry analysis of HSC marker (B) PROX1 and stem cell marker (C) OCT3/4. *p < 0.01, **p < 0.001, ***p < 0.0005, and ns: not significant.
Figure 3.9. Confocal analysis of HSC marker expression in ADSCs treated with vehicle, VEGF-C, and shear stress. (A-E) PROX1. (F-J) F-actin. (K-O) Merged images. (A, F, K) Vehicle-treated ADSCs as the control. (B, G, L) ADSCs treated with VEGF-C. (C, H, M) ADSCs with shear stress. (D, I, N) ADSCs co-treated with VEGF-C and shear stress. (E, J, O) Primary HSCs as the positive control for comparison. Scale bar = 100 µm.

Figure 3.10. Confocal analysis of HSC marker expression in ADSCs treated with VEGF-C and shear stress. (A-E) CD31 and (F-J) α-SMA. Scale bar = 100 µm.
These protein level expression changes were also captured in the immunocytochemistry analysis after 11-day treatment with VEGF-C, shear stress, or both (Fig. 3.9-11). Among these conditions, ADSCs co-treated with VEGF-C and shear stress exhibited a similar protein expression profile to primary HSC cells, including aligned cytoskeleton F-actin organization, increased lymphatic marker PROX1 (compare Fig. 3.9D to E), decreased stem cell marker OCT3/4 (compare Fig. 3.10D to E), and comparable expression pattern of vascular marker CD31 and trabecular meshwork marker αSMA (compare Fig. 3.11D to E). These results confirmed that the synergistic treatment with VEGF-C and shear stress can induce ADSC differentiation into HSC-like cells.
3.3.3 Co-culture with HTM on SU-8 scaffolds for Schlemm’s canal-like cells differentiation

In addition to biological and mechanical cues such as VEGF-C and fluidic shear stress, we also investigated the role of cellular microenvironment in directing HSC-like differentiation of ADSCs. In vivo, HSC endothelial cells form a layer underneath the trabecular meshwork tissue, exchanging signaling through the basement membrane and regulating aqueous humor outflow together. As described previously, the trabecular microenvironment was mimicked by culturing primary HTM cells on microfabricated, well-defined, porous SU8 to recreate 3D HTM-scaffold constructs. The 3D HTM grown on the SU8 scaffold could support primary HSC cells to grow into a continuous cell layer on the other side of the SU8 scaffold to form a 3D HTM/HSC construct (Torrejon, 2015). Similarly, the 3D HTM also allowed ADSCs to grow and differentiate on the other side of the SU8 scaffold to form a 3D HTM/ADSC-derived HSC-like construct.

To evaluate whether these ADSC-derived HSC-like cells could regulate outflow, a perfusion study was performed using a porous SU8 scaffold as the cell culture substrate. Perfusion results for HTM alone and co-culture with HSC cells and ADSC-derived HSC-like cells decreased outflow facility significantly for the co-culture when compared to HTM alone, indicating that the addition of HSC or ADSC-derived cell layer to the HTM outflow model could simulate the flow resistance from the HSC endothelial lining in vivo. Interestingly, the outflow facilities of both ADSC-derived HSC-like cells and primary HSC cells were very similar (Fig. 3.12J), suggesting ADSCs co-cultured with 3D
HTM could be a potential alternative to the HTM/HSC construct for mimicking the ocular outflow tract.

**Figure 3.12. Co-culture with 3D HTM-scaffold constructs facilitate HSC-like differentiation of ADSCs.** (A-I) SEM images of cells grown on micropatterned, porous SU-8 scaffolds. Left panels: scale bar = 200 µm. Middle panels: scale bar = 20 µm. Right panels: scale bar = 2 µm. (A-C) 3D HTM cells alone. (D-F) HSC cells co-cultured with 3D HTM. (G-I) ADSCs co-cultured with 3D HTM. (J) Perfusion studies of the outflow facility of 3D HTM, HTM/HSC and HTM/ADSC-derived HSC-like constructs that could mimic the trabecular outflow tract. **p < 0.001, ***p < 0.0005, and ns: not significant.

Immunocytochemistry analysis was further performed to examine HTM and HSC markers in these 3D HTM, HTM/HSC and HTM/ADSC-derived HSC-like constructs (Fig. 3.13-14). 3D HTM alone expressed HTM markers of myocilin and αSMA (Fig. 3.13A), but did not express HSC marker of PROX1 (Fig. 3.14A). As predicted, HTM/ADSC-derived HSC-like constructs expressed PROX1 at a level similar to that of HTM/HSC constructs (Fig. 3.14 B-C). A cross-sectional view of the confocal z-stack showed two distinct layers of cells, with only the HTM cells expressing myocilin (Fig. 3.13).
Figure 3.13. Confocal analysis of HTM markers expression in 3D HTM, HTM/HSC and HTM/ADSC-derived HSC-like constructs after perfusion studies. Expression of HTM markers of (A-C) myocilin and (D-F) αSMA, co-stained with (G-I) phalloidin-stained F-actin, along with the merged crossectional view. Scale bar = 50 µm.

Figure 3.14. Confocal analysis of HSC markers expression in 3D HTM, HTM/HSC and HTM/ADSC-derived HSC-like constructs after perfusion studies. Expression of HSC markers of (A-C) PROX1 and (D-F) vimentin, co-stained with (G-I) phalloidin-stained F-actin, along with the (J-L) merged crossectional view.
Finally, we examined whether the 3D HTM/ADSC-derived HSC-like construct could respond to an agent that increased flow resistance. Dexamethasone is known to increase flow resistance and IOP, and was used to modulate outflow facility.

Figure 3.15. DEX-induced responses in HTM/HSC and HTM/ADSC-derived HSC-like constructs. Expression of HSC markers (A-D) PROX1 and (E-H) Vimentin co-stained with phalloidin to label (I-L) F-actin and DAPI to label nuclei shown in (M-P) merged view. (E-H) White arrows indicate DEX-associated CLAN formations. Scale bar = 50 µm.

Immunocytochemistry analysis showed that treatment with 100 nM DEX for 7 days induced formation of cross-linked actin network (CLAN) (Fig. 3.15J and L, white arrows) and increased ECM protein collagen IV and fibronectin expression in both HTM/
HSC and HTM/ADSC-derived HSC-like constructs (Fig. 3.16). Furthermore, DEX treatment seems to have decreased PROX1 and vimentin expression in both HSC and ADSC co-cultures (Fig. 3.15).

**Figure 3.16.** DEX-induced responses in HTM/HSC and HTM/ADSC-derived HSC-like constructs. Expression of ECM protein marker (A-D) collagen IV and (E-H) fibronectin co-stained with phalloidin to label (I-L) F-actin and DAPI to label nuclei shown in (M-P) merged view. White arrows indicate DEX-associated CLAN formations. Scale bar = 50 µm.

For analysis of outflow resistance, both HTM/HSC and HTM/ADSC-derived HSC-like constructs were first treated with 100 nM dexamethasone for 3 (Fig. 3.17A) or 7 days (Fig. 3.17B), and then placed in the perfusion chamber. The outflow facility in both co-cultures decreased, although the 7-day dexamethasone treatment induced a more
significant response in both constructs (Fig. 8B), indicating that HTM/ADSC-derived HSC-like constructs have a similar drug response compared to HTM/HSC constructs.

Figure 3.17. Perfusion studies of the outflow facility of HTM/HSC and HTM/ADSC-derived HSC-like constructs after (A) 3-days and (B) 7-day of DEX treatment. *p < 0.01, **p < 0.001, ***p < 0.0005.
3.4 Discussion

Schlemm’s canal cells play a critical role in regulating aqueous humor removal through formation of intracellular giant vacuoles and pores, making them an attractive target for drug interventions. However, high throughput, in vitro preclinical screening of potential drugs requires a large quantity of cells and the scarcity of HSC cells is a bottleneck. To overcome this roadblock in drug discovery, we have attempted to develop an alternative source of HSC-like cells for drug screening purposes. In this study, we investigated the feasibility of differentiating ADSCs into Schlemm’s canal-like cells using VEGF-C, shear stress and HTM co-culture on a microfabricated, porous scaffold. Our goal is to differentiate cells that express both vascular (e.g., CD31, VEGFR2) and lymphatic markers (e.g., PROX1, VEGFR3), and exhibit ability to regulate fluidic outflow in response to IOP-modulating agents.

ADSCs are easily obtainable adult stem cells with high expandability and fibroblast-like morphology. Previous studies have shown that co-stimulation of VEGF-A and fluid shear stress could induce formation of cord-like structures, and expression of vascular endothelial markers after 7 days (Fischer et al., 2009; dela Paz et al., 2011; Colazzo et al., 2014). Indeed, similar to previous studies, three-day post-differentiation, treated ADSCs become more spindle-like and begin to align in the periphery of the culture dish (Fig.3.3). After 11-day treatment, robust cords-like structures, several millimeters in length, could be observed using optical microscopy in cultures treated with both VEGF-A and shear stress (Fig.3.3H). While treatment with shear stress also induced cord-like formation, it was not nearly as robust as the co-treatment (Fig. 3.3G). In
addition to morphological changes, both shear stress and co-treatment with VEGF-A induced a significant increase in the expression of vascular endothelial markers, CD31 and VEGFR2 (Fig. 3.4D and H). CD31, also known as platelet endothelial cell adhesion molecule-1, is expressed at high levels at junctions between adjacent endothelial cells (Givens et al., 2016). The VEGF-A/VEGFR2 is the most prominent ligand-receptor complex in the VEGF system and is responsible for endothelial cell proliferation, migration, survival, and new vessel formation during angiogenesis (Abhinand et al., 2016). Both CD31 and VEGFR2 are part of a mechanosensory complex that elicits many signaling pathways as a response to fluid shear stress (Givens et al., 2016). Therefore, it is no surprise that both growth factor and dynamic stress are necessary to achieve successful endothelial differentiation. These results indicated that shear stress is critical for inducing endothelial differentiation of ADSCs and confirmed previous findings that the combined effect of VEGF-A and shear stress are synergistic in promoting endothelial differentiation (Fischer et al., 2009).

Due to Schlemm’s canal’s unique identity (lymphatic & vascular), we decided to use a modified protocol for differentiating ADSCs into HSC-like cells. Previous research showed that short-term treatment with VEGF-C induced PROX1 expression but not VEGFR-3 in ADSCs (Yan et al., 2011). VEGF-C, a lymphangiogenic growth factor and its receptor, VEGFR-3, are essential for SC development. Delivery of VEGF-C into the adult eye resulted in sprouting, proliferation, and growth of HSC endothelial cells, and was associated with a trend toward a sustained decrease in IOP in adult mice (Aspelund et al., 2014). Recently, VEGFR3 was shown to be a constituent of the mechanosensory
complex containing CD31, VE-Cadherin, and VEGFR2. At the onset of shear, VEGFR2 and VEGFR3 are recruited to the mechanosensory complex by the transmembrane domain of VE-Cadherin, followed by phosphorylation of both VEGFR2 and VEGFR3 (Coon et al.; 2015; Givens et al., 2016). These studies suggest that maintenance of Schlemm’s canal identity and function may depend on shear stress-induced VEGFR3 expression and downstream signaling.

We determined that treatment with VEGF-C, shear stress, or both, changes the cell morphology from fibroblast-like to elongated and spindle-like (Fig. 3.6A-J). Gene analysis showed that PROX1 is significantly upregulated in VEGF-C alone treatment, similar to a previous finding (Yang et al., 2015). Shear stress also unregulated PROX1, while co-treatment with VEGF-C and shear stress further upregulated PROX1 in comparison to ADSCs. It has been shown that 12-hour–long laminar flow can physically associate KLF4, a key shear stress–responsive transcription factor, with the PROX1 regulatory region in blood endothelial cells, potentially explaining the up-regulation we observed under the shear treatment (Park et al. 2014). Similar to PROX1, VEGFR3 expression is upregulated synergistically by VEGF-C and shear stress co-treatment (Fig. 3.7), confirming that VEGFR-3 expression is responsive not only to its native ligand, but also shear stress. Western blot analysis (Fig. 3.8) and immunofluorescence detection (Fig. 3.9-10) of the Schlemm’s canal-specific markers suggests that VEGF-C alone and co-treatment induced PROX1 expression in ADSC-derived HSC-like cells is comparable to that in primary HSC cells. These results are evidence that combined treatment of VEGF-C and shear stress can induce a Schlemm’s canal-like phenotype in ADSCs.
Aside from shear stress, the inner wall endothelium of Schlemm’s canal also experiences basal-to-apical flow. This dynamic environment makes it necessary for HSC cells to have strong cellular adhesion to their basement lamina, which include collagen IV, fibronectin, and laminin. These proteins act as structural support, and compensate for mechanical strain, especially around the cell periphery (VanderWyst et al., 2011; LeBleu et al., 2007). We reasoned that since the Schlemm’s canal’s ability to form giant vacuoles and pores depends heavily on basement membrane stiffness, creating an in vitro microenvironment with in vivo-like substrate stiffness, composition, and porosity is critical not only for maintaining Schlemm’s canal phenotype in vitro, but also for guiding stem cell differentiation. Previous research by our group demonstrated that bioengineered HTM on microporous SU8 scaffolds exhibit several in vivo-like characteristics, such as expression of HTM markers, secretion and remodeling of surrounding ECM, and most importantly, response to IOP-altering agents (Torrejon et al., 2013, 2016; Dautriche et al., 2015). Co-culturing ADSCs with this HTM construct could provide the appropriate biological and structural cues to guide ADSC differentiation. To assess whether the co-cultured HTM/ADSC-derived HSC construct possesses the ability to regulate fluid flow, we perfused the construct at various flow rates and monitored the resistance to flow. Notably, both HTM/HSC and HTM/ADSC-derived HSC-like constructs showed pore-like structures at the sub-micron scale, similar to intracellular pores observed in vivo (Braakman et al., 2016). In addition to morphological similarity, both constructs provided similar resistance to flow, indicating comparable tissue permeability (Fig. 3.12J). Immunocytochemistry confirmed that the HTM/ADSC-derived HSC-like construct not
only forms a confluent monolayer that is distinct from the HTM cells (Fig. 3.13-14), but expresses levels of PROX1 comparable to those of HSC cells in HTM/HSC constructs. To determine whether these two constructs behave similarly in terms of flow modulation, they were treated with dexamethasone to induce a decrease in outflow facility. Both constructs showed such a decrease, indicating that ADSC-derived HSC-like cells provide a similar drug response to HSCs when cultured with HTM cells.

Although promising, the potential of using ADSC-derived HSC-like cells in glaucoma drug screening will require further examination. For example, it is important to determine the long-term fate of ADSC-derived HSC-like cells either with or without coculturing with HTM, as well as their physiological responses to other agents that are known to affect outflow facility. Numerous previous studies have demonstrated the success of using stem cell differentiation techniques for ocular regeneration (Stern et al., 2018). The combination of stem cell products with tissue engineering not only offers great potential for tissue and organ repair, but also scalable and modular 3D cell culture systems for cost-efficient stem cell expansion, differentiation, and high-throughput drug screening.
3.5 Conclusion

In conclusion, we have demonstrated the feasibility of differentiating ADSCs into Schlemm’s canal-like cells using VEGF-C, fluidic shear stress, and co-culturing with 3D HTM-scaffold constructs. The differentiated ADSCs expressed characteristic Schlemm’s canal markers, PROX1 and VEGFR3. Furthermore, 3D HTM/ADSC-differentiated HSC-like constructs exhibited similar cell morphology, orientation, and coverage on microfabricated porous SU8 scaffolds, as well as outflow facility comparable to HTM/HSC cells, with and without dexamethasone treatment. These findings show that ADSC-derived HSC-like cells have the potential to serve as an alternative cell source to recreate the ocular outflow tract for \textit{in vitro} glaucoma drug screening.
3.6 References


4.1 Introduction

Fluid shear stress is a flow-induced frictional force per surface area that plays an important role in regulating endothelial cell behaviors, gene expression, and physiological homeostasis (Baeyens et al., 2016; Hahn et al., 2009). Every organ system in the human body has developed distinct tolerances and downstream signaling events to varying levels of mechanical stimulation. For endothelial cells in the arterial network, high levels of shear stress created by blood flow are normal and atheroprotective, while such levels would lead to apoptosis in cardiomyocytes (Malek et al., 1999). Therefore, the ability to sense a change in shear stress level and return it to normal is critical for maintaining homeostasis of the organ system. Endothelial cells have developed a complex, yet sophisticated mechanism using shear-responsive kinases, GTPases, ion channels, and other mechanosensors to adapt to changes in shear stress level (Zhou et al., 2014; Zhang et al., 2011). Although the exact transduction mechanism is still unclear, consensus among researchers is that activation of mechanotransducer proteins, endothelial NO synthase (eNOS), and subsequent production of nitric oxide (NO) plays a central role in mediating vasodilation and homeostasis (Hsieh et al., 2014; Fleming et al., 2010; Predescu et al., 2005).
Schlemm’s canal cells are specialized endothelial cells that line the collector duct of the ocular conventional outflow pathway (Ramos et al., 2007). The primary function of the Schlemm’s canal is to drain aqueous humor into the circulatory system, while maintaining the blood-aqueous barrier integrity ((Johnson et al., 2006). The inner wall Schlemm’s canal cells experience both basal-to-apical pressure gradient generated by the flow of aqueous humor, as well as shear stress calculated to reach levels comparable to those found in major arteries (2-20 dynes/cm²) (Ethier et al., 2004; Fautsch et al., 2006). Such high shear stress is suspected to result from rising intraocular pressure (IOP) and narrowing of the Schlemm’s canal lumen. Recently, shear stress-induced NO production was observed in both ex vivo perfusion of mouse eyes and cultured primary human Schlemm’s canal cells (Stamer et al., 2011), suggesting the presence of an endogenous feedback loop within the Schlemm’s canal cells that detects changes in IOP, possibly through mechanosensing and NO signaling, and regulates outflow facility so as to restore physiological IOP (Schneemann et al., 2002).

Currently, NO-donating agents are being investigated as potential treatments for glaucoma, a chronic eye condition often characterized by elevated IOP. However, drug screening efforts are limited by the availability of donor eyes, reproducibility, and the cost of ex vivo perfusion set up. While in vitro studies are cheaper, they require a large quantity of primary donor cells and often oversimplify the cell-drug interaction. Microfluidic chips circumvent many of these issues by providing a cost-effective, high-throughput screening platform that mimics the physiological and pathological
microenvironment by integrating 3D cell culture and mechanical stimulations (Chi et al., 2016).

Previously, our lab developed a micropatterned, porous scaffold-based 3D trabecular meshwork culture system that enables measurement of outflow facility of human trabecular meshwork (HTM) and human Schlemm's canal (HSC) cells in vitro, using a basal-to-apical perfusion chamber. While this set-up provided valuable information about the outflow facility, it does not allow for the study of shear stress effects on the outflow tissue. Commercially available devices for applying shear stress, such as the parallel plate flow chamber, do not allow the basal-to-apical perfusion capability needed for outflow tissue studies.

In this study, we set out to develop a dual-flow microfluidic device that provides both basal-to-apical and shear flow to the bioengineered HTM/HSC-scaffold construct. This device could be used to study the morphological, pharmacological and physiological properties of the outflow tissue under dynamic microenvironment, as well as a high-throughput screening platform for glaucoma drugs.

The dual-flow fluidic device was modeled, simulated, and experimentally validated by measuring the effect of different shear rates on the behavior of HSC cells or co-culturing of HTM with HSC cells grown in microporous SU-8 scaffolds. Parameters known to be directly affected by shear stress, such as the expression of the endothelial eNOS, cell alignment, and outflow facility were analyzed.
4.2 Materials and Methods

4.2.1 Perfusion chamber design

The design of the dual-flow perfusion chamber needs to satisfy multiple requirements, including (i) both basal-to-apical (BtA) and shear flow, (ii) the desired shear stress between 0.2 to 2 Pa, (iii) ability to image via an inverted microscope, and (iv) easy assembly and reusability.

![Figure 4.1. Cross-sectional view of the dual-flow perfusion chamber.](image)

Figure 4.1. Cross-sectional view of the dual-flow perfusion chamber. It includes (A) support plates attached to the (B) shear and (C) BtA chambers, and a cell-scaffold construct sandwiched in between. (E) Addition of inlets/outlet with the direction of flow labeled in blue arrowheads.

The chamber was designed according to the above-mentioned criteria, and was assembled out of several components: (a) the top and bottom support plates made out of poly(methyl methacrylate) (PMMA) slabs, (b) the BtA chamber made out of
polydimethylsiloxane (PDMS), (c) the shear chambers made out of machined PMMA sheets, and (d) the porous SU-8 scaffold that is sandwiched between the BtA and shear chambers. This modular configuration makes each component reusable, so that only the SU-8 scaffold needs to be replaced (Fig. 4.1).

Figure 4.2. Schematic of the dual-flow perfusion system setup. (A) Perfusion system with Inlet 1 (BtA flow) connected to a pressure transducer and a monitor that provide real-time measurement of back pressure. Separate syringe pumps provided different flow rate for the inlet 1 and inlet 2 (shear flow). Perfused medium exits through the outlet and is collected into a waste bottle.
The fully assembled perfusion system will include a perfusion chamber with an integrated pressure transducer (Edwards Lifesciences, Irvine, CA) that continuously measures the transmembrane pressure of the cell-scaffold construct in real-time (Fig. 4.2A). Inside the perfusion chamber, the cell-scaffold construct will contain either HSC cells alone or co-culture of HTM with HSC cells.

### 4.2.2 COMSOL Simulations

To optimize microfluidic chamber design, COMSOL Multiphysics software (COMSOL Inc., Burlington, MA), specifically the computational fluid dynamics module, was used to model the shear and BtA chambers, as well as the porous SU-8 scaffold. (Fig. 4.3A).

**Figure 4. 3. COMSOL model of the dual-flow perfusion device.** (A) Shear chamber is 10 mm long, with entrance/exit channel width of 1 mm and main shear channel width of 2 mm. Basal-to-apical (BtA) chamber has 3 mm diameter and 2 mm thickness. The desired shear chamber height is determined through simulation. (B) Close up of the porous SU-8 scaffold with 12-µm pores, located at the interface between shear and BtA chambers.
The Navier-Stokes equations for momentum were solved assuming laminar, incompressible Newtonian fluid flow. A no-slip boundary condition at the inner walls of the microfluidic chamber, laminar inflow, and zero outlet pressure were applied to the computation. The height of the shear chamber is determined through a parameter sweep from 20 to 500 µm, while the flow rates needed to generate the desired wall shear stress of 0.2 to 2 Pa were determined through a parameter sweep from 10 to 1500 µl/min. For simplicity, the simulation focused on the intersection between the BtA and shear chamber.

4.2.3 Microbeads Validation of COMSOL Simulation

Polystyrene microbeads (10 µm, Polybead, Polysciences, Inc., Warrington, PA) were used to validate the shear stress simulations for the 200 µm shear chamber. 10 µl of microbead solution was diluted in 5 ml of deionized water and pulled into a 10 ml syringe connected to the shear chamber inlet. Shear flow rates of 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, and 1000 µl/min were applied for at least 5 minutes to ensure the flow has reached steady state. Then a Nikon TS100-F inverted microscope (Micro Video Instrument, Avon, MA) was used to auto capture a video of the microbeads flowing through the shear channel. At least 25 microbeads were randomly selected for calculation of average velocity. To calculate the experimental shear stress, average velocity for each flow rate was plugged into the derived form of Newton’s law of viscosity, shown here:

$$\tau = \frac{6 \mu Qh}{W \delta^3}$$
4.2.4 Perfusion Chamber Fabrication

The chambers were designed using the free online 3D modeling software, TinkerCad, and optimized iteratively through COMSOL simulation. The final shear chamber design was fabricated from PMMA powder using a customized wafer debonder instrument to form an ultra-thin transparent sheet. The thickness of the PMMA sheet can be controlled by altering the temperature and compressive pressure of the debonder plates. BtA and shear chamber features were created by machining. Inlets and outlets were drilled into the PMMA support plates and debris was removed with Scotch tape to prevent clogging. Chambers and support plates were first sterilized under UV for 1 hour prior to assembly with the scaffold-cell construct.

4.2.5 Primary Human Schlemm’s Canal Cell Culture

Primary HSC cells were kindly provided by Dr. Stamer at Duke University. The protocol for HSC cell extraction from postmortem human eyes was described in detail elsewhere (Stamer et al., 1998). Extracted cells were shipped overnight in T-25 flasks filled with DMEM/Low glucose (Life Technologies; Carlsbad, CA) with 10% FBS (Atlas Biologicals, Fort Collins, CO) and 1% penicillin/streptomycin. Upon arrival, the culture medium was replaced with fresh medium and flasks were stored in the incubator at 37°C and 5% CO₂. Cells were fed every other day. Cells were trypsinized and passaged at 1:3 ratio when reaching 80% confluent. All studies were conducted using cells before the 6th passage.
4.2.6 Primary Human Trabecular Meshwork Cell Culture

Primary HTM cells were isolated from donor tissue rings discarded after penetrating keratoplasty in the Danias laboratory. Isolation of these cells was performed under an IRB-exempt protocol approved by the SUNY Downstate IRB. Isolation and culture conditions were as previously described (Torrejon et al., 2013). All HTM cell strains were characterized for expression of αB-crystalline and α-smooth muscle actin (αSMA) before experiments. HTM cells were initially expanded in 75 cm² cell culture flasks coated with 1% gelatin and fed every 48 hours with 10% FBS (Atlas Biologicals) in Improved MEM (Corning Cellgro, Manassas, VA) with 1% gentamicin. All studies were conducted using cells before the 5th passage.

4.2.7 Microfabrication of SU-8 Scaffolds

The microporous, free-standing SU-8 scaffolds used for cell culture were microfabricated as described previously (Torrejon et al., 2013). Briefly, a release layer was first spin-coated onto the silicon wafer and baked between 120–150°C. Next, SU-8 2010 negative-tone photoresist (MicroChem Corp., Newton, MA) was spin-coated onto the release layer to a thickness <5 μm, baked at 95°C, then cooled to room temperature. The photoresist was exposed to UV-light (140 mJ/cm²) through a custom-designed mask with the desired pattern, baked at 95°C, and developed in PGMEA (MicroChem Corp.) Finally, the scaffolds were removed from the release layer, rinsed with isopropyl alcohol, and air dried.
4.2.8 Co-culture of HTM and HSC Cells on SU-8 Scaffolds

Prior to use in cell culture, SU-8 scaffolds were mounted on aluminum tape rings, UV sterilized, coated with 1% gelatin to promote cell attachment. Primary HTM cells were seeded at a density of 50,000 cells/scaffold and cultured in an appropriate medium for 7 days. For co-culture, the scaffold was flipped upside-down and seeded with either human ADSC or HSC cells for an additional 7-10 days or until confluency.

4.2.9 Immunocytochemistry

Samples were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked using 5% bovine serum. Samples were subsequently incubated with the appropriate primary antibodies, rabbit anti-eNOS, and secondary antibodies, mouse anti-rabbit Alexa Fluor 647. These samples were also labeled with 488 phalloidin to reveal F-actin cytoskeleton and further counter-stained with 40,6-diamidino-2-phenylindole (DAPI) to reveal cell nuclei. Laser scanning confocal microscopy was performed using a Leica SP5 confocal microscope, and images were acquired at 63X magnifications using an oil-immersion objective. Confocal images were processed using Leica LasAF software, and all confocal images within a given experiment were imaged and captured using the same laser intensity and gain settings in order to be able to compare intensities across samples.

4.2.10 ImageJ Analysis

F-actin cytoskeleton was revealed using phalloidin (Life Technology) labeling and co-stained with DAPI to reveal cell nuclei. Cell alignment was quantified using the
ImageJ plugin, OrientationJ Analysis and Distribution, which creates a histogram of cell orientation based on the confocal images. The standard deviation of the histogram was calculated to represent the alignment index, where high alignment index indicates random orientation and low alignment index indicates orientation in a similar direction.

4.2.11 Statistical Analysis

All experimental data, unless otherwise specified, were expressed as means ± standard error of mean from at least three independent experiments. Multiple comparisons were performed using two-way ANOVA. p-values of <0.05 were taken to indicate statistical significance.
4.3 Results

4.3.1 COMSOL Simulation of Flow Profit in the Dual-flow Microfluidic Device

COMSOL simulations were used to visualize the dynamic microenvironment inside the dual-flow fluidic device, consisting of a shear and a BtA chamber (Fig. 4.3).

![Figure 4.4. Velocity profile of the dual-flow perfusion device.](image)

(A-B) Without the SU-8 scaffold, the simulation reveals a well-developed flow throughout the device. (C-D) When the SU-8 scaffold is incorporated, the flow from the BtA chamber is interrupted at the pores. (B and D) Magnified view of the center of the shear device, indicated by the black dotted box. White arrows indicated the direction of fluid flow; the size of the arrows is proportional to flow velocity; red lines are streamlines.

For simplicity, we focused on velocity and shear stress profile at the center of the shear chamber and its interaction with the SU-8 scaffold and its 12 µm pores (Fig. 4.4). Without the SU-8 scaffold, the velocity profile of the BtA and shear flow in the chamber display a fully developed laminar flow for all simulated chamber heights and shear stress conditions (Fig. 4.4A and B). With the scaffold incorporated, the velocity streamlines
from the BtA chamber were interrupted and segmented by the pores (Fig. 4.4C-D). No dead zone was observed in the device.

Figure 4.5. Parameter sweep of shear chamber height and flow rates to determine the wall shear stress experienced at the SU-8 interface. Representative examples of (A) wall shear stress contour at the main shear chamber and (B) wall shear stress profile along the dashed line.

In addition to velocity profiles, we also calculated the shear stress at the interface between SU-8 scaffold and shear chambers. A parameter sweep was performed with shear
chamber height and shear flow rate as variables to obtain a comprehensive understanding of the shear stress profile.

Figure 4.5 shows the representative simulation results when a height of 200 µm and flow rates of 100, 500, and 1000 µl/min were evaluated. The shear stress profile along the dotted line showed that flow rates between 300 and 1000 µl/min could generate the desired shear stress range of 0.2 to 2 Pa (Fig. 4.5B). Comparing the shear stress profile of all simulated parameters demonstrated that while all heights simulated could generate the desired shear stress, height above 200 µm requires much larger flow rate, while height below 200 is too sensitive to the increase of flow rate, making it difficult to control (Fig. 4.6). Therefore, we chose to use 200 µm as the shear chamber height.

![Figure 4.6. Composite result of parameter sweep. Graph comparing the effects of height (50, 100, 200, 300 µm) and flow rate (10, 50, 100, 200, 400, 600, 800, 1000 µl/min) on shear stress. Gray box indicates the desired shear stress region.](image)

4.3.2 Experimental validation of COMSOL simulation
To validate the COMSOL simulation results, we used microbeads to calculate the shear stress experimentally. The velocity of 20 microbeads per flow rate was averaged and plugged into the Navier-Stokes equation. The experimental results were very close to the COMSOL simulation (Fig. 4.7). The difference at higher flow rates was due to the inability of the camera to capture the beads flow.

![Figure 4.7. Experimental validation of simulation data using Polybeads perfused through a shear chamber with 200 µm height.](image)

(A) Screenshot of beads moving through the device. (B) Graph comparing simulation results with experimental average of 20 microbeads per flow rate.

### 3.3 Effect of shear stress on HSC-scaffold constructs

To test the feasibility of using the dual-flow chamber to study effects of shear stress on the outflow tissue, HSC-scaffold constructs were assembled into the dual-flow chamber and subjected to three different shear levels, 0, 0.1 and 1 Pa. After 12 hours, expression of eNOS and F-actin organization was evaluated via immunocytochemistry (Fig. 4.8). As expected, the expression of eNOS increased with the increase in shear stress (Fig. 4.8B, F, and J).
Figure 4.8. Confocal imaging of HSC constructs under various shear stress. The HSC-scaffold construct was perfused in the dual flow chamber for 12 hours at 0 (A-D, static), (E-F) 0.1, and (I-L) 1 Pa. Expression of (B, F, J) eNOS (red) and (C, G, K) F-actin (green) were evaluated.

ImageJ analysis of the fluorescence intensity revealed that shear stress of 1 Pa has significantly greater eNOS expression than both 0 Pa static control and 0.1 Pa conditions (Fig.4.9A). Furthermore, the 1 Pa shear stress condition appears to have altered the morphology of the HSC cells (Fig. 4.9C) and their nuclei (Fig. 4.9B). ImageJ analysis of the DAPI staining revealed that the nuclei became more elongated and aligned with the direction of flow after 12 hours, a phenomenon that is not observed in the static and 0.1 Pa shear stress condition.
Figure 4.9. ImageJ quantification of immunocytochemistry analysis of HSC cells. (A) eNOS expression. (B) Alignment index of DAPI-stained nuclei (C) Alignment index of phalloidin-labeled F-actin cytoskeleton. A high alignment index, like that of the 0 Pa shear stress, represents high randomness in cell orientation. *p < 0.01, **p < 0.001, ***p < 0.0005, ****p < 0.0001.

Figure 4.10. Perfusion studies of HSC-scaffold construct at 0 (static), 0.1 and 1Pa. (A) Graph of flow rate versus transmembrane pressure in HSC-scaffold construct measured by the dual-flow perfusion device after 12 hours of perfusion. BtA flow rates are 2, 4, 8, 16 µl/min for 3 hours each, while shear flow rates are constant at 0, 0.1, or 1 Pa. (B) Outflow facility of HSC-scaffold construct. **p < 0.001, ****p < 0.0001.

Perfusion study was conducted to evaluate the transmembrane pressure of the HSC-scaffold construct in response to increasing BtA flow rates and shear stress (Fig. 4.10A). The back pressure results were recorded continuously for 12 hours and used for calculating outflow facility. Pressure drop was clearly observed under 1 Pa shear stress.
when compared to static or 0.1 Pa of shear stress. Outflow facility also increased in cell-scaffold constructs that experience the highest level of shear stress (Fig. 4.10B).

### 4.3.4. Effect of shear stress on outflow facility of co-cultured HTM/HSC-scaffold constructs

To further examine whether shear stress could affect outflow facility of the outflow tissue, we conducted perfusion studies using co-cultured HTM and HSC on the SU-8 scaffold as shown previously (Fig. 4.2B).

![Figure 4.11. Perfusion of co-cultured HTM/HSC-scaffold construct at 0 (static), 0.1 and 1 Pa.](image)

(A) Graph of flow rate versus transmembrane pressure in the HTM/HSC-scaffold construct measured by the dual-flow perfusion device after 12 hours of perfusion. BtA flow rates are 2, 4, 8, 16 µl/min for 3 hours each, while shear flow rates are constant at 0, 0.1, or 1 Pa. (B) Outflow facility of the co-cultured HTM/HSC-scaffold construct. *p < 0.01, **p < 0.001.

The co-culture constructs were subjected to BtA flow rates of 2, 4, 8, and 16 µl/min for 3 hours at each flow rates, while simultaneously experiencing either 0, 0.1 or 1 Pa shear stress. The co-culture constructs subjected to 1 Pa shear stress showed a significantly higher outflow facility than both the control and 0.1 Pa conditions,
indicating that increase in shear stress could improve outflow facility of the co-cultured HTM/HSC construct.
4.4 Discussion

To date, only one other study utilized microfluidics to examine the effect of shear stress on the Schlemm’s canal cells (Ashpole et al., 2014). Using a commercially available µ-slide (Ibidi, Martinsried), Ashpole et al. investigated the relationship between NO production and shear stress levels in cultured HSC cells. This study confirmed that the HSC cells have a similar response to shear stress as other vascular endothelial cells, specifically in its ability to increase NO production and align with the direction of flow. This study provided direct proof for the shear-induced feedback loop within HSCs that could play a central role in regulating IOP. Something missing from this study is the direct linkage between shear stress, NO and outflow facility. To begin testing for this relationship, a perfusion system is needed that includes both basal-to-apical perfusion and shear flow, as well as a porous scaffold to allow co-culture of HTM and HSC cells. There are currently no commercially available devices to accommodate such requirements.

In this study, we set out to provide a computational and experimental platform for the study of 3D artificial outflow tissue under dynamic fluid flow. Computational fluid dynamic modeling was used to design and optimize the dual-flow microfluidic device, and to mimic the shear stress generated by aqueous humor flow through the Schlemm’s canal.

To experimentally validate the shear stress effects of the microfluidic device, latex microbeads were perfused through the chamber at different flow rates. The movement of beads across the shear chamber was captured in a time-lapse video using an inverted microscope. The velocity of microbeads under different shear flow rate was used to
calculate the experimental shear stress using the derived Navier-Stokes equation. The simulation results closely resemble the experimental data, within the range of 0-500 µl/min, beyond which the microbead movement became blurry and difficult to quantify.

There is a strong correlation between NO levels in the perfusate and eNOS levels in the cells, so we directly probed for eNOS expression using confocal microscopy. We found that high shear stress up-regulated eNOS expression in HSC cells, which in turn activated downstream events to increase the outflow facility of the tissue. These results are consistent with those in previous studies in mouse models that showed overexpression of eNOS increased outflow facility (Stamer et al., 2011).

Furthermore, cytoskeletal realignment to the direction of flow was also observed within the cell-scaffold construct. Epstein et al. (1999) showed prominent stress fibers running through the entire HSC cell, with no preferential accumulation of F-actin bundles in the periphery of the cell. This was also observed in our 1 Pa shear stress samples. The absence of F-actin bundles could explain the increase in outflow facility, since the stress fibers are free to rearrange according to the pressure gradient. The outflow facility increased significantly under high shear stress for both HSC alone and HTM/HSC co-culture samples. As expected, the outflow facility was higher in HSC alone than the co-cultured sample, due to the absence of the HTM layer that could provide additional flow resistance. This small difference in our recorded data indicates that our dual-flow perfusion system is quite sensitive, and could be used for drug screening purposes in the future.
4.5 Conclusion

We have developed a microfluidic setup comprising of a basal-to-apical flow and shear flow for mimicking the dynamic microenvironment of the outflow tissue. This device enabled *in situ* live imaging and pressure measurements of the bioengineered HSC cell-scaffold or co-cultured HTM/HSC-scaffold construct while experiencing perfusion and drug treatment. The device is easily assembled and reusable. The shear stress conditions were specifically tuned to include the range suitable for HSC cells. The *in vitro* drug screening capabilities of this device have great potential to expedite glaucoma drug discovery and research, in particular for the field of HSC cell biology.
4.6 References


SUMMARY

Primary open angle glaucoma (POAG) is a complex disease that inflicted by age, gender, race, and family history. Currently, lowering the intraocular pressure (IOP) is the major therapeutic strategy for POAG. Due to genetic differences between patients, several medicines are in need and finding the perfect “cocktail” without adverse side effects can be challenging. Topical medication such as eye drops has been used as the primary treatment for managing IOP and preventing further vision loss. Most of currently existing medication act on reducing the aqueous humor production. Therapeutic agents that directly targeting the pathological site — trabecular meshwork tissue are still in preclinical or early clinical trial, and will likely encounter similar variation in effectiveness due to patient variability. There is a great need of in vitro model of outflow tissue for higher throughput drug screening and testing. Stem cell technology provides a new avenue to improve the drug discovery process. For example, stem cells derived from patients can be used for evaluating drug effectiveness and potential adverse effects, or the adult stem cells could be differentiated and transplantation to rescue dysfunctional tissues in vivo.

Primary HTM was previously used to create a cell-scaffold construct for drug testing. Unlike primary HTM, which are usually obtained from donor eyes and are limited in supply, iPSC-derived trabecular meshwork (iPSC-TM) cells are obtained by genetically reprogramming patient’s own fibroblasts into pluripotent stem cell-like state.
These cells are not only less labor-intensive compared to isolated primary cells, but also autologous for purposes of cell therapy. In particular, iPSC-TM cells have already shown promise in lowering IOP after transplantation into mouse models. In order to test the feasibility of using iPSC-TM cells to create 3D trabecular meshwork tissue, we cultured human iPSC-TM cells on microfabricated, well-defined, porous SU-8 scaffolds, examined cell morphology, HTM marker expression, and phagocytic activity and outflow function, and evaluate drug responsiveness to stored treatment in comparison with primary HTM. In chapter 2, we demonstrated that iPSC-TM cells provide an invaluable source for basic glaucoma research, drug discovery, and cell therapy.

The inner wall of the human Schlemm’s canal (HSC) works synergistically with the JCT region of trabecular meshwork to provide the most resistance to AH outflow. In glaucomatous Schlemm’s canal tissue, pore density is significantly lower than normal due to increased tissue stiffness, which then leads to increased outflow resistance and IOP. Currently, Schlemm’s canal research is bottlenecked by cell scarcity and difficulty in isolation. In chapter 3, we introduced a novel method to differentiation adipose-derived stem cells (ADSCs) into HSC-like cells. ADSCs are used because of its abundance in the human body and previous successes in differentiating into other cell types such as osteoblast, neural, and most importantly, vascular and lymphatic endothelial cells, which are related to the identity of HSC cells. Here we demonstrated that the combination of VEGF-C, shear stress, and co-culture with HTM cells on SU-8 scaffolds could induce stem cell differentiation into HSC-like cells. ADSC-derived HSC-like cells recapitulate morphology, protein expression, dexamethasone response and outflow facility of primary
HSC cells, and could become a valuable alternative cell source for future glaucoma research.

In the previous chapter, we showed that shear stress is a critical component in induction of HSC marks PROX1 and VEGFR3. Shear stress experienced by HSCs are on the same order of magnitude as major arteries. Therefore, decreased AH flow could lead to decreased HSC function overall. The current perfusion system for outflow studies only include basal-to-apical flow, but no shear flow. In chapter 4, we created a dual flow perfusion system to allow both shear and basal-to-apical flow to be studied simultaneously. For proof of concept, we use HSC on SU-8 scaffold to evaluate the effect of shear stress on its outflow facility. eNOS is a mechanosensing protein that regulates vasodilation through nitric oxide. We confirmed the hypothesis that increasing shear stress activated eNOS signaling and increased outflow facility, indicating the critical role HSC plays in IOP regulation. Altogether, this project emphasizes the merit of using stem cell, tissue engineering, and microfluidic technology to create an in vitro, dynamic outflow tissue for physiological studies and high-throughput drug screening.

Future Direction

Future works for the project should include diversifying the therapeutic agents tested for a more comprehensive drug response profile. Currently, only dexamethasone was used to validate that the stem cell-derived trabecular meshwork model responds to drug as expected because it is such a well studied pharmacological agent with well known effects on the outflow tissue. However several new agents have been developed,
such as ROCK inhibitors and NO-donating compounds, that specifically target the outflow tissue, and would serve as a better gauge of drug responses.

Another aspect of the project that could be improved upon is the addition of a transepithelial electrical resistance (TEER) sensor into the microfluidics chamber to monitor membrane resistance in response to flow and/or drug treatment. The TEER sensor, when connected to an EVOM voltmeter, measures membrane resistance and can be used to evaluate the barrier integrity of the HSC-scaffold construct. A similar setup has been used to study the blood-brain barrier, gastrointestinal tract, and pulmonary tissues in real-time. The sensor should be fabricated on transparent substrates, such as plexiglass, to allow live imaging of cells. The sensor itself should be made of a non-toxic and biocompatible metal, such as gold or silver. It is expected shear stress and NO-induced vasodilation will increase the porosity of the HSC layer, thereby decrease the barrier resistance.