

Exploring the Cytoskeleton and Apoptosis Mechanisms in Fuchs Endothelial Corneal Dystrophy Using *In Vitro* Biomimetic Models and Immortalized FECD Cells

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Applied Science

in

Chemical Engineering

Waterloo, Ontario, Canada, 2023

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Chapter 1 consists of the content from a review manuscript which is co-authored by Wesley Luu (overall structure), Myagmarsend Enkhbat (*In vivo* section revision), and myself (content, *In vitro and ex vivo* models). My Supervisor, Professor Evelyn Yim, assisted us greatly in structuring and coordinating the manuscript. Our collaborators from Singapore Eye Research Institute, Dawn Neo and Dr. Gary S L Peh, also provided valuable insights and improvements to the manuscript.

Abstract

Fuchs endothelial corneal dystrophy (FECD) is a corneal endothelium (CE) and Descemet's membrane (DM) dysfunction symptom, which is the most common corneal endothelial disease. Despite the long history of clinical assessments regarding FECD, the only clinical treatment available currently is corneal transplantation. Therefore, a study to further explore the complex pathology of the FECD is required to develop novel therapies. Clinically, FECD is a condition indicated by the gradual loss of corneal endothelial cells (CEnCs) and the development of abnormal accumulations of extracellular matrix (ECM) on Descemet's membrane, known as guttae. By utilizing a patient-derived immortalized FECD corneal endothelial cell line FECD-SV-54F-73 and an *in vitro* synthetic guttata (s-guttata) model with varying dimensions, an *in vitro* model was constructed aiming to recapitulate the *in vivo* abnormal cell-ECM interactions in FECD. With this model, we hypothesize that the FECD cells seeded on the s-guttata patterns exhibit higher cytoskeleton contractility and early apoptosis rate. In addition, we speculate that the healthy CEnC line B4G12 on the synthetic guttata would undergo the endothelial to mesenchymal transition (EndMT) process, which is also observed in FECD pathology. The FECD 54F CEnCs had a significant increase in early apoptosis and cytoskeletal response in 20x20x20 μm (diameter-spacing-height) s-guttata samples. Furthermore, these apoptotic cells were localized around the s-guttata with smaller diameters and higher densities. However, after seven days, no significant upregulation of EndMT-related gene expressions (*ZEB1* and *SNAIL*) was detected in the cells seeded on the 20x20x20 μm sample compared to the unpatterned control.

In addition, by employing a curved contact lens, we also aim to explore the cell migration mechanism of the CEnCs and hypothesize that the wound healing rate of the FECD cells is higher than the healthy corneal endothelial cell line B4G12. Interestingly, we found that while the FECD 54F (2.35% of total wounded area per hour) cells migrated faster than the B4G12 (1.41% per hour) cells on the concave side of the gelatin methacrylate + (GelMA+) contact lens, B4G12 had a significantly higher migration speed on flat PDMS (3.02% of per hour) and TCPS (2.74% per hour) substrate surfaces when compared with FECD 54F (2.9% per hour on PDMS, 1.73% per hour on TCPS). However, further studies are required for the curved samples as there were not sufficient sample replicates for statistical analysis. A lithography process was also developed to explore whether the effect of micro-topography can affect cell behavior on curved surfaces. While the sub-micron patterns on the silicon wafer were successfully created via maskless photolithography, the subsequent soft lithography process that required the

transfer of the designs from a flat substrate onto the curved surface was delicate and required more improvements for subsequent cell experiments on a non-planar curved surface.

In general, by applying various instruments and analytical approaches, this study aims to contribute further to comprehending FECD pathophysiology and improve the current stages of FECD clinical treatment approaches.

Acknowledgements

I would like to express my sincerest gratitude to my supervisor, Professor Evelyn Yim, for her guidance and continuing support throughout my research. Her expertise and encouragement have always been instrumental in shaping the direction of this manuscript. Most importantly, I express my gratitude to her for giving me the chance to learn and grow under her guidance, particularly during such uncertain times.

I would also like to thank my thesis committee members, Professor Valerie Ward and Professor Elisabeth Prince, for their patience during the scheduling process and valuable constructive criticism regarding the content of this thesis.

I would like to extend my appreciation to Dr. Jodhbir Mehta, Dr. Gary S L Peh, Dawn Neo, and all our collaborators from Singapore Eye Research Institute (Singapore); their expertise and collaboration have significantly strengthened the depth of this work and the construction of the review manuscript. I would like to extend my appreciation to our collaborators Dr. Stephan Ong Tone and Dr. Judy Yan from Sunnybrook Health Sciences Centre, for their invaluable contributions in providing the FECD 54F cell line and offering crucial assistance throughout the experimental process.

I would also like express my gratitude to my collaborators from the Institute for Quantum Computing (University of Waterloo, Canada), who helped me in learning and improving my micro fabrication and lithography skills.

I would also like to give special thanks to my dedicated lab mates at the Regenerative Nanomedicine Lab. Their collaborative and inspiring environment played a significant role in this project. Their assistance in suggesting potential improvements in my methods and processes and their words of encouragement have been invaluable in this academic journey.

I extend my heartfelt appreciation to my parents for their constant financial support, understanding, and love.

I am indebted to my friends who provided encouragement, motivation, and a welcome distraction when needed. Your friendship has made this academic journey more memorable and enjoyable.

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Chapter 1

Introduction and Literature Review

1.1 Cornea physiology and structures

The cornea, the clear front part of the visual system, serves as the eye's essential defense against infections and structural support. It is also a crucial optical system component, combined with tear film to serve as the anterior refractive surface. Its clarity and functionality result from a combination of factors, including its complex structures and physiological components. It comprises five layers: the corneal epithelium, Bowman's layer, stroma, Descemet's membrane, and corneal endothelium (as shown in Figure 2) [1, 2]. The characteristics of these five layers (two acellular and three cellular layers) are summarized in **Table 1**. To better understand the FECD pathology and FECD model development, special attention was taken toward the stroma, Descemet's membrane, and corneal endothelium.

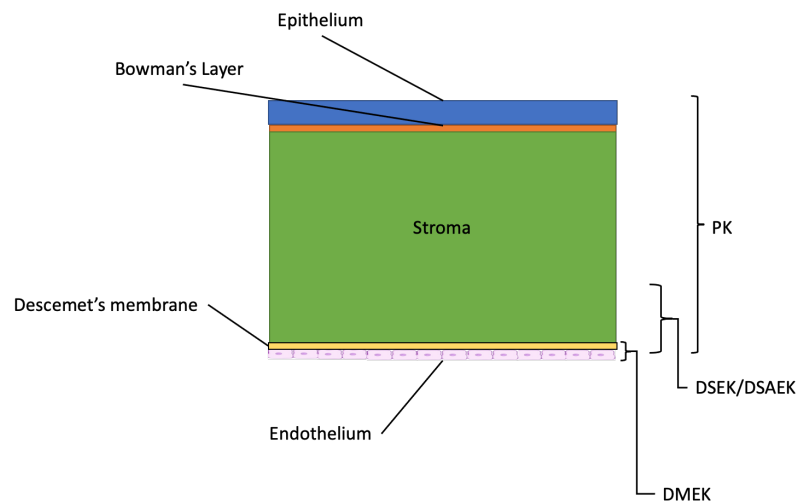


Figure 1. Cornea Structure and their respective surgical sections required (adapted from [1], size adjusted according to the thickness of each section.)

Table 1. Characterization of five layers of the cornea.

Structural Name	Prime Characteristic	Cellular/acellular	Main function	Developmental origin	Ref.
Epithelium	Formed in three distinct cell layers: superficial cells, wing cells, and basal cells	Cellular (epithelial cells)	Provides 2/3 of the refractive power for the eye, protects against pathogens and toxins, and frontal barrier	Derived from surface ectoderm at 5 – 6 weeks during development	[2]
Bowman's layer	An acellular layer made up of collagen fibrils	acellular	Provides structural support to the corneal epithelium	Becomes noticeable at 13 and 19 weeks of embryo development	[2, 3]
Stroma	The bulk of the cornea structure, highly organized	Cellular (primary type: keratinocytes)	Provides corneal transparency and mechanical support	During the second wave of neural crest migration (at seven weeks), after primitive endothelium has formed	[2]
Descemet's membrane (DM)	Structurally formed by the anterior banded layer	acellular	Supports the corneal	The anterior layer was formed before	[2]

	(ABL) and the posterior non-banded layer (PNBL)		endothelium structure	birth at the 8-week stage. Endothelial cells continuously secrete Descemet's membrane before and after birth	
Endothelium (CE)	A monolayer of cells hexagonal in shape that span along the DM	cellular (endothelial cells)	Keep the clarity of the entire cornea by maintaining the corneal hydration dynamically via a "pump-leak" process	Derived from Neural crest monolayer of cells	[2]

1.1.1 Epithelium

The corneal epithelium consists of three distinct cellular layers: superficial cells, wing cells, and basal cells, from the frontal layer to the posterior. The superficial epithelial cells combined with the tear film provide most of the eye's refractive power and protect against chemicals, microbes, and toxins. The superficial cells also maintain tight junctions to prevent foreign materials from penetrating deeper into the cornea. Posterior to the superficial cells are wing cell layers, which also have tight junction mechanisms with 2-3 layers of cells. Basal cells are essential for producing wing and superficial cells and for the cells to epithelial basement membrane attachment, which prevents the epithelium from separating from the cornea [2].

1.1.2 Bowman's layer

The Bowman's layer is a layer in the cornea composed of collagen fibrils with no cells. It is located between the epithelium basal membrane and the stroma. The precise function of the Bowman's layer in the cornea is yet not fully understood. However, it is assumed that it helps maintain the shape of the cornea and provides protection to the subepithelial nerve plexus [4].

1.1.3 Stroma

The corneal stroma is posterior to the Bowman's layer, the most substantial portion of the cornea thickness (85-90%). The stroma mainly comprises highly organized collagen fibres, proteoglycans, and extracellular matrix (ECM). The primary structural components of stromal collagen consist of type I and V collagen. These fibres are densely packed into fibrils, and adjacent parallel fibrils come together to create layers known as lamellae. The human corneal stroma typically contains between 200 and 250 lamellae arranged roughly perpendicular. This organization allows the cornea to minimize the scattering of incident light and maintain its mechanical integrity. In addition, the interactions among various ECM components in the stromal layer also play a part in maintaining the mechanical strength, shape, and avascularity of the cornea [2, 5].

1.1.4 Descemet's Membrane

The Descemet's Membrane (DM) is a basement membrane without cells. Structurally, it can be divided into two distinct sections: the anterior banded layer (ABL) and the posterior non-banded layer (PNBL). The ABL is created before birth with around three μm thickness, while the PNBL is produced after birth. Over time, the PNBL, which is secreted by the endothelial cells, can increase in thickness to approximately ten μm [2]. The primary role of Descemet's Membrane (DM) is to offer structural support to the corneal endothelial cells. These cells are attached to the DM, generating and reacting to the biophysical signals from DM, implying a bidirectional relationship between the cell monolayer and the membrane. It is worth noting that many types of corneal endothelial dystrophy involve an abnormal DM microenvironment [6].

1.1.5 Endothelial layer

The most posterior layer of the cornea is the endothelium, consisting of a monolayer of hexagonal cells that extend across Descemet's Membrane (DM) and are held together by numerous hemidesmosomes. These corneal endothelial cells originate from the embryonic neural crest and, once formed, tend to

remain quiescent and lack mitotic ability *in vivo* [4]. In early development, the cells are constructed as a cubical shape, which then flattens and adheres closely to each other; anterior to the flattened cells, a layer forms that later becomes the DM. The endothelial cells then continue to flatten and stabilize at approximately four μm in adulthood [2]. The CEnCs density and the hexagonal cell percentage decreases with age, declining from 3000-4000 cells/ mm^2 to approximately 2600 cells/ mm^2 , and the percentage of hexagonal cells decreases from around 75% to 60% throughout a person's lifetime. [7].

The primary role of corneal endothelium is to maintain corneal clarity by actively removing fluids from the other layers, thus ensuring a relatively dehydrated state in the stroma. This process is also known as a pump-leak process, where the fluid transports from the stroma to the aqueous humor. This function is maintained by many Na^+/K^+ -ATPase pumps that actively transport sodium (Na^+) and potassium (K^+) ions across the basolateral membrane, leading to high intracellular K^+ and extracellular Na^+ concentrations. Sodium (Na^+) ions pass through the endothelial layer into the aqueous humor and form an ionic gradient, which leads to the removal of fluids from the stroma through passive osmosis [2].

It is important to note that endothelial cell density and morphology can significantly affect the function of corneal endothelium. Cell number reduction can be connected to factors such as aging, trauma, inflammation, and diseases like Fuchs endothelial dystrophy. In addition, understanding the composition, development, and surface properties of the DM is crucial for FECD research, as DM and CEnC together form a bidirectional system.

1.2 Abnormal cornea endothelium physiology

In individuals with FECD, significant alterations are observed in the physiology of all five corneal layers. FECD typically occurs in the 50-60 years group and has a higher occurrence in females. Initially, the endothelium undergoes an accelerated loss of cells, starting at the central region. Subsequently, the remaining cells in the peripheral area migrate to the cell loss site, causing them to lose their healthy hexagonal shape, further inducing polymorphism (increased variation in cell shape) and polymegathism (variation in cell size). The decrease in endothelial cell counts and cell function also reduces the number of available ion pump sites. Eventually, the endothelium gradually loses its pump function and cannot sustain corneal deturgescence. This reduction in functional ability leads to corneal edema and vision loss [8].

In FECD, the extracellular deposits accumulate abnormally on the DM, a phenomenon associated with the severity of the disease [9-11]. During the progression of the FECD, these accumulations start to

form observable deposits known as guttata, becoming a characteristic feature of FECD. These deposits can also accumulate over time, increasing in number and dimensions [10, 12]. Guttata is primarily located in the central region of the DM and can lead to alterations in the shape and size of the endothelial cells in the DM. In the progression of FECD, guttata can spread from the central DM towards the peripheral region, continuously damaging the CEnCs and corneal endothelium pump function [13]. In cases of late-onset FECD, abnormal collagen accumulates and forms posterior banded and fibrillar layers posterior to Descemet's Membrane [14]. In severe cases, a posterior collagenous layer may also develop [10].

Once the corneal endothelial cell counts fall below a critical threshold at approximately 500 cells/mm², the rate of fluid removal from the stroma may be compromised, and the dynamic function of the corneal endothelium begins to deteriorate. This deterioration causes the gradual accumulation of excess fluid in the corneal stroma (stromal edema) [2, 15]. The corneal stromal edema then leads to focal thickening in the cornea, significantly impacting visual acuity [4]. The condition may progress without treatment, eventually causing stromal keratocytic cell death and subepithelial fibrosis. Ultimately, FECD in the advanced stage can lead to corneal blindness [9].

1.3 Pathology and etiology of FECD

While extensive research has been conducted to explore the pathogenic pathways of FECD, existing theories are still unable to converge to all the instances of the disease [15]. Some identified mutations have been associated with FECD: transcription factor 4 (TCF4), collagen VIII α 2-subunit chain (COL8A2), and Solute carrier family 4-member 11 bicarbonate transport protein (SLC4A11). In addition, researchers have uncovered pathogenesis and pathophysiology mechanisms such as the endothelial-mesenchymal transition (EndMT), cell senescence, oxidative stress, and ECM microenvironment.

1.3.1 Genetic-related FECD pathology

Approximately 37-39 percent of FECD patients have hereditary autosomal dominant mutations [15]. It has been presumed that several genetic mutations can trigger the development of the FECD. For example, COL8A2 is one of the mutations closely related to the early onset form of FECD [14]. Other genes, such as TCF4, SLC4A11, Zinc finger E-box-binding homeobox 1 (*ZEB1*), Lysyl oxidase-like 1 (LOXHD1), and ATP/GTP binding protein-like 1 (AGBL1), are more closely associated with the late-

onset form of the FECD. The *TCF4* mutation is known to be the most prevalent gene mutation [13]. These genes are involved in extracellular matrix (ECM) deposition, cell apoptosis, ion transportation, intercellular contact, and cell proliferation.

In the following section, two of the common FECD-associated gene mutations will be reviewed and summarized. Notably, the clinical manifestations of FECD are genetically heterogeneous; the expression level varies between cases, and the penetrance is incomplete amongst patient populations [9].

1.3.1.1 Transcription factor 4 mutation

The *TCF4* gene is located on chromosome 18 and encodes the protein transcription factor 4 (TCF4). It is reported that the trinucleotide repeats expansion (TNR) of intronic cytosine–thymine–guanine (CTG) may be associated with the increased risk of FECD [16].

The primary mechanism is hypothesized to be the CTG repeats forming transcript aggregates in the nucleus, leading to the sequestration of Muscleblind-like 1 (MBNL1) and MBNL2, which are essential factors in messenger RNA (mRNA) splicing. Subsequently, this causes mis-splicing events downstream [17, 18]. Notably, the resulting mis-splicing also generates mutant transcripts affecting properties related to cytoskeletal protein binding or cell adhesion [19].

Notably, 79% of all FECD-phenotype patients exhibited CTG repeat expansion in a US Caucasian cohort [13]. Additional studies have reported similar findings in northern European groups [20]. However, the prevalence of CTG TNR-associated FECD in Asian ethnicities was relatively lower [21, 22]. These variations demonstrate the complexity involved in FECD and *TCF4* mutation.

TCF4 may also be linked to endothelial-to-mesenchymal transition (EndMT) in FECD, as the *TCF4* gene is the coding sequence of the E-protein family (E2-2) closely related to EndMT. To briefly explain, EndMT is a process where endothelial cells change their phenotype into mesenchymal-like cells [23].

A significant part of the experimental setup and analysis of this thesis is based on a cell line derived from a 54-year-old female FECD patient donor, who is identified with a single allelic 73-repeat expansion of the CTG sequence in the *TCF4* gene (FECD-SV-54F-73) based on a genetic analysis from blood samples [24].

1.3.1.2 COL8A2

The COL8A2 gene encodes for collagen VIII $\alpha 2$ -subunit short-chained collagen, which is secreted by corneal endothelial cells (CEnCs) and deposited on the Descemet's Membrane (DM) [25]. It is worth noting that, instead of being associated with late on-set FECD, Mutations in COL8A2 are associated with early-onset FECD [25, 26]. One of the mutations, L450W, leads to increased collagen deposition on the anterior banded layer (ABL) of the DM. This causes a significant increase in ABL thickness and continued deposition of collagen, fibronectin, and laminin after birth in posterior DM [27]. Another mutation, the Q455K mutation, is linked to early-onset FECD and is associated with endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) [28, 29]. The study also suggested that in the mutant murine models, the DM of lower stiffness may influence the progression of endothelial cell function [28].

1.3.2 FECD cells and microenvironment

While approximately 37-39 percent of patients exhibit familial genetic linkage (closely located genes on the same chromosome inherited together amongst generations), the pathogenesis of FECD may be combinatorial, and most clinical cases are sporadic [9, 15]. The clinical data collected and analyzed from these sporadic occurrences of FECD can be concluded as abnormal cell behavior, impaired corneal endothelium function, and apoptosis. The central question of this issue asks whether cell dysfunction, pump dysfunction, or the development of an abnormal microenvironment primarily contribute to cell death. Consequently, these pathological events have led to exploring EndMT, cell senescence, and the guttata as potential primary mechanisms in FECD pathogenesis.

1.3.2.1 Abnormal ECM (Guttata)

A key characteristic of FECD is the excessive and abnormal secretion of ECM, which is responsible for guttata formation. The presence of guttata can impact cell function, depending on guttata size and density. In 2016, Rizwan and colleagues discovered that synthetic guttata pillars with diameters, spacing, and height of 20 μm and guttata with 40 μm diameters could disrupt the formation of primary CEnC monolayers [30]. guttata of smaller heights and lower density did not obstruct monolayer formation. Similarly, Kocaba *et al.* observed a comparable outcome regarding how CEnCs respond to guttata size and density in *ex vivo* tissue samples at different diseased stages [31]. However, they found that guttata did not lead to intercellular dysfunctions, such as DNA damage, defective reactive oxygen

species (ROS) response, mitochondrial dysfunction, and unfolded protein response (UPR), implying that the sole presence of guttata may not entirely encompass the characteristics of FECD pathogenesis.

1.3.2.2 Abnormal Cell

1.3.2.2.1 Endothelial-to-Mesenchymal Transition (EndMT)

The primary characteristics of FECD involve the thickening of Descemet's Membrane (DM) with the gradual formation of guttata. The mechanism underlying this pathophysiology has been associated with EndMT, a variant of epithelial-to-mesenchymal transition (EMT) [32]. In the EndMT process, endothelial cells transform from an endothelial phenotype to a fibroblastic one, losing the normal hexagonal phenotype and cell junction [33]. Subsequently, the EndMT process also induces the excessive deposition of extracellular matrix (ECM) and the loss of cellular apical-basal polarity, ultimately leading to changes in corneal endothelial cell motility [34, 35].

In FECD, proteins such as collagen I and III, fibronectin, and transforming growth factor betas (TGF β) were present in CEnCs adjacent to the guttata [35]. During the EndMT process, transforming growth factor betas (TGF β) play a critical regulatory role. Activation of TGF β upregulates *ZEB1* and *Snail*, which are EndMT-inducing genes. When these genes are found in high expression levels in FECD, the production of ECM proteins Collagen type I and fibronectin also upregulates [32]. It is also suggested that other pathways, like FGF-2, may regulate the expression of *ZEB1* and *SNAIL* [36].

1.3.2.2.2 Cell senescence

One of the indicators of cell senescence is the loss of proliferative capacity. In healthy cells, senescence is a natural process during normal physiological development. However, for chronic diseases like FECD, the dysfunctional interaction between CEnCs and ECM is also linked to cell senescence and cell death. [9].

Researchers have speculated that premature cell senescence may be involved in the pathogenesis of FECD. Matthaei *et al.* [37] and White *et al.* [38] have both demonstrated senescence in CEnCs by using the Col8a2 knock-in mouse model and UV-A radiation exposed models, respectively, both of which caused significant upregulation of the gene encoding the cyclin-dependent kinase inhibitor 1A protein (CDKN1a, also known as p21, linked to mechanisms of cell cycle arrest) and p53 level. The researchers also proposed a series of mechanisms associated with senescence: when there is persistent

DNA damage, CEnCs become arrested in the G2/M stage, leading to premature cell senescence, which then induces a profibrotic phenotype marked by ECM secretions and EndMT in CEnCs, as observed in FECD [38].

1.3.2.2.3 Oxidative stress and EndMT

Oxidative stress is believed to play a role in the FECD etiology [9], primarily due to the cornea's constant exposure to UV light and the high metabolic activity involving oxygen throughout life [9, 39]. In normal CEnCs, when there is an excess of reactive oxygen species (ROS), oxidative stress triggers a series of cellular defense mechanisms, which include the triggering of antioxidant production and apoptosis pathways [9]. In FECD-affected cells, however, the paths may be bypassed in the aging process [40, 41], leading to the down-regulation of specific transcripts in the FECD endothelium [42, 43].

To further explore the pathogenesis of oxidative stress in FECD, Katikireddy *et al.* used a combination of TGF-B and menadione on HCECs. The study proves that ROS, more specifically menadione, could induce EndMT. The impact of ROS levels might be further increased by the deficiency of NQO1, a protein responsible for protecting against oxidative stress [33]

1.4 Current and potential future treatments of FECD

The primary treatment for FECD currently involves corneal transplantation. More recently, however, new and promising treatments are emerging as potential alternatives to traditional transplantation. For instance, cell injection and drug treatments are being developed and tested on various models [9].

1.4.1 Keratoplasty

In the past, penetrating keratoplasty has been a critical surgical intervention for FECD, but it is also associated with post-surgical complications [44]. Emerging tools and devices have recently led to huge advancements that improved surgical procedures.

Two of the more recent methods are Descemet's Stripping Endothelial Keratoplasty (DSEK) and Descemet's Membrane Endothelial Keratoplasty (DMEK); DSEK requires removing the DM and CE monolayer and inserting the donor graft containing posterior stroma, DM, and endothelium. DMEK is similar to DSEK, except the donor graft consists of only Descemet and Endothelium. DMEK surgery

is more complex than DSEK due to the complex preparation, delicate insertion, and unrolling of the monolayer graft. [45].

Descemetorhexis Without Endothelial Keratoplasty (DWEK) has been an emerging technique in the past decade. The treatment involves stripping of the FECD host DM and CEnCs without graft or cell injection. It entirely relies on the patient's host peripheral cells to migrate to the wounded site [46]. DWEK has a relatively longer recovery time. In addition, DWEK is only feasible for mild to moderate FECD patients [47].

Soh *et al.* implemented a new technique called Descemet membrane transplantation (DMT), as DM is critical in wound healing of CE monolayer and functionality compared to stroma only [48]. By transplanting solely an acellular DM in the absence of a donor endothelium, this technique could utilize a large portion of rejected donor corneas (due to low cell count) as the DM is grafted without cells.

1.4.2 Cell-based and drug-based treatments

1.4.2.1 Cell-based treatments

Due to the limited number of compatible donor cornea grafts, alternative options are developing, such as cell injection therapy or tissue-engineered graft replacement. Cell injection faces challenges such as cell aggregate formation and adherence issues [49]. ROCK Y-2763 inhibitor in the form of eye drops was proven to promote cell proliferation as well as enhanced adhesion of CECs onto recipient tissue when utilized with cell-injection therapy [50]. Feline CECs tested the combination of cell injection with Y-27632 ROCK inhibitor [51]. Injection of human CECs with ROCK inhibitor in people with bullous keratopathy showed repopulation of cell density and restoration of normal corneal thickness [52].

Peh *et al.* created an expanded corneal endothelial cell graft using a dual-media approach [53]. The group went further to prove that the delivery of corneal endothelial cells could be done by both tissue-engineered endothelial keratoplasty (TE-EK) or corneal endothelial cell injection (CE-CI) [54].

1.4.2.2 Drug-based Treatments

Alternatively, developing drug-based therapies can enormously reduce the invasiveness of the FECD treatment. These drugs include ROCK inhibitors [55-58], sulforaphane [40], antioxidant medications [59, 60], TGF- β inhibitors [35], and RNA antisense oligonucleotides [61], all of which hold potential for advancing the treatment of FECD.

One of the widely tested drugs is the Rho-associate protein kinases (ROCK) inhibitor. More specifically, studies have proven that the ROCK inhibitor Y-27632 can enhance cell adhesion, prevent apoptosis, and improve cell proliferation in CECs [62]. These changes in cellular behavior demonstrate that the ROCK inhibitor can promote the healing of endothelial wounds and ultimately achieve corneal transparency. Initial studies have already been carried out using animal models to assess these effects [55-58].

1.5 Models used to recapitulate the pathophysiology of FECD and cornea

The development of FECD models considers a wide range of factors, from molecular mechanisms to the microenvironment. The models have been created with different objectives, reflecting the complex nature of FECD's pathology and etiology. In the next section, the strengths, and weaknesses of each FECD model will be discussed, and the motivations of these models will be discussed.

1.5.1 *In vitro* models

From the late 20th century, scientists have been involved in experiments aimed at creating fully functional human corneas. The tissue-engineered corneal models are expected to be useful for innovative drug testing and other chemical studies. They are also expected to eventually reduce the dependence on animal models. Currently, ongoing research may also offer alternative approaches to address the need for donor-tissue transplantation [63].

Together with the progress in constructing tissue-engineered human corneas *in vitro*, different models have also been developed for studying FECD. Apart from more complex models involving microenvironment or tissue samples, cell models have also been developed to study FECD *in vitro*.

Table 2. Summary of characteristics of various *in vitro* FECD models

Model Species	Prime Characteristic	Pathological & physiological recapitulation	Pros	Cons	Ref.
Primary HCEnCs	<i>In vitro</i> expansion of primary healthy and FECD cells	Cell genetic mutation, molecular changes in cells	Closest recapitulation of the HCEnCs <i>in vivo</i>	Limited proliferative ability. Primary FECD CEnCs have low yield due to low cell viability.	[51, 61, 64-70]
Immortalized HCEnCs	Viral oncogenes, Cellular Oncogene, RNA Interference, CRISPR/dCas9 immortalized FECD, and healthy cells	Cell genetic mutation, molecular changes in cells	Induced proliferative ability with active ion transport and full confluence. Less batch variability compared to primary cells	Less accurate biological information, highly dependent on donors, different from <i>in vivo</i> environment	[24, 30, 32, 33, 35, 40, 70-81]
Primary HCEnCs on biomimickin	Hot embossed topographical patterns	Recapitulate the impaired DM and CEnCs	Ability to control guttata size, morphology,	Not a complete representation	[30, 82]

g synthetic	microenviron	stiffness, and	of <i>in vivo</i>
guttata	ment <i>in vitro</i>	spacing.	environment.
	by mimicking	Surfaces can	
	guttata	be replicated,	
	structure.	not reliant	
		upon animal	
		donors.	

1.5.1.1 Primary Human FECD CEnCs

CEnCs have a very limited capacity for proliferation within the cornea. To address this limitation *in vitro*, various methods have been created, including the dual media approach [67] and substrate (topography)-supported approach [68, 69]. Nonetheless, achieving a significant mitotic ability while preserving the endothelial phenotype and gene expressions *in vitro* remains challenging [64]. On the other hand, it has been reported that the *in vitro* culture of FECD CEnCs could lose the FECD phenotype and recover to healthy CEnCs phenotype [65]. On the positive side, however, *in vitro* expansion of primary human CEnCs may provide the closest representation to the *in vivo* CEnCs [70].

Primary human CEnCs have been predominantly used in experiments related to cell expansion and the study of cell behavior. Several studies have focused on CEnCs expanded from patient samples with FECD [51, 65, 66]. For instance, Zaniolo *et al.* obtained FECD cells from the central cornea, specifically from patients in advanced stages of the disease. They were able to culture CEnCs from 18 out of 29 samples successfully. The study's findings revealed that FECD CEnCs from patients absent of a fibrillar layer on the DM exhibited higher success rates for *in vitro* culture [65]. However, it is essential to note that only 12 out of 18 specimens' CEnCs could restore or maintain their endothelial morphology; the remaining six samples exhibited a fibroblastic-like (elongated cell morphology) phenotype. All CEnCs from younger donors showed endothelial morphology, suggesting that both age and the fibrillar layer in the DM could affect culture results. Nonetheless, this study indicates that FECD DM still contains CEnCs that can proliferate in *in vitro* culture [65]. Zaniolo *et al.* also observed that phenotypical changes occurring after *in vitro* cell culture are not exclusive to FECD but also occur in healthy CEnCs [65]. This result implies that an *in vitro* cell culture and an *in vivo* corneal endothelium might become different [65].

Primary FECD cell cultures *in vitro* often undergo an “alteration” where they lose their distinctive FECD characteristics and tend to revert to the typical phenotype of healthy CEnCs. Multiple studies have reported the restoration of various cell functions in these cultures, including the recovery of telomere length, mitochondrial DNA (mtDNA) levels, and a balance between oxidants and antioxidants [65, 66]. This change in phenotype may result from a variety of factors, including the natural selection of healthier cells, the presence of proliferative growth factors in the culture medium, the absence of a toxic microenvironment, or a combination of all these factors (or two of these factors combined) [65, 66].

1.5.1.2 Immortalized human CEnCs

While expanding healthy or FECD primary CEnCs is still challenging, utilizing immortalized CEnCs instead could enable researchers to obtain preliminary data without the challenges of primary cell culture. However, immortalized cell lines pose an issue of altered cell behavior and response [71-74], especially when studying cell proliferation or drug response [30].

The immortalized CEnCs also possess some differences when compared to the primary CEnCs. Some cell lines have different phenotypes [71, 72] or partially impaired pump functions, or both in some cases [73, 74]. Cell lines differ from each other in terms of gene and protein expressions [70]. Nonetheless, immortalized CEnCs are still widely used in *in vitro* FECD modelling [9, 30].

1.5.1.2.1 Immortalized CEnC line from primary HCEncs

Immortalized CEnCs, such as SV40 (simian virus) Large T-antigen induced cell lines, are the first viral oncogene to immortalize HCEncs [71]. The survivability of CEnCs from both young and old donors was higher after the transfection. However, the SV40 cell lines have distinctive characteristics not observed in the normal HCEncs [71]. Another method involves using SV40 Small T-antigen. This immortalized cell line is called HCEnc-12; the hexagonal shape could be maintained after the transfection. Two other subtypes, HCEnc-B4G12 and HCEnc-H9C1, could also be sub-cloned and cultured. B4G12 is a closer recapitulation of the *in vivo* HCEncs with polygonal shape and monolayer formation, whereas the H9C1 is closer to the HCEncs during the developing or transitional stage [71, 72].

In a study of oxidative stress in FECD pathogenesis, Jurkunas *et al.* demonstrated the oxidant-antioxidant imbalance in FECD CE and normal CE and the increased oxidized DNA damage in FECD

CE using donor tissues [40]. They also used SV40-transfected CEnCs with an *in vitro* oxidative stress induction to support the findings from CEs. Aligned with the observation from CE, the immortalized cell lines showed a down-regulation of transcript nuclear factor erythroid 2-related factor 2 antioxidant (Nrf2, a vital antioxidant defence activation transcription factor) and oxidative DNA damage [40]. In 2016, Rizwan *et al.* initially used HCEnC-B4G12 as a CEnC line for a preliminary study on monolayer formation, migration, and expansion on a biomimicking synthetic guttata microenvironment *in vitro* before a more specific investigation with primary cells [30].

Another technique for immortalization is DNA transduction with the human Telomerase Reverse Transcriptase gene (hTERT) [78]. This expression may restore telomerase activity by introducing hTERT, an enzyme that synthesizes and maintains telomeres. The high proliferative and low senescence HCEnC-21T cell line is created using this immortalization process [78].

One example of using HCEnC-21 is the research conducted by Katikireddy *et al.* on studying the effects of oxidative stress on EMT in FECD [33]. The researchers observed that menadione (MN) could induce ROS generation, EndMT and FECD-related markers upregulation, and cellular remodelling involved in FECD pathogenesis [33].

1.5.1.2.2 Immortalized FECD cell line

Similar to the primary healthy and FECD CEnCs, *in vitro* immortalized FECD cells (iFECDs) possess some similarities but have distinctive differences compared to healthy immortalized CEnCs (iHCEncs) *in vitro*. Both iHCEncs and iFECDs could form normal monolayers and contact-inhibiting hexagonal cells similar to the *in vivo* CEnCs [24, 32]. However, findings have shown that the iFECD cell line is not precisely identical to iHCEncs in terms of FECD-related ECM and EndMT gene expressions [24, 32].

As early as 1997, He *et al.* attempted to develop CEnC culture by immortalizing FECD CEnCs. The CEnCs from FECD patient CE were transduced with a disabled retrovirus coding for human papillomavirus type 16 transforming oncoproteins E6/E7. Unexpected to the researchers, the transduced FECD cells also had comparable morphology, protein patterns, extended life span, and proliferation rate with normal cells treated with E6/E7. However, the quantitative differences were still observed in protein expression levels [73].

In 2015, the Koizume research group first generated immortalized CEnCs from healthy donors and FECD patients using SV40 large T antigen and hTERT to produce iFECDs and iHCEnCs. They showed that EndMT-related gene expressions (*ZEB1* and *SNAIL*) and excessive ECM protein production (e.g., type I collagen and fibronectin) in the iFECD line were significantly upregulated [32]. Subsequently, in a study of the TGF- β pathway in FECD, the Koizume group used both iFECDs and iHCEnCs as *in vitro* cell models to support their observation that the expression level of transforming growth factor- β (TGF- β) isoforms and TGF- β receptors are higher in FECD patients' CE. Using the iFECDs, they also found that TGF- β induced the formation of unfolded protein and the downstream apoptosis pathway following unfolded protein response (UPR) [35].

The Jurkunas group has also generated immortalized cells from human donor cells with SV40 T antigen. The iFECDs and HCEnC-21T healthy cell lines were used to detect the activation of mitophagy pathways, which plays an essential role in the loss of mitochondrial mass in FECD [81]. The iFECD line showed a substantial decrease in mitochondrial mass resulting from autophagy. They then further explored that the expression of Mfn2 (Mitofusin 2) was also downregulated, which is essential for mitochondrial function and antioxidant-oxidant balance [81].

In a later study by Ong Tone *et al.* (2021), the Jurkunas group found that the iFECDs cell behavior was not matched to iHCEnCs. Moreover, they also discovered that the cells retained the TCF4 CTG mutation after the immortalization of the cells from the patient with the mutation [24]. Subsequent experiments have also revealed that iFECDs were migratory and had fibroblastic morphology. More specifically, the iFECDs displayed more fibroblastic features through filamentous actin staining when compared to iHCEnCs, while the cell-cell tight junction was detected from both cell lines through ZO-1 staining. The mean migration speed, mean maximum speed and wound healing rate of iFECDs were all significantly faster than the healthy cell line [24].

1.5.1.3 Primary HCEnCs on biomimicking synthetic guttata

Rizwan and colleagues have developed a biomimicking synthetic guttata model, an *in vitro* model constructed based on the clinical observation of the FECD microenvironment. The guttata microstructure is synthesized from polystyrene materials. The guttata model with various dimensions and structures was used to resemble different stages of the FECD microenvironment [30]. These ranged from 10 to 80 μm in guttata diameter, spacing between nodules, and height with shapes of either pillars

or domes. It provides insights into engineering *in vitro* FECD model with controlled dimensions and shapes.

To validate the CEnC monolayer reformation ability of the FECD DM after using the ROCK inhibitor pharmacotherapy with cell injection treatment, they expanded primary CEnCs on the synthetic guttata model to investigate monolayer formation. When the height, diameter, and spacing of the guttata reached 20 μm , the seeded primary CEnCs would fail to form the monolayer [30].

Moreover, samples with larger diameters (40 μm) also showed similar results. The patterns with lower heights showed a higher rate of monolayer recovery; this indicates that the diameter, spacing, and height of the guttata could be significantly affected the CEnC monolayer recovery, suggesting that the patient's recovery after the pharmacotherapy and cell injection therapy would be more effective in early-stage FECD patients but may not be as successful for the late stage FECD patients [30].

Regarding cell migration, dome structures were shown to improve migration speed and lower directionality for CEnCs than pillar structures [30]. Other synthetic materials, such as hydrogels and gelatin methacrylate (GelMA), can recapitulate the DM microenvironment *in vivo* with varied stiffness [82].

1.5.1.4 3D *in vitro* corneal models

While there is a lack of non-planar *in vitro* models specifically designed to investigate the pathology of FECD, several studies have utilized a curved surface *in vitro* further to recapitulate the unique non-planar physiology of the cornea. In a study conducted by Postnikoff *et al.*, a stratified human corneal epithelium model has been developed *in vitro*, utilizing a commercially available contact lens and immortalized corneal epithelial cells. After cell culture for seven days, the samples were cultured in an air-liquid interface for another seven days for cell stratification and differentiation. The model was stratified into 3-5 layers of epithelial cells and subsequently used to test commercial ophthalmic solutions [83].

Gouveia introduced a novel approach for the development of biofunctionalized and curved human corneal stromal equivalents. By utilizing a convex-shaped agarose lens, the cells seeded on the periphery of the wells surrounding the convex lens could migrate up toward the center of the lens. The study demonstrates that curvature is sufficient to induce alignment of corneal stromal cells. Additionally, the curved tissues can also support the growth, stratification, and differentiation of corneal

epithelial cells. This work demonstrated that curvature alone could significantly affect the corneal cells' behavior [84].

Isaacson *et al.* have utilized 3D bioprinting techniques to create a curved corneal stroma. They used sodium alginate and methacrylated type I collagen as bio-inks and combined the bioinks with human corneal stromal cells. The bioinks were then injected into a 3D printed plastic support structure with a concave shape recapitulating the cornea curvature. The researchers have shown that 3D bioprinting is a feasible method to engineer artificial corneal structures *in vitro* [85].

1.5.2 *Ex vivo* corneal tissues

To understand how the microenvironment affects CEnC behavior, *ex vivo* models have been developed to recapitulate the phenotypical change and progression of the FECD DM and endothelium. Various *ex vivo* models have been utilized to study the FECD, and in these models, cell migration, cell-cell interaction, cell death, wound healing, and other investigations were conducted. Current *ex vivo* models for FECD research are summarized in **Table 3**.

Table 3. Summary of characteristics of various *ex vivo* FECD model species

Model Species	Prime Characteristic	Pathological & physiological recapitulation	Pros	Cons	Ref.
FECD <i>ex vivo</i> corneal tissue with cells	DM and CEnCs isolated from Stroma	FECD microenvironment, ECM	A close representation of the FECD <i>in vivo</i> , cells reside in native tissue	Not reusable, limited cell amount	[40, 59, 81, 86-88]
Normal <i>ex vivo</i> corneal tissue with cells	DM and CEnCs isolated from Stroma	Introducing oxidative stress via chemical or physical approach	A close representation of the normal environment <i>in vivo</i>	Not reusable	[24]
Mouse corneal tissue	Whole mount corneas, with CEnCs	Recapitulating human <i>ex vivo</i> corneal tissue	Great potential for early-stage preliminary studies	N/A	[40]

FECD <i>ex vivo</i> corneal tissue with seeded immortalized cells	Decellularize d tissue seeded with HCEnC-21T	FECD microenvironme nt, ECM	A close representati on of the FECD <i>in vivo</i> . Studies using FECD DM with healthy cells are possible	HCEnC-21T may not be the closest representation of the diseased cell state; <i>ex vivo</i> tissue is not reusable	[31]
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1.5.2.1 Human *ex vivo* corneal tissue

Human *ex vivo* corneal tissues provide the closest representations of the corneal endothelial cell-DM interactions. Unlike immortalized CEnC lines, *ex vivo* tissues can only be obtained for single-use experimental studies. Similar to primary cells, *ex vivo* tissues have limited availability as they have the same scarcity as the cadaveric donor corneas. Cadaveric donor corneas with high quality are also mainly used for cornea transplantation, thus further limiting the number of available specimens for *ex vivo* experiments [9, 89]. *Ex vivo* corneal tissue has been used to study oxidative stress, cell apoptosis, cell migration, and surgical procedures in FECD [24, 59, 87, 88, 90].

Halilovic *et al.* conducted a study using normal and FECD *ex vivo* corneal tissues to investigate cell morphology and oxidative stress. The researchers initially observed higher levels of oxidative stress-induced DNA damage in iFECDs (discussed in section 1.5.1.2.2) and *ex vivo* FECD samples, compared to immortalized CEnCs and *ex vivo* corneal tissue cells. They then compared these findings with the menadione (MN, a substance that induces cellular oxidative stress) treated healthy immortalized CEnCs [59]. They observed that when normal CEnCs were exposed to oxidative stress *in vitro*, they exhibited a rosette-like phenotype similar to the cells observed in FECD *ex vivo* specimens. Further analysis revealed that the MN-treated immortalized CEnCs also showed oxidative damage to mtDNA and nuclear DNA (nDNA), mitochondrial fragmentation, and cellular apoptosis, consistent with the observations made in FECD samples [59].

Cell apoptosis in FECD was further explored by Ziaei *et al.* [87]. They utilized *ex vivo* FECD corneal tissues to study the nuclear factor erythroid 2-related factor 2 (Nrf2) as an antioxidant defence mechanism in FECD. FECD cells had a deficiency in the Nrf2 antioxidant defence mechanism, while sulforaphane (SFN) could enhance the nuclear translocation of Nrf2 and reduce cell apoptosis. Furthermore, the researchers also found that the cell density in the *ex vivo* FECD tissue was limited. Hence, the subsequent study of the SFN mechanism was not performed in tissue samples but in cell lines instead; this implies the possible cell viability issue when using the FECD *ex vivo* model [87].

Cell migration is crucial for the FECD disease fate, which could also be studied using *ex vivo* models. Ong Tone *et al.* conducted a study using *ex vivo* models to investigate cell migration in the progression of FECD [24]. To examine cell migration, the researchers utilized human corneal tissues with both healthy and FECD (with guttata) conditions. Firstly, they collected DM with the endothelial layer from donor corneas from FECD and healthy individuals. Specimens (with cells) were then transfected with a green fluorescent protein (GFP) using a lipid nanoparticle (LNP), and the mean migration speed of the cells was calculated with an automated live cell tracking system. As a result, CEnCs in the FECD sample had a higher mean migration speed (for low cell density areas) than normal CEnCs [24]. The cells in FECD specimens also migrated in single-cell behavior instead of a "*monolayer spreading*," which describes the concerted movement of adjacent cells closing the wound [24, 90].

A recent study evaluated the potential FECD treatment using laser ablation on FECD *ex vivo* DM [88]. The excimer laser was originally employed for glaucoma surgery. The laser could ablate the diseased tissue guttata on the *ex vivo* FECD DM. After the surgical procedure, the DM and cells were expanded in a medium supplemented with ROCK inhibitor ripasudil. The resulting cell migration and expansion led to the initiation of wound closure, which was nearly completed after 26 to 38 days. This study using FECD DM as a model has demonstrated a potential solution to treat the abnormal ECM in FECD DM [88].

1.5.2.2 Animal *ex vivo* corneal tissue

Due to the global shortage of the availability of human corneal tissues, cornea specimens from other animal species, such as porcine corneal tissue and mouse *ex vivo* tissue, are considered a potential alternative for FECD study, as they are more readily available [40, 91, 92] since they offer great potential for early-stage preliminary studies before being extended on human *ex vivo* corneal tissues [40, 59].

Mouse *ex vivo* cornea tissue has been employed for oxidative stress studies [40]. Jurkunas *et al.* did a simple experiment in which *ex vivo* mouse corneal tissues were treated with varying concentrations of H₂O₂, following cell morphology and viability were evaluated. The results highlighted that oxidative stress led to cell morphological changes and apoptosis in both early and late-onset forms, which mirror a potential FECD pathogenesis mechanism [40].

1.5.2.3 *Ex vivo* decellularized tissue

To further study the pathogenesis role of guttata in FECD, Kocaba *et al.* used FECD *ex vivo* tissue without cells. The researchers developed a combinatorial model constructed from decellularized FECD *ex vivo* tissue and an immortalized cell line HCEC-21T [31] by seeding the HCEC-21T on decellularized FECD tissues. They found that small diameter (mean, 10.5 µm) guttata did not inhibit the proliferation of the CECs. Whereas medium diameter guttata (mean, 21.1 µm) induced rosette-like structures, and the large guttata (mean, 31.8 µm) caused the same rosette pattern with increased TUNEL-positive cells. Both medium-diameter guttae and large-sized guttae have induced upregulations of EndMT and senescence markers [31]. Most importantly, they found that the mRNA expression of EndMT-inducing genes: *Alpha-smooth muscle actin* (α SMA), *N-cadherin*, and *SNAIL* gene were significantly upregulated in cells surrounding the large guttae when compared to the cells adjacent to the small guttae or on the normal DMs. In addition, the expression of *NOX4* was upregulated in medium and large guttata samples. These results highlighted the importance of the guttata dimension in FECD. However, several known FECD intercellular markers were not found in this model. Hence, the microenvironment may not be the only factor influencing FECD pathogenesis *in vivo*. In addition, the immortalized cell line used may not be the most accurate representation of *in vivo* FECD since HCEC-21T is a healthy HCEC line known to have good proliferative ability [31, 78].

Goyer *et al.*, on the other hand, utilized CECs from end-stage FECD patients and seeded the cells onto the decellularized DM side of the normal cornea to construct a corneal endothelium model. The subsequent analysis of the engineered FECD tissue shows a significantly higher presence of fibronectin compared to the tissues seeded with healthy cells, implying that fibronectin may be involved in the early pathogenesis of the disease prior to the buildup of laminin and type IV collagen on DM [93].

1.5.3 *In vivo* models

In vivo animal models are crucial for FECD studies, as they allow for preclinical testing of drugs, treatments, and cell transplantation, as well as investigating the pathogenesis of the disease. However, not all animal models are equally suited for these purposes. While some species, such as rabbits, bovines, and a limited number of murine models, have higher proliferation rates during CEnC recovery, others, including primates (humans and monkeys), felines, and porcine, have limited proliferation rates. Therefore, animal models like rabbits and felines are typically used to study the functionality of primary corneal endothelial cells rather than to study FECD. It is essential to be clear about the purpose and limitations of each animal model when designing and interpreting experiments for FECD research. This section summarizes the utilization of different *in vivo* models based on the species.

Table 4. Summary of characteristics of various *in vivo* FECD model species.

Model Species	Prime Characteristic	Pathological & physiological recapitulation	Pros	Cons	Ref.
Murine (mouse)	Exposure to ultraviolet light	Recapitulate cellular DNA damage induced by UVA irradiation	Model for oxidative damage through ROS, <i>in vivo</i> recapitulation of the diseased environment	UVA exposure is considered the sole environmental factor in FECD induction, while many genes are involved in FECD development	[94, 95]
	Mutation in COL8A2 gene (including Q455K and	Genetic mutation of early-onset form of FECD	Model for early-onset FECD	Not representative of late-onset FECD	[25, 26, 28, 29, 96]

L450W variants)					
Feline	Partial DM removal <i>in vivo</i>	Recapitulate impaired DM and CEnCs	Does not proliferate <i>in vivo</i> . Cell density and corneal thickness are similar to humans. Human xenograft transplantations into felines are well tolerated.	Not a full recapitulation of human DM and CEnCs <i>in vivo</i>	[51, 97, 98]

1.5.3.1 Murine models

Murine (mouse) models are one of the generic models in the FECD study. These models offer simple methodologies and straightforward techniques. In particular, UVA-induced FECD, post-irradiation morphological imaging, and cell density measurement are all practically achievable.

By using the C57BL/6 wild-type (WT) mice, Liu *et al.* developed an FECD animal model by ultraviolet A (UVA) irradiation to explore the mechanisms of environmental and sex-dependent factors in FECD [94]. In another study, the same strain of mouse was used to investigate a link between CEnC senescence and EndMT with UVA light stress on the eye [95]. They found that UVA caused G2/M phase cell cycle re-entry for *in vivo* CEnCs. The arrested senescent CEnCs then exhibited higher expression of EndMT genes, resulting in a profibrotic morphology. These findings indicate a clear link between cell cycle status and EndMT.

UVA-irradiated murine models help elucidate apoptosis mechanisms related to cell loss caused by oxidative stress. However, the genetic heterogeneity of FECD and the genetic differences between humans and mice are among the limitations. Genetic heterogeneity cannot be induced via only UVA irradiation, but genetically modified mice have been established to recapitulate and examine genetic

mutations involved in FECD [29, 99]. One of the examples is a genetically engineered homozygous Col8A2^{Q455K/Q455K} mutant mice knock-in model, pathologies like cell loss and guttata formation were observed [25, 26]. Although not all the phenotypical changes were observed in the genetically engineered murine models (e.g., DM thickening), the study still provides an essential perspective as the early pathogenesis and pathophysiology of FECD are impossible to be studied using human tissues [25, 26, 100].

1.5.3.2 Feline models

The feline cornea is a valuable animal model for *in vivo* FECD research because of its similarity to human corneal endothelium as non-proliferative *in vivo*. Feline CEnCs compensate for denuded areas by cell enlargement and migration, similar to human CEnCs [97, 98]. Cell density and corneal thickness are comparable to human corneas [97, 98].

The feline model represented the first known *in vivo* model where human FECD CEnCs were transplanted as xenografts [98]. In a study conducted by Haydari *et al.*, human FECD CEnCs were expanded on decellularized stromal carriers and then transplanted into the eyes of felines. After seven days of transplantation, the corneal endothelium restored its pump function and transparency.

Interestingly, the recovery rate of FECD cells in the feline eyes was better than in human patients with similar symptoms, even though some feline corneas had mild to moderate FECD symptoms post-transplantation. However, the transplanted FECD specimens still displayed increased thickness, more fibrillar substance accumulation, and reduced cell densities, all characteristics resembling the FECD model [98].

This study introduced a "living" *in vivo* FECD model, opening up possibilities for developing novel therapies using human FECD cells in an *in vivo* setting. The model can potentially study FECD cell dysfunction, molecular pathophysiology, and apoptosis without FECD DM. Notably, the research suggested the feasibility of autografts for FECD patients, as it demonstrated partial recovery of FECD CEnC function in the feline model.

The feline model is also helpful in the preclinical evaluation of CEnC injection and ROCK inhibitor administration [51]. The researcher found that cell injection therapy may not be ideal for the incompletely functional endothelium, as the ROCK inhibitor can stimulate the wound healing mechanism with the host peripheral CEnCs.

Chapter 2

Hypothesis and Objectives

The pathogenesis of Fuchs endothelial corneal dystrophy (FECD) is a complex and heterogeneous disease that can be affected by multiple factors, which could be categorized as genetic and exogenous factors [9]. The currently clinically approved treatment for FECD is corneal transplantation, which requires donor cornea that is consistently in shortage. In the progression of FECD, the corneal endothelial cell count will gradually decrease, and the abnormal deposition of extracellular matrix (ECM) will eventually form a guttae structure [8, 10, 101]. The guttata structure will progressively grow on DM and continuously damage the corneal endothelium, further causing cell number reduction and cell dysfunction [8-10, 101]. It has been proven that guttata formation is associated with CEnCs morphology loss and excessive apoptosis [102]. However, the pathogenesis of the FECD, specifically the mechanisms of dysfunctional cells and its interactions with the abnormal microenvironment, has not been fully elucidated. Previous research conducted in the Regenerative Nanomedicine Lab has suggested that with varying dimensions and density of biomimicking synthetic guttata (s-guttata) patterns, normal CEnCs behaviors can be significantly altered. Studies have explored CEnCs' response to the s-guttata model in monolayer formation, apoptosis, and migration behavior [30]. Therefore, follow-up studies were conducted based on the hypothesis that the cytoskeletal response and apoptosis rate of the FECD corneal endothelial cells were affected by the guttata structures, which play an essential role in the pathogenesis of FECD. Therefore, the following sub-aims were formulated:

1. To investigate how the dimensions of guttata influence apoptosis in FECD corneal endothelial cells.
2. To examine the potential cytoskeleton mechanisms of the FECD cells with guttata

To further conduct follow-up studies on the FECD CEnCs pathophysiology, the migration pattern and wound closure rate of the FECD and normal immortalized cells were studied on a concave surface, thus formulating the following sub-aims:

3. To perform migration study of the FECD and normal CEnC line on the concave surface
4. To develop curved lens patterning method to affect and control cell behaviors

Chapter 3

FECD and B4G12 CEnC apoptosis and cytoskeleton mechanisms

3.1 Introduction

The most posterior layer of the cornea is the corneal endothelium, consisting of a monolayer of hexagonal cells that extend across the Descemet's Membrane (DM) [4]. Corneal endothelium, together with the DM, form a bidirectional system. From the clinical observations, the CE undergoes cell loss and formation of ECM depositions called guttata; the pump function will then be compromised, and the corneal dehydration will no longer be maintained [8]. During the progression of the FECD, the extracellular deposits start to be accumulated on the DM; these accumulations then form observable deposits known as guttata, in which their dimensions and density are associated with the severity of the disease [9-12]. Guttata initially appears in the central region of the DM. During the progression, guttata can spread toward the peripheral region, continuously damaging the CEnCs and DM [13]. The progressive deposition of guttata and their dimensional changed due to the progression of FECD have been studied and observed extensively in clinics [102-104]. It has been proven that guttata formation is associated with CEnCs morphology loss and gradual cell loss [102]. However, the role of guttata in FECD pathophysiology and its interaction with the native FECD cells during the disease progression has not been fully understood yet. In addition, the effect of guttata dimensions on corneal endothelial cells also remains unclear.

The dysfunctional cell behaviors in FECD can be classified into several categories, including oxidative stress, endothelial to mesenchymal transition, senescence, and apoptosis [9]. Several markers and gene expressions have been used to study these cell dysfunctional events, and a previous study has demonstrated an upregulation of EndMT (*α SMA*, *N-cadherin*, and *SNAIL*) and senescence markers, excessive apoptosis, and phenotypical changes in FECD [12, 24, 31, 32, 59]. By employing the techniques of immortalization, the diseased CEnCs from the FECD patients were able to be maintained and expanded *in vitro* [24, 32, 35, 73, 81]. These immortalized FECD cells provided a novel platform suitable for studying the pathology and etiology of FECD [24, 32, 35, 81]. However, a model that can further elucidate the combinatorial effect of toxic FECD-ECM interaction has not yet been developed.

Previously, past members have developed a synthetic biomimicking guttata (s-guttata) model with varying dimensions, providing a model to explore how guttatas affect the migration, monolayer

formation, apoptosis, and cytoskeleton contractility of normal CEnCs [30]. In this chapter, the s-guttata model was employed further to study the pathogenesis and pathophysiology of the FECD. By utilizing the s-guttata patterns with an immortalized FECD cell line, the role of the guttata microenvironment in the FECD cell dysfunction, including excessive apoptosis, EndMT, and senescence, can be further understood. This chapter also tries to recapitulate the advanced stage of FECD under the combinatorial effects of the dysfunctional cell and toxic microenvironment.

3.2 Materials and Methods

3.2.1 Cell culture

3.2.1.1 Healthy human corneal endothelial cells (B4G12, DSMZ, Germany) cell culture

Human corneal endothelial cells (B4G12, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany, between passage 20-40) were grown in T25 culture flasks in DMEM media (high glucose DMEM [+] 4.5 g/L glucose, L-glutamine. [-] sodium pyruvate) containing 10% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin-Streptomycin (Gibco). The cells were maintained in an incubator at 37°C with 5% CO₂, and the media was refreshed every other day until the cells reached confluence. For cell passage and experiments, cells were detached using 0.25% trypsin (Gibco) and then seeded onto substrates with the appropriate cell density.

All the study protocol was approved by the University of Waterloo Institutional Research Board (IRB: 43952) and conducted in accordance with the tenets of the Declaration of Helsinki.

3.2.1.2 FECD human corneal endothelial cells (FECD-SV-54F-73) cell culture

An immortalized FECD human corneal endothelial cells were generously provided by Dr. Stephan Ong Tone (Sunnybrook Health Sciences Centre, Sunnybrook Research Institute, Toronto, Canada). These cells were obtained from a 54-year-old female FECD patient undergoing endothelial keratoplasty [24]. Genetic analysis performed by Ong Tone *et al.* indicated that this FECD cell line has maintained the single allelic 73 repeat expansion mutation of the CTG sequence in the TCF4 gene [24].

FECD 54F were cultured in Chen's medium (OptiMEM-I; Invitrogen) containing 8% fetal bovine serum (FBS, Gibco), five ng/ml epidermal growth factor (Millipore), 100 mg/ml bovine pituitary extract (MP Biomedicals, USA), 200 mg/l calcium chloride (Sigma-Aldrich), 0.08% chondroitin

sulphate (Toronto Research Chemicals, Canada), 50 mg/ml gentamicin (Gibco), and 1:100 diluted antibiotic/antimycotic solution (Gibco).

To prepare the corneal endothelial cells for cell passage and experiments, they were detached using 0.05% trypsin (Invitrogen) at 37°C and 5% CO₂ for 5 minutes. Subsequently, the cells were centrifugated at room temperature for 3 minutes at 100 g to form a cell pellet. These corneal endothelial cells were carefully mixed into prewarmed Chen's media through gentle mechanical trituration.

For cell culture, the T25 cell culture plate was precoated with undiluted FNC Coating (Athena ES, Baltimore, USA) for approximately 3-5 mins. Cells were then seeded and incubated at 37° C, 5% CO₂. Corneal endothelial cells were incubated for approximately 48 hours at 37° C, 5% CO₂. Between changing the media, cells were replated every 4-5 days.

3.2.2 PDMS and polystyrene synthetic guttata fabrication

PDMS (Polydimethylsiloxane, Sylgard, Dow, USA) solution was prepared with a 10:1.05 base to curing agent ratio. After thorough mixing and degassing, PDMS was applied to the silicon wafer molds and placed in a vacuum desiccator for 1 hour to ensure the removal of all trapped air. After degassing, PDMS with silicon mold was incubated in the oven for polymerization at 60°C overnight. Subsequently, the uncured PDMS with the silicon mold was placed in an oven and left to crosslink overnight at 60°C. The polymerized PDMS molds were then detached from the silicon wafer. The following dimensions were created with PDMS soft lithography:

Table 5 Dimensions of fabricated s-guttatas

Diameter (μm)	Spacing between s-guttatas (μm)	Height (μm)	Assigned name
20	20	10	20x20x10
20	20	20	20x20x20
40	80	10	40x80x10
40	80	20	40x80x20

Subsequently, these molds underwent plasma treatment for 60 seconds using 80% O₂ gas and 70 Watts of power (Zepto, Diener, Germany). Following the treatment, the molds were coated with an anti-stiction layer (using (1H,1H,2H,2H)-perfluorodecyl trichlorosilane (FDTS, Sigma)). To remove any excess reagent, the PDMS molds were replicated at least five more times. These molds can then be used as master molds for creating polystyrene (PS) substrates.

Polystyrene (PS) with 2 cm x 2 cm dimensions was cut from the petri dish (VWR International), washed with ethanol, and allowed to air dry. These pieces were then embossed with patterned PDMS molds at 220°C and a pressure of 0.3–0.5 MPa for 90 seconds. After this period, the heating system was turned off, and the samples were left off the stage with pressure on for an additional 10 minutes. Unpatterned PDMS molds were employed to create control PS samples. To observe the dimensions of the guttata pattern and potential defects, the samples were examined using the LEXT OLS5000 3D measuring laser microscope (Olympus, Japan).

3.2.3 Cell seeding for the staining experiments

The patterned and unpatterned PS samples were cleansed with ethanol, allowed to air dry, and plasma treated using 80% O₂ gas and 70 Watts (Zepto, Diener, Germany) for 60 seconds. Following plasma treatment, these samples underwent sterilization under UV light within a biosafety cabinet for one hour. Cells were then seeded directly onto the samples at 300,000 cells/cm². The cells were cultured for 24 hours before staining works.

3.2.4 Apoptosis and cytoskeletal staining

3.2.4.1 Early apoptosis staining assay and quantification

After 24 hours of cell seeding on the patterned PS surface, cell media was aspirated from the samples, and the cells were rinsed twice for three minutes with 1x Phosphate-buffered saline (PBS). Subsequently, each sample was incubated in the dark for 15 minutes with a 50µl Annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂ in PBS, pH 7.4) solution containing 2µl Annexin V conjugate dye (A13201, ThermoFisher) and 1µl propidium iodide (PI) (100 µg/ml, P1304MP, ThermoFisher). Following incubation, the cells were washed twice with Annexin binding buffer and then fixed for 15 minutes in 4% PFA containing 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂. The cells were rinsed twice with Annexin binding buffer again and then incubated with DAPI (Invitrogen, USA) at a 1:1000 dilution with 1% goat serum for 20 minutes. After two additional washes

with Annexin binding buffer, the cells were mounted onto the glass coverslip with the mounting solution (3:7 PBS to Fluoromount-G (Invitrogen) ratio) for observation.

The samples were captured using a Zeiss immunofluorescence microscope at a 20x magnification with z-stack imaging at approximately 2-5 μm intervals, starting from the basal plane to the apical plane of the cells. Image analysis was carried out using Fiji (ImageJ), and each channel image was merged using the z-project option with average intensity projection. Further adjustments in brightness and contrast were made using Fiji software for further analysis.

In the Fiji software, cell quantification was conducted using the original images. The cell population was determined by manually configuring the threshold to eliminate the background from different channels, and the stained cells were measured using either the "analyze particle" option or manual counting if there were overlapping cell signals.

3.2.4.2 Cytoskeletal immunofluorescence staining assay and quantification

The cells were washed three times with 1x PBS, each rinse lasting 1 minute. Following this, they were fixed with 4% PFA for 15 minutes. Subsequently, the cells underwent permeabilization in a 0.1% Triton-X (Sigma) solution in 1x PBS for 1 hour at room temperature. This was followed by overnight blocking with 1% BSA and 10% goat serum in PBS at 4°C.

After the blocking step, the cells were incubated with Alpha-smooth muscle actin (αSMA) Mouse monoclonal primary antibody (1:200, ab7817, Abcam) in 1% goat serum in PBS overnight at 4°C. The samples were washed with a washing medium (composed of 0.05% Triton-X and 1% goat serum in 1x PBS) to reduce background interference. For the secondary antibody, the cells were incubated with the anti-mouse 546 conjugate (1:750, Invitrogen) for 1 hour at room temperature in the dark. Subsequently, cells were treated with DAPI (1:1000, Invitrogen) and phalloidin (1:500, Invitrogen) for 20 minutes before two final washes with washing medium to prepare for immunofluorescence (IF) microscopy.

The samples were imaged using a Zeiss immunofluorescence microscope at 20x and 40x magnification, with z-stack imaging capturing intervals of 2-5 μm , starting from the basal to the apical plane. Image analysis was conducted using Fiji (ImageJ), and all images in a single channel were merged using the z-project option with average intensity projection. Further adjustments in brightness and contrast were performed using this software.

3.2.5 Cell location quantification

In Fiji software, cell quantification was carried out using the original images. As previously mentioned, the images were stacked using the z-project option with average intensity projection. The threshold was manually tuned for the stacked channels related to PI, Annexin, and α SMA to eliminate background interference. After merging the staining channels with the brightfield image displaying guttata topography patterns, two distinct assessments were performed. Firstly, cells with positive signals contacting the s-guttatas' boundary were manually counted and analyzed. The positive signals dispersed between the s-guttata were also manually counted and examined independently.

3.2.6 Statistical analysis for FECD staining samples

To assess statistical significance, a two-way ANOVA with Tukey's multiple comparisons was applied across all the samples; all the samples were compared with the control and each other. Every experiment had at least three biological replicates ($n > 3$), with two technical replicates within each experiment. In each biological replicate, a minimum of two images per technical replicate were analyzed, and each image comprised a minimum of 300 cells. The threshold for statistical significance was set at $p \leq 0.05$, and the data are presented as means $\pm$ standard deviation (SD).

3.2.7 Semi-quantitative PCR

Quantifications of the two EndMT inducing genes Zinc finger E-box-binding homeobox 1 (*ZEB1*) and Zinc finger protein *SNAIL* (*SNAIL*) gene expression were done with RT-qPCR, using StepOne™ Real-Time PCR System (4379216 Applied Biosystems™).

Forward and reverse primer sequences were referenced from Lee *et al.* [36] and designed as custom RxnReady™ Primer Pool Integrated DNA Technologies (IDT) for *SNAIL* (forward: 5'-AAGATGCACATCCGAAGCCAC-3', reverse: 5'-GGGCACTCAGGAGGGAATTCC-3'), *ZEB1* (forward: 5'-TTAGTTGCTCCCTGTGCAGTTA-3', reverse: 5'-TGTGTGAGCTATAGGAGCCAGA-3'). The primer of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward: 5'-CTGACTTCAACAGCGACACC-3', reverse: 5'-AGGGGTCTACATGGCAACTG-3') has been selected as the housekeeping gene expressions for B4G12 CEnCs samples.

Blank and 20x20x20 samples were seeded with B4G12 in 100,000 cells/cm² for seven days, along with a negative control seeded directly onto a tissue culture well plate with the same cell density and culture

time. To ensure enough yield of the RNA for the subsequent cDNA synthesis, every RNA sample collected was pooled from two specimens.

RNA isolation was conducted using the RNeasy® Mini Kit (74104 QIAGEN), referring to the manufacturer's instructions. Subsequently, cDNA strands were synthesized utilizing the iscript™ cDNA Synthesis Kit (1708890 Bio-Rad), following the manufacturer's protocol. Finally, the amplification and semi-quantification of specific gene regions were performed using PowerTrack™ SYBR™ Green® Master Mix (A46111, applied biosystems), according to the manufacturer's instructions, with the exception that the cDNA stock concentration was used at 100 ng/μL (10ng/ μL for each well). Each measurement was conducted in triplicate for every experimental group.

To assess statistical significance, a two-way ANOVA with Tukey's multiple comparisons was applied across all the samples; all the samples were compared with the control and each other. N.S. denotes that there is no significant difference. The threshold for statistical significance was set at $p \leq 0.05$, and the data are presented as means $\pm$ standard deviation (SD). Data were presented with a mean $\pm$ standard deviation of the triplicate in a single trial run.

3.3 Results

3.3.1 Fabrication of guttatas with different dimensions

By employing a method well established in previous literature [1], the biomimicking synthetic guttata model with varying dimensions was fabricated on PS using a heat imprinting technique. In this method, 2cm x 2cm polystyrene was heat-embossed with a patterned PDMS mold to generate surface patterns (**Figure 2**). To accurately recapitulate guttata structures *in vivo* during the different stages of FECD, all the spacing was set to be larger than 20 microns. The height of the guttata was set as 10 and 20 (more advanced FECD) microns, with the diameter of the guttata set into 20 and 40 μm (**Figure 2**).

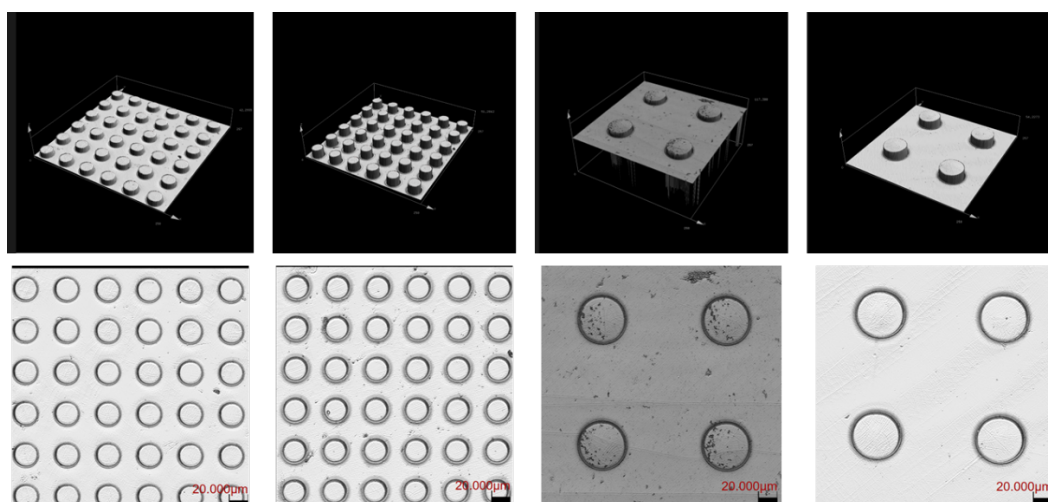


Figure 2. Representative images of patterns on TCPS with Diameter x Side to Side spacing x Height. Scale bar: 20 µm. (50X)

3.3.2 FECD cells show early-stage apoptosis on guttata

It is known that the FECD-inflicted CEnCs can become apoptotic. Previously, lab members had hypothesized that guttatas played a role in cell apoptosis. The experimental results suggested that by utilizing immortalized normal CEnC line B4G12 under a cell density of 300,000/cm², the s-guttata model of different dimensions can induce early-stage apoptosis after 24 hours and 48 hours of seeding. This phenomenon was most significant in the 20-micron height samples, and the 20x20x20 samples showed the highest apoptotic rate amongst all the s-guttata and unpatterned samples on day 2 [105]. However, the effect of FECD cell molecular interactions and cell pathophysiology on the guttata microenvironment remained unclear.

Table 6 Annexin V+ and PI+ cell population on different s-guttata patterns on Day 1 and Day 2 from previous studies [105]

Day 1	UP	20x20x10	20x20x20	40x80x10	40x80x20
Annexin V+ (%)	4.70%	4.20%	5.30%	4.90%	5.90%
PI+ (%)	1.50%	2.00%	1.50%	1.60%	1.60%
Day 2					
Annexin V+ (%)	1.80%	3.04%	4.27%	3.83%	3.48%
PI+ (%)	0.45%	1.07%	1.22%	1.21%	1.42%

To further explore the FECD mechanism and how a diseased cell would interact with the guttata, an immortalized FECD CEnC line, FECD-SV-54F-73, has been utilized and tested on the s-guttata substrates. The cell apoptosis was analyzed through Annexin V/PI staining after 24 hours of seeding (**Figure 3**). After 24 hours, the expression level of Annexin V was higher in all the patterned samples (except the pattern 40x80x20 was not significant, $p = 0.0769$). The Annexin V⁺ signal also appeared to be most significant in the 20x20x20 sample, which aligned with B4G12 cell results. The 20x20x20 sample also demonstrated a significantly higher Annexin V level compared to the 20x20 sample with 10-micron heights, implying that the height plays an important role in affecting the apoptotic rate for the small diameter samples (**Figure 3B**). The 40x80x10 vs. 40x80x20, however, appeared to be not significantly different in Annexin -V levels.

On the other hand, the PI⁺ signals, however, were not significantly different in most patterned samples compared to the unpatterned control. Only 20x20x20 has a slightly higher PI⁺ signal when compared to the control. Generally, the first 24-hour results suggest that at a 300,000/cm² cell seeding density, s-guttata can induce early-stage apoptosis in FECD cells (**Figure 3B**).

To analyze the effect of s-guttata on the cell apoptosis mechanism, the specific location of the apoptosis relative to the guttata was also analyzed (**Figure 3C**). After 24 hours, patterns with smaller diameter and spacing (20x20x20, 20x20x10) have significantly higher Annexin V positive signal touching the guttata patterns, with $87.69 \pm 5.74\%$ of Annexin V positive cells in the 20x20x10 sample, and $81.42 \pm 7.85\%$ in 20x20x20 sample. Most of the PI⁺ cell population in both of these samples were also located around the guttata patterns. On the other hand, for 40x80x10 and 40x80x20 samples, there were significantly fewer Annexin V and PI-positive cell populations adjacent to guttata. For the 40x80x10 pattern, only $20.67 \pm 11.03\%$ of Annexin V positive cell population were found to be around the pillars, and there were $24.77 \pm 14.68\%$ guttata touching Annexin V positive signal for the 40x80x20 sample. Similarly, there was less PI-positive cell population surrounding the guttata in 40x80x10 and 40x80x20 patterns (**Figure 3C**).

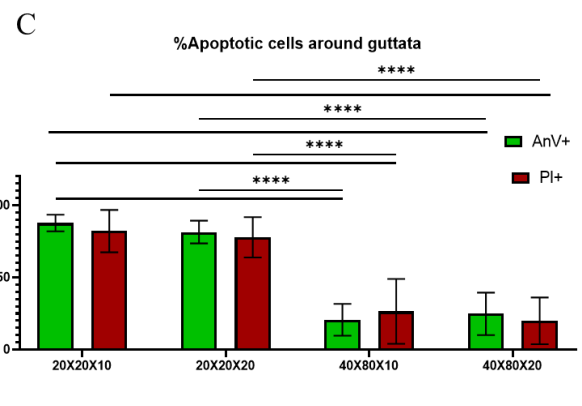
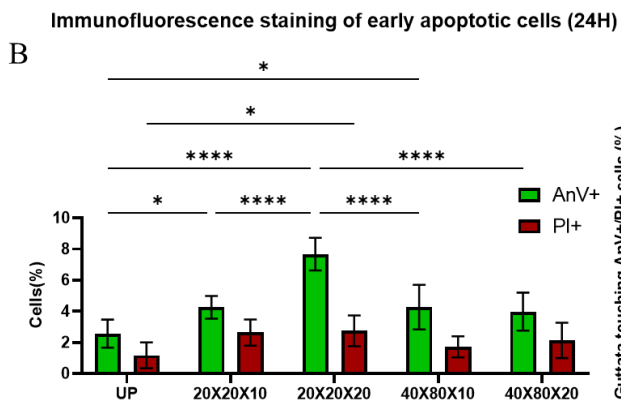
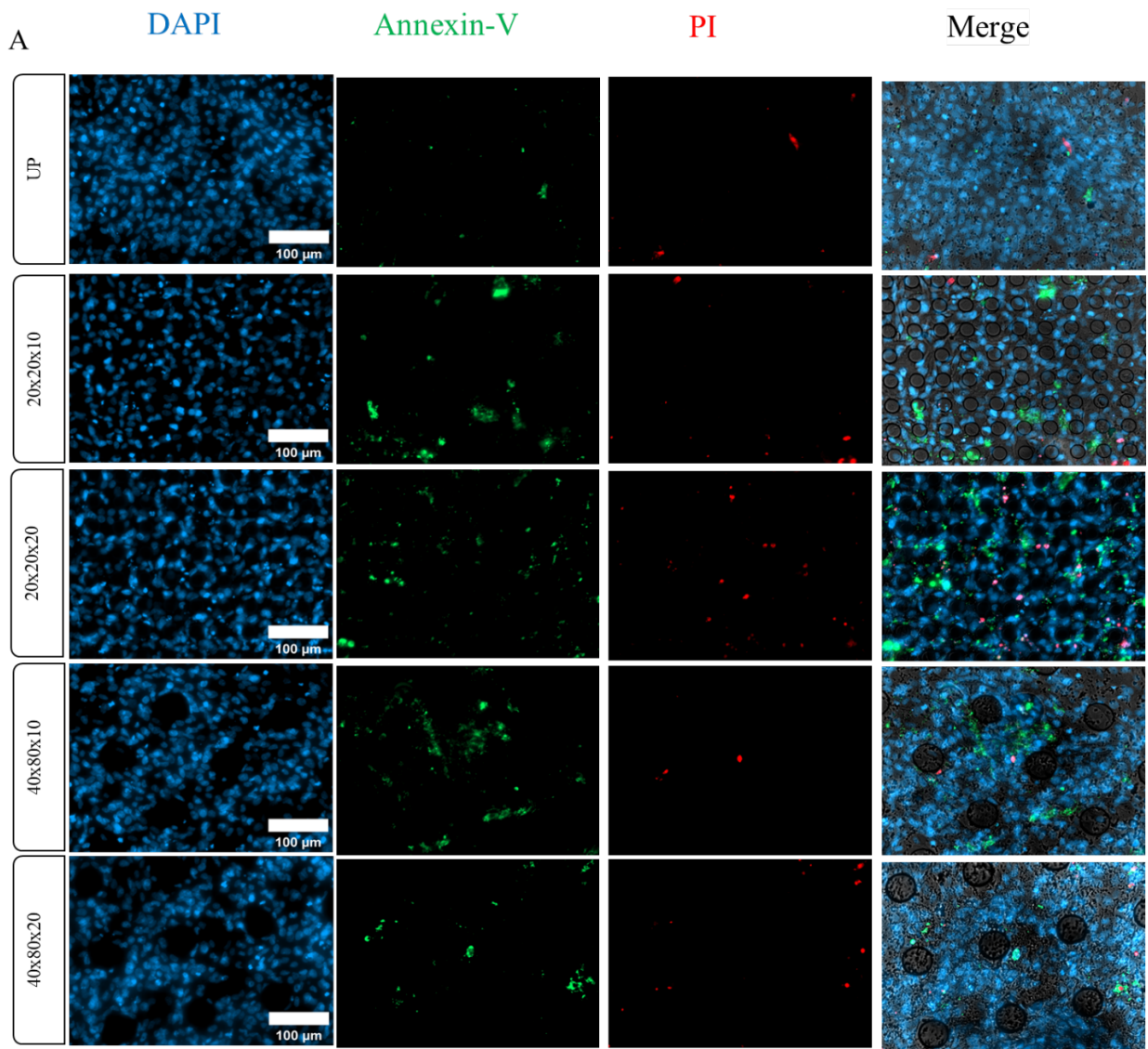


Figure 3 (on page 49). FECD 54F on s-guttata shows higher early-stage apoptotic marker expression on Day 1 at 300,000/cm² seeding density (n = 3) **A.** Representative IF images of Annexin V+ and PI+ staining of CEnCs seeded on s-guttata substrates. Scale bar: 100μm, 20X (after modifying the threshold) **B.** Bar graph representing Annexin V + and PI + cell population on different s-guttata patterns. **C.** Bar graph representing Annexin V + and PI + cell population that is localized around guttata. (*P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.001; *****P < 0.0001)

3.3.3 Cell cytoskeletal contractility around the guttata

From the previous section, the direct apoptotic phenomenon was observed on the FECD cells with the biomimicking synthetic guttata model of varying dimensions. More importantly, in 20x20x10 and 20x20x20 samples, the Annexin V and PI-positive cells mostly surrounded the guttata, implying a potential mechanism between the guttata toxic microenvironment and FECD 54F cells. Apart from the excessive apoptosis event observed in the FECD, one of the proposed phenomena is that the cells may have a higher cytoskeleton stress around guttata, which can be detected by increased expression of several cytoskeleton markers. However, the effect of FECD cell molecular interactions and cell pathophysiology on the guttata microenvironment remained unclear. Previously, lab members had hypothesized that guttatas might contribute to the upregulation of the cytoskeleton contractility in normal CEnCs (**Table 7**). The experimental results indicated that in the B4G12 cells seeded s-guttata sample, the cytoskeleton stress was increased around the guttata on day 2 [105].

Alpha-smooth muscle actin (α SMA) has been recognized as one of the EndMT-inducing gene expressions and the markers of cellular dysfunction in FECD [31, 32, 106]. Filamentous actin (F-actin) has also been used to detect phenotypical changes in the FECD *in vitro* cell and *ex vivo* specimens [24]. In this section, α SMA and F-actin were chosen as the visualization for the cytoskeletal stress marker. The α SMA positive signals in each sample were further quantified by setting up a threshold and cell counting (**Figure 4A**).

Table 7 α SMA+ cell population on different s-guttata patterns on Day 2 from previous studies [105]

αSMA+ total	UP	20x20x10	20x20x20	40x80x10	40x80x20
α SMA+ (%)	16.6%	18.9%	44.0%	50.9%	39.6%
αSMA+ cell localized around the guttata					
α SMA+ (%)	N/A	61.0%	73.9%	32.9%	74.4%

After being seeded on an s-guttata substrate for 24 hours, patterns with 20 microns guttata height (20x20x20, 40x80x20) both showed a statically higher α SMA signal compared to the unpatterned control (**Figure 4B**). $9.61 \pm 1.58\%$ of the cells in the 20x20x20 sample were α SMA+ cells, and $91.02 \pm 4.14\%$ of these positive signals were localized around the 20-micron guttata. As for the 40x80x20 sample, while the α SMA+ cells accounted for $7.197 \pm 1.355\%$ of the total population, the positive signals around the guttata were only at $45.39 \pm 17.13\%$ of the total positive signals. The 20x20x10 sample showed a similar amount of α SMA+ cells ($4.59 \pm 1.23\%$) when compared to the unpatterned ($3.92 \pm 0.86\%$); nonetheless, most of the positive cells on 20x20x10 were still adjacent to the guttata ($86.24 \pm 6.14\%$), unlike their counterparts that have higher diameters and spacings (40x80 samples). It is worth noting that in the 10 μ m height samples, the F-actin signal was observable on top of the guttata structure, while in the 20 μ m samples, the F-actin signal was only detected on the basal plane (**Figure 4C**).

Under high magnification (63X), cells on the unpatterned blank sample substrate had more cells with observable long actin fibres and a relatively lower intensity of α SMA (**Fig 5**). On 20x20x20 patterns, cells surrounding s-guttata showed stronger α SMA expression, with α SMA curved and wrapped around the guttata, indicating a contractile phenotype. There were also cells expressing the higher intensity of the F-actin around the guttata. Interestingly, in the 20x20x10 sample, the F-actin structure was mostly undisrupted. At the same time, in the other s-guttata patterns, especially in the higher pillar patterns, the F-actin appeared to be disrupted around the guttata. Meanwhile, the cells that were not directly in contact with guttata had relatively uniform α SMA and F-actin expression. The data suggest that cells surrounding s-guttas can exhibit higher cytoskeletal stress and contractility.

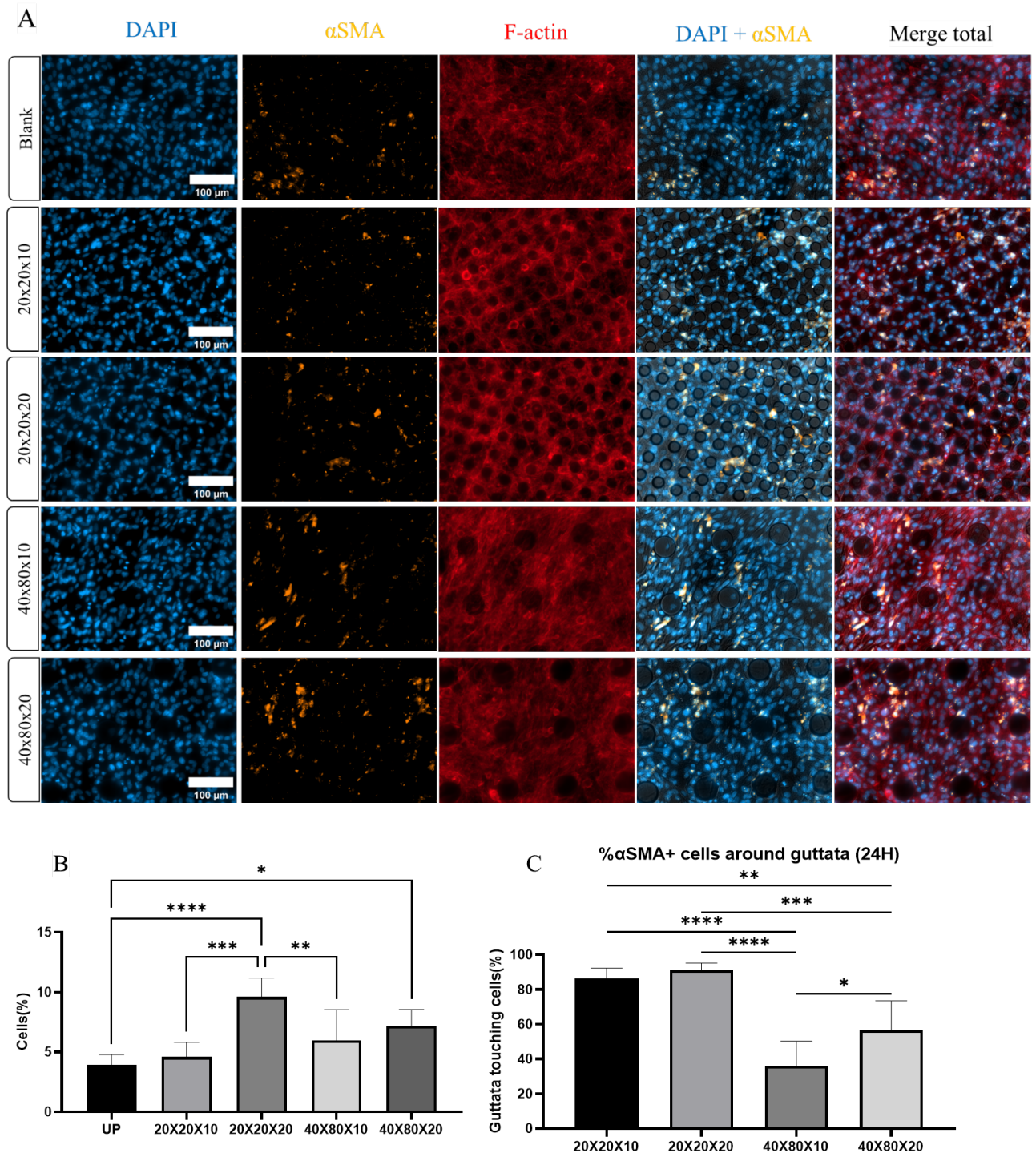


Figure 4. FECD 54F on s-guttata shows higher cytoskeleton expression at 300,000/cm² seeding density (n = 3) **A.** Representative IF images of α SMA and F-actin staining of CENCs seeded on s-guttata

substrates. Scale bar: 100 μ m, 20X (after modifying the threshold) **B.** Bar graph representing α SMA+ cell population on different s-guttata patterns **C.** Bar graph representing α SMA+ population that is localized around guttata. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001)

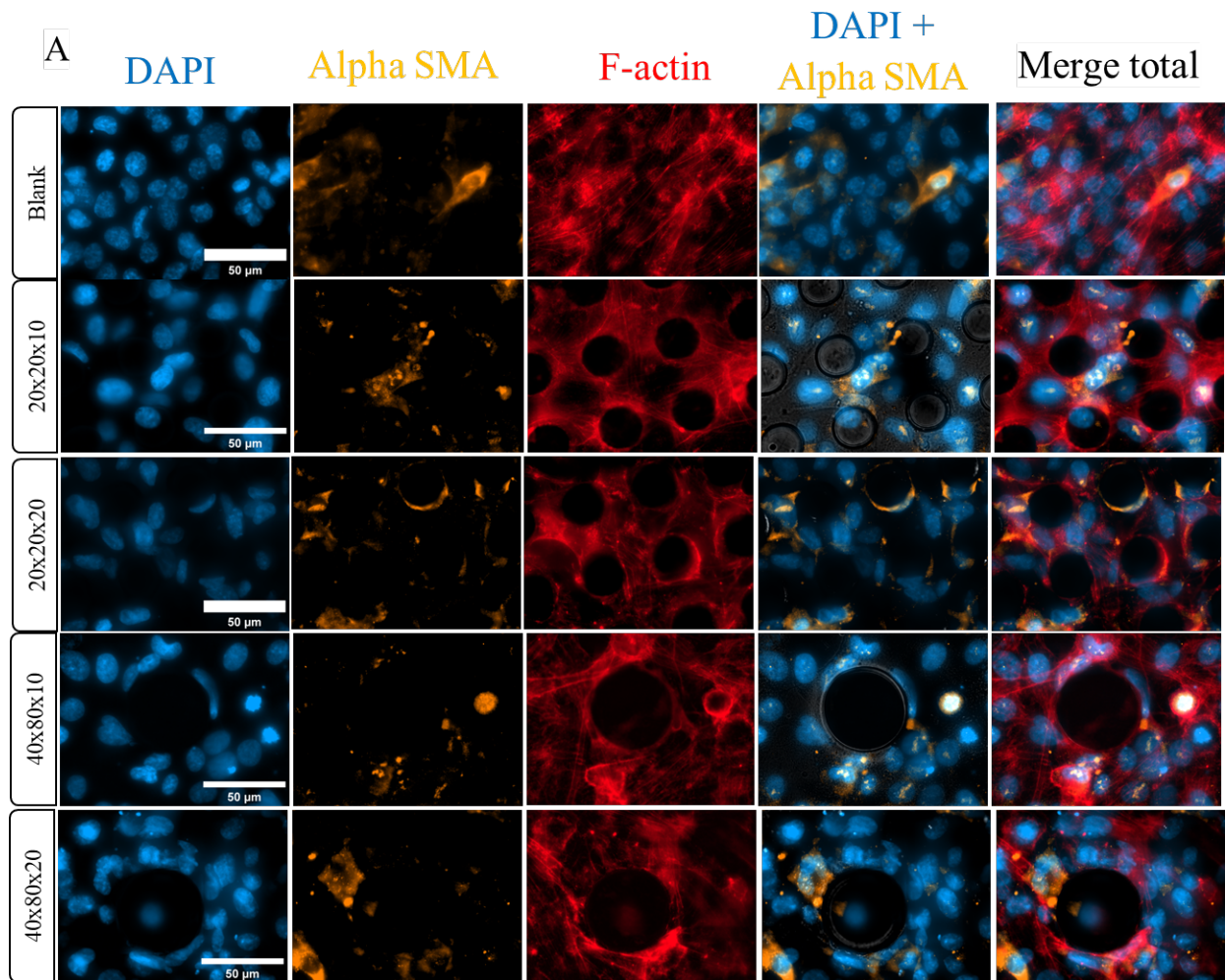


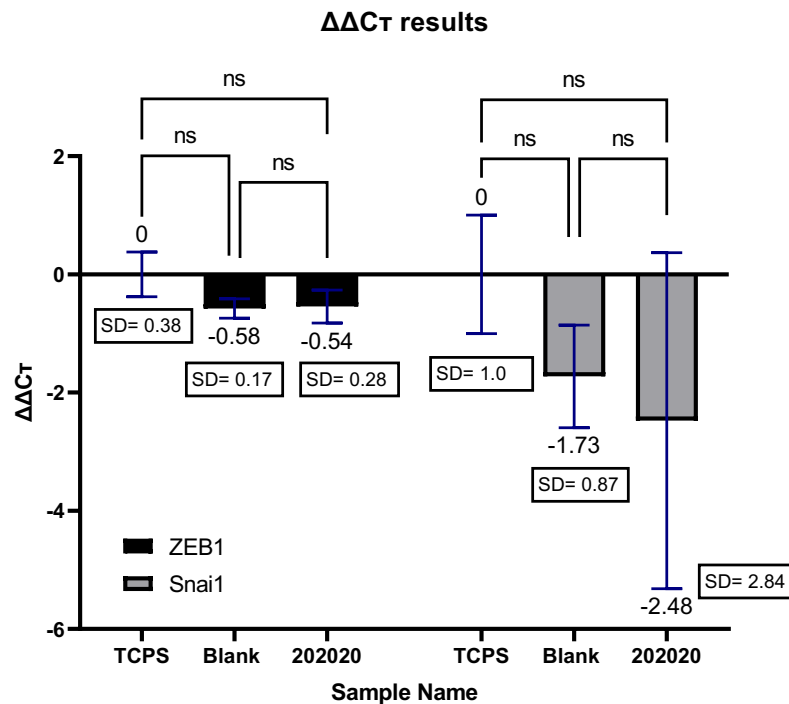
Figure 5 FECD 54F on s-guttata show higher cytoskeleton expression at 300,000/cm² seeding density (63X oil lens)

3.3.4 Normal B4G12 Cells did not show significant upregulation of EndMT expressions on s-guttata

To further demonstrate the cell cytoskeletal alternations and their connection with the EndMT observed in FECD, as shown by previous studies [31, 32], trial experiments were conducted using semi-quantitative PCR. EndMT-inducing genes *SNAIL* and *ZEB1* were chosen as the test sequences. The

unpatterned and s-guttata samples 20x20x20 were selected as the test samples, and the B4G12 cell line was used. The cells were directly seeded on a 24-well tissue culture plate as the negative control, and the GAPDH was set as the housekeeping gene.

After seven days of cell culture on the TCPS, unpatterned and patterned 20x20x20 substrate, the samples went through RNA isolation, cDNA synthesis, and the qPCR, as mentioned in section 3.2.7. As shown in **Figure. 6**, the mean relative fold expression of *ZEB1* expression was slightly higher in the unpatterned and 20x20x20 samples, but they are not statistically significant (ns $P > 0.05$). On the other hand, due to the high variability (STD) amongst the *Snai1* test wells, the difference between the results was not significant even though the mean relative fold expression of the unpatterned and 20x20x20 sample was higher than the TCPS negative control.



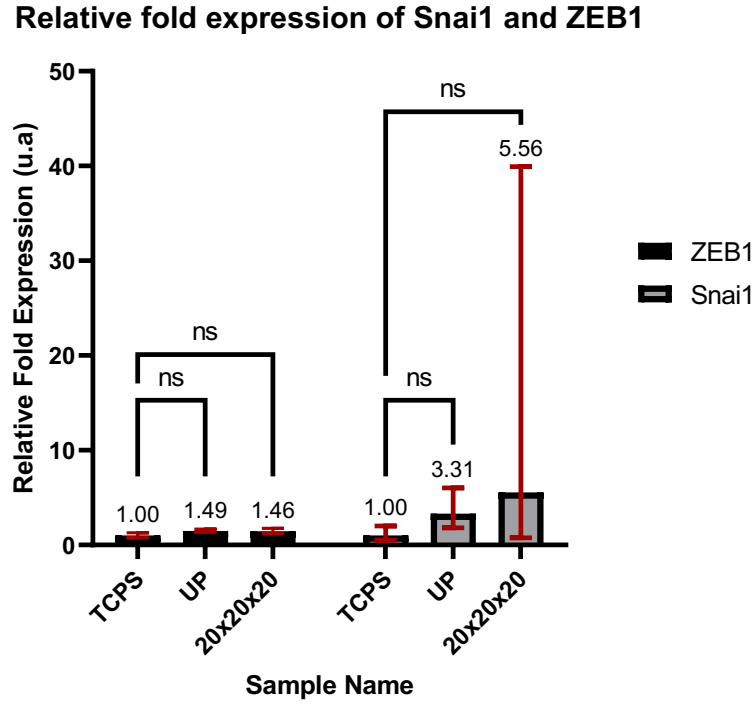


Figure 6. Relative fold expression and $\Delta\Delta C_T$ results of *SNAIL* and *ZEB1* in B4G12 on s-guttata. The STD values in the relative fold expression was calculated as upper limit and lower limit from the $\Delta\Delta C_T$ results. Both *SNAIL* and *ZEB1* expressions did not show significant upregulation. Both upper limit and lower limit are listed in the Appendix A. (ns $P > 0.05$)

3.4 Discussion and conclusion

The fabrication of s-guttata using the PDMS is a well-established soft lithography and heat imprinting process used extensively in previous studies [30, 82]. The sample guttata in micron sizes can be easily replicated using a hot embossing procedure. The replicated polystyrene substrates are consistent with its PDMS master molds. It is worth noting that the polystyrene substrates may not be a full representation of the guttata microenvironment *in vivo*. For instance, Young's modulus of native DM (varied between 50 kPa and 2.57 MPa due to differences in testing methods [6]) differs from the PS (3GPa [107]), and the cells' biophysical response to the PS might differ from the native FECD DM.

CEnCs are derived from the embryonic neural crest and are arrested in the terminally differentiated stage; thus, it has been proposed that the FECD may be similar to neurodegenerative diseases, which

are prone to oxidative stress, reactive oxygen species (ROS), and subsequent excessive apoptosis [108, 109]. While the FECD CEnCs could proliferate and even form similar phenotypes to normal CEnCs and generate cell-cell tight junctions *in vitro* [24], there are still distinctive differences in FECD cells [24, 32, 35, 81]. By utilizing the s-guttata with an immortalized FECD cell line, the role of the guttata microenvironment in the FECD cell dysfunction, including excessive apoptosis, EndMT, and senescence, can be further understood. In addition, utilizing an FECD cell line on s-guttata substrates allows us to provide a closer representation of an advanced stage FECD *in vitro*.

A varying level of the early apoptotic phenomenon was observed by utilizing the biomimicking guttata *in vitro* model with the FECD 54F cell line. The Annexin -V/PI staining demonstrated that the sample 20x20x20 had the highest level of early-stage apoptosis, almost 2.5 times higher than the unpatterned control. In addition, almost all the patterned samples showed apoptotic rates higher than the control. The samples with smaller diameters and spacings (20x20 samples) also showed a significantly higher portion of cells localized around the guttata, demonstrating a strong correlation between apoptosis and the guttata. However, in the 40x80 samples, the apoptosis patterns observed had a lower correlation to the guttata structure. Compared to the past experiments conducted with normal B4G12 cell line [105], the FECD 54F cells seeded on s-guttata samples displayed a higher level of early apoptosis on day 1, significantly different from the unpatterned control. This observation suggests that the FECD cells were more susceptible to microenvironment toxicity, leading to higher apoptosis levels after short seeding.

In general, the apoptosis pathway is a complex mechanism that involves both extrinsic and intrinsic pathways [9]. More experiments may need to be conducted to study the underlying mechanism of early-stage apoptosis in the diseased FECD cells.

Apart from the excessive apoptosis observed in FECD, the pathophysiological events that have been widely observed in the FECD are the phenotypical changes of the FECD cells and EndMT processes [9]. Several markers and gene expressions have been utilized to study these cell dysfunctional events. Previous study has demonstrated that the gene expression level of *α SMA*, *N-cadherin*, and *SNAIL* were significantly upregulated when the immortalized healthy CEnC line HCEC-21T were seeded and cultured on a decellularized FECD *ex vivo* tissue with large guttae [31]. On the other hand, the expression level of *ZEB1* and *SNAIL* was also significantly upregulated in the immortalized FECD CEnCs [32]. Furthermore, researchers have also observed the fibroblastic morphology of the FECD CEnCs [24]. However, a model that can further study the combinatorial effect of abnormal FECD cells

and toxic microenvironments has not been developed yet. To further visualize the cytoskeletal stress and fibrogenesis of the FECD, α SMA and F-actin were chosen as the markers. It has been shown that all the patterned samples had a relatively higher number of strong α SMA expressing cells (α SMA+) except the 10 μ m height sample; this implies that the guttata height may be the significant factor affecting the FECD cells' cytoskeletal response. The 20x20x20 sample has shown the highest number of α SMA+ cells, which is also the sample pattern with the highest early apoptosis rate. In addition, both samples with 20 μ m diameter and 20 μ m spacings have most of the α SMA+ cells surrounding the guttatas.

On the other hand, the F-actin signals in the s-guttata sample also showed a more fibroblastic morphology. In conclusion, a higher level of cytoskeleton stress was observed under the s-guttata patterns with higher pillars, and the morphology of the cells was also affected by the toxic microenvironment. The result implies that the guttata height may be one of the major factors affecting the EndMT process and morphological changes of FECD cells.

When comparing the cytoskeleton response of FECD 54F (after 24 hours) with B4G12 cells on s-guttata after 48 hours, although the population of α SMA+ cells was significantly higher in the B4G12 sample, a similar trend was observed [105]. These population differences between the two cell lines could be due to the extended cell culture time (48 hours instead of 24 hours). It is also possible that the past member used a different threshold when analyzing the B4G12 samples. The B4G12 α SMA+ cells % on pattern 40x80x10, however, was significantly higher when compared with the FECD 54F sample with the same pattern [105].

As mentioned previously, *ZEB1* and *SNAIL* were used as the marks of EndMT in FECD cells [32] [35]. In the semi-quantitative PCR experiment, the *ZEB1* relative fold expression was not significant between samples; this may have resulted from the relatively short period (7 days) of seeding for an immortalized normal cell line to go through significant EndMT process and having a statically significant difference compared to negative control. On the other hand, the relative fold expression of the *SNAIL* has a huge variability in the same samples; this may be due to the two peaks on the *SNAIL* melt curve, meaning there were two amplifications in all the samples, making the results inaccurate. Further experiments and more replications are required for further analysis. FECD 54F diseased cell line can also be included in the gene expression experiment for more comparison studies.

Chapter 4

Human corneal endothelial cell migration and wound healing, topographical pattern fabrications on non-planar surface

4.1 Introduction

The cornea is a transparent tissue with a horizontal and vertical diameter of 11.5 mm and 10.5 mm in the average adult human eye [110], respectively. It is an aspheric optical system with a relatively flat periphery and steeper center region. Amongst 36 normal eyes, the average anterior radius of curvature is 7.82 mm [111, 112], and the average posterior radius of curvature is approximately 6.15 mm [112].

In this chapter, to further study the FECD and normal CEnCs wound closure and cell migration behavior, the experiment was performed on both flat substrates and concave surfaces fabricated from contact lens mold. PDMS (Polydimethylsiloxane) was used to replicate a concave surface from the contact lens to prepare the substrates for cell seeding. A crosslinked biocompatible hydrogel GelMA+ was also fabricated using a contact lens master mold and was used as a seeding substrate for both CEnC lines. The mechanical properties of each material utilized as the seeding substrates were also compared and discussed in the conclusion section, along with a comparison to the native Descemet's membrane (DM).

PDMS elastomer has been widely used in a broad range of biomedical applications due to its biocompatibility, transparency, and strong mechanical properties [113]. Therefore, PDMS was initially employed on the concave surface for the CEnC wound healing experiment. More importantly, as the methods of controlling the cell fate with micro or nano-topographical cues are gaining more interest in biomaterials study, PDMS has been extensively employed in the field using soft lithography techniques [30, 82, 113].

Photolithography is another method extensively utilized in micro-or nano-topographical patterning [113]. Maskless photolithography was ultimately chosen as a micropatterning tool to generate topographical patterns on a flat substrate (silicon wafer). Compared to masked lithography, maskless lithography is proven to be ideal for smaller batch experiments with a relatively lower cost and difficulty [114]. To investigate whether cell behavior can be influenced and controlled by micro-topographical cues on the non-planar surface, a series of fabrication methods that involve maskless lithography and PDMS micro-molding were employed.

Gelatin methacrylate (GelMA) is a bioactive hydrogel material that started gaining interest in tissue engineering and biomedical engineering due to its good compatibility, decent mechanical properties, and microfabrication feasibility [115]. Apart from tissue engineering applications, single-cell studies, drug and gene delivery, and bio-sensing experiments can also be performed via GelMA [115]. However, regular GelMA lacks tunable biodegradability and mechanical properties; therefore, previous members have developed an enhanced GelMA+ material to improve these properties [82]. By applying a hybrid (sequential physical and then UV) crosslinking method, GelMA+ has improved its mechanical strength compared to regular GelMA. The compressive modulus of the 30% GelMA+ is at around 233.3kPa, which is known to be 4.1 times higher than the GelMA made with the same concentration. Similarly, the 10% GelMA is 4.9-fold lower than the 10% GelMA+.

Apart from the GelMA+'s great mechanical strength and biocompatibility, CEnCs were assumed to adhere more easily onto the GelMA+ hydrogel since it contains cell adhesion ligands [82], making it a suitable substrate for corneal endothelium cell-related experiments *in vitro*. Furthermore, GelMA+ is also a transparent material, making it easy for cell imaging.

4.2 Materials and methods

4.2.1 Cell culture

FECD 54F cells and B4G12 cells were cultured, as mentioned in section 2.2.1.

4.2.2 TCPS, PDMS, and GelMA+ cell migration & wound healing assay

4.2.2.1 Preparation of PDMS films and for cell blocking

PDMS (Polydimethylsiloxane, Sylgard, Dow, USA) solution was prepared with a 10:1.05 base to curing agent ratio. After thorough mixing and degassing, PDMS solution was applied and spin-coated onto a glass slide at 500 rpm for 30 seconds to reach 200 microns thickness. Subsequently, PDMS was crosslinked for 4 hours at 60 °C. After cross-linking, the PDMS film was removed from the glass slides. To prevent cell attachment on flat surfaces, the PDMS strips were prepared approximately 1 mm wide and 5 mm long in size. For the PDMS blocks applied to concave surfaces, the films were cut into 1-mm and 2-mm diameter circles. The strips and circular blocks were then utilized to block cell attachment on flat TCPS, concave PDMS, and concave GelMA+ samples.

4.2.2.2 TCPS flat surface cell seeding and wound healing assay

FECD 54F cells and B4G12 cells were cultured, as mentioned in section 2.2.1. After reaching 80% confluency, cells were collected from the T25 cell culture plate and directly seeded on a 24-well Cell Culture Plate (VWR® Multiwell). PDMS strips were sterilized by autoclave and placed at the center of the TCPS plate to block cell attachment. TCPS plates and PDMS strip samples were sterilized under UV light in a biosafety cabinet for an hour. The samples were coated with an FNC coating mix (Athena ES, AES0407, Baltimore, USA) for at least 5 mins. Cells were seeded directly on the samples at 200,000 cells/cm² or 300,000 cells/cm². Cells were cultured for 24h or until forming a monolayer.

Subsequently, PDMS strips were gently lifted from the TCPS samples, and samples were washed with media once to remove any unattached cells. Time-lapse images of the cell migration were captured every 2 hours using a microscope (Zeiss) until the wound was healed. After cell imaging, the samples were cultured for several days to allow additional observation.

4.2.2.3 PDMS concave lens & flat surface cell seeding & wound healing

Similarly, FECD 54F cells and B4G12 cells were cultured, as mentioned in section 2.2.1. The PDMS (Polydimethylsiloxane) solution was first prepared with a 10:1.05 base to curing agent ratio. A concave PDMS was then prepared by pouring PDMS into a container and placing the original contact lens convex side facing the PDMS solution shown in **Figure 7**. After detaching from the lens master mold (**Figure 8a**), the concave PDMS lens was washed with 100% ethanol and air dried completely. Subsequently, PDMS samples and blocks were plasma treated for 120s at 80% O₂ gas and 70 Watts (Zepto, Diener, Germany). DI water was immediately added to plasma treated PDMS to maintain the hydrophilicity, and PDMS samples were sterilized for an hour under the UV light in a biosafety cabinet. Before seeding, the samples were washed with 70% ethanol and washed twice with PBS. PDMS circular blocks were sterilized by autoclave and placed at the center of the PDMS concave sample to block cell attachment. The samples were then coated with the FNC coating mix (Athena ES, AES0407, Baltimore, USA) for at least 5 mins. Then, cells were seeded directly on the samples at 200,000 cells/cm² or 100,000 cells/cm². Cells were cultured for 24h or until forming a monolayer.

Subsequently, PDMS circular blocks were gently lifted from the TCPS samples; samples were washed with media once to remove unattached cells. Time-lapse images of the cell migration were captured every 2 hours using a microscope (Zeiss) until the wound healed. After cell imaging, the samples were cultured for several additional days to allow additional observation.

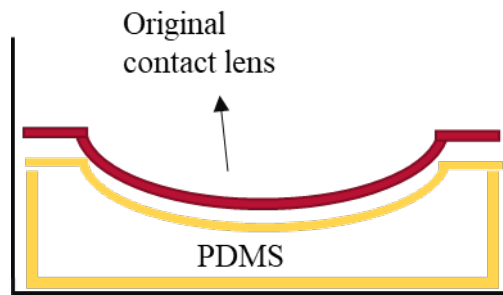


Figure 7 Concave-shaped PDMS fabrication by using the original contact lens

4.2.2.4 30% GelMA+ concave lens & flat surface cell seeding & wound healing

Gelatin methacrylate (GelMA) was produced following a two-step procedure. Initially, 10% w/v gelatin (Type A, Sigma Aldrich) was dissolved in 1x PBS at 50°C until fully dissolved. Then, 20% methacrylic anhydride (Sigma Aldrich) was slowly added to the solution at the same temperature. After the complete addition of the anhydride, the reaction continued for another hour. DI water at 40°C was introduced into the reaction and allowed to mix thoroughly for 5 minutes. The resulting mixture was poured into 50ml centrifuge tubes and centrifuged at 5000 rpm for 5 minutes at room temperature. The supernatant was transferred into 12-14kDa dialysis tubes (Sigma Aldrich) and placed in DI water for five days at 37°C. The water was changed 3-5 times daily. After five days, the GelMA solution was poured into 50ml centrifuge tubes and stored at -80°C for one day. Subsequently, this solution was lyophilized to obtain GelMA foam, stored at -80°C. The degree of methacrylation will be measured using the TNBSA assay.

To create GelMA+, the curing solvent was prepared with 1x PBS containing 0.5% w/v Irgacure 2959 (BASF, Germany) at 60°C. 30% GelMA foam was then dissolved in the curing solvent after the foam had fully dissolved. Subsequently, 100 μ L of GelMA solution was pipetted onto the concave side of the contact lens mold (**Figure 8a**) and combined with another contact lens mold, as shown in **Figure 8b**. After adding the solution, the setup was incubated for at least 1 hour at 4°C. Later, the solution was crosslinked using UV irradiation (360-480 nm) with an intensity of 32 mW/cm² (HTLD-100x100-365) for 60 seconds. The crosslinked GelMA+ hydrogels were carefully removed from the lens mold after being fully immersed in PBS and incubated in 1x PBS at room temperature. For the 30% GelMA+ lens to be ready for cell experiments, the samples were placed in a 50 mL falcon tube filled with PBS. 50 mL tubes were then placed onto the tube rotator, and the media was changed 4-5 times per day. After 2-3 days, the lens was sterilized and stored in a 20mL scintillation vial filled with PBS.

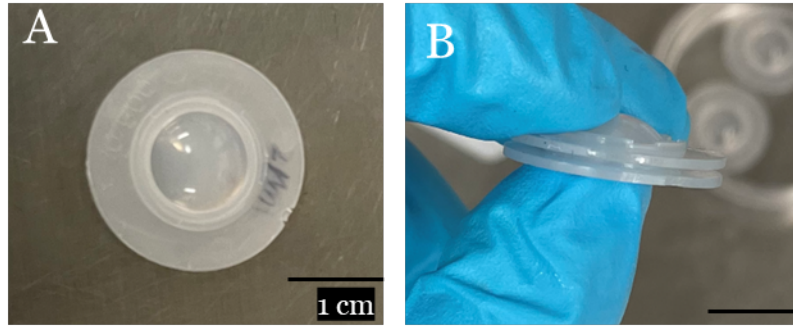


Figure 8. Image of the contact lens mold, two lens mold can be combined A. Single contact lens master mold, B. two contact lens molds coupled together forming a chamber for hydrogel molding.

The GelMA+ was then coated with the FNC coating mix (Athena ES, AES0407, Baltimore, USA) for at least 5 mins. Then, cells were seeded directly on the samples at 200,000 cells/cm² density. Cells were cultured for at least 24 hours or until forming a monolayer.

Subsequently, PDMS circular blocks were gently lifted from the samples; samples were washed with media once to remove unattached cells. Time-lapse images of the cell migration were captured every 2 hours using a microscope (Zeiss) until the wound healed. After cell imaging, the samples were cultured for several days to allow additional observation.

4.2.2.5 Cytoskeletal immunofluorescence staining assay and quantification

FECD 54F cells and B4G12 cells were stained as mentioned in section 3.2.4.2

4.2.3 Photolithography and soft-lithography fabrication on non-planar surface

4.2.3.1 Pattern design for maskless photolithography

To conduct maskless photolithography, the patterns can be designed directly in the pattern design software. Several radial and spiral-shaped topographical pattern designs were developed to prepare a proposed topography-guided model. KLayout was used as pattern designing software, and the patterns were designed by using the KLayout macro function primarily, which was developed using Python. The code was adapted from the document written by Greg Holloway (Institute for Quantum Computing, IQC) and modifications were made to the spiral and radial pattern designs (**Figure 9**) [116].

```

def produce_impl(self):

    # This is the main part of the implementation: create the layout

    # fetch the parameters convert to database units
    inner_r_dbu = self.inner_r / self.layout.dbu
    outer_r_dbu = self.outer_r / self.layout.dbu

    # compute the spiral
    pts = []
    da = math.pi * 2 / self.n
    dr = (self.width+self.spacing)/self.n/self.layout.dbu
    current_radius = inner_r_dbu
    current_angle = 0
    num_p = 0
    while current_radius < outer_r_dbu:
        pts.append(Pya.Point.from_dpoint(pya.DPoint(current_radius * math.cos(current_angle), current_radius * math.sin(current_angle))))
        current_radius += dr
        current_angle = (current_angle+da)%(math.pi*2)
        num_p += 1
    if num_p >= 8000:
        self.cell.shapes(self.l_layer).insert(pya.Path(pts,self.width/self.layout.dbu))
        num_p = 0
        pts = [pts[-1]]

    # create the shape
    self.cell.shapes(self.l_layer).insert(pya.Path(pts,self.width/self.layout.dbu))

```

Figure 9. A snapshot of the Python program that was used to generate spiral patterns, the complete code can be found at <https://gist.github.com/g2hollow> [116]

The double spiral pattern has a path length of 2-micron spacing and 2-micron width for the paths; the total radius of the spiral is around 2.5 mm, and the inner radius is 5 microns. To further enhance the entrance path of the topographic pattern, two spirals were incorporated together to form a double spiral pattern (**Figure 10**).

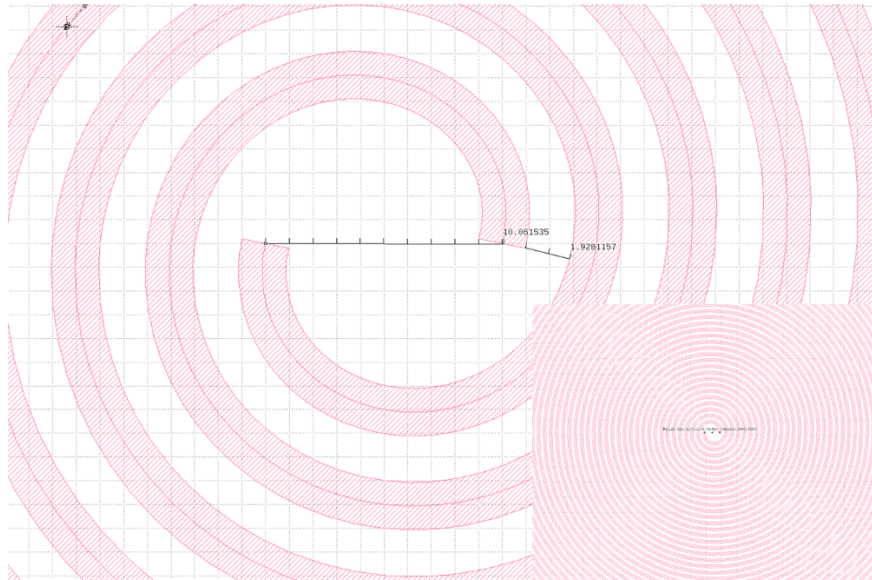


Figure 10. Center of the double spiral pattern design images taken in KLayout; the smaller image demonstrates the more zoomed-out image

Similarly, similar procedures were followed for the designing of radial patterns. A “parametric array” micro was used to generate lines of the same width in the radial topography (**Figure 11**). For the simple radial pattern, the outer radius is around 7 mm, and there were 128 lines with 2-micron width (**Figure 11a**). A denser radial pattern has also been prepared, with the same line width at 2 microns and the spacing of each layer higher at the outer radius and lower (~2 microns) at the inner radius; there were a total number of nine layers for the dense radial pattern (**Figure 11b, c, d**). A radial-spiral pattern was also designed to recapitulate and combine both the spiral and radial characteristics (**Figure 11e, f**).

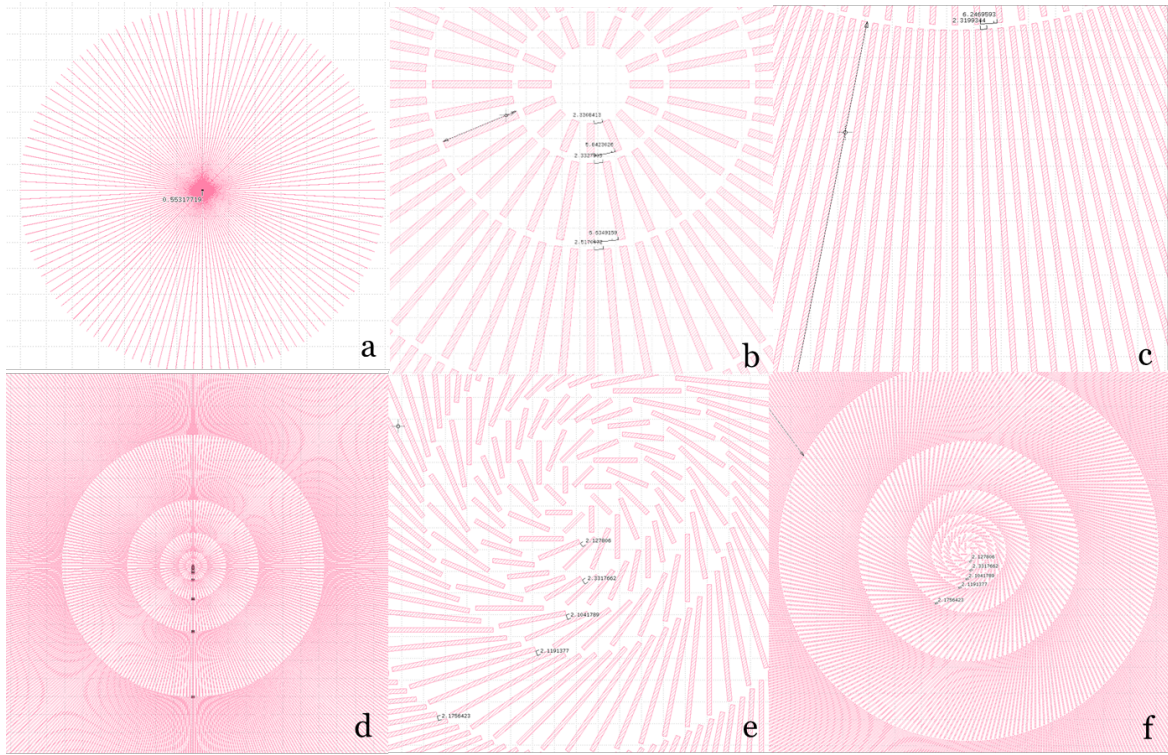


Figure 11. Images of radial pattern designs taken in KLayout. The simple radial pattern (a). Enlarged images of the denser radial (b, c) and the dense radial pattern on a bigger scale (d). Amplified (e) and zoom out (f) image of the radial-spiral pattern

4.2.3.2 Maskless photolithography

As demonstrated in schematics **Figure 6**, the silicon (Si) wafers were initially cut into 2x2 cm pieces by using a diamond cutter to prepare the maskless photolithography. The Si substrate was then cleaned

with O₂ plasma (YES-CV200RFS O₂ Plasma Asher). An HMDS priming (YES-310TA) was subsequently performed to help with the photoresist adhesion. Subsequently, Shipley S1811 positive photoresists were spin coated and soft-baked onto the Si substrate. To write the pattern onto the photoresist, the sample was placed into the MLA150 (Heidelberg) maskless photolithography system, 405 nm UV light was used for the writing, for ideal results to be produced for 2 microns line-shaped patterns on S1811, a dose of 100 mJ/cm² and a defocus were tuned to -2 in the process. Eventually, to develop the S1811 photoresist, a positive photoresist MF-319 developer was used. The Si wafer samples were then ready for the subsequent soft lithography process. The images were taken with a LEXT OLS5000 3D measuring laser microscope (Olympus, Germany) to observe the resolution.

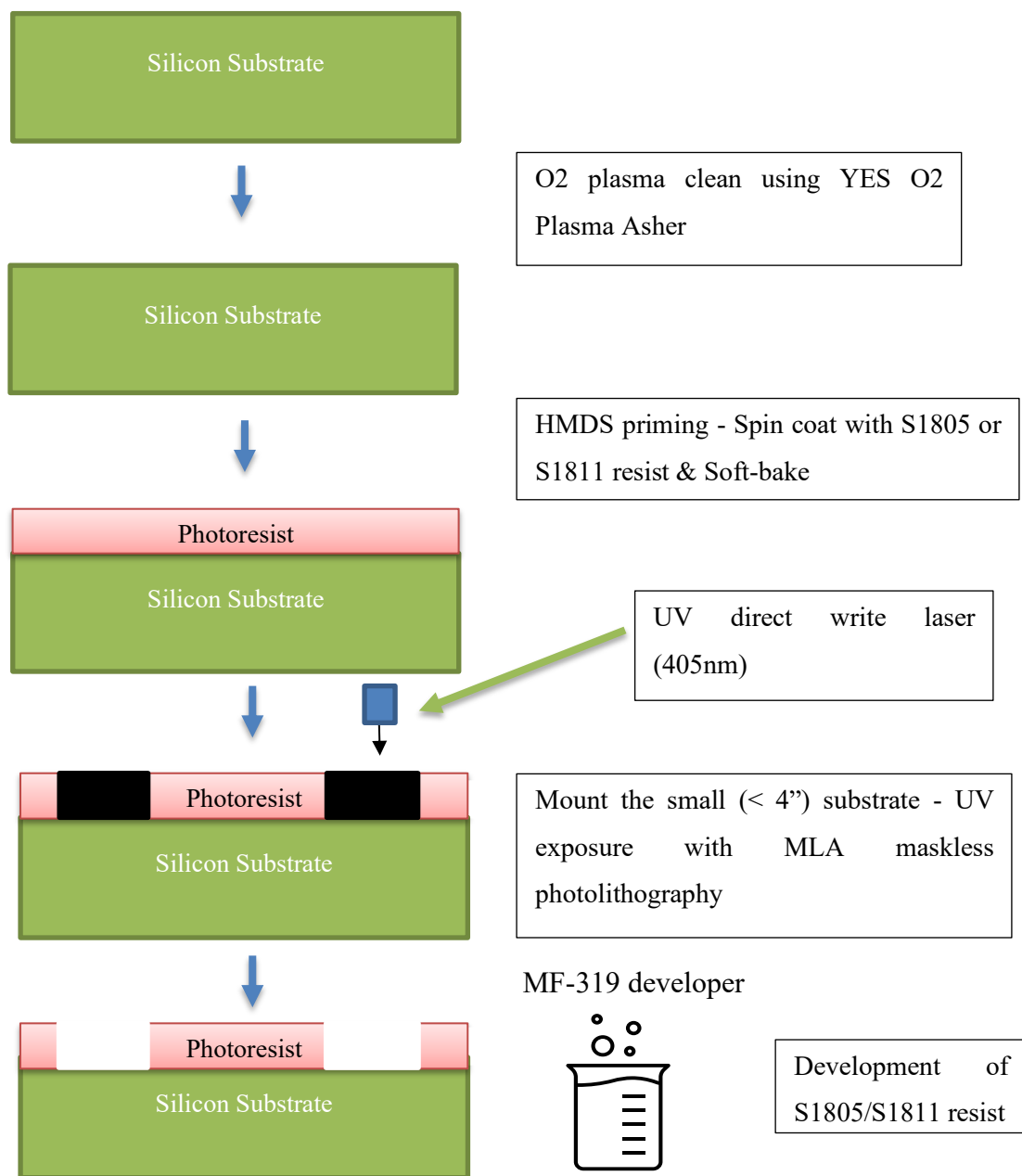


Figure 12 Schematic of the maskless photolithography procedure conducted in the QNC cleanroom

4.2.3.3 Soft lithography on PDMS lens samples

The brief procedures for the soft lithography are demonstrated in schematic **Figure 13**. To be more specific, the PDMS (Polydimethylsiloxane) solution was prepared with a 10:1.05 base to curing agent ratio. A concave PDMS was then prepared by pouring the PDMS onto the convex side of the original contact lens shown in **Figure 8**. After overnight incubation at 60°C, the PDMS concave mold was coated with (1H,1H,2H,2H)-perfluorodecyl trichlorosilane (FDTS, Sigma), an anti-stiction layer. Similarly, the silicon wafer developed in section 3.2.4.2 was coated with the same anti-stiction layer overnight, and the PDMS solution with a 5:1 base to curing agent ratio was then introduced onto the patterned Si wafer. After the same curing procedure, the PDMS molds were detached from the silicon wafer and coated with silane to prepare the PDMS patterned films with around 20 – 40 microns thickness. The patterned PDMS films were gently cut into suitable sizes and shapes to be applied onto the center of the convex PDMS mold, and the convex side was plasma treated for 120s at 80% O₂ gas and 70 Watts in advance (Zepto, Diener, Germany) to improve film attachment to the mold. After having films attached to the lens, the samples were salinized and added to the PDMS solution. After the curing process, the detached PDMS concave lens imprints patterns on the center area.

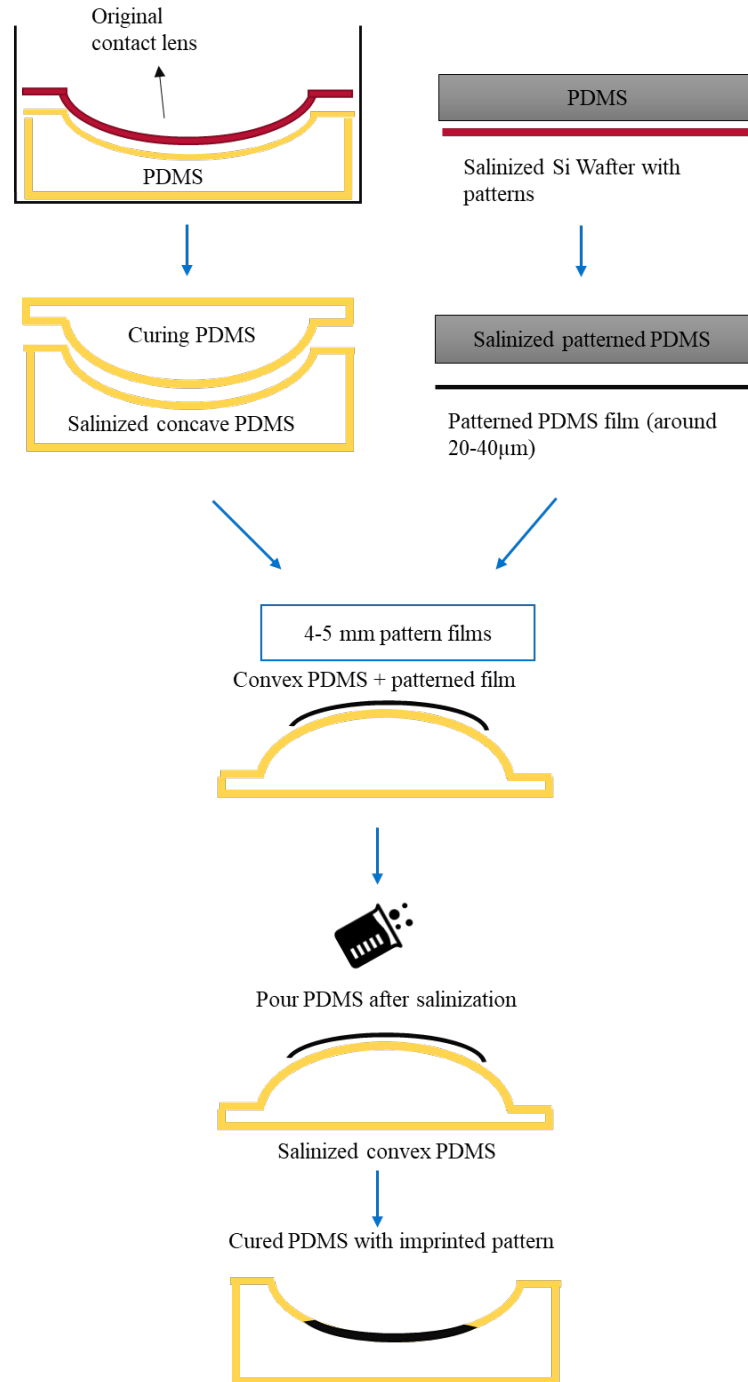


Figure 13 Schematic of the soft photolithography procedure conducted with PDMS

4.3 Results

4.3.1 Cell migration on tissue culture plate, flat PDMS, and concave PDMS

In a previous study, Ong Tone *et al.* demonstrated that the mean migration speed and the maximum migration speed of the FECD CEnCs (FECD-SV-54F-73) were higher than normal CEnCs (HCEC-SV-67F-17) *in vitro* [24].

To further explore the FECD cell mechanisms and compare the results with the previously conducted results, the cell migration of an FECD cell line FECD-SV-54F-73 and a normal cell line B4G12 were compared. Different substrates were utilized, including flat TCPS, curved and flat PDMS, and GelMA+ contact lens (Appendix B, Figure 24). All the PDMS and GelMA samples were coated with same FNC coating mix. The wound closure rate of FECD and normal cells were initially compared and calculated on the 24-well tissue culture plate, a linear trendline was plotted, and the slope was analyzed. It has been observed that when the B4G12 cells were seeded on FNC coated TCPS, the mean wound closure rate was the fastest (2.92% of total wounded area per hour), while the FECD 54F has the lowest wound recovery rate (1.73% per hour) on the TCPS (**Figure 14C**). In addition, the wound closure rate of the B4G12 sample without the FNC coating (2.74% per hour) was slightly lower than the coated sample (**Figure 14C**). **Figure 14B** also demonstrated differences in terms of the migration pattern during the wound closure, similar to what Ong Tone and colleagues have observed [24, 90]. The cells in FECD specimens migrated more individually in a spindle shape instead of a more organized epithelial-type collective migration closure, as seen in normal cell line B4G12.

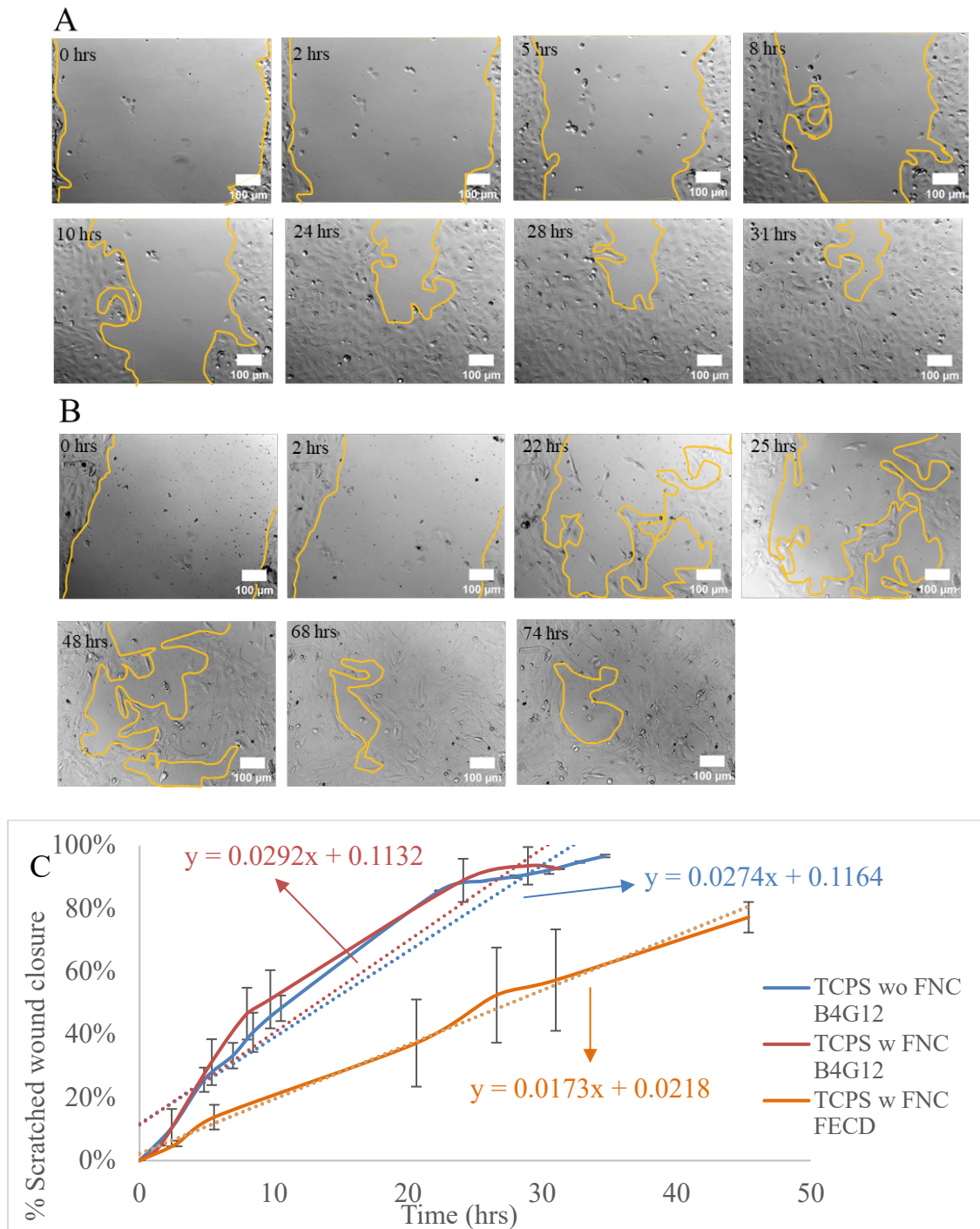
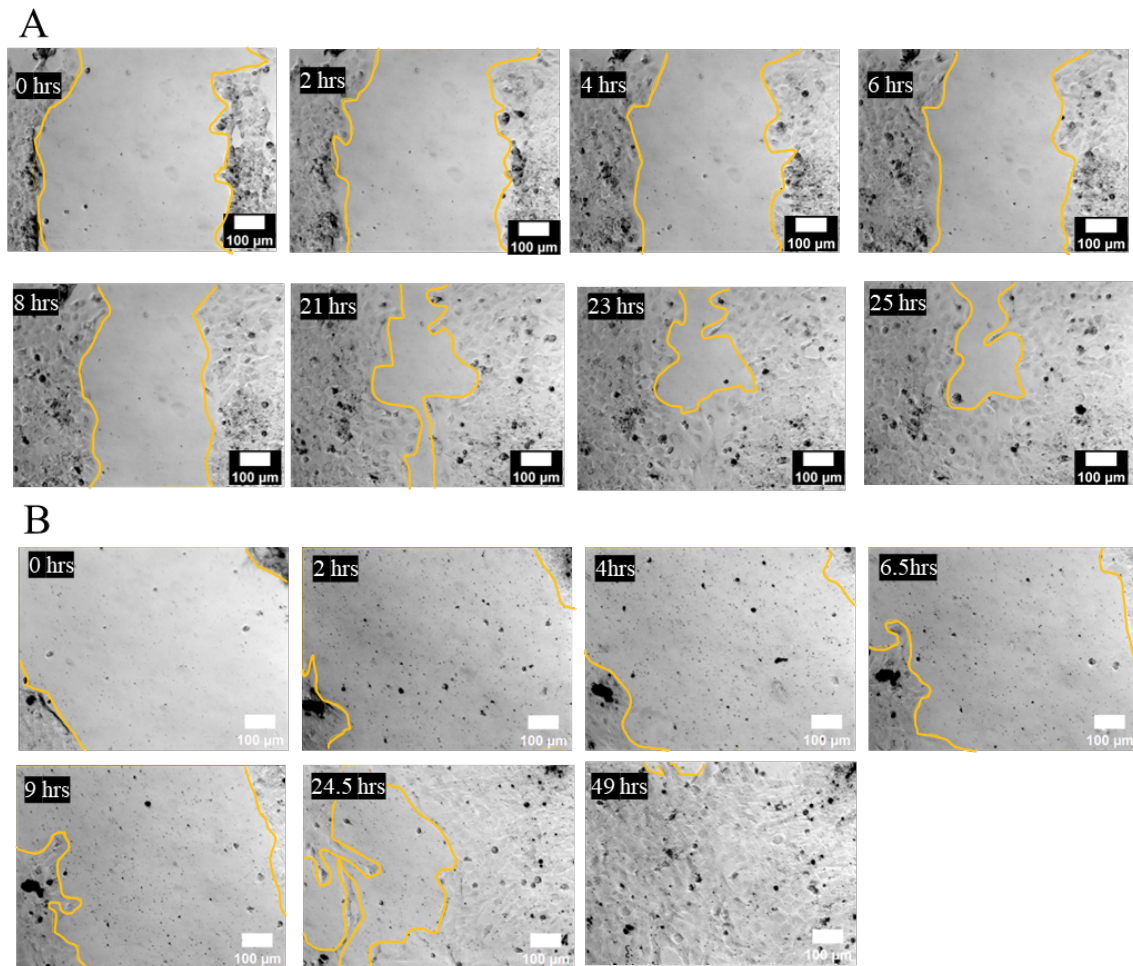


Figure 14 Higher CEnC migration speeds in confluent conditions in B4G12 compared to FECD CEnCs. **A.** B4G12 normal CEnCs on TCPS (with FNC coating). **B.** FECD 54F normal CEnCs on TCPS

(with FNC coating). C. Line graph showing FECD 54F cell migration speed compared with normal CEnCs B4G12 detected by closure of scratch area (n = 2)

To further recapitulate the corneal endothelial cells migration pattern *in vivo*, a concave lens PDMS model was utilized (**Figure 14**). However, the poor cell adhesion issue had been reoccurring during the trials between the FECD 54F cell line and PDMS concave lens substrate. Therefore, FECD cells were only tested on flat PDMS and subsequently tested on GelMA+ contact lens substrate. The wound closure rate of FECD 54F (2.9% of total wounded area per hour) and B4G12 (3.02% per hour) was similar to the flat PDMS, with the linear trendline slightly higher on B4G12 samples (**Figure 15C**). On the other hand, for FECD cell samples, the rate of wound closure on the flat PDMS substrate was faster than on the flat tissue culture plate.



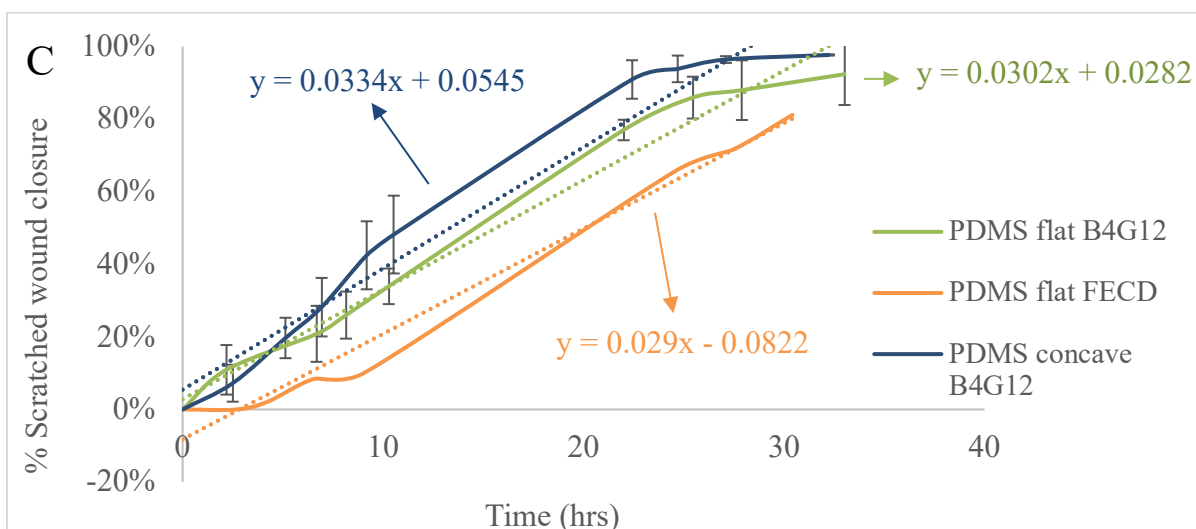


Figure 15 B4G12 and FECD CEnCs migration speeds in confluent conditions **A.** B4G12 CEnCs on PDMS (with FNC coating). **B.** FECD 54F CEnCs on PDMS (with FNC coating). **C.** Line graph showing FECD 54F cell and normal CEnCs B4G12 migration speed detected by closure of scratch area ($n = 2$, for FECD 54F on flat PDMS $n = 1$, image taken in 10X)

A few more trials have been conducted on the concave PDMS lens using the B4G12 cell line (**Figure 16**). A circular PDMS film with around 1mm diameter and 200-micron thickness was placed on the center of the concave PDMS as a block and then gently lifted after cells formed a monolayer. Time lapse images were taken every 2 hours until the wound closed. The wound closure rate of the B4G12 cells on concave PDMS was compared with flat PDMS. The differences in time points were not significant. However, the trendline suggested that the B4G12 on concave samples (3.34% per hour) migrated slightly faster than the cells on flat PDMS samples (**Figure 15C**). More individual cell migration was also observed on the concave sample, whereas the cells on flat PDMS migrated together as a monolayer similar to the TCPS B4G12 samples.

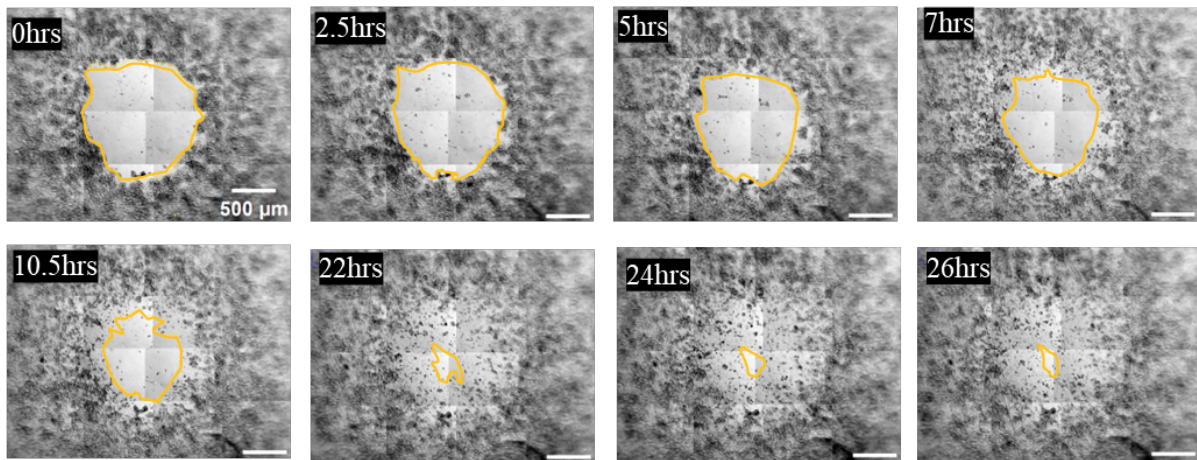


Figure 16 10X Stitch image of B4G12 CEnC migration on concave PDMS (with FNC coating) in confluent conditions.

4.3.2 Cell migration on GelMA+ contact lens

To further explore the potential mechanisms of FECD cell migration patterns and their differences with the B4G12 cells, a hydrogel GelMA+ was utilized as the seeding substrate for the CEnC lines. GelMA+ has been proven to adhere more easily to the GelMA+ hydrogel since it contains cell adhesion ligands [82]. **Figure 17** shows that the FECD cells (2.35% per hour) migrated faster than the B4G12 (1.41% per hour) cells on the 30% GelMA+ contact lens samples (**Figure 17C**). B4G12 cells also had a slower wound closure rate in the GelMA+ lens compared to the PDMS concave samples. Similar to previously tested substrates, the FECD cells in GelMA+ also migrated more individually than B4G12. In addition, FECD and B4G12 cells migrated more individually when compared to the flat samples.

To study the cytoskeleton response of the two cell lines, the GelMA+ samples were also stained with phalloidin after wound closure. **Figure 18** shows that F-actin structure in the FECD 54F sample exhibited a more fibroblastic morphology when compared to the B4G12 cells.

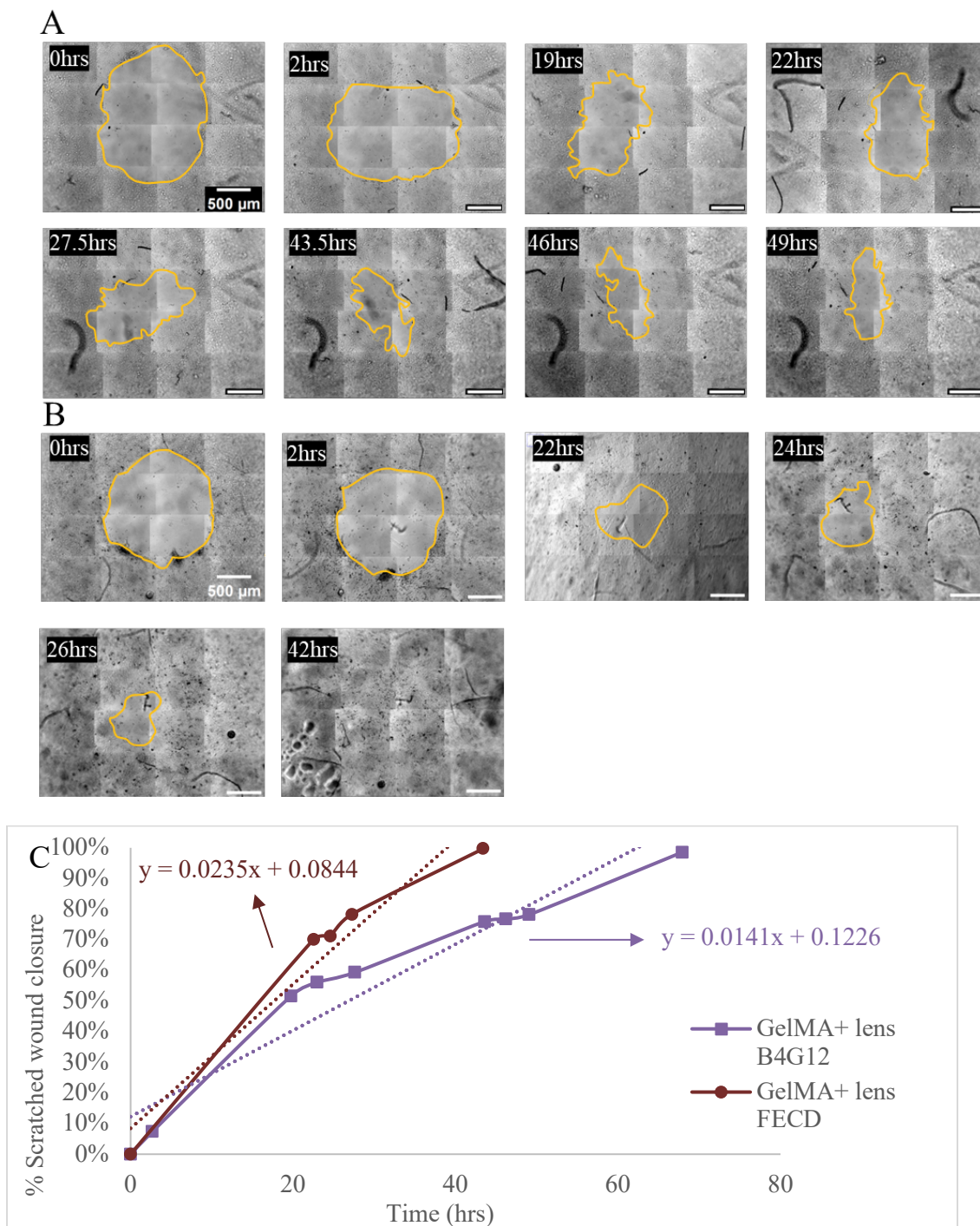


Figure 17 B4G12 and FECD CEnC migration speeds in confluent conditions on 30% GelMA+ contact lens (with FNC coating). **A.** 10X Stitch image of B4G12 CEnCs on 30% GelMA+ contact lens. **B.** 10X

Stitch image of FECD 54F CEnCs on 30% GelMA+ contact lens. **C.** Line graph showing FECD 54F cell and normal CEnCs B4G12 migration speed detected by the closure of scratch area ($n = 1$)

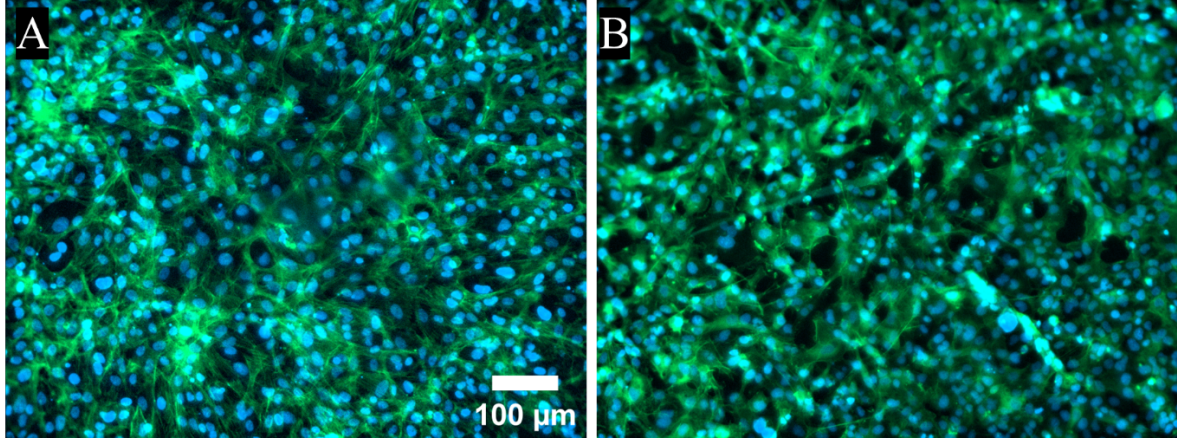


Figure 18. Representative IF images of F-actin (green) and DAPI staining of CEnCs at the center of the GelMA+ contact lens. Scale bar: 100μm, 10X **A.** FECD 54F sample **B.** B4G12 sample

4.3.3 Photolithography and soft lithography of patterned concave lens

As mentioned previously, one of the important developing fields in biomaterial and biomedical study is manipulating the surface micro or nano-topographical cues to impact cell fate [82]. To further explore whether cell behaviors can be altered on a non-planar surface through micro-topographical patterning, a soft lithography protocol was developed using PDMS micro-molding and maskless photolithography. Firstly, by utilizing maskless photolithography, the patterns were imprinted onto the silicon wafer; the simple radial pattern (**Figure 18A**), dense radial (**Figure 16B**), and the double spiral (**Figure 16C**) pattern was chosen to be fabricated, the subsequent soft lithography processes were then conducted as demonstrated in **Figure 13**. As **Figure 18 A, B, and C** show, the pattern after maskless photolithography obtained a similar feature size and a good resolution. However, the subsequent PDMS patterns and the patterns imprinted onto the non-planar surface have all experienced a certain degree of damage or defects, as shown in **Figure 18 D, E**. After the PDMS micro-molding procedure, the spiral patterns have been mostly intact; the radial patterns, however, have shown the lower resolutions, underdeveloped areas, and some degree of contamination.

However, during the last pattern transfer process, the films more significant than 5 mm diameters could not be transferred without an obvious bubble-shaped defect. The feature pattern resolution was

maintained in smaller diameter films, and no huge bubble-shaped defects were detected on the final concave PDMS products after the demolding (**Figure 19**).

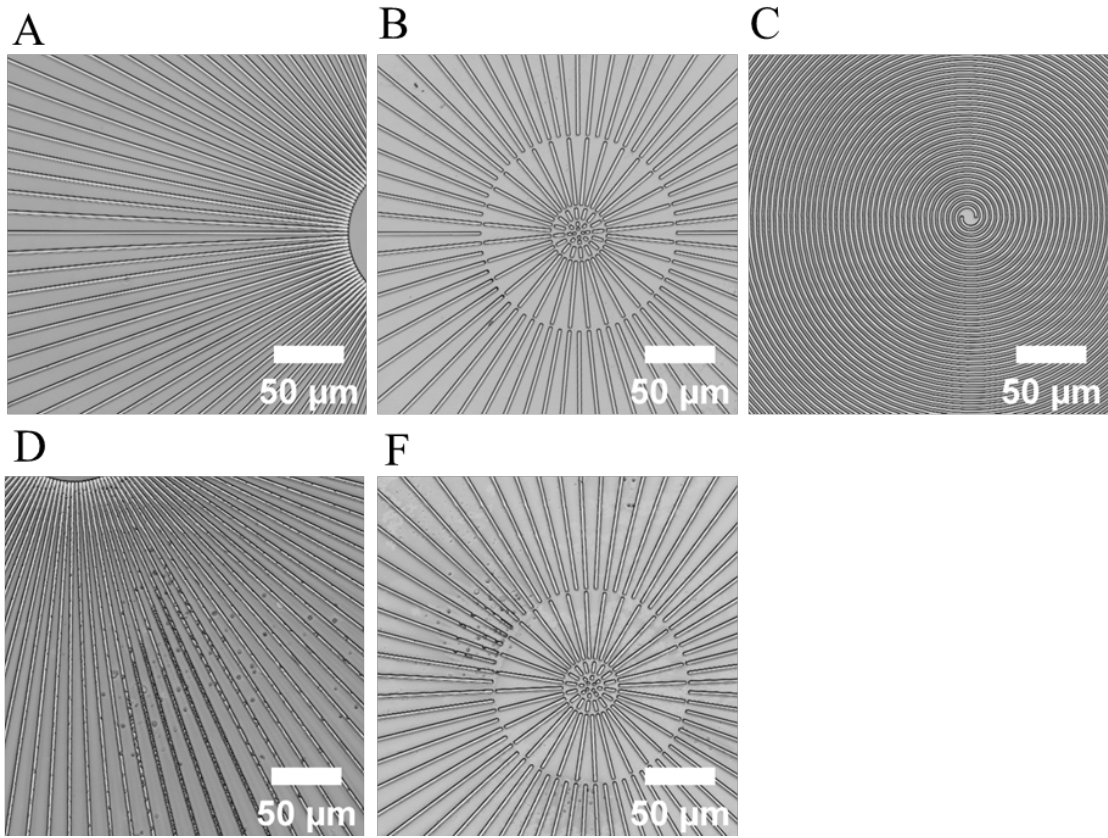


Figure 19 Images of patterns on the original Si wafer, 50X. A, B, C. Patterns before the soft lithography fabrication (A. Simple radial, B. Dense radial, C. Double spiral) D, F. Patterns after the soft lithography procedure, defects were observed after fabrication, no significant defects were observed on the double spiral sample (D. Simple radial, F. Dense radial). Scale bar: 50 μm

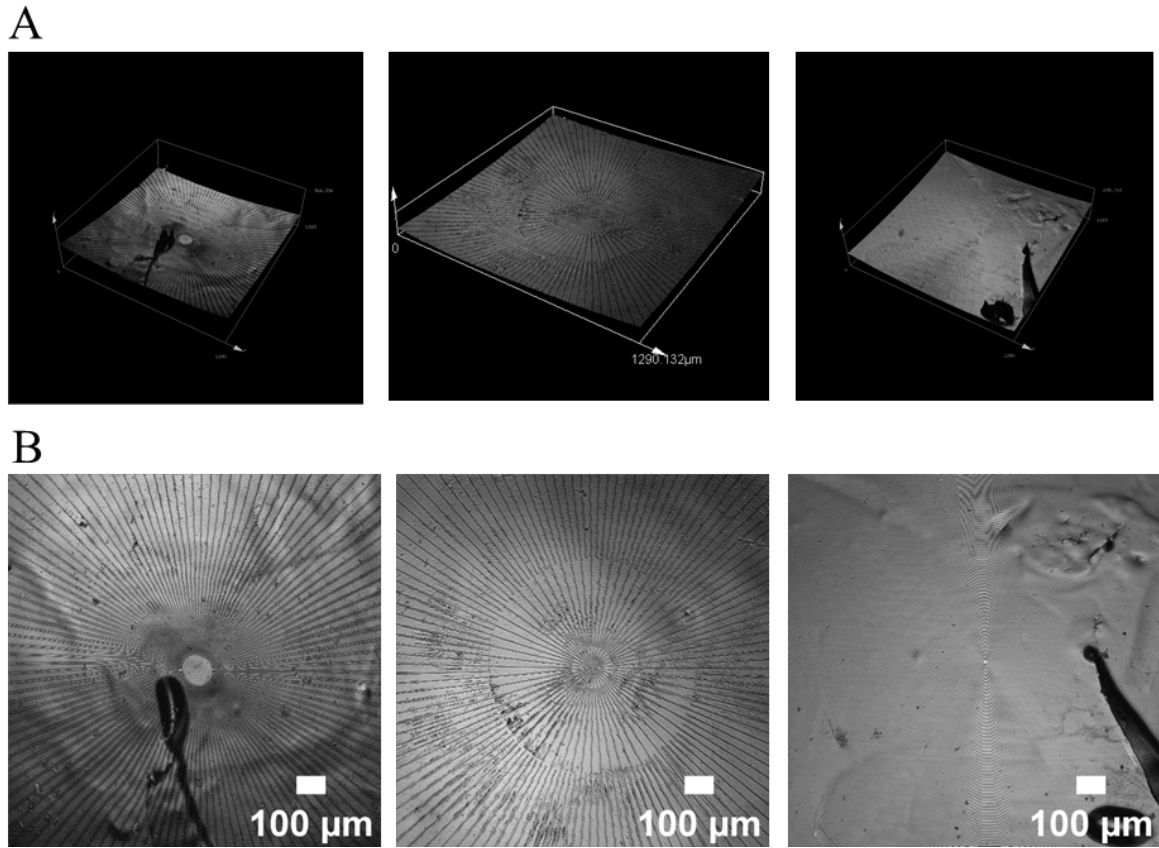


Figure 20. Images of patterns on the concave lens surface. Defects can be observed on the patterns; the cuts and the bubble-shaped defects were due to the pattern transfer process. (10X) A. 3D view of the patterns on the concave surface B. Top-down view of the patterns on the concave surface. Scale bar: 100 μm

4.4 Discussion and Conclusion

4.4.1 CEnCs wound healing on concave surfaces

The corneal endothelial cells (CEnCs) originate from the embryonic neural crest and remain in the terminally differentiated stage, which lacks mitotic ability *in vivo* [4]. During the progression of FECD, the endothelium initially undergoes cell loss, starting at the central region. The remaining cells in the peripheral area then migrate to the cell loss site, causing them to lose their healthy phenotype [8]. Apart from the spontaneous cell migration mechanisms, several currently used clinical surgeries (DWEK) [117, 118] and testing drugs also heavily rely upon the migration of peripheral or injected CEnCs [52,

57]. Several studies have focused on CEnC migration behavior in normal DM and healthy cells [118, 119]. However, there have been limited models representing the migration of FECD CEnCs in DM. On the other hand, as previously mentioned, an *in vivo* model has demonstrated the feasibility of using a xenogeneic human FECD cell graft in a feline cornea [98], implying the potential possibility of utilizing an autograft tissue engineering therapy for FECD patients in the future [98]. Therefore, constructing the FECD cell migration model on a concave surface may help better understand the wound healing mechanisms in FECD patients and potentially provide a theoretical background for developing the tissue engineered autograft for FECD patients.

Previous studies conducted by Ong Tone *et al.* demonstrated that the FECD CEnCs possess a higher migration speed in *ex vivo* FECD tissues and *in vitro* tissue culture plate surfaces when compared to normal CEnCs [24]. In this section, a novel concave platform was utilized for the wound healing cell migration study. FECD 54F had a relatively slower migration rate in TCPS but faster on PDMS and GelMA+. The normal CEnC line B4G12 showed a faster wound closure rate than the FECD 54F when cultured on a flat tissue culture plate. On PDMS samples, the migration speed was also similar between the two cell lines. However, when seeded on the 30% GelMA+ surface, B4G12 samples showed significantly slower migration speed than the FECD cell sample and other B4G12 samples cultured on different substrates. In contrast to the findings reported in the earlier study [24], the FECD cells migration speed in our studies were not necessarily faster than the normal cell line on all the substrates. This discrepancy may be due to the different normal cell lines used [24]. Instead of using normal CEnCs B4G12, Ong Tone *et al.* utilized a self-developed healthy CEnC line HCEC-SV-67F-17 [24]; this cell line may also be used in future studies for further verification.

It is worth noting that both FECD 54F cells and B4G12 cells have different wound closure rates and migration patterns on different substrates. For instance, FECD 54F migrated slower in TCPS but faster on GelMA+ and vice versa for the B4G12 cells, which migrated slower on the GelMA+ substrate, implying that both cell lines may have different behaviors on substrates with different surface chemistry. In addition, given that Young's modulus of native DM is varied between 50 kPa and 2.57 MPa [6], the effect of substrate stiffness and substrates' curvature also remained unclear. 30% GelMA+ has a compressive modulus of 233.3kPa [82], whereas TCPS has Young's Modulus of 3GPa [107], and the PDMS (10:1 ratio) has the compressive modulus ranging between 117.8 and 186.9 MPa [120]. The B4G12 may already be well adapted for the experiment on the tissue culture surface. In contrast, the FECD 54F cell line may be more comfortable migrating on a substrate with lower mechanical strength.

However, it is also important to mention that the trial experiment on the GelMA+ contact lens was only conducted once; further verification may be required to analyze the cell's behavior on the concave and flat GelMA+ surface.

PDMS concave surface was initially used for the wound healing test on the curved surface; however, this substrate was later opted out and changed to GelMA+, mainly due to the limited adhesion of FECD 54F cells to the PDMS surface. During the surface treatment of PDMS before the cell seeding, hydrophilicity may gradually decrease during the handling process. On the other hand, there might be residual uncured PDMS on the substrate, which could be potentially toxic to the cells, affecting the cell viability and adhesion. It is also possible that the FNC coating mix on the substrate may not be enough for the FECD 54F cells to properly adhere, a small volume of FNC coating mix may need to be added to the cell culture to improve the adhesion.

For better imaging, the GelMA+ lens samples were required to be stabilized; a PDMS mold replicating the concave side of the contact lens was used as a sample holder. However, although the relative location of the lens can be fixed, the samples may change angle and dislocate during the stitching image mode, making it difficult to take images with better resolution. The technique for stabilizing the GelMA+ can be further improved. The surface curvature and the thickness of the PDMS and GelMA+ samples can also be the contributing factor to the inconsistent resolution in the stitch images.

4.4.2 Micro-topographical patterning on lens surface

To study whether the cell behavior on a curved platform can be further affected by manipulating the surface micro or nano-topographical cues, a soft lithography process was developed involving the utilization of PDMS micro-molding and maskless photolithography. As lithography has been widely used in fabrication of microstructures, the non-planar, low defect patterning of the microstructures were recognized to be challenging [121, 122]. Maskless photolithography has produced consistent samples with high resolution. However, during the PDMS micro-molding process, the samples may be mishandled, which subsequently affected the cured PDMS patterns resolution and consistency. Regarding the transferring of the patterns from a flat substrate onto a non-planar surface, various methods have been developed previously. However, these methods typically involve either a complex procedure that includes casting ultraviolet-curable liquid polyurethane (PU) [121] or a comprehensive process known as Decal Transfer Lithography (DTL) involving silicon deposition and etching [122].

Eventually, after several rounds of iteration and improvements, a process was developed that only required PDMS micro-molding techniques. This process involved transferring the patterns from a thin, flat PDMS film (25-40 microns) onto a non-planar convex side of the PDMS lens. The fabrication outcome was somewhat consistent with a smaller diameter (5 mm) and some sacrificial cutting on a portion of the patterned films. More iterations of the experiment methods and handling protocols may be required to eventually produce a more ideal outcome for cell experiments.

Chapter 5

Conclusion and Future Works

5.1 Cytoskeleton and apoptosis pathophysiology in FECD

The FECD is a complicated diseased state that can be affected by multiple factors [9]. The abnormal deposition of the ECM and subsequent guttae formation are hallmarks of FECD. As the current treatment for FECD is donor transplantation, further understanding the FECD pathogenesis and its connection with the dysfunctional cell-ECM interactions is crucial for developing drugs and clinical procedures [9, 30, 123]. The biomimicking synthetic guttata developed by former lab members has provided a wide range of research opportunities [30], including cell migration, monolayer formation, and apoptosis studies. In this study, the FECD immortalized cell line was utilized on the s-guttata model; compared to the normal B4G12 CEnCs, it was assumed that this FECD cell line could provide closer recapitulation of the native FECD condition. The results have shown a higher rate of early-stage apoptosis in the guttata patterned samples, which also aligned with the results conducted using a normal B4G12 CEnC line. In the future, more assessments may be conducted using the FECD cell line, including adding a 48-hour early apoptosis assay and flow cytometry for the positive signal analysis; late-stage apoptotic assay can also be conducted through the s-guttata and FECD cells.

Other FECD pathophysiology, including the phenotypical changes, cytoskeleton response to the guttata, and the EndMT, were also explored by combining the FECD cell and s-guttata patterns *in vitro*. It was shown that the guttata could induce the FECD cell line to form a more contractile phenotype and undergo potential fibrogenesis in the s-guttata patterned samples. On the other hand, the PCR results did not show significant EndMT expression upregulation in the B4G12 samples within seven days. Given that B4G12 is a healthy cell line, more studies can be conducted using FECD cells to study the potential pathways and mechanisms of EndMT.

5.2 Human CEnCs migration on concave contact lens model

In a previous study conducted by Ong Tone *et al.*, both *in vitro* live cell imaging and wound healing assay demonstrated a higher migration speed and wound closure rate in FECD CEnCs, respectively [24]. To further expand the study, a concave surface has been used to study both the cell migration behavior and the wound healing rate. It was observed that the FECD cell migration was higher on a 30% GelMA+ contact lens sample when compared to the healthy B4G12 sample; however, on the flat

TCPS, B4G12 migrated faster than the FECD 54F cell line. Apart from the apparent difference in terms of the cell lines used, it is possible that both the substrate's shape and stiffness can affect the cell adhesion and migration behavior. However, it is worth noting that the study is still in the preliminary stage as the migration assay on GelMA+ has only been conducted once. Since the cells have only been assessed on curved 30% GelMA+ once, it is also worthwhile to conduct additional tests on flat GelMA+ to further examine whether the migration patterns and wound healing rate of CEnCs on curved GelMA+ were mainly affected by the curvature or the chemical properties of the substrate.

Further study and experiments can be helpful to further elucidate cell migration behaviors and potential cell mechanotransduction mechanisms on different cell lines. On the other hand, the poor adhesion of the FECD 54F cell onto the PDMS surface may have resulted from the loss of hydrophilicity in the handling process before the cell seeding. Increasing the ratio of curing agent in the PDMS mixture and having a longer PDMS curing time can further minimize the chance of having uncured PDMS affecting cell proliferation. Secondly, the FNC coating mix can be added into the medium before seeding and after removing the PDMS block to further enhance the proliferation, which was proven helpful for the GelMA+ samples.

5.3 Micro-topographical contact lens model fabrication

A lithography process was developed to transfer the microtopography patterns onto the non-planar surface to further study the potential of topography in affecting cell behaviors. This lithography process involves using maskless photolithography and PDMS micro-molding procedures. The patterns can be replicated onto the concave surface and used for subsequent cell experiments as a master mold; however, the process may still need reiterations to improve the pattern quality and avoid the resolution loss in the process as much as possible. Potentially, the Decal Transfer Lithography (DTL) process may be possible in the cleanroom, which involves the silicon deposition on a convex surface and subsequent PDMS imprint and reactive-ion etching (RIE) [122].

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Appendix A

Melt curve of the RT-qPCR assay and fabrication of the biomimetic guttata structure

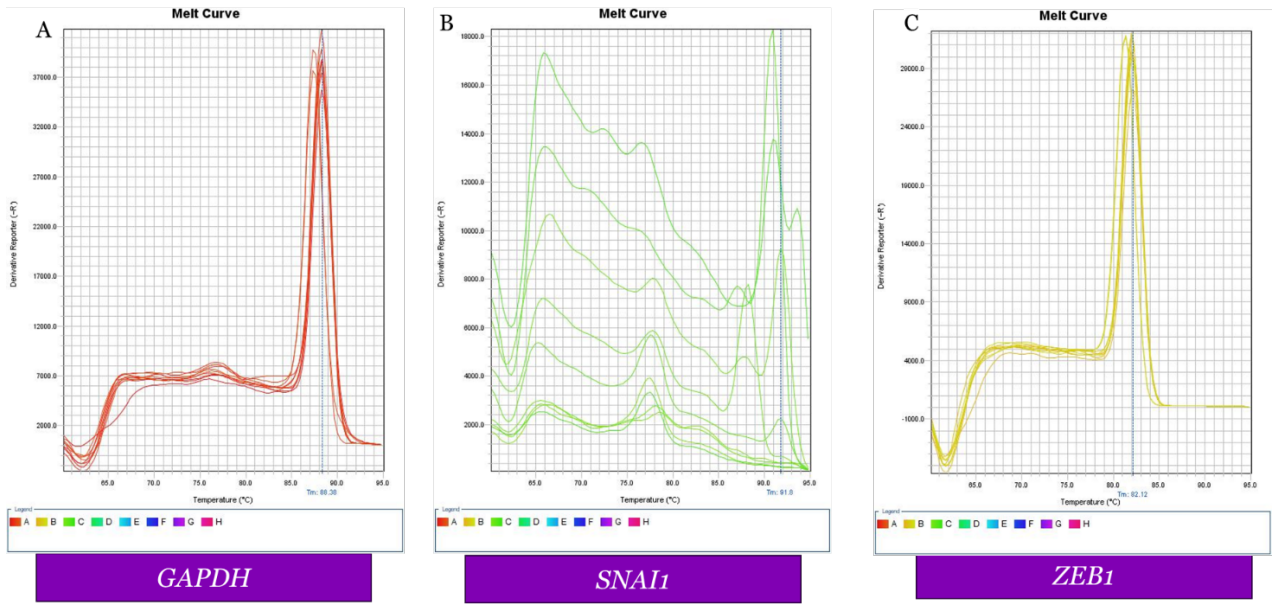


Figure 21. Melt curve of the B4G12 samples from RT-qPCR, A. melt curve of the housekeeping gene *GAPDH*. B. melt curve of the test gene *SNAIL*, showing two melt curves in all samples (B4G12 on TCPS, blank, and 20x20x20), the amplification at 65°C may represent a primer dimer or a non-specific amplification. C. Melt curve of the test gene *ZEB1* showing only one amplification in all samples.

Table 8 Mean, upper limit, and lower limit of the RT-qPCR samples

	ZEB1			SNAIL		
	Mean	Upper limit	Lower limit	Mean	Upper limit	Lower limit
TCPS	1	1.30095445	0.76866642	1	2.00138387	0.49965427
UP	1.49256236	1.67407447	1.33073078	3.30713979	6.0353423	1.81218779
20x20x20	1.45665458	1.76749826	1.20047788	5.56187252	39.9356093	0.77460759

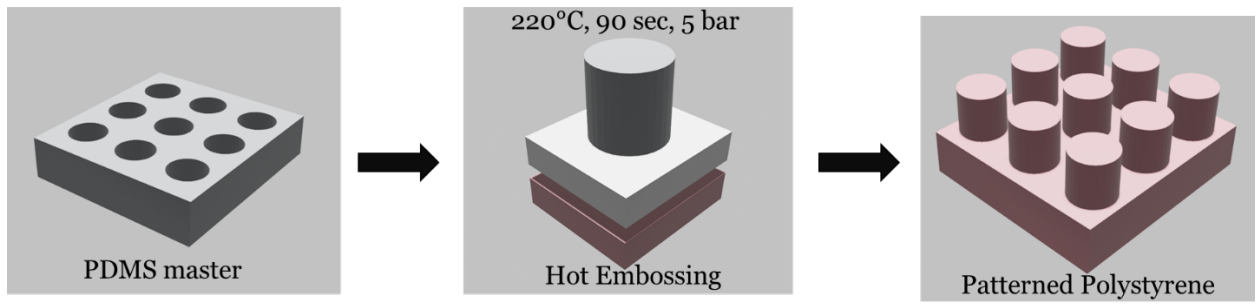


Figure 22. Simple schematic of the patterned polystyrene fabrication method (models generated with Blender).

Appendix B

FECD 54F and B4G12 cells migration study

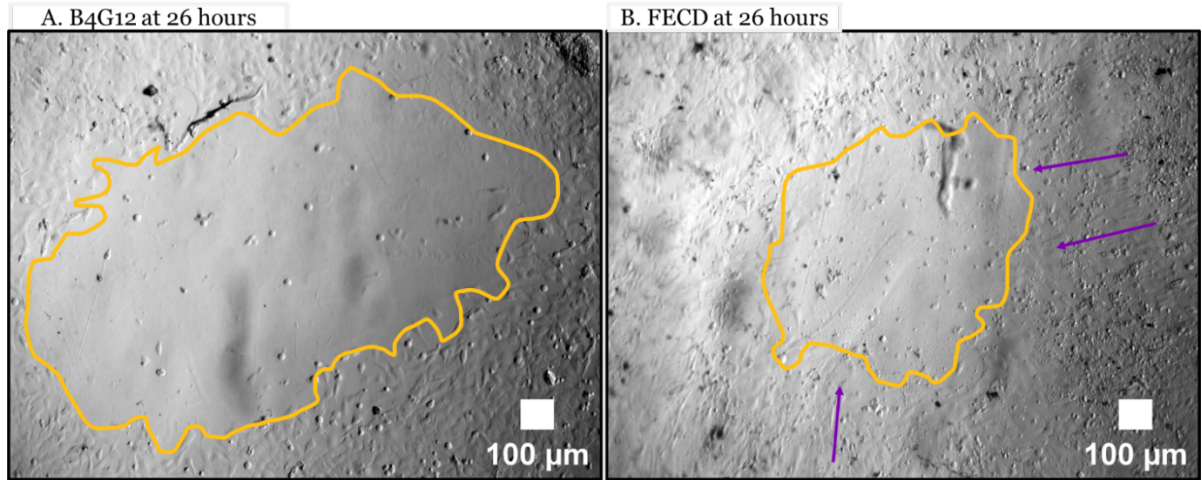


Figure 23. Images of the CEnCs on 30% GelMA+ contact lens after 26 hours of wound healing. A. B4G12 on GelMA+ after 26 hours. B. FECD 54F on GelMA+ after 26 hours; cells in some regions were along the direction of curvature, likely to contribute to the wound closure, same patterns were also observed in other time points.

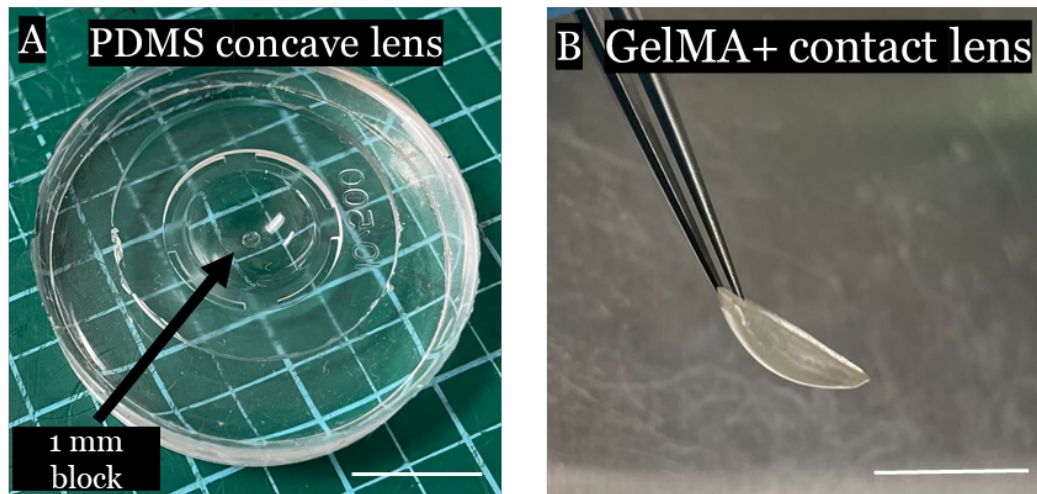


Figure 24. Images of the concave substrates used for the cell migration study. A. PDMS concave lens. Before cell seeding, a 1mm diameter circular block was placed at the center of the lens. After cell confluence, the blocks were removed to initiate the cell wound healing. B. A 30% GelMA+ lens.