ANGLIA RUSKIN UNIVERSITY

FACULTY OF SCIENCE AND TECHNOLOGY

EVALUATION OF CORNEAL ENDOTHELIAL CELL THERAPY USING AN *IN VITRO* HUMAN CORNEAL MODEL

KOSTADIN DIMOV ROLEV

A thesis in partial fulfilment of the
requirements of Anglia Ruskin University
for the degree of Doctor of Philosophy (PhD)

This research program was carried out in the Department of
Biomedical and Forensic Sciences,
in collaboration with the Vision & Eye Research Unit

Submitted: November 2017

ACKNOWLEDGEMENTS

I would like to thank my wife Celine Zhang for her love and support throughout the entire time of this work. And I would like to express my sincere gratitude to my supervisory team Prof. Madhavan Rajan, Dr. Alexandra Chittka, Dr. Linda King and Dr. Peter Coussons for their support, advice and significant contribution to this work. I am also grateful to Anglia Ruskin University for generously providing the funding for this project, without which this work would not have been possible.

ANGLIA RUSKIN UNIVERSITY

ABSTRACT

FACULTY OF SCIENCE AND TECHNOLOGY

DOCTOR OF PHILOSOPHY

EVALUATION OF CORNEAL ENDOTHELIAL CELL THERAPY USING AN *IN VITRO* HUMAN CORNEAL MODEL

KOSTADIN ROLEV

NOVEMBER 2017

Aim: To establish an *in vitro* human corneal decompensation model and to use it for the evaluation of a cell-therapy approach for treating corneal endothelial (CE) disorders and to test the expression profile of positive regulatory domain proteins (PRDMs) as potential markers for corneal endothelial cells (CECs).

Materials and Methods: Human cadaveric corneas were obtained from Bristol and Manchester Eye Banks, UK. A CE decompensation model was established by removal of the Descemet's membrane (DM)/Endothelium complex from donor corneas and placing them in air-interface organ culture. The corneal thickness was used as a surrogate measure of CE function and was measured using Optical Coherence Tomography (OCT). Decompensated corneas were subjected to cultured endothelial cell therapy using immortalized HCEC -12 cells (group 1), primary human corneal endothelial cells (hCECs) at 0 passage (group 2) and hCECs at passage 2 (group 3) with defined seeding cell density. The effect on stromal de-swelling in cell therapy treated corneas was assessed 3, 7 and 10 days post-transplantation followed by histological evaluation. In addition, expression of PRDM genes in the corneal endothelium was undertaken using reverse transcriptase polymerase chain reaction (RT-PCR), immunocytochemistry and immunohistochemistry.

Results: Organ culture of human cadaveric corneas in air-interface following the selective removal of the DM/Endothelium complex resulted in stromal thickness of 903.6 ± 86.51 μm, whereas normal corneas maintained a physiological thickness of 557.51 ± 72.64 μm. When transplanted directly onto the posterior corneal stroma the human CECs were able to attach and achieved physiological corneal thickness of 458.91 ± 90.07 μm, 489.65 ± 94.62 μm and 613.7 ± 94.62 μm for cell therapy groups -1, -2 and -3 respectively. The study identified PRDMs 1, 2, 4, 5 and 10 in the human CE and revealed a differential expression between normal CE and cultured hCECs.

Conclusion: Removal of the DM/Endothelium complex from cadaveric human corneas held in air interface organ culture resulted in corneal endothelial decompensation. Direct transplantation of cultured primary hCECs to bare posterior corneal stroma devoid of DM resulted in the formation of an endothelial monolayer and restoration of stromal hydration to physiological thickness, substantiating the role of cell therapy to treat corneal endothelial disorders. The identification of PRDM proteins in the human corneal endothelium paves the way for future studies to understand their role in hCEC proliferation control.

Key words: Corneal endothelium – Human corneal endothelial cell culture – Corneal endothelial cell transplantation, PRDM proteins.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DECLARATION

This thesis is submitted to Anglia Ruskin University for the degree of Doctor of Philosophy in Biological Sciences. I (Kostadin Dimov Rolev) declare that this thesis under the supervision of Prof. Madhavan Rajan, Dr. Linda King, Dr. Peter Coussons and Dr. Alexandra Chittka, is my own work, except where noted or referenced. This thesis or any parts of it have not been submitted for any previous degree or award.

LIST OF ABBREVIATIONS

AJs	Adherens junctions
Akt	Protein kinase B
AM	Amniotic membrane
APCM	Acellular porcine corneal matrix
α-SMA	α-smooth muscle actin
ATPase	Adenosine triphosphatase. An enzyme that breaks down adenosine triphosphate into adenosine diphosphate and a free phosphate ion
B4G12	A clonal subpopulation derived from the Human Corneal Endothelial Cells-12 (HCEC-12) immortalized cell line
BCEC-ECM	Bovine corneal endothelial cell-derived extracellular matrix
bFGF	Basic fibroblast growth factor
BM-MSCs	Human bone marrow-derived mesenchymal stem cells
BMP	Bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium ion
CDC25A	Cell division cycle 25A
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
cDNA	Complementary deoxyribonucleic acid
CE	Corneal endothelium; corneal endothelial
CECs	Corneal endothelial cells
CCT	Central corneal thickness
CO_2	Carbon dioxide
COL8A2	Collagen, type VIII, alpha 2
Cx43	Connexin43
CS	Chondroitin sulfate
Cl^-	Chloride ion
cMycER^{TAM}	c-Myc transcription factor/mutant estrogen receptor
DAB	3,3'-diaminobenzidine
DEPC	Diethyl pyrocarbonate
DM	Descemet's membrane
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DMEK	Descemet's Membrane Endothelial Keratoplasty
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DSAEK	Descemet's Stripping Automated Endothelial Keratoplasty
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	Dithiothreitol
E2F	E2F transcription factor
ECACC	European Collection of Cell Cultures
E-cadherins	Epithelial cadherins
ECD	Endothelial cell density
ECGF	Endothelial cell growth supplement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGM-2	Endothelial growth medium-2
Erk1/2	Extracellular signal-regulated kinase 1/2
ESC	Embryonic stem cells

ESC-CM	Embryonic stem cell-conditioned medium
F99	Ham's F12 mixture with Medium 199 in a 1:1 ratio
FBS	Foetal bovine serum
FCED	Fuchs' corneal endothelial dystrophy
FGF-2	Fibroblast growth factor-2
FGF	Fibroblast growth factor
FN	Fibronectin
FNC-coating mix	Fibronectin-collagen coating mix
FREP	Faculty Research Ethics Panel
G1 phase	Gap 1 phase of the cell cycle
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
GPCRs	G-protein-coupled receptors
H^+	Hydrogen ion
H&E	Haematoxylin and eosin staining
HCEC-12	Human Corneal Endothelial Cells-12 immortalized cell line
hCECs	Human corneal endothelial cells
HCO^-_3	Bicarbonate ion
HEPES	(*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*)
HGF	Hepatocyte growth factor
HRP	Polymeric horseradish peroxidase
HSC	Haematopoietic stem cell
HTA	Human Tissue Authority
K^+	Potassium ion
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL-1β	Interleukin-1β
iPS	Induced pluripotent stem cells
ICE	Iridocorneal endothelial syndrome
LED	Light-emitting diode
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LM-5	Laminin-5
M	Mole (it is the unit of measurement in the International System of Units (SI) for amount of substance)
mM	Millimole (equals 0.001 moles. It is the unit of measurement in the International System of Units (SI) for amount of substance)
MCECs	Monkey corneal endothelial cells
MDS1	An isoform of PRDM3
MECOM	An isoform of PRDM3
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium chloride
MSCs	Mesenchymal Stem Cells
MSC-CM	Mesenchymal stem cell-conditioned medium
Na^+	Sodium ion
N-cadherins	Neuronal cadherins
NGF	Nerve growth factor
NF-kB	Nuclear factor-kappaB
º	A degree of arc
ºC	Degrees Celsius
Oct 3/4	Octamer-binding transcription factor 3/4
OCT	Optical coherence tomography
p15	Cyclin-dependent kinase 4 inhibitor B
p16	Cyclin-dependent kinase 4 inhibitor 2A
p18	Cyclin-dependent kinase 4 inhibitor C
p19	Cyclin-dependent kinase 4 inhibitor 2D
p21	Cyclin-dependent kinase 2 inhibitor 1

p27	Cyclin-dependent kinase inhibitor 1B
p75 NTR	Neutrophin receptor
PAPCM	Posterior acellular porcine corneal matrix
Pax-6	Paired box protein Pax-6
PBS	Phosphate buffered saline
P-cadherins	Placental cadherins
PCM-DM	Pericellular matrix prepared from human decidua-derived mesenchymal stem cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDL	Poly-d-lysine
PFA	Paraformaldehyde
pH	Potential of hydrogen (it is a numeric scale used to specify the acidity or basicity of an aqueous solution)
PI3K	Phosphoinositide 3-kinase
PPCD	Posterior polymorphous corneal dystrophy
PRDMs	Positive regulatory domain zinc finger proteins
RAFT	Real architecture for 3D tissues
Rb	Retinoblastoma protein (tumor suppressor)
RCFM	Retrocorneal fibrous membrane
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse-transcriptase polymerase chain reaction
+RT	Plus reverse-transcriptase
-RT	Minus reverse-transcriptase
SCGSs	Spherically curved gelatin hydrogel sheets
SD	Standard deviation
SHEM	HEPES-buffered DMEM and Ham's F12 in 1:1 ratio
SLC4A11	Solute carrier family 4, sodium borate transporter, member 11
SMAD	Small body size Mothers against decapentaplegic protein
Sox-2	Sex determining region Y-box 2
S-phase	Synthesis phase of the cell cycle in which DNA is replicated
SPMs	Superparamagnetic microspheres
SV40	Simian vacuolating virus 40
T25	A cell culture flask with a surface area of 25 cm^2
T75	A cell culture flask with a surface area of 75 cm^2
TGF-β	Transforming growth factor-β
TGFBI	Transforming growth factor, beta-induced
TJs	Tight junctions
V	Volt (unit for electric potential)
VE-cadherins	Vascular endothelial cadherins
VEGF	Vascular endothelial growth factor
Wnt-1	Wnt proto-oncogene family member 1
ZO-1	Zona occludens-1

1. CHAPTER 1 – INTRODUCTION

1.1. Chapter overview

This chapter is an introduction to the corneal endothelium (CE), its function, pathological conditions that affect it, the current treatment methods used to manage such conditions, their limitations and research background of the development of a cell-therapy approach as a novel treatment method for corneal endothelial decompensation. It also includes current research advances in corneal endothelial cell culture and transplantation testing, and discusses some of the limiting factors in the development of a cell therapy approach. An outline of the aims of this study is presented at the end of this chapter.

1.2. The human corneal endothelium

The human cornea is the anterior-most part of the eye and being at the forefront of the visual apparatus, it is optically transparent and permeable to light. The cornea has five main layers: the epithelium, the Bowman's layer, the corneal stroma, the Descemet's membrane (DM) and the corneal endothelium (CE). The CE is a monolayer of cells located on the posterior side of the cornea, which separates the corneal stroma from the aqueous humour, and has a barrier and pump function, regulating stromal hydration (See Figure 1).

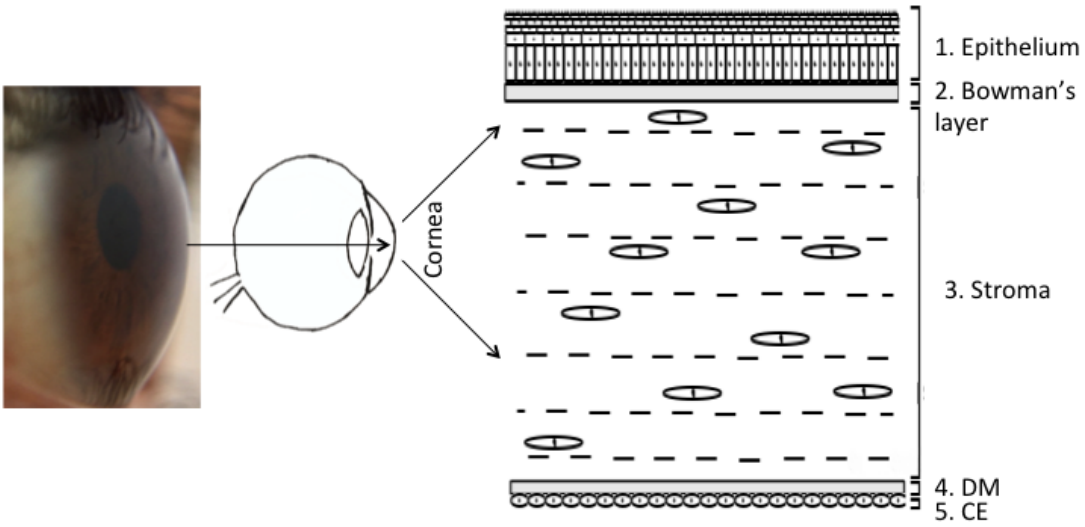


Figure 1 – The cornea is the front part of the eye and consists of five layers: (1) epithelium - the outer-most layer of the cornea that has a protective function; (2) Bowman's layer - serves as a basement membrane or a scaffold for the corneal epithelium; (3) the stroma - the largest corneal layer, responsible for the mechanical robustness of the cornea; (4) the Descemet's membrane (DM) - serves as a scaffold for the corneal endothelium; (5) the corneal endothelium (CE), which acts as a barrier and controls the fluid transport between the stroma and the aqueous humour, and keeps the cornea transparent by regulating the stromal hydration.

1.2.1. Pre-natal and post-natal development of the human corneal endothelium and of the Descemet's membrane

During human embryonic development the CE originates from the neural crest, which itself is derived from the neural tube, mesoderm and surface ectoderm (Hayashi, et al., 1986). Prior to the 8th week of gestation mesenchymal cells are loosely arranged on the posterior surface of the cornea but by the end of the 8th week

they start forming a monolayer and the anterior banded layer of the DM is already in place, and by the 5th month of gestation it significantly increases in thickness and consists of multi-layered collagen lamellae, which by the 8th month of gestation become more compact (Wulle, 1972). The fluid transport mechanism of the CE also develops during the embryonic stage with gap junctions already in place by the 16th gestation week, and it becomes fully functional by the 20th gestation week (Wulle, 1972). The DM is comprised of two layers: during its embryological development the CE secretes and deposits collagen type VIII on the posterior surface of the cornea and that forms the anterior banded layer, while the posterior non-banded layer starts to appear after birth and is composed of collagen type IV (Marshall, et al., 1993). The anterior banded layer could have up to 30-40 layers and has a thickness of about 3.5 μm at birth, but as the posterior non-banded layer appears and grows after birth, the DM size could reach about 12.5 μm by 60 years of age (Murphy et al., 1984b). Corneal endothelial cells (CECs) have the capacity to secrete collagen type IV (Kay, 1989) and the thickness increase of the posterior non-banded layer throughout the lifetime of the individual suggests that the CECs continuously secrete and deposit collagen type IV to form their basement membrane.

The CECs have a hexagonal shape and the structural arrangement of the collagen type IV in the posterior non-banded layer of the Descemet's membrane forms a hexagonal lattice (Jakus, 1956) [See Figure 2]. This hexagonal lattice acts as a scaffold for the corneal endothelial cells and plays a crucial role in the maintenance of corneal transparency (Hassell and Birk, 2010). There appears to be a strong correlation between the DM and the corneal endothelial cell morphology and they

seem to influence each other. For example, Park Kay, et al., (1985) showed that under the influence of polymorphonuclear leukocytes CECs change their morphology to a fibroblastic phenotype and start secreting collagen type I instead of collagen type IV which is responsible for abnormal collagen deposition on the DM (Kay, et al., 1982). On the other hand, it has been demonstrated that the presence of an intact DM supports CEC regeneration after wounding and stimulates the expression of a hexagonal morphology compared to when the DM has been peeled (Chen, et al., 2017a and Soh, et al., 2016, Bhogal, et al., 2017). Interestingly, Hanson, et al., (2017) found that the DM was able to support the differentiation of human embryonic stem cells into corneal endothelial-like cells *in vitro* that expressed Na^+/K^+ ATPase and ZO-1. Very new findings have revealed very interesting insights about the CEC morphology. It appears that the shape of the CECs is different on their apical side facing that aqueous humour in comparison with their basolateral side facing the DM. It has been demonstrated (Hirsch, et al., 1977) and recently confirmed (Harrison, et al., 2016 and Chen, et al., 2017b) that the basolateral side facing the DM has an elaborate multipolar shape whereas the traditional hexagonal shape is only present on the apical surface facing the aqueous humour. These novel discoveries in the shape and morphology of the CE will give new insights in understanding how the CE performs its physiological function and how it interacts with the DM.

All these findings taken together seem to point that the CECs are responsible for the secretion and deposition of the DM all the way from the embryonic development up to adulthood, but the DM is also influencing the CEC morphology, migration,

regenerative capacity and differentiation of the CECs. Ali, et al., (2016) described such a bidirectional relationship between the CECs and the DM and how they affect each other in health and disease.

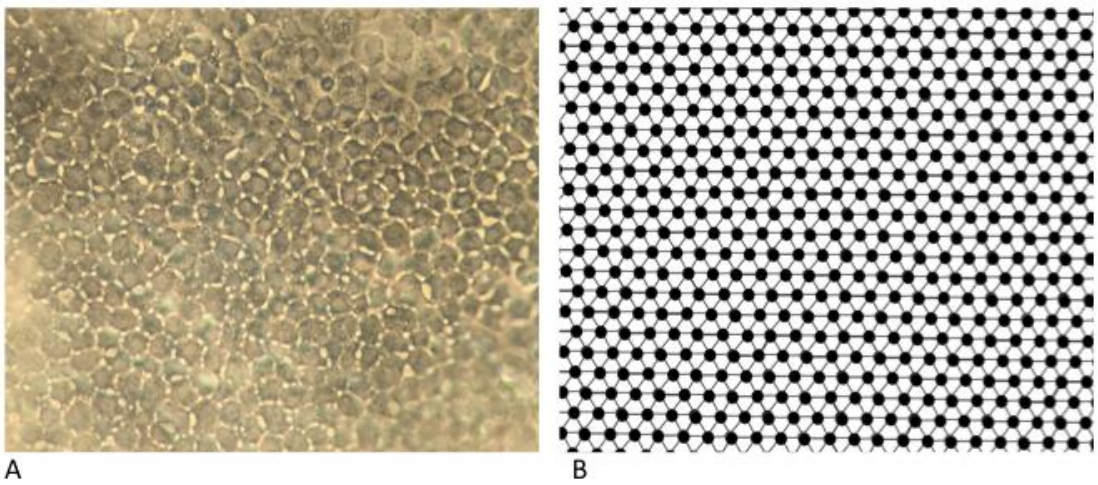


Figure 2 – Morphology of the human corneal endothelial cells [phase-contrast microscopy: x400] (A). The spatial arrangement of collagen type IV in the posterior non-banded layer of the Descemet's membrane resembles a hexagonal lattice, which is secreted by the corneal endothelial cells after birth and serves as an anchorage substrate [Graphical representation] (B).

The human corneal endothelial cells (hCECs) are hexagonal and have a cell density of 3,400 to 5,632 cells/mm^2 in 6 days to 11 months old infants, 2,658 to 3,638 cells/mm^2 in 4-12 years old children, 1,962 cells/mm^2 in a 31-year-old man and 1,864 cells/mm^2 in a 67-year old man (Sherrard, et al., 1987). Capella and Kaufman (1969) found that in infant (0-2.5 years), child (3-15 years), adult (15-65 years) and aged (65+) groups the surface area of the cornea and the cell area are increasing in children, suggesting that the endothelial cell count decrease during childhood is possibly due to the growth of the cornea. This notion has been confirmed in recent

years (Elbaz, et al., 2017). However, there is also some evidence that the CE cell number is decreasing with age from the embryonic stage throughout adulthood due to cell loss independently from the growth in size of the cornea (Murphy, et al, 1984a).

1.2.2. Corneal hydration control

1.2.2.1.CE function: the pump-leak mechanism

Theodor Leber in 1873 first demonstrated that the corneal endothelium controls the flow of fluid into the corneal stroma (Stocker and Reichle, 1974). Later studies on the corneal hydration control confirmed that the corneal endothelium plays the most significant role in the corneal swelling and the control of corneal hydration (Maurice and Giardini, 1951). It has been experimentally demonstrated that the CE resists the uptake of fluid into the stroma through an active-transport mechanism, which is highly dependent on the presence of oxygen (Davson, 1955). Mishima and Kudo, (1967) confirmed that the primary site of active transport is in the endothelium and later it was shown that it is capable of pumping fluid against a head of pressure (Maurice, 1972). In another study it was shown that corneal thickness could be reversed to normal in the presence of adenosine and glutathione and this was dependent on sodium, bicarbonate and oxygen, which suggests that the active pump function of the CE is performed by an adenosine triphosphatase (ATPase) (Dikstein and Maurice, 1972). A later study confirmed the importance of the Na^+/K^+ ATPase for the pump function in human corneas (Geroski, et al., 1984). It is now generally accepted that the CE has an active ion transport activity and regulates the fluid exchange between the corneal stroma and the aqueous humour, controlling the

stromal hydration and corneal transparency (Bourne, 2003). Under normal physiological conditions the human cornea is maintained in a dehydrated state and has a thickness of about 523 ± 39 μm (Martola and Baum, 1968). The pump and barrier function of the CE are highly dependent on the presence of calcium ions (Ca^{2+}) and this barrier can be disrupted by perfusion with Ca^{2+}- free medium, keeping the corneas completely immersed in medium (Eye Bank storage conditions) or perfusion of the cornea at a high pressure (Kaye, et al., 1968 and 1973).

The mechanism of fluid transport regulation is termed the pump-leak mechanism. Ion transport exists both on the apical and on the basal side of the CE. The corneal endothelial cells express a number of voltage-gated ion channels, such as Na^+ channels, K^+ channels, Cl^- channels and Ca^{2+} channels in addition to calcium-permeable transient receptor potential channels (Mergler and Pleyer, 2007). They also express Cl^-/HCO^-_3 exchangers, Na^+/H^+ exchangers, $Na^+/K^+/2Cl^-$ co-transporters, Na^+/K^+ ATPase active pumps (Srinivas, 2010) and aquaporins (Bonanno, 2012). Through these ion transport proteins the CE is able to generate an ion flux that drives the fluid transport from the stroma towards the aqueous humour and nutrients from the aqueous humour to the stroma and epithelium. The Na^+/K^+ ATPase is responsible for the active ion exchange of $3Na^+$ outwards from the cells and $2K^+$ ions inwards and is crucial for normal CE function. It creates an ion flux, which facilitates water movement across the cell membrane through the CE (Bonanno, 2012) and counterbalances the fluid leak, which occurs from the aqueous humour towards the stroma as a result of the stromal swelling pressure (Srinivas, 2010) (See Figure 3).

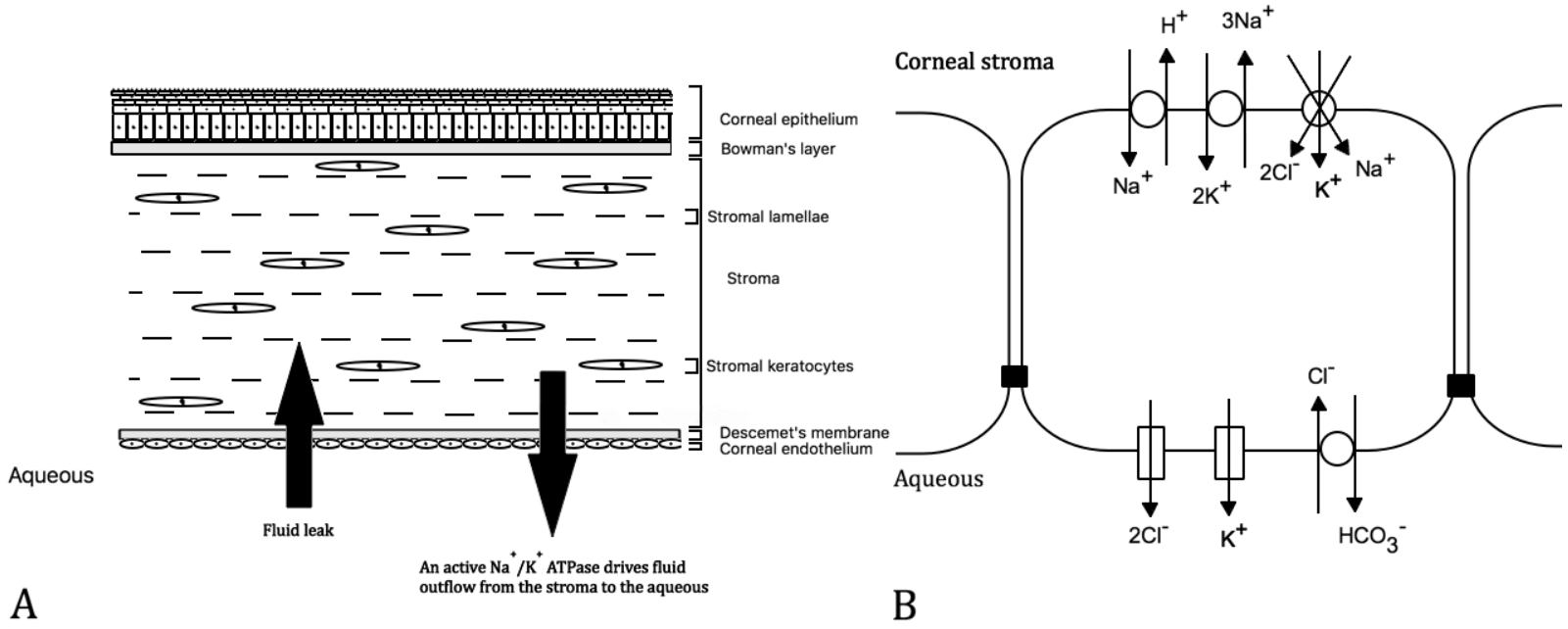


Figure 3 – The pump-leak mechanism of fluid transport through the corneal endothelium (CE): the swelling pressure of the stroma draws fluid from the aqueous humour into the stroma. The CE creates a flux of actively transported ions and the movement of fluid follows the flux of the actively transported ions (A), corneal endothelium ion channels, exchangers, co-transporters and active ion pumps - basal (facing towards the stroma), facing towards the corneal stroma, and apical (facing towards the aqueous), facing towards the aqueous humor (B).

1.2.2.2.Stromal swelling pressure

The stromal swelling pressure drives the fluid leak that occurs from the direction of the aqueous humour towards the stroma. The stromal swelling pressure is the tendency of the stroma to swell as a result of its mechanical composition and is affected by the concentration of proteoglycans (Fischbarg and Maurice, 2004), which play an important role in corneal transparency (Massoudi, et al., 2016). Therefore, in the absence of a functional CE the stroma would swell as a result of the stromal swelling pressure, which equates to 60 mm Hg (Tuft and Coster, 1990). However, since the corneal stroma is sandwiched between the epithelium and endothelium, a negative fluid pressure is created through the active ion transport that counteracts the stromal swelling pressure and causes the corneal thickness to decrease (Dohlman, et al., 1962).

1.2.2.3.Corneal endothelial permeability

The corneal endothelial cells express a number of intercellular junctions, such as tight junctions, adherens junctions, gap junctions, ion transporter proteins and water channels (aquaporins) that enable them to perform their physiological fluid transport function. The intercellular junctions are responsible for mediating cell adhesion, cell-cell communication, and barrier function, and also have a role in the control of proliferation (Zhu, et al., 2008; Zhu, et al., 2012).

1.2.2.3.1.Tight junctions and adherens junctions

Tight junctions (TJs) are connections between the corneal endothelial cells at the apical side and seal the spaces between each individual cell, thus blocking the free flow of fluid. This creates a barrier between the aqueous humour and the stroma and permits the CE to tightly control stromal hydration. The tight junctions are composed of zona occludens-1 (ZO-1), occludins and claudins (Anderson and Itallie, 1995). ZO-1 is an intracellular protein that binds to actin and anchors the cytoplasmic components of the claudins and occludins to the cell cytoskeleton. The claudins and occludins have one cytoplasmic component, four trans-membrane domains and two extracellular loops. The extracellular loops form connections with the claudins and occludins of adjacent cells. Another type of intercellular junction that plays an important role in maintaining the physiological function of the CE is the adherens junction (AJs), which is composed of catenins and cadherins.

Catenins and cadherins act in forming cell-cell adherens junctions by interacting with actin filaments. Cadherins are responsible for Ca^{2+} dependent cell adhesion and cell proliferation and come in several different types that are expressed by the human corneal endothelium *in vivo*, such as neuronal cadherins (N-cadherins), epithelial cadherins (E-cadherins), vascular endothelial cadherins (VE-cadherins), and placental cadherins (P-cadherins) [Zhu, et al., 2008]. Cadherins have a cytoplasmic component, a trans-membrane component and an extracellular component and in the presence of Ca^{2+} they bind to the extracellular component of the cadherins expressed by adjacent cells and form cell-cell connections. The human CE also expresses α-, β-, γ-, p120- and p190-catenins. α-Catenin is a cytoplasmic

protein, which binds to actin and to β-catenin. β-Catenin and p120 bind to the cytoplasmic component of the cadherins. α- and p190-catenins have actin cytoskeleton remodeling function, β- and γ-catenin control Wnt signaling and p120-catenin plays a role in cell-cell adhesion (Zhu, et al., 2008).

1.2.2.3.2.Gap junctions

Gap junctions are clusters of intercellular channels composed of connexin proteins that allow the direct connection of the cytoplasm of two cells, thus enabling cell-cell transfer of ions and small molecules, such as cyclic adenosine monophosphate (cAMP) and calcium (Ca^{2+}) ions, between cells and participate in intercellular signaling (Keven Williams and Watsky, 2002). A type of transmembrane protein called connexin, which forms hexamers or connexons, forms the gap junctions. Two connexons assemble together to form an intercellular channel (Goodenough and Paul, 2009).

1.3. Corneal endothelial disorders leading to corneal endothelial cell decompensation

The corneal endothelium and its clinical implications have been of interest for a long time especially with regards to corneal endothelial degeneration that leads to corneal opacity (Stocker, 1953). The human corneal endothelial cells are generally considered post-mitotic and are not capable of replacing damaged or lost cells via mitosis. Normally humans have enough corneal endothelial cells to last for a lifetime but sometimes CE decompensation might arise due to some factors, such as:

- Previous surgeries, such as cataract surgery that can lead to iatrogenic damage the CE.
- Fuchs' corneal endothelial dystrophy (FCED) and posterior polymorphous corneal dystrophy (PPCD), both of which can be inherited (Klintworth, 2009).
- Iridocorneal endothelial syndrome (ICE).
- Viral infections, such as herpes simplex infections that affect the cornea.
- Injury, such as mechanical injury to the cornea or to the endothelium.
- Raised intraocular pressure as a result of glaucoma or other ocular disease.

The progression of Fuchs' dystrophy is characterized by the appearance of guttae on the posterior side of the Descemet's membrane due to abnormal collagen deposition and by corneal endothelial cell degeneration resulting in loss of barrier and pump function and leading to corneal opacity and blindness. Abnormal collage deposition on the posterior side of the DM usually occurs post-grafting, post-disease or could be post traumatic (Sherrard and Rycroft, 1967a) and is very likely to occur if the corneal endothelium gets damaged (Sherrard and Rycroft, 1967b). In ICE and PPCD extensive damage to the corneal endothelial cells can be observed (Laganowski, et al., 1991). As a result of CE decompensation the cornea may become swollen and oedematous, which leads to corneal blindness. The barrier and pump function of the CE can still be maintained after some degree of cell loss by an increase in cell size (Zavala, et al., 2013). The critical cell density, below which the physiological function of the corneal endothelium starts to fail, is 300-500 cells/mm^2, which results in corneal decompensation and corneal opacity (Tuft and Coster, 1990),

leading to corneal blindness. Corneal endothelial decompensation affects the corneal endothelial cell morphology and triggers abnormal changes in cell size and shape, such as pleomorphism (cell shape variation) and polymegethism (cell size variation). Loss of normal corneal endothelial cell function is usually preceded by abnormal morphological changes and a reduction in cell number in the CE monolayer. In addition to gradual corneal endothelial cell loss, DM thickening also occurs as a result of CE decompensation caused, for example, by Fuchs' dystrophy. Corneal endothelial decompensation may arise from trauma or injury to the CE but also some genetic mutations have been associated with the development of Fuchs' dystrophy, including mutations in genes such as *SLC4A11* (Sodium-mediated transporter, Solute carrier family 4, sodium borate co-transporter, member 11), *TGFBI* (Transforming growth factor, beta-induced, 68kDa), and *COL8A2* (Collagen, type VIII, alpha 2) (Iliff, et al., 2012; Gottsch, et al., 2005a and 2005b). Thickening of the DM and guttae formation as a result of abnormal collagen deposition are caused by Fuchs' dystrophy. However, an additional retrocorneal fibrous membrane (RCFM) on the posterior side of the DM may appear as a result of damage to the CE due to previous eye surgery (Jakobiec and Bhat 2010). In this case of abnormal collagen deposition, the DM itself seems to maintain normal integrity and normal thickness; however, a new collagenous layer, which closely resembles the corneal stroma in its structure but is much thicker than the DM, appears. Corneal endothelial cells that are isolated from RCFM and cultured *in vitro* have a spindle-shaped morphology and secrete collagen type I instead of collagen type IV (Kay, et al.,

1982; Park Kay, et al., 1985; Kay, 1989). The secretion of collagen type I may cause the formation of a RCFM (Leung, et al., 2000).

1.4. In vivo proliferative capacity of the human corneal endothelium

The human CE is generally considered post-mitotic and unable to heal itself through proliferation. However, CE proliferation *in vivo* does not keep pace with cell loss even though growth factors are present in the aqueous humour and on the DM, such as fibroblast growth factor-2 (FGF-2), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF) (Araki-Sasaki, et al., 1977; Joyce, 2003). This unresponsiveness of the CE to growth factors could be attributed to the effect of transforming growth factor-β2 (TGF-β2) present in the aqueous humour, which has been shown to suppress CE proliferation (Chen, et al., 1999) and also inhibits FGF-2-induced corneal endothelial cell proliferation (Lu, et al., 2006). Wound repair in the CE occurs by cell enlargement and migration rather than cell proliferation. Hence treatment of corneal endothelial disorders relies on a corneal transplant.

1.5. Current methods for treating corneal endothelial disorders

The Australian Corneal Graft Registry Report 2015 shows that penetrating keratoplasty is the most common technique for treating corneal endothelial disorders, which also shows the highest success rate for transplant survival (Williams, et al., 2015). However, currently Descemet's Stripping Automated Endothelial Keratoplasty (DSAEK) is the preferred method for treating corneal endothelial disorders since it is less invasive. Some other techniques have emerged such as as micro-thin DSAEK that allows the use of a thinner graft (Thomas, et al., 2013; Melles, et al., 2002) and Descemet's Membrane Endothelial Keratoplasty

(DMEK). These procedures are generally very efficient at restoring normal vision and show very good long-term results (Rajan, 2014). However, all these treatment methods are completely reliant on availability of donor tissue. Due to the donor shortage, which limits the treatment of corneal blindness especially in some parts of the world, a novel approach needs to be developed that could potentially eliminate the need for donor tissue. As a result a cell-based therapy approach has been actively researched as an alternative new treatment. Some non-transplant requiring treatment methods have emerged recently, such as the use of ROCK inhibitor eye drops (Okumura, et al., 2017). More recently, however, some researchers are challenging the notion about the *in vivo* capacity of the CE to regenerate itself and have proposed a non-transplant requiring treatment that only involves surgically removing the diseased central CE and DM and allowing the peripheral CE to renew and regenerate the central part that was removed (Van, et al., 2017).

1.6. Cell-based therapy approach as a novel treatment for corneal endothelial disorders

The use of cultured corneal endothelial cells for transplantation has been explored as early as the1970s and has been shown to be a promising method for restoring normal CE function (Jumblatt, et al., 1978; Gospodarowicz, et al., 1979 and Jumblatt, et al., 1980). However, the clinical application of this technique is limited by the cell source and culture of the cells for provision of cells and the transplantation technique (Maurice, et al., 1981). A number of research groups in recent years have worked towards developing a cell-based treatment method that involves isolating and culturing hCECs *in vitro* and using them for cell transplantation and treatment of

CE disorders (Engelmann, et al., 1999 and Böhnke, et al., 1999, Aboalchamat, et al., 1999, Chen, et al., 2001, Mimura, et al, 2004a, Sumide, et al, 2006, Koizumi, et al., 2007, Patel, et al., 2009, Joyce, et al., 2012, Levis, et al, 2012, Ju, et al, 2012, Zhang, et al., 2014, Okumura, et al., 2014a, Bostan, et al., 2016, Yamaguchi, et al., 2016, Okumura, et al., 2016). This new treatment method has several advantages over the current treatment methods. For example, it is not limited by donor availability and could potentially be less invasive.

There are a number of factors that are currently hindering the development of cell therapy, which include the lack of an established protocol for long-term *in vitro* culture and expansion of hCECs, and suitable experimental models for testing CE transplantation in human corneas. It has been shown that hCECs can enter the cell cycle when cultured *in vitro* using optimized culture environment consisting of culture media supplemented with growth promoting supplements, such as foetal bovine serum (FBS), and growth factors (Joyce, et al., 2003). The main aspects of cell therapy development that current research is mainly focused on include:

- Testing and establishing a protocol for effective CE cultivation.
- Establishing suitable experimental models for testing CE transplantation.
- Optimizing CE transplantation techniques with translational value to future clinical trials.

1.6.1. Human corneal endothelial cell culture as a source for cell-based therapy

Isolation and culture of primary hCECs is considered to be the primary cell source for cell transplantation and cell therapy use, and therefore a lot of focus has been put

into developing an efficient method for successful hCEC isolation and culture in current literature.

1.6.1.1.Human corneal endothelial cell isolation

1.6.1.1.1.Enzymatic dissociation

A number of different enzymes, such as trypsin/EDTA, dispase and collagenase (Engelmann, et al., 1988; Chen, et al., 2001; Ishino, et al., 2004; Li, et al., 2007; Peh, et al., 2011; Choi, et al., 2014), have been used by researchers and compared for their effectiveness in isolating hCECs from human corneas. And the authors have concluded that collagenase shows the best results followed by trypsin/EDTA, therefore these two will be considered as methods of choice for primary hCEC isolation in this study.

1.6.1.1.1.1.From whole corneas without peeling the Descemet's membrane

Engelmann, et al. (1988) reported that the most effective way for isolating the corneal endothelial cells from whole corneas is by first exposing the cells to a high concentration of collagenase (0.5 %) for 90 min followed by exposure to a low collagenase concentration (0.04 %) for an additional 1020 min (17 hours) but this method seemed to produce contamination with other corneal cell types, such as stromal keratocytes.

1.6.1.1.1.2.Peel-and-digest method

More recently researchers have used the so called peel-and–digest method for hCEC isolation, which involves peeling the Descemet's membrane/Endothelium complex first, then isolating the cells by enzymatic dissociation (Li, et al., 2007; Ishino, et al.,

2004; Chen, et al., 2001; Peh, et al., 2011). Peh, et al. (2013) suggested that the initial seeding density is a very important factor for successful culture and sub-culture of hCECs, and indicated that a seeding density of 10,000 cells/cm^2 yields best results for initiating primary hCEC cultures when using the peel-and-digest method.

1.6.1.1.2.Primary explant culture

Primary explant culture has also been utilized for hCEC isolation and culture by Choi, et al. (2014) and Walshe and Harkin (2014) reported that serial explant culture might be a suitable method for isolating corneal endothelial progenitor cells, consequently it could be used for isolation of corneal endothelial progenitor cells.

1.6.2. Proliferative capacity of cultured human corneal endothelial cells

Corneal endothelial cells are arrested in Gap 1 (G1) phase of the cell cycle and, therefore, do not replicate *in vivo* (Joyce, et al., 1996). This is due to contact inhibition, lack of effective growth factor stimulation and TGF-β2 suppression of S-phase entry (Joyce, 2012). Both TGF-β and contact inhibition mediate their effect through the action of cyclin-dependent kinase inhibitors p15, p16, p18, p19, p21 and p27 and lead to the degradation of cyclin D and cyclin E thus preventing the cell to progress to S phase of the cell cycle [See Figure 4].

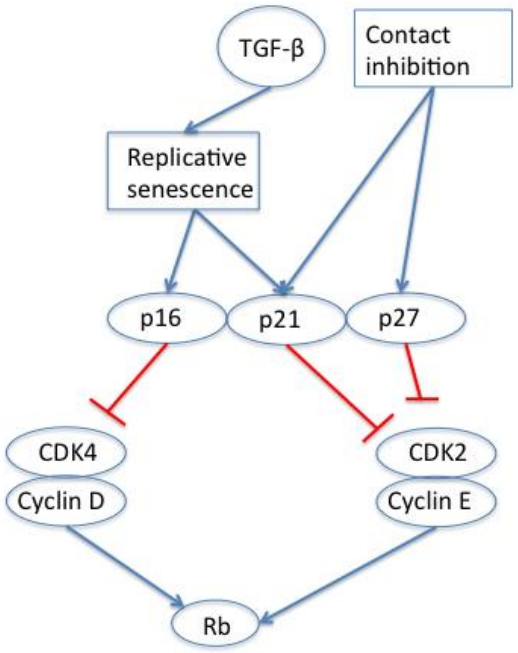


Figure 4 – The human corneal endothelial cells are arrested in Gap 1 (G1) phase through the action of transforming growth factor-β (TGF-β) present in the aqueous humour and also as a result of cell-cell contact, which causes contact inhibition. TGF-β causes replicative senescence through cyclin-dependent kinase inhibitors p16 and p21, which in turn inhibit cyclin dependent kinase 2 and 4 (CDK2 and 4) and trigger cyclin D and cyclin E degradation. Contact inhibition has a similar effect and causes CDK2 inhibition via p21 and p27 resulting in cyclin E degradation. This causes human corneal endothelial cells to be unable to complete the cell cycle and progress to the synthesis phase (S-phase) in which DNA is replicated.

Joyce and Harris (2010) also demonstrated that p16 and p21 participate in the negative regulation of the cell cycle in cultured human corneal endothelial cells and cause senescence in culture. However, research shows that when using optimized culture conditions, corneal endothelial cells can be stimulated to proliferate in

culture to some extent (Joyce and Zhu, 2004). A number of growth factors and culture media supplements have been used for hCEC culture such as:

- Fibroblast growth factor-2 (FGF-2) – 10, 12.5, 200 ng/ml (Rieck, et al., 1995; Nayak and Binder, 1984; Senoo and Joyce, 2000a)
- Epidermal growth factor (EGF) - 6.25 - 50 ng/ml (Nayak and Binder, 1984; Engelmann and Friedl, 1989; Senoo and Joyce, 2000a)
- Endothelial cell growth supplement (ECGS) – 25 ng/ml, 12.5 μg/ml (Nayak and Binder, 1984; Engelmann and Friedl, 1989)
- Transforming growth factor-β1 (TGF-β1) – 1 ng/ml (Rieck, et al., 1995)
- TGF- β1 + FGF-2 (Rieck, et al, 1995)
- Chondroitin sulfate (13.5 – 25 mg/ml) (Yue, et al., 1989)
- Foetal bovine serum (FBS) (Engelmann and Friedl, 1989; Senoo and Joyce, 2000a)
- High-calcium (Li, et al., 2007)
- L-Ascorbic acid 2-phosphate (Shima, et al., 2011)
- Hepatocyte growth factor (HGF) (Shima, et al., 2011)
- Laminin-5 (Yamaguchi, et al., 2011)

Engelmann and Friedl (1989) performed clonal growth assays using cells (passages 3 – 9) isolated from a 48-year-old donor cornea and (passages 6 – 10) from a 30-year-old donor cornea. In the same study the researchers tested the effect of a number of culture medium supplements and mitogens on hCEC proliferation such as: FBS, human serum, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), endothelial cell growth supplement (ECGS), nerve growth factor

(NGF), TGF-β and FGF-2. Out of all these they found that human serum and FBS gave the best results. Joyce and Zhu (2004) also reported that EGF had a stimulatory effect on hCEC proliferation, although NGF did not have any effect. Senoo and Joyce (2000a) reported that and ethylenediaminetetraacetic acid (EDTA) had a stimulatory effect on proliferation as well as FGF-2 (Joyce, 2003), however, FGF-2 seems to be also capable of causing a morphological change to a spindle-shaped morphology in CECs (Gu, et al., 1996), whereas TGF-β had only a negative effect on proliferation (Chen, et al., 1999). Therefore, based on the above-mentioned studies FBS or human serum will be used as an essential culture medium supplement for hCEC culture based on availability.

1.6.3. Human corneal endothelial cell culture conditions

Optimized culture conditions are another very important aspect of primary hCEC culture. Basal culture media that are most commonly used include MEM (Blake, et al., 1997), DMEM (Ishino, et al., 2004), F99 (Ham's F12/Medium 199 in a 1:1 ratio) (Engelmann and Friedl, 1989), Opti-MEM-I (Joyce and Zhu, 2004), SHEM (Li, et al., 2007), EGM-2 (Choi, et al., 2010) supplemented with foetal bovine serum, growth factors and various culture media supplements (See Table 1).

Table 1 - Media used for culture of corneal endothelial cells.

Primary hCECs	Cornea storage conditions	Culture media	Culture media supplements	Substrate used	Reference
Human	Not specified	L-Valine free formulation of F99	5% FBS, 20 µg/ml ascorbic acid, 20 µg/ml bovine insulin, 2.5 µg/ml transferrin, 0.6 ng/ml sodium selenite, 10 ng/ml bFGF	0.5-1mg/ml collagen IV, 10µg/ml laminin, 15µg/ml fibronectin, 0.01% gelatin, 1% chondroitin sulfate	Engelmann and Friedl, 1989; Engelmann, et al., 1995
Human	Optisol GS	DMEM	10% FBS, 2 ng/ml bFGF, 50 U/ml penicillin, 50 µg/ml streptomycin	Collagen IV	Ishino, et al., 2004
Human	Optisol GS at 4ºC	Opti-MEM-I	8% FBS, 20 ng/ml NGF, ng/ml EGF, 20 µg/ml ascorbic acid, 200 mg/L calcium chloride, 100 µg/ml pituitary extract, 50 µg/ml gentamicin, 1 x antibiotic/antimycotic , 0.08% chondroitin sulphate	FNC coating mix	Joyce and Zhu, 2004
Human	Optisol at 4ºC	SHEM	0.5 % DMSO, 2 ng/ml EGF, 5 µg/ml insulin, 5 µg/ml transferrin, 5 µg/ml selenium, 0.5 µg/ml hydrocortisone, 1 nM cholera toxin, 50 µg/ml gentamicin, 1.25 µg/ml amphotericin B	No coating	Li, et al., 2007
Human	Optisol	Opti-MEM-I, ESC-CM	25% ESC-CM, 8 % FBS, 40 ng/ml FGF, 5 ng/ml EGF, 20 ng/ml NGF, 20 µg/ml ascorbic acid, 0.005% human lipids, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 1% RPMI-1640 multiple vitamin solution, 50 µg/ml gentamicin, and antibiotic/antimycotic solution (1:100)	None	Lu, et al., 2010
Human	Not specified	DMEM (low glucose), DMEM	1. 15% FBS, 2.5 mg/L amphotericin B, 2.5 mg/L doxycycline, 2 ng/ml bFGF 2. Serum free	1. BCEC-ECM 2. LM-5	Yamaguchi, et al, 2011
Human (Fuchs' dystrophy)	Optisol-GS on ice	OptiMEM-I	8% FBS, 5ng/ml EGF, 20ng/ml NGF, 20µg/ml ascorbic acid, 0.08% CS, 100 IU/ml penicillin.	FNC coating mix	Zaniolo, et al., 2012
Human	Not	F99	5% Fetal calf serum (FCS), 100 µM sodium L-	5 mg/ml	Sheerin, et al., 2012

	specified		ascorbate, 20 µg/ml bovine insulin, 2.5 µg/ml transferrin, 40 pM sodium selenite, 10 ng/ml bFGF	chondroitin sulfate and 5 µg/ml mouse laminin	
Human	Optisol-GS at 4ºC	F99	5 % FBS, 20 µg/ml ascorbic acid, 1x Insulin, transferrin, selenium, 1x anti-biotic/anti-mycotic and 10ng/ml bFGF	FNC coating mix	Peh, et al., 2013
Human	Optisol at 4ºC	MSC-CM	8 % FBS, 5ng/ml EGF, 20 µg/ml ascorbic acid, 200 mg/L calcium chloride, 0.08 % CS, 50 µg/ml gentamicin	FNC coating mix	Nakahara, et al., 2013
Human	Not specified	EGM-2, DMEM	1. 10 % FBS, EGF, VEGF, FGF, IGF, hydrocortisone, gentamicin, amphotericin-B; 2. 10 % FBS, FGF;	Collagen type IV, Fibronectin, FNC coating mix	Choi, et al., 2014
Primate	Not specified	DMEM	10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 ng/ml FGF-2	FNC coating mix	Okumura, et al., 2013
Bovine	N/A (fresh corneas used)	SHEM	5% FBS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 50 unit/ml penicillin, 50 µg/ml streptomycin, 250 ng/ml amphotericin B, 0.5% DMSO, 2 ng/ml (human epidermal growth factor) hEGF, 1nM cholera toxin	No substrate used	Chou, et al., 2014

Note: Foetal bovine serum (FBS); Epidermal growth factor (EGF); Nerve growth factor (NGF), Chondroitin sulfate (CS); Dimethyl sulfoxide (DMSO); fibronectin-collagen coating mix (FNC); Mouse embryonic stem cell conditioned medium (ESC-CM); bone marrow-derived mesenchymal stem cell conditioned medium (MSC-CM); Dulbecco's modified Eagle's medium (DMEM); Endothelial growth medium-2 (EGM-2); HEPES-buffered DMEM and Ham's F12 in 1:1 ratio (SHEM); Mouse embryonic stem cell-conditioned medium (ESC-CM); Descemet's membrane (DM); Bovine corneal endothelial cell-derived extracellular matrix (BCEC-ECM); Laminin-5 (LM5); basic fibroblast growth factor (bFGF).

1.6.3.1.Stem cell conditioned media

Bone marrow-derived mesenchymal stem cell conditioned medium (MSC-CM) (Nakahara, et al., 2013) and mouse embryonic stem cell conditioned medium (ESC-CM) (Lu, et al., 2010) have also been used in hCEC culture, showing very good results in stimulating proliferation. Cultured hCECs using MSC-CM maintain expression of CE functionality markers, Na^+/K^+ ATPase and ZO-1 suggesting that the cells still maintain normal corneal endothelial cell pump function. This culture medium was shown to mediate its positive effect on hCEC proliferation through the downstream activation of PI3K/Akt signaling that leads to p27 and p21 degradation and cyclin D1 activation and cell cycle progression to S-phase. This signaling pathway has been shown to play a role in CEC proliferation in other studies as well (Lee and Kay, 2003). Therefore, MSC-CM was chosen for use in this study also because it seemed to yield better results for primary culture initiation compared to F99 with 10% FBS. However, research results are still inconclusive and currently the search for the best-optimized culture medium condition for hCEC culture is still ongoing. However, a new study more recently compared the effect of bone marrow-derived endothelial progenitor cell conditioned medium, bone marrow-derived mesenchymal stem cell conditioned medium (MSC-CM) and corneal stromal cell conditioned medium and found that the corneal stromal cell conditioned medium had the best effect on rat corneal endothelial cells (Zhu, et al., 2016). It has also been revealed that the stromal keratocytes release a range of substances including growth factors/cytokines, extracellular matrix (ECM) components, and kinases, which support normal corneal development and

homeostasis (Zhang, et al., 2017). These are very important findings and suggest that the corneal stromal keratocytes play an extremely important role in supporting the CE and could give new insights for optimizing hCEC culture introducing stromal keratocyte conditioned medium or feeder layers.

1.6.3.2.Substrates used in human corneal endothelial cell culture

The extracellular matrix plays a very significant role in the cell shape and proliferation of different cell types including the corneal endothelium (Gospodarowicz, et al., 1978). Therefore, when endeavouring to optimize culture conditions for hCEC culture, many researchers have examined the effect of different extracellular matrices, substrates and culture dish coating substrates, including:

- Different culture dish plastic (Li, et al., 2007; San Choi, et al., 2013; Yamaguchi, et al, 2011)
- Bovine corneal endothelial cell-derived extracellular matrix (BCEC-ECM) (Engelmann and Friedl, 1989; Blake, et al., 1997)
- Chondroitin sulfate (CS) (Engelmann and Friedl, 1989)
- Laminin (LM) (Engelmann and Friedl, 1989; San Choi, et al., 2013)
- CS/LM mix (Engelmann and Friedl, 1989)
- Fibronectin (FN) (Engelmann and Friedl, 1989; San Choi, et al., 2013)
- Gelatine (Engelmann and Friedl, 1989)
- Collagen type IV (Engelmann and Friedl, 1989; San Choi, et al., 2013; Sumide, et al., 2006; Choi, et al., 2014)
- Fibronectin-collagen (FNC) coating mix (Zhu and Joyce, 2004; Engler, et al., 2009; San Choi, et al., 2013)

- Laminin-5 (Yamaguchi, et al., 2011)
- Collagen type I (Engler, et al., 2009; San Choi, et al., 2013; Gruschwitz, et al., 2010)
- Collagen type I/Fibronectin mixture (Engler, et al., 2009)

A study by Engelmann, et al. (1988) compared the clonal growth of hCECs seeded on different substrates: plastic, laminin, laminin/chondroitin sulphate mix, chondroitin sulphate and gelatine. Laminin/chondroitin sulfate coating was reported to be yielding the highest clonal growth of the hCECs. Moreover, a later study by Engelmann and Friedl, 1989 demonstrated that BCEC-ECM greatly surpassed all other substrates tested in its effect on proliferation, including gelatine, chondroitin sulfate, laminin/chondroitin sulfate mix, fibronectin and collagen type IV in its stimulatory effect on proliferation. In addition, the proliferation effect on hCECs was very significantly enhanced when each substrate was used in combination with fibroblast growth factor (FGF) supplemented medium. Choi, et al. (2014) tested and compared the effect of collagen type IV, fibronectin and fibronectin-collagen mix (FNC) on the effective establishment of primary corneal endothelial cell cultures and reported that there was no statistically significant difference between them. However, the FNC mix showed an observable trend for the highest success rate in primary culture initiation. Numata, et al. (2014) demonstrated that collagen type IV, collagen type I, and fibronectin coating substrates did not have any significant growth-promoting effect on CECs. However, pericellular matrix prepared from human decidua-derived mesenchymal stem cells (PCM-DM) showed a significant effect. Based on the findings from the above-mentioned studies, BCEC-ECM and

PCM-DM seem to have the highest capacity to stimulate proliferation of CECs in culture. However, research results remain inconclusive and the search for the ideal substrate for use in hCEC culture is still ongoing.

1.6.3.3.Rho-associated protein kinase inhibitor Y-27632 in human corneal endothelial cell culture

Okumura, et al. (2009) were the first to use a ROCK inhibitor Y-27632 as a corneal endothelial cell survival and attachment enhancer. Rho-associated protein kinase signaling pathway is known to affect cell adhesion and plays a role in actin cytoskeleton remodeling and stress fiber formation and is involved in the cell cycle control. Signals from the extracellular matrix (ECM) and ligands activate the Rho kinase via integrins, receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs) and downstream activation of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The Rho kinase pathway regulates cell mobility, stress fiber formation and cell cycle progression (Schwartz, 2004).

ROCK inhibitor Y-27632 has a positive effect on corneal endothelial cell yield in culture (Okumura, et al., 2014a) and facilitates transplantation (Okumura, et al., 2012). Y-27632 mediates its effect through the PI3K and up-regulates cyclin D expression, in addition to up-regulation of cell division cycle 25A (CDC25A) enabling cyclin D/cyclin-dependent kinase 4 (CDK4) and cyclin E/cyclin-dependent kinase 2 (CDK2) complexes to inactivate retinoblastoma (Rb) and p27, thus activating E2F leading to progression to S phase of the cell cycle (Okumura, et al., 2014a). This furthermore confirms the importance of PI3K signaling as an important

pathway that controls human corneal endothelial cell proliferation. In addition to its positive effect on cell proliferation Yamamoto, et al. (2012) found that it also protects the expression of normal CE morphology. Another study by Pipparelli, et al. (2013) demonstrated, however, that Y-27632 did not stimulate hCEC proliferation but could enhance cell attachment.

1.6.3.4.Negative factors affecting human corneal endothelial cell proliferation

1.6.3.4.1.Contact inhibition

The corneal endothelial cells form a monolayer in which all cells are very closely packed together and retain close cell-cell contact, which induces contact inhibition and stops the corneal endothelial cells from proliferating (Joyce, et al., 2002; Joyce, 2003). Disruption of cell-cell contact by EDTA has been shown to promote hCEC proliferation, especially from old donors (Senoo and Joyce, 2000b). Therefore, this suggests that using enzymatic dissociation as a method for corneal endothelial cell isolation may represent a suitable technique for breaking the cell-cell contacts. Releasing the cells from contact inhibition prior cell culture potentially makes them more susceptible to the effect of growth promoting molecules present in the culture medium.

1.6.3.4.2.Transforming growth factor-β2

Another factor that has experimentally been demonstrated to negatively affect corneal endothelial cell proliferation is TGF-β2 (Joyce, 2012). TGF-β signaling is a major pathway that controls corneal endothelial cell morphology and proliferation

and mediates its effect through SMAD proteins. TGF-β or bone morphogenic proteins (BMPs) stimulation can activate a number of downstream signaling cascades including Rho signaling, phosphoinositide-3 kinase/Akt (PI3K/Akt) signaling and extracellular signal-regulated kinase 1/2 (Erk1/2) signaling all of which have been shown to play a significant role in the control of proliferation in human corneal endothelial cells. Joyce and Zieske (1997) demonstrated the expression of TGF-β receptor in human corneal endothelial cells. TGF-β2 was found to be a mitotic inhibitor for corneal endothelial cells (Chen, et al., 1999). In the aqueous humour the presence of TGF-β2 results in suppression of corneal endothelial cell proliferation and G1 phase arrest. Exogenous TGF-β2, when added to cultured corneal endothelial cells, has the ability to suppress their proliferation (Chen, et al., 1999). Zhu, et al. (2012) demonstrated that TGF-β1 could induce a morphological change in human corneal endothelial cells and cause them to acquire a spindle-shaped phenotype. Additionally, TGF-β1 stimulates the expression of α-smooth muscle actin (α-SMA) by cultured hCECs, which is a differentiation marker of myofibroblasts (Zhu, et al., 2012). TGF-β is another potential target for further research on primary human corneal endothelial cell proliferation and some TGF-β receptor inhibitors seem to preserve the normal corneal endothelial cell morphology in culture (Okumura, et al., 2013).

1.6.3.4.3.Age

In a study using hCEC *ex vivo* culture, Choi, et al., (2014) indicated that age and hCECs isolation methodology most directly affect the quality of the cells grown. The results of this study further show that primary cells isolated from donors below

the age of 30 have the highest rate of culture success and the highest potential to reach confluence and be sub-cultured. However, other studies, such as Zhu and Joyce (2004) successfully cultured human corneal endothelial cells isolated from both young and old donors on a 6-well plate pre-coated with undiluted FNC Coating Mix. Up to 4 passages were attained, using basal media OptiMEM-I supplemented with 8% FBS, EGF 5 ng/mL, NGF 20 ng/mL, pituitary extract 100 μg/mL, ascorbic acid 20 μg/mL, calcium chloride 200 mg/L, 0.08% chondroitin sulfate, gentamicin 50 μg/mL, and antibiotic/antimycotic solution diluted 1:100 demonstrating that the current culture media confirmations do not allow continuous human corneal endothelial cell culture beyond several passages. This poses a limitation to the development of a cell-therapy approach for treating corneal endothelial disorders. In order to tackle this limitation of inadequate proliferative capacity of the human corneal endothelial cells some researchers have attempted to identify and isolate a human corneal endothelial progenitor cell with high proliferation potential.

1.6.4. Human corneal endothelial progenitor cells

The peripheral corneal endothelium has been the subject of much interest as potentially harbouring corneal endothelial progenitor cells that are capable of proliferation and renewal of the corneal endothelium. Such cells could prove to be very useful for culture and expansion due to their ability to proliferate, and could potentially be used to generate cells for transplantation and cell-based treatment of corneal endothelial disorders.

Some studies have successfully demonstrated that the corneal endothelial cells from the periphery retain mitogenic activity, whereas the corneal endothelial cells from

the central cornea do not (Bednarz, 1998). Konomi, et al. (2005) found that the cells from both these regions retain proliferative capacity. However, the peripheral region showed a higher capacity for proliferation. On the other hand, another study by Choi, et al. (2014) comparing the central and peripheral endothelium had a better success rate of primary cell culture initiation from the central region. Whikehart, et al. (2005) reported that in the periphery of the cornea, some cells that were undergoing mitosis, especially after wounding, migrated towards the wound site as demonstrated by 5-bromo-2-deoxyuridine (BrdU) incorporation. Some researchers have tried to locate a potential stem cell niche and found that stem cell markers, such as nestin, telomerase, octamer-binding transcription factor 3/4 (Oct 3/4), Wnt proto-oncogene family member 1 (Wnt-1), Paired box protein Pax-6 (Pax-6), Sex determining region Y-box 2 (Sox-2), are expressed after wounding and removal of the central corneal endothelium within the regions of the Schlemm's canal, trabecular meshwork, transition zone and peripheral endothelium, after wounding of organ cultured corneas (McGowan, et al., 2007).

1.6.4.1.Isolation and culture of human corneal endothelial progenitor cells

A number of research groups have reported successful isolation and cultivation of hCEC progenitor cells as a potential cell source that could be cultured more easily compared to normal mature hCECs. These cells have been isolated based on neural crest and stem cell marker expression, high proliferative capacity and sphere-forming properties.

Yokoo, et al. (2005) found that within the CE there are cells with sphere-forming capacity that express neural crest and mesenchymal markers - nestin and α-smooth

muscle actin (α-SMA) - and were able to isolate and culture them. In another study Hara, et al. (2014) isolated hCECs that expressed p75NTR (neurotrophin receptor), a neural crest marker, which had a higher proliferative capacity compared to other hCECs that did not express p75NTR (neurotrophin receptor). These cells were cultured as corneal endothelial progenitors, which could potentially be used for cell transplantation. Hirata-Tominaga, et al. (2013) reported that leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), a stem cell marker, is exclusively expressed in the periphery of the CE. The LGR5-expressing cells had very high proliferative capacity and could be cultured *in vitro* more easily compared to hCECs, which did not express LGR5.

Current research on identifying and culturing human corneal endothelial progenitor cells is still ongoing since there is a huge demand in the field for effective culture of primary human corneal endothelial cells. Positive regulatory domain zinc finger proteins (PRDMs) have been shown to regulate proliferation, differentiation and senescence of haematopoietic stem cells, embryonic stem cells (ESC) and induced pluripotent stem cells (iPS) (Fog, et al., 2012; Zhang, et al., 2011; Kinameri, et al., 2008) and their expression and role in the human cornea has not been well studied with the exception of PRDM5 that was reported to be involved in extracellular matrix development (Burkitt Wright, et al., 2011). Therefore, it would be of great interest to explore the role of these proteins in human corneal endothelial cell proliferation and stem cell properties. In this study a complete expression profile of PRDMs was generated and demonstrated that a number of PRDMs are expressed in human corneas and cultured human corneal endothelial cells. This unlocks

possibilities for future studies of the roles and function of these proteins in the control of proliferation and stem cell capacity in the human corneal endothelial cells and can potentially lead to finding new ways of enhancing corneal endothelial cell culture that can be used in cell-therapy approach for treating corneal endothelial disorders.

1.7. Current methods used for testing corneal endothelial cell transplantation

Human corneal endothelial cell transplantation involves isolating and culturing primary hCECs from human donor corneas and using them for transplantation. In the most current published literature the preferred isolation method of corneal endothelial cells is the peel-and-digest method where the Descemet's membrane/Endothelium complex is peeled and the cells are enzymatically dissociated prior to primary culture initiation (Peh, et al., 2011 and 2015b; Okumura, et al., 2016). Basal culture media, such as DMEM (Okumura, et al., 2016), F99 (Ham's F12/Medium 199 in a 1:1 ratio) [Peh, et al., 2015b] and OptiMEM-I (Nakahara, et al., 2013) are currently being used for propagation of corneal endothelial cells in combination with FNC coating mix. ROCK inhibitor Y-27632 has also been used and studied extensively in recent years and has shown some potential for enhancing corneal endothelial cell adhesion and culture (Okumura, et al., 2012; Okumura, et al., 2016; Pipparelli, et al., 2013 and Peh, et al., 2015b). The most commonly associated signaling pathway involved in inducing human corneal endothelial cell proliferation through growth factor and ROCK inhibitor stimulation is the PI3K signaling pathway with downstream activation of cyclin D and Erk 1/2

(Nakahara, et al., 2013; Okumura, et al., 2014a). Therefore, in this study F99 and Opti-MEM I (used as a basal medium for the preparation of MSC-CM), were used for culture of immortalized and primary hCECs and all primary hCECs were isolated using the peel-and-digest method.

Two of the most common methods for hCEC transplantation include: 1) cell suspension transplantation, where a suspension of corneal endothelial cells is being injected into the anterior chamber of the eye and 2) carrier substrate transplantation, where the hCECs have been pre-cultured on a carrier substrate and are delivered to the anterior chamber of the eye via this carrier substrate. Human corneal endothelial cell transplantation has been the subject of investigation by many researchers in recent years using a number of *in vivo* and *in vitro* models.

1.7.1. In vivo animal models for corneal endothelial cell transplantation

In order to study and test the potential of cell-based therapy to restore corneal endothelial cell function, a suitable model is needed that allows the assessment of the post-cell therapy outcomes and determines the safety of the procedure. This would allow researchers to accurately compare the effects of cell therapy on the corneal thickness and corneal transparency to the effects of CE decompensation and enable them to test whether cell therapy is capable of restoring normal CE function and normal corneal thickness and transparency to corneas that have previously had CE decompensation.

Han, et al. (2013) described a methodology for establishing an *in vivo* mouse model of corneal endothelial decompensation using cryoinjury. The researchers used the corneal thickness as a surrogate measure of corneal endothelial function and

measured it using anterior segment optical coherence tomography (OCT) over a 21-day period. Abnormally high corneal thickness in this case would testify for an abolished corneal endothelial cell function. This swollen phenotype can therefore easily be compared to cell-therapy treated animals. Comparison of the corneal thickness between the treated and non-treated eyes in this case would give evidence of restored corneal endothelial cell function.

Most of the current research on corneal endothelial cell-therapy approach has been done using animal models (See Table 2). In experimental research the successful outcome of cell therapy could be evaluated based on several factors including:

1) Morphology of the cultured corneal endothelial cells (CECs), observed by phase-contrast microscopy prior to transplantation.
2) CE functionality marker expression (Na^+/K^+ ATPase and ZO-1).
3) Corneal transparency, observed by a slit lamp microscope post-transplantation.
4) Central corneal thickness (CCT), measured by ultrasound pachymetry post-transplantation.
5) Cell morphology (hexagonal) relative to the normal *in vivo* CE morphology, observed *in vivo* by noncontact specular microscopy and electron microscopy post-transplantation.
6) Histological observation of the CE post-transplantation.

Therefore, a successful outcome of corneal endothelial cell transplantation could be determined by an effective recovery of normal corneal endothelial pump function evidenced by restoration of corneal transparency and normal corneal thickness.

Table 2 – *In vivo* animal models for corneal endothelial cell transplantation reported in literature.

In vivo animal model	Cells used for transplantation	Substrate used as a carrier for cell transplantation	Transplantation technique	Outcome	References
Rabbit	Rabbit corneal endothelial cells – 2-5x10^3 cells/ml	Rabbit corneal button with intact Descemet's membrane (DM)	Full-thickness corneal button graft	19 clear grafts – maintained transparency up to 30 days.	Jumblatt, et al., 1978
Rabbit	Bovine corneal endothelial cells – 1.5x10^6 cells	Rabbit corneal button with intact DM	Full-thickness corneal button graft	Clear grafts for over 100 days	Gospodarowicz, et al, 1979
Rabbit	Human corneal endothelial cells (hCECs) - 6x10^3 cells/mm^2	Denuded amniotic membrane (AM) was placed on rabbit corneal buttons - no DM	Full-thickness corneal button/AM graft	Transparent grafts with little oedema 4 days post-transplantation	Ishino, et al, 2004
Rabbit	hCEC cell sheet harvested from temperature-responsive dishes – 3x10^6 cells	Rabbit central corneal button	Corneal button/cell sheet graft	Thin and clear corneas up to 7 days after transplantation	Sumide, et al, 2006
Rabbit	Rabbit corneal endothelial cells – 1x10^7 cells/ml	No carrier substrate	Cell seeding directly on native DM then encapsulated with self-cross-linked composite hydrogel formed in situ	Clear grafts up to 60 days after transplantation	Liang, et al, 2011
Rabbit	CEC-like cells from ESC - 2x10^5 cells/mm^2 for 4h	Posterior acellular porcine corneal matrix (PAPCM lamella) with DM	CEC-like cells/PAPCM transplantation	Corneal thinning up to 28 days	Zhang, et al., 2014

Rabbit	Rabbit corneal endothelial cells – $2x10^5$ cells in 200µl DMEM	No carrier substrate	Cell injection +/- 100 µM Y-27632 ROCK inhibitor directly on native DM	Cell injection with Y-27632 produced corneal transpatency	Okumura, et al., 2012
Rabbit	hCECs – $1x\ 10^6$ cells in 300µl DMEM	Collagen type I sheet coated with fibronectin	Transplantation of collagen sheet	Restored corneal transparency	Mimura, et al., 2004b
Rabbit	hCECs	Collagen sheet with or without Viscoat® (seeded with 40,000 cells/mm^2 for 3 weeks)	DM removed. Collagen sheet transplantation	Viscoat® improved cell viability of the transplanted hCECs	Yamaguchi, et al., 2016
Rabbit and Primate	Corneal endothelial cell-like cells from skin-derived precursors and B4G12 - $2.0x10^5$ or $4.0x10^5$ cells	No carrier. The DM was left intact to serve as a substrate for the injected cells.	Cell injection in 100 µl or 50 µl human endothelial serum-free medium supplemented with 3.2 µg or 1.6 µg of Y-27632	Restored corneal transparency	Shen, et al., 2017
Primate	Monkey corneal endothelial cells	Collagen type I - $5\text{-}10x10^2$ cells/mm^2 or No carrier substrate– $8x10^4$ cells in 50µl;	Collagen carrier inserted and attached to DM by air injection or Cell suspension injection to native DM	Collagen carrier/MCECs maintained corneal transparency up to 6 months after surgery; Cell injection did not produce corneal transparency;	Koizumi, et al, 2007
Primate	Monkey corneal endothelial cells – $2x10^5$ cells in 200µl DMEM	No carrier substrate	Cell injection +/- 100µM Y-27632 ROCK inhibitor directly on native DM	With or without Y-27632 corneal transparency was achieved	Okumura, et al., 2012
Primate	Monkey corneal endothelial cells - $5x10^5$ cells in 200µl	No carrier substrate	Cell injection +/- 100µM Y-27632 ROCK inhibitor directly on	The presence of ROCK inhibitor promoted corneal transparency	Okumura, et al., 2016

	DMEM; hCECs - $5x10^5$ cells in 200µl OptiMEM-I;		native DM		
Primate	Monkey corneal endothelial cells	Spherically curved gelatin hydrogel sheets (SCGSs)	Transplantation of sheet (4mm of central DM removed)	Restored corneal transparency	Kimoto, et al., 2014
Feline	Bovine corneal endothelial cells – $1x10^5$ cells/60µl	Feline corneal button – no DM	Full-thickness penetrating keratoplasty	Thin and transparent grafts. Immune host response by 9^{th} postoperative day	Bahn, et al, 1982
Feline	Feline corneal endothelial cells – 250,000-285,000 cells per cornea	Devitalized human corneas – with DM	Full-thickness corneal transplantation	Thin and transparent grafts 7 days after grafting	Proulx, et al, 2009
Feline	Feline corneal endothelial cells – $2x10^5$ – $1x10^6$	No carrier used (cell transplantation directly on native DM)	Cell injection with Y-27632 ROCK inhibitor	Incompletely functional CE	Bostan, et al., 2016
Rat	hCECs– $1x10^6$ cells/300µl DMEM	Rat corneas with DM	Full-thickness grafting	Thin and transparent grafts for 1 month after transplantation	Mimura, et al, 2004a
Rat	Immortalized human corneal endothelial cells (B4G12) - $8.5x10^4$ cells or 3000 cells/mm^2	No carrier used (cell transplantation directly on native DM)	Cell injection	Restored corneal transparency	Ju, et al, 2012

Note: Human corneal endothelial cells (hCECs); Acellular porcine corneal matrix (APCM); Descemet's membrane (DM); Corneal endothelial cell-like cells from embryonic stem cells (CEC-like cells from ESC); Posterior acellular porcine corneal matrix (PAPCM); Descemet's membrane endothelial keratoplasty (DMEK); Spherically curved gelatine hydrogel sheets (SCGSs); Monkey corneal endothelial cells (MCECs); Rho-associated protein kinase (ROCK); Dulbecco's modified Eagle's medium (DMEM).

1.7.2. In vitro animal models for corneal endothelial cell transplantation

A number of studies have reported that *in vitro* cultured corneal endothelial cells can be successfully transplanted into various *in vitro* models and show positive effects on diminishing corneal oedema and restoring normal corneal thickness (See Table 3).

Table 3 – *In vitro* animal models for corneal endothelial cell transplantation reported in literature.

***In vitro* corneal endothelial cell transplantation studies**	**Cells used for transplantation**	**Substrate used as a carrier for cell transplantation**	**Transplantation technique**	**Outcome**	**References**
***In vitro* cultured porcine eyes**	Immortalized human corneal endothelial cells (B4G12)	Plastic compressed collagen type I (RAFT) seeding density – 2000-4000 cells/mm^2 for 4-14 days	Pull-through/air-bubble technique similar to DSAEK procedure	The RAFT constructs successfully attached to the recipient ex vivo porcine eye. hCEC function was not reported.	Levis, et al, 2012
APCM lamellae with DM	Immortalized human corneal endothelial cells (B4G12)	No carrier	Cell injection - Seeding density - $1.5x10^5$ cells/cm^2 for 4h	APCM thickness decrease	Ju, et al, 2012

Note: Real architecture for 3D tissues (RAFT); human corneal endothelial cells (hCECs); acellular porcine corneal matrix (APCM); Descemet's membrane (DM).

1.7.3. In vitro corneal endothelial cell transplantation in organ cultured human corneas

Johnson and Tschumper (1987) previously described a method for *in vitro* culture of human cadaveric corneas, which involved culturing corneas in a moist chamber allowing the epithelial side of the cornea to maintain contact with the air while the endothelial side could be continuously perfused with culture medium. A modification of this model was used for corneal endothelial cell transplantation studies by Patel, et al. (2009) to test a technique using superparamagnetic microspheres (SPMs) incorporated hCECs and transplanting them to corneal stroma of cadaveric human corneas. Another type of set-up for air-interface organ culture of human corneas has previously been reported by Rajan, et al. (2005) that mimicked the normal corneal environment where the cornea keeps in contact with the air but without using continuous perfusion.

In vitro organ cultured human donor corneas have also been used successfully in cell transplantation studies aiming to test the plausibility of a cell-therapy approach for treating corneal endothelial disorders (Engelmann, et al., 1993 and 1999; Aboalchamat, et al., 1999; Chen, et al., 2001; Patel, et al., 2009). They have had success in demonstrating efficient cell attachment, morphology and monolayer formation post-transplantation (Böhnke, et al., 1999) and restoring normal CE function based on the corneal de-swelling measured by ultrasound pachymetry (Aboalchamat, et al., 1999; Engelmann, et al., 1999), (See Table 4).

Table 4 - *In vitro* cultured human corneas used for corneal endothelial cell transplantation studies.

hCEC transplantation model	Descemet's membrane	Cells used for transplantation	Substrate used as a carrier for cell transplantation	Transplantation technique	Outcome	References
Human donor corneas	Intact	Human corneal endothelial cells	No	Cell suspension transplantation on the DM	Achieved a cell density of 1800 cells/mm^2. FGF improved monolayer formation. Function not assessed.	Engelmann, et al., 1993
Human donor corneas	Intact	Human corneal endothelial cells - 150,000 – 700,000 cells per cornea	No	Cell suspension transplantation on the DM	1850 cells/mm^2 density. Stromal de-swelling reached physiological levels. 24 h perfusion experiment.	Engelmann, et al., 1999 and Böhnke, et al., 1999
Human donor corneas	Intact	Immortalized human corneal endothelial cell line (HCEC-12) - 50,000 or 200,000 or 500,000 cells/200µl	No	Cell suspension transplantation on the DM	2086 - 2293 cells/mm^2 density. Corneal thickness measured by pachymetry showed normal corneal thickness within physiological	Aboalchamat, et al., 1999

					levels. 8-12 h perfusion experiments.	
Human donor corneas	Intact	Human corneal endothelial cells - $5x10^5$ cells/ml	No	Cell suspension transplantation on the DM	Cells formed a monolayer and expressed ZO-1 within 7 - 14 days after transplantation; Function not assessed.	Chen, et al., 2001
Human donor corneas	Removed	Human corneal endothelial cells (SPMs incorporated) - 300,000 – 1,000,000 cells per cornea	No	Cell suspension transplantation directly on the stroma	SPMs incorporated hCECs successfully attached and formed a monolayer on the corneal stroma; Function not assessed.	Patel, et al., 2009
Human donor corneas	Intact	Human umbilical cord blood mesenchymal stem cells	No	Cell suspension transplantation on the DM	Expressed ZO-1, N-cadherin and endothelial-like phenotype; Function not assessed.	Joyce, et al., 2012

Note: corneal endothelial cells (CECs), acellular porcine corneal matrix (APCM), Descemet's membrane (DM), corneal endothelial cell-like cells from embryonic stem cells (CEC-like cells from ESC), posterior acellular porcine corneal matrix (PAPCM), Descemet's membrane endothelial keratoplasty (DMEK), mesenchymal stem cells (MSCs), superparamagmetic spheres (SPMs).

1.7.4. Limitations of using animal models for corneal endothelial cell transplantation testing

The rabbit has been used the most in the research field as an animal model for cell therapy testing based on the largest number of articles reporting rabbit studies compared to any other animal model. Other animal models that have also been used include rat, feline and primate. However, the suitability of some of these models has been questioned because the CE of some species seems to possess proliferative capacity, therefore the results generated with these models might not be directly applicable to humans, whose CE is unable to regenerate itself by proliferation *in vivo*. Some species whose CE has been shown to possess proliferative capacity are rabbit (Van Horn, et al., 1977; Mimura, et al., 2005) and rat (Tuft, et al., 1986), which potentially makes these models unsuitable for cell therapy studies since their own regenerative capability might mask any effects from the cell transplantation treatment. Joyce, et al., (1996a) showed that cyclin E in rabbits was located in the cytoplasm of rabbit corneal endothelial cells but in humans it was found in the nucleus, which mediates its effect through protein kinase C (Graham, et al., 2000). This difference of cellular distribution of cyclin E is possibly the reason why rabbit corneal endothelial cells are able to overcome the G1-phase arrest and progress to the S-phase of the cell cycle.

The CE of a number of other animal models, such as feline (Van Horn, et al., 1977) and primate (Van Horn and Hyndiuk 1975) seems to possess similar non-proliferative characteristics as the human CE. This makes them a more suitable platform for cell therapy testing compared to rabbit and rat models. Some positive

outcomes of hCEC transplantation to restore vision have been demonstrated in primates. In a primate model Koizumi, et al. (2007), after mechanically scraping the native corneal endothelium from four eyes of four different animals, transplanted collagen sheets with monkey corneal endothelial cells (MCECs) and used ultrasound pachymetry to measure corneal thickness over a period of 6 months. As a control they transplanted a collagen sheet without MCECs in one eye of another animal after corneal endothelial dysfunction by mechanically scraping the corneal endothelium. Their results showed that within 3 to 6 months after the transplantation the cell-therapy treated eyes restored their transparency and returned to almost the same corneal thickness as before the experiment, whereas the control remained opaque, with corneal thickness larger than 1200 μm. The researchers pointed out a limitation of their corneal thickness measurement approach, stating that the pachymetry method used was not capable to detect any corneal thickness if the values were above 1200 μm.

However, probably the biggest limitation of animal models remains the use of materials of animal origin making the generated data not directly relevant to humans. Therefore the development of a suitable human model for CE transplantation studies that can be directly applicable to humans is necessary.

1.7.5. Limitations of using human donor corneas for cell transplantation studies

The use of human donor corneas in research has several advantages over the use of various animal models. The translational value of results that could be extrapolated from laboratory studies to clinical studies is one of the prime benefits over animal

corneas or *in vivo* animal studies where proliferation potential is higher in certain species. However, there are also certain limitations of using human donor corneas such as:

1) Availability.
2) Lack of good quality of human cadaveric donor corneas.
3) Advanced age of donor corneas available for research studies.
4) Long post-mortem time intervals.
5) Limitation of organ culture preservation.
6) Lack of an intact closed anterior chamber.
7) Poor understanding of stromal behavior in immersion and air-interface organ culture.

Human donor corneas for research are available as excised tissue with a scleral rim and this forms one of the limitations because of the lack of an intact anterior chamber. In order to overcome this limitation several research studies have attempted to mount these human donor corneas in artificial anterior chambers with irrigation to mimic aqueous humour circulation. However, the transfer from immersion to air-interface organ culture is poorly qualified in relation to stromal behaviour seen in human corneas. We have attempted to overcome this knowledge gap by conducting specific experiments in this thesis to form a basis to undertake physiological assessment of human corneas with or without cell therapy.

Despite these limitations we have attempted to use human donor corneas from eye banks for their translational value and to understand and to address the gap in knowledge regarding the stromal behaviour of human corneas in culture. By

carefully considering the limitations it is possible to form strategies using human donor corneas for cell therapy research, which could have the advantage of convincing ethical bodies when it comes to undertaking clinical experimentation in live humans. It is with this primary motive that we have undertaken all our experimentation using human donor corneas including *ex vivo* generated human corneal endothelial cell cultures.

1.8. Summary of review of literature

This literature review in relation to corneal endothelial cell cultures and their potential in cell therapy has identified a few areas that need to be addressed before human endothelial cell therapy can be used for treating corneal endothelial disorders in humans. They are as follows:

- There is a lack of a well-documented and established *in vitro* human corneal model to test strategies for human corneal endothelial cell transplantation.
- There is a lack of clarity related to methodology of cell therapy application and the requirement of the Descemet's membrane for cell attachment.
- Poor understanding and absence of a suitable methodology to test the physiological function of transplanted cultured endothelial cells.
- There is a lack of understanding of the mechanisms that govern hCEC proliferation, differentiation and maintenance.

1.9. Specific aims of this study

In order to evaluate the plausibility of using cell therapy as a treatment for corneal endothelial (CE) disorders in humans and to address some of the limitations that are

currently hindering the clinical use of corneal endothelial cell therapy, this study aimed:

- To set up and validate an *in vitro* human corneal model of CE decompensation.
- To test the capacity of hCECs to attach, form a monolayer and restore normal corneal endothelial cell function after transplantation directly to posterior corneal stroma in the absence of a DM.
- To study the expression profile of positive regulatory domain zinc finger proteins (PRDMs) in human corneas and cultured human corneal endothelial cells.

1.10. Ethics approval

The research project was granted ethics approval by the Faculty Research Ethics Panel (FREP), Faculty of Science and Technology, Anglia Ruskin University, Cambridge, UK in May 2011 for a period of 3 years. It was extended in June 2013 and was further extended in February 2015 until 21st May 2017. Reference number: RESC080. A license was obtained from the Human Tissue Authority (HTA), UK for the handling of human tissue such as human cadaveric corneas.

2. CHAPTER 2 – ESTABLISHING AND VALIDATION OF AN *IN VITRO* HUMAN CORNEAL MODEL OF ENDOTHELIAL DECOMPENSATION

2.1. Chapter overview

In this chapter the use of cadaveric human donor corneas as an *in vitro* model of CE decompensation was tested. The thicknesses of normal and decompensated corneas were compared after incubation in air-interface organ culture for an incubation period of 12 hours and up to 14 days. The corneal thickness was used as a surrogate measure of CE function and was measured using Zeiss Visante OCT. It was confirmed experimentally that the absence of a Descemet's membrane (DM)/Endothelium complex in the decompensated corneas resulted in an oedematous phenotype throughout the incubation period, whereas the presence of an intact DM/Endothelium complex in the normal corneas was sufficient to maintain normal physiological corneal thickness. The results from this chapter show that this *in vitro* model can be used for testing the presence or absence of a functional corneal endothelial cell monolayer, thus is a useful platform for cell-therapy evaluation in future studies.

2.2. Brief introduction

Cell-based approach for treating corneal endothelial decompensation has been investigated in animal models, such as rabbit (Jumblatt, et al., 1978; Gospodarowicz, et al., 1979), primate (Koizumi, et al., 2007; Okumura, et al., 2016), and feline (Bostan, et al., 2016). Important aspects of cell transplantation, such as the capacity

of the transplanted cells to proliferate, form attachment, maintain viability and restore a functional corneal endothelial monolayer need to be tested in order to confirm the feasibility and safety of such treatment before it can be applied to humans. In cell therapy research studies, several characteristics are regarded as determinants of a successful outcome after transplantation of cultured CECs, namely corneal endothelial cell attachment, morphology and monolayer formation, corneal transparency, and the ability to achieve physiological corneal thickness. Using a variety of animal models recent research studies have yielded very valuable insights and have confirmed the plausibility of using a cell-based therapy for treating corneal blindness by showing that after transplantation *in vitro* cultured CECs can attach and form a functional CE monolayer and restore corneal transparency and normal corneal thickness. However, using animal models for cell therapy studies has certain limitations due to the CE of some animals, such as rabbits possessing a high proliferative and self-regenerating capacity. Therefore, the research data obtained through animal models might not be directly applicable to humans.

In order to address issues that are currently limiting cell therapy research studies with this study we established an *in vitro* human model of CE decompensation by surgically removing the DM/Endothelium complex from human donor corneas, which would allow cell therapy testing in a human model in the absence of a DM. It was hypothesized that after a period of time in air-interface organ culture corneas with intact DM/Endothelium would experience a reduction in thickness to a physiological level, whereas decompensated corneas (DM/Endothelium removed) would remain oedematous. The *in vitro* model was validated for future use in hCEC

transplantation studies by testing the effect of the presence or absence of the DM/Endothelium complex on the corneal thickness by measuring the corneal thickness using Zeiss Visante anterior segment OCT. The experiment results confirmed that this *in vitro* model could be used as a suitable platform for demonstrating the presence or absence of normal corneal endothelial function within 12 hours and up to 14 days of air-interface corneal organ culture.

2.3. Materials and methods

2.3.1. List of materials

Human corneas from deceased human donors were obtained from Bristol and Manchester Eye Banks, UK with consent for research studies; Foetal bovine serum (FBS) – (F0804-500ML), Sigma Aldrich; Trypsin/EDTA – (T3924), Sigma Aldrich; 0.06% VisionBlue™ (trypan blue); Barron artificial anterior chamber – Barron Precision Instruments; BD Plastipak 10 ml syringes – (SYR188), Medisave; Zeiss Visante OCT.

2.3.2. Cornea storage conditions

In the eye bank and prior to experimentation all corneas were stored in immersion organ culture using Eagle's Minimal Essential Medium (MEM) supplemented with 2 % foetal bovine serum (FBS), at 37^{o}C, 5 % CO_2 in a humidified incubator (relative humidity – 95%.

2.3.3. Cornea selection

Corneas that were received from the eye bank were randomly allocated for DM/Endothelium removal in order to create a CE decompensation model.

2.3.4. Inducing corneal endothelial decompensation in human donor corneas

An *in vitro* model of corneal endothelial decompensation was prepared by removing the native Descemet's membrane/Endothelium complex as follows:

1) The corneoscleral rim was placed with the endothelial side facing upwards on a trephine base and was stained for 30 sec with VisionBlue® (trypan blue) vital stain (See Figure 5).

Figure 5 – A cornea placed endothelium side up on a trephine base. Corneal endothelial cell viability was tested using VisionBlue® (trypan blue) vital stain.

2) After that the corneal endothelium was washed with un-supplemented basal culture medium F99 or OptiMEM-I.
3) The corneal endothelium was maintained in a water bath to avoid drying.
4) The corneoscleral rim was held in place using forceps.
5) The Descemet's membrane was cut in a circular motion using a surgical knife up to the transition zone or Schawalbe's line that separates the endothelium from the trabecular meshwork and was peeled from the stroma by using forceps. Care was taken not to disturb the underlying stroma whilst scoring the Descemet's membrane.

6) The stroma was wiped with a sterile spear to ensure all remaining Descemet's membrane was completely removed.

Human corneas with intact DM/Endothelium complex were used as a positive control for normal CE function as part of the *in vitro* model validation process. Human donor corneas were dissected using a low magnification stereo zoom microscope (See Table 5).

Table 5 – Leica S6E stereo zoom microscope specifications.

Specifications	
Light	Benchtop Stand and Simple light-emitting diode (LED) Reflected Light
Magnification Power	6.3x – 40x, continuous zoom
Eyepiece	10X
Maximum field of view	36.5 mm
Product Type	Microscope stereozoom

- 0.06 % VisionBlue™ (trypan blue) vital staining was used to assess the CE viability (See Figure 6);
- Corneas with low CE viability were excluded from the control group of normal CE function [See Figure 6 (D)].

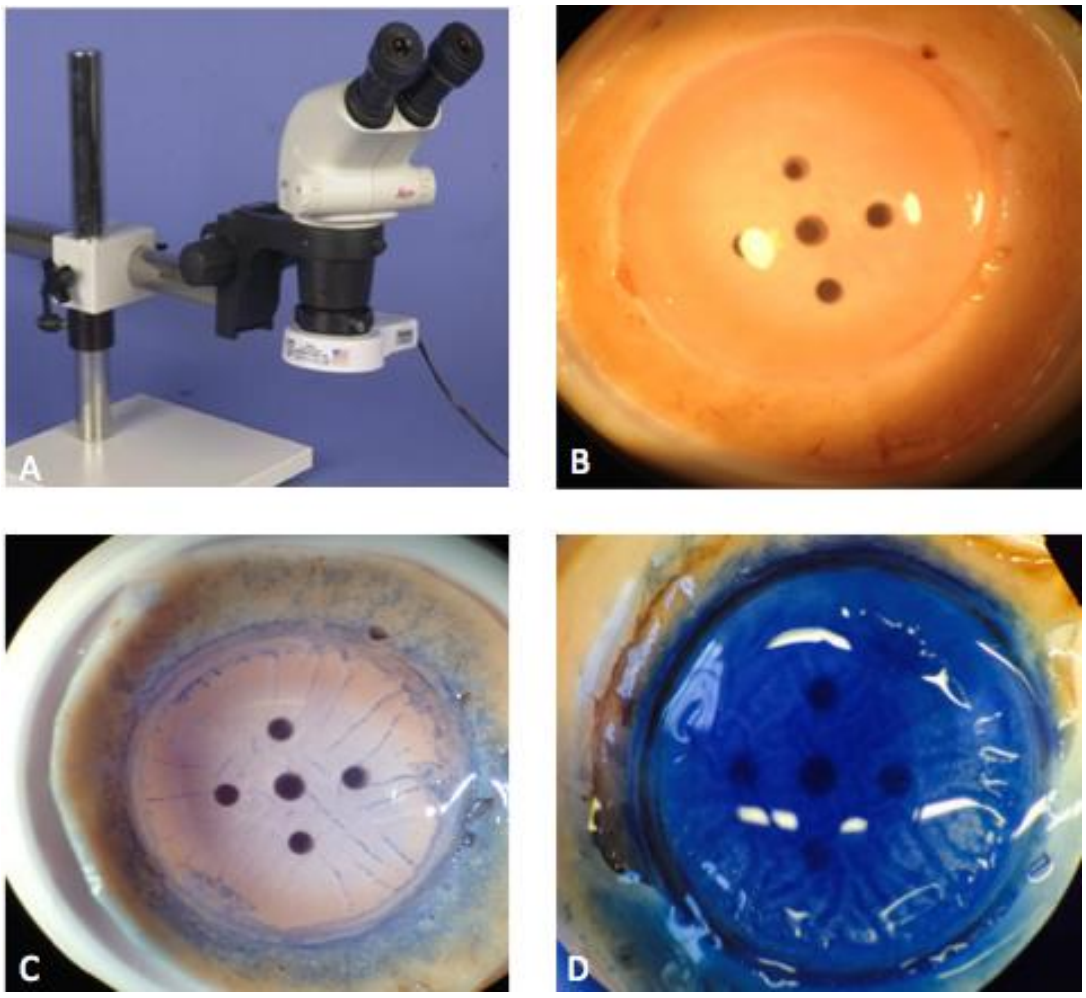
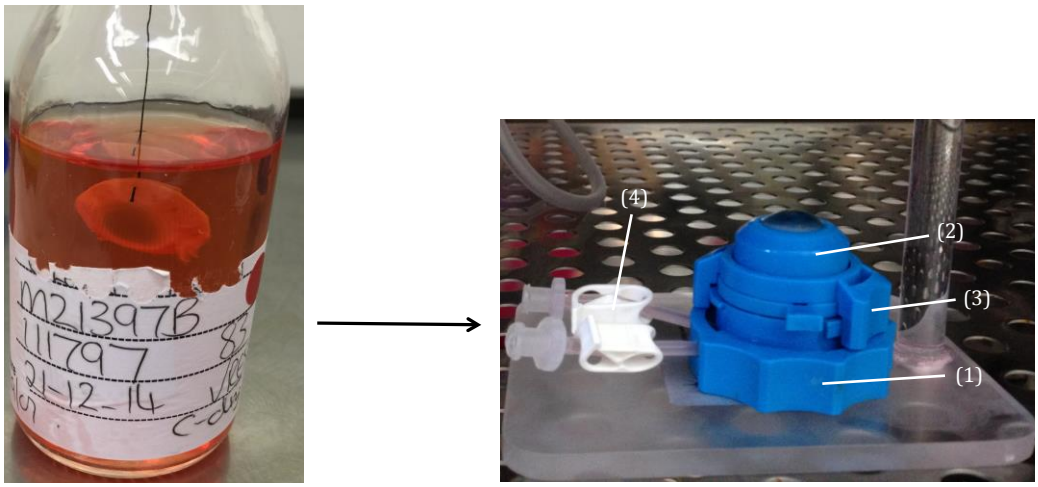


Figure 6 – Leica S6E stereo zoom microscope used for cornea dissection (A). A cornea is placed endothelial side up and is prepared for trypan blue cell viability assessment (No staining; x200) [B], cornea with high corneal endothelial cell viability [0.06 % VisionBlue® (trypan blue) staining for 30 sec; x200] [C], cornea with low corneal endothelial cell viability [0.06 % VisionBlue® (trypan blue) staining for 30 sec; x200] [D].

All corneas were kept in immersion organ culture except for when they were taken out for CE viability assessment and DM/Endothelium complex removal. After these processes were completed the corneas were transferred from immersion and into air-interface organ culture as described below.

2.3.5. In vitro air-interface organ culture of normal and decompensated human corneas

In vitro air-interface organ culture of human corneas is a technique, which allows the corneas to be cultured in conditions that mimic normal physiological conditions where the epithelial side of the cornea maintains contact with the air (air-liquid interface). A Barron artificial anterior chamber was used, which consists of three parts: a base, a tissue retainer and a locking ring. The base contains two port connectors with pinch clamps, allowing fluid injection using a syringe. The tissue retainer holds the cornea in place. The locking ring is used to lock the tissue retainer against the base. The anterior chamber can hold a donor cornea with a scleral rim diameter of 14-18 mm. The tissue retainer has a 12.5 mm opening, which allows air contact with the epithelial side of the cornea. Once the cornea is secured to the artificial chamber, culture medium can be injected through the port connectors. After the chamber is filled with medium the port connectors are closed using the pinch clamps. This model allows exchanging of the culture medium by injection of fresh medium through the port connectors when necessary and also allows controlled moisturizing of the epithelial side of the cornea. This *in vitro* system for air-interface organ culture of decompensated human corneas was used as an *in vitro* corneal model of CE decompensation and was incubated in an incubator at 37^{o}C, 5% CO_2 and 95% relative humidity (See Figure 7).



Immersion organ culture
(Eye Bank storage conditions)

Air-interface organ culture

Figure 7 – Eye bank human corneas were received in immersion organ culture storage conditions. Barron artificial anterior chamber consisting of a base (1), tissue retainer (2), a locking ring (3) and pinch clamps (4) for opening and closing the perfusion tubes (purchased from Barron Precision Instruments) with a 12.5 mm opening, which allows the epithelial side of the cornea to be in contact with the air during organ culture was used for air-interface organ culture of human donor corneas. The corneas were incubated in an incubator at 37ºC, 5% CO_2 and 95% relative humidity.

Fresh pre-warmed culture medium F99 (37ºC) was injected into the Barron artificial anterior chamber every 1 hour (12-hour experiment) and every 24 hours (14-day experiment) by releasing the pinch clamps on both port connectors while the base was kept in an upside-down position in order to avoid deflation of the cornea. The epithelial side of the corneas was moistened with 200 µl of medium every 1-hour (12-hour experiment) and every 24 hours (14-day experiment). The corneal thickness was measured using Zeiss Visante OCT every 1-hour (12-hour

experiment), and every 24 hours for 14 days (14-day experiment). The time periods for media exchange and moistening of the epithelial side of the cornea were based on observation of corneal capsizing occurring roughly every hour, therefore the media on the posterior side of the cornea was exchanged every hour followed by moistening of the epithelial side with fresh culture medium and this was kept consistent throughout the experiment.

2.3.5.1.Air-interface organ culture: 14-day incubation

2.3.5.1.1.Human donor corneas

A total number of n=8 corneas were used for this experiment and were separated into groups: normal and decompensated.

2.3.5.1.1.1.Normal and decompensated corneas

Normal human donor corneas with an intact DM/Endothelium complex (n=4) were used as a normal CE function control. Human donor corneas that had their DM/Endothelium complex removed (n=4) were used as an *in vitro* model of CE decompensation (See Table 6).

Table 6 – Corneal characteristics (14 days in air-interface organ culture).

	Normal (n=4)	Decompensated (n=4)
DM/Endothelium complex	Intact	Removed
ECD (cells/mm^2)	1718 ± 306.31 (range – 1500-2172)	1820.5 ± 433.81 (prior to DM removal)
Age (years)	68.25 ± 3.30 (range – 64-72)	70.75 ± 5.3 (range – 64-75)
Period in storage prior to experiment (days)	34.5 ± 6.45 (range – 25-39)	29.75 ± 10.11 (range – 21-39 days)

Note: There was no statistically significant difference between the normal and decompensated groups when ECD, age and period in storage were compared. Endothelial cell density (ECD); Descemet's membrane (DM). Cornea donor information is available in APPENDIX 1- Table 18.

2.3.5.2.Air-interface organ culture: 12-hour incubation

2.3.5.2.1.Human donor corneas

9 corneas were used in this experiment and were separated into groups. Two of the corneas were used once as part of the normal group with intact DM/Endothelium complex and then after DM/Endothelium removal they were used in the decompensated group, hence equating to 11 samples in total (See APPENDIX 1 Table 16).

2.3.5.2.1.1.Normal and decompensated corneas

Normal human donor corneas (n=6) with intact DM/Endothelium complex were used as normal CE function control. Human donor corneas whose DM/Endothelium complex was removed (n=5) were used as an *in vitro* model of CE decompensation (See Table 7).

Table 7 – Corneal characteristics (12 hours in air-interface organ culture).

	Normal (n=5)	Decompensated (n=6)
DM/Endothelium complex	Intact	Removed
ECD (cells/mm^2)	2002 ± 357.99 (range - 1670-2500)	N/A
Age (years)	67.83 ± 20.52 (range - 29-89)	52.8 ± 22.18 (range – 29-76)
Period in storage prior to experiment (days)	39.66 ± 13.6 (range - 29-57)	31.4 ± 3.28 (range - 29-35)

Note: There was no statistically significant difference between the normal and decompensated corneas when the age and period in storage were compared. The DM/Endothelium complex of the decompensated corneas was completely removed prior to the experiment. Endothelial cell density (ECD); Descemet's membrane (DM).

2.3.5.3.Optical Coherence Tomography assessment of corneal endothelial cell function

The Zeiss Visante OCT is able to scan the entire surface of the cornea and to acquire a cross-section image at specific orientations, ranging between 0º-180º, 45º-225º, 90º-270º and 135º-315º. It also has inbuilt software that allows corneal thickness measurements to be done at multiple locations, providing an accurate and precise method for assessment of whole corneal thickness without observer or selection bias (See Figure 8).

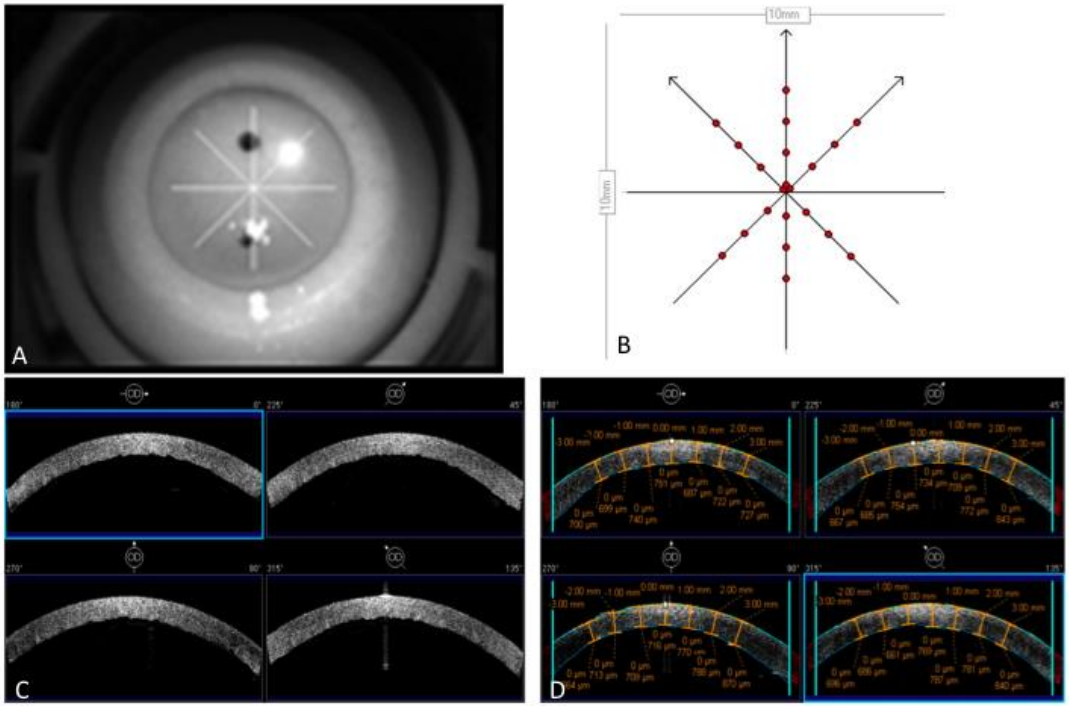


Figure 8 – Zeiss Visante Optical Coherence Tomography (OCT). The thickness of each cornea was acquired and corneal cross-section images were generated at four different orientations – 0º-180º, 45º-225º, 90º-270º and 135º-315º (A and B), corneal cross-section image acquisition (C), a total of 28 measurements across a 6 millimeter diameter of the central cornea were done and the average value ± standard deviation (SD) was used as a representative of the corneal thickness (B and D).

2.3.5.3.1.Initial corneal thickness measurement

Before starting the experiment the human donor corneas were kept in immersion organ culture conditions as described in section 2.3.2. After the DM/Endothelium complex was removed to create a CE decompensation model both the normal and the decompensated corneas were transferred into air-interface organ culture as described in section 2.3.5. Immediately after the corneas were transferred from

immersion into air-interface organ culture an initial corneal thickness measurement was performed using Zeiss Visante OCT. Corneal cross-section images were acquired for each individual cornea at specific orientations or quadrants – 0^{o}-180^{o}, 45^{o}-225^{o}, 90^{o}-270^{o} and 135^{o}-315^{o}. For each cornea 7 individual pinpoint measurements across a 6 mm diameter in each of the 4 quadrants were taken using the Zeiss Visante OCT analysis mode. Therefore, a total of 28 measurements were done at different locations for each cornea. The average value of the 28 measurements was used to represent the corneal thickness of each cornea. Normal CE function could be demonstrated based on the amount of thickness de-swelling and physiological thickness level achieved after transfer into air-interface organ culture.

2.3.5.3.2.Subsequent corneal thickness measurements

Subsequent corneal thickness measurements were done at regular time intervals every 1-hour for the experiment lasting 12-hour incubation period and every 24 hours for the 14-day incubation period experiment.

2.3.6. Histology

Histology was used to confirm the successful complete removal of the DM/Endothelium complex from human donor corneas used as a CE decompensation model. The corneas were fixed in 10% formalin for 10 min at room temperature and were stored at 4^{o}C overnight. Paraffin embedding, haematoxylin and eosin (H&E) staining and sample analysis was done in Addenbrooke's Hospital, Cambridge, UK.

2.3.7. Statistical analysis

Single factor ANOVA test was used for statistical analysis to determine if there was a statistically significant difference between the normal and decompensated corneal groups after a period of air-interface organ culture. Confidence levels were set at 95%. $P<0.05$ was considered statistically significant. Post hoc t tests (Bonferroni corrected) were used to compere the corneal thickness of the different groups of corneas at specific time points throughout the incubation period. The error bars in all graphs and bar charts represent the mean ± standard deviation (SD).

2.4. Results

2.4.1. Optical Coherence Tomography assessment of corneal endothelial cell function during air-interface organ culture: 14-day incubation

There was no statistically significant difference in endothelial cell density (ECD) prior to DM/Endothelium complex removal, the age and the storage period prior to experimentation between the normal and decompensated corneas used in the 14-day incubation experiment. Both the normal and the decompensated corneas expressed oedematous phenotype at the start of the experiment as a result of being stored in immersion organ culture conditions before the experiment. The initial thicknesses at time point 0 of the normal and the decompensated corneas were 876.41 ± 88.62 µm and 918.68 ± 98.27 µm respectively. After 24 hours of *in vitro* air-interface organ culture the normal corneas showed a 33.32 ± 6.89 % decrease from the initial thickness reached a thickness of 582.59 ± 70.31 µm. This was statistically significant ($p<0.001$) compared to the decompensated corneas, which only showed a 4.21 ± 6.62 % decrease and maintained an oedematous phenotype with 879.73 ± 109.58 µm

thickness over the same period. The normal corneas maintained a near-normal physiological thickness of 581.25 ± 7 μm between 24 hours and 4 days of incubation. Between the 4th and the 14th day the normal corneas maintained an average thickness of 666.87 ± 57.06 μm (23.01 ± 6.47 % decrease from their initial thickness). Between 24 hours and 14 days the decompensated corneas expressed a severe oedema and maintained an average thickness of 826.32 ± 23.46 μm (9.87 ± 2.5 % decrease from their initial thickness). A single-factor ANOVA test showed that there was a statistically significant difference between the normal corneas and the decompensated corneas when the mean thicknesses (μm) and the mean percentage thickness decrease (%) were compared (See Figure 9 and Figure 10). The normal corneas showed a thickness decrease to normal physiological levels, which gradually started to increase after 72 hours of incubation, whereas the decompensated corneas had an oedematous phenotype throughout the entire incubation period (See Figure 11). All corneal thickness (μm) and percentage decrease (%) values are available in APPENDIX 1 - Table 19.

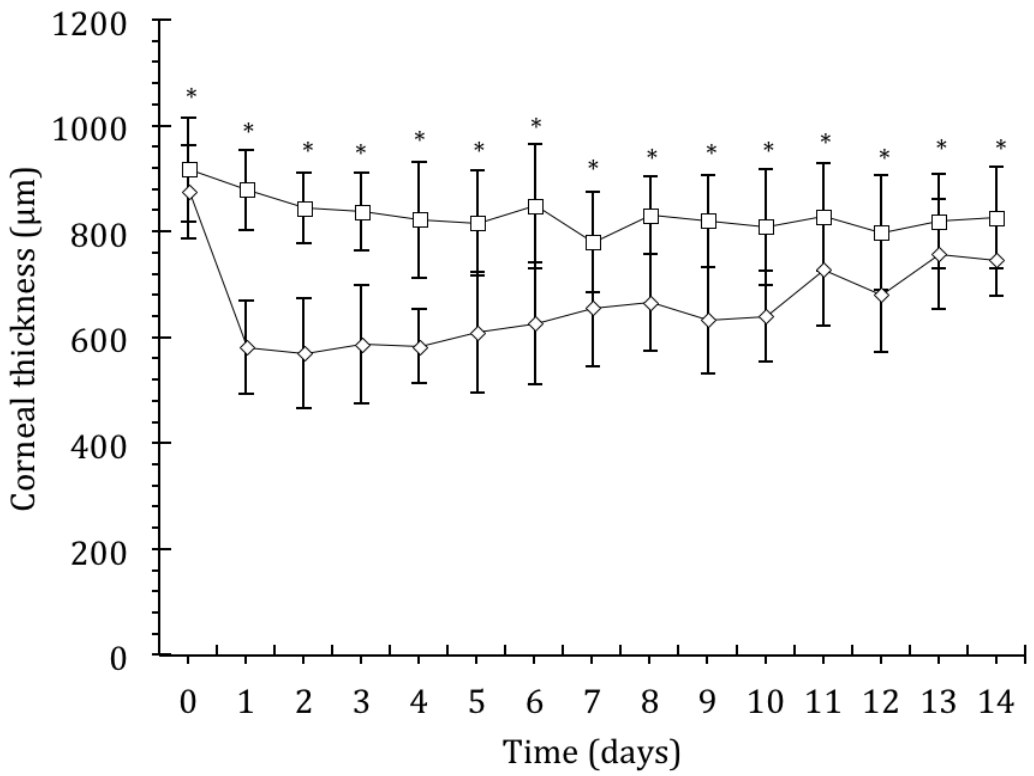


Figure 9 – Stromal de-swelling of *in vitro* organ cultured human corneas during a 14-day incubation period. Normal corneas with endothelial cell density of 1718 ± 306.31 cells/mm^2 (n=4) [◇] reached a normal physiological thickness level of 582.59 ± 70.31 µm and maintained a thickness of 666.87 ± 57.06 µm until the end of the 14-day incubation period. The endothelial cell density was not measured at the end of the experiment. The decompensated corneas showed signs of severe oedema with thickness of 826.32 ± 23.46 µm throughout the same period (n=4) [□]. A single-factor ANOVA test showed that there was a statistically significant difference between the means of the normal and decompensated corneas ($p<0.05$ was considered statistically significant). *Post hoc t test (Bonferroni correction) revealed statistically significant difference between all time points.

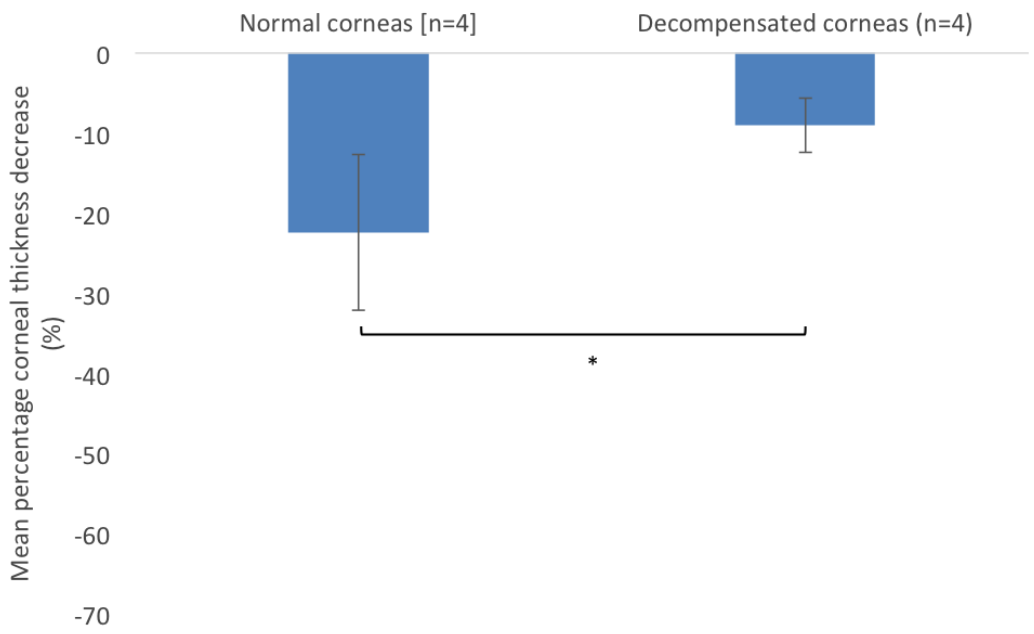


Figure 10 – Stromal de-swelling assessment of *in vitro* organ cultured human corneas during a 0-14 day incubation period. The normal corneas displayed an average 23.01 ± 6.47 % decrease, whereas the decompensated corneas only showed a 9.87 ± 2.5 % decrease. A single-factor ANOVA test revealed that there was a statistically significant difference between the normal and the decompensated corneas. *p=0.000003 ($p<0.05$ was considered statistically significant).

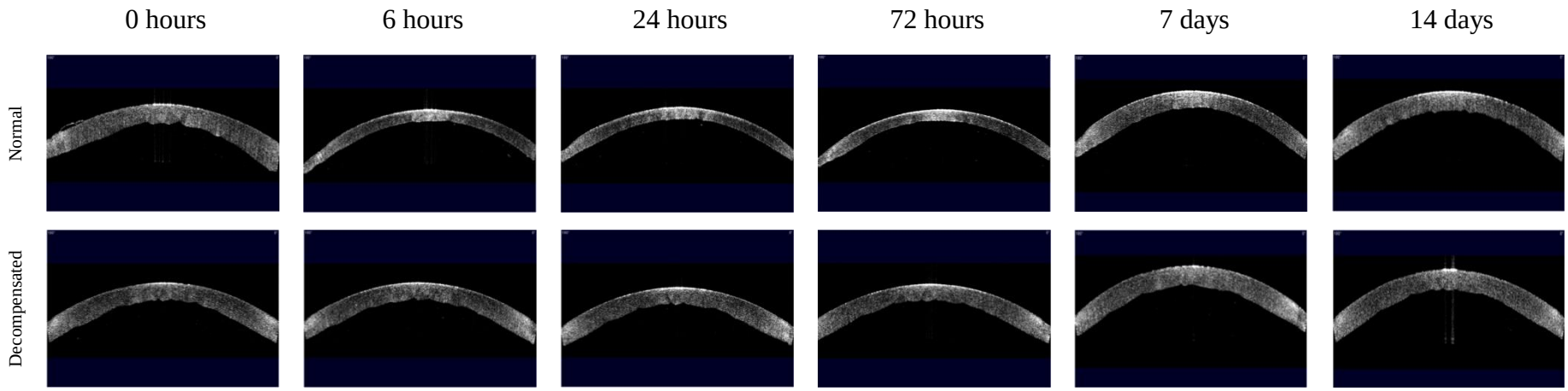


Figure 11 - Optical Coherence Tomography: images of normal (n=4) and decompensated (n=4) corneas cultured at air-interface organ culture. The normal corneas show a reduction in thickness to normal physiological levels at 6, 24 and 72 hours, however, an oedematous phenotype is observable after 7 and 14 days of air-interface organ culture. Corneal endothelial cell count was not done at the end of the experiment. The decompensated corneas displayed an oedematous phenotype throughout the entire experiment.

2.4.2. Optical Coherence Tomography assessment of corneal endothelial cell function during air-interface organ culture: 12-hour incubation

There was no statistically significant difference between the age and the storage period prior to experimentation in the corneas used in the 12-hour incubation experiment. Both the normal and the decompensated corneas expressed oedematous phenotype at the start of the experiment as a result of being stored in immersion organ culture conditions prior to the experiment. The initial thickness at time 0 of the normal corneas (n=6) was 1092.74 ± 108.03 μm and 1128.51 ± 98.91 μm for the decompensated corneas (n=5). At 12 hours the normal corneas, placed into air-interface organ culture, experienced a thickness decrease of 48.27 ± 9.78 % from its initial value and reached a normal physiological thickness of 557.51 ± 72.64 μm. While the thickness of the decompensated corneas decreased only by 19.27 ± 10.87 % from their initial thickness, they maintained an oedematous phenotype with thickness of 903.6 ± 86.51 μm throughout the entire incubation period. A single-factor ANOVA test showed that there was a statistically significant difference between these two groups (See Figure 12 and Figure 13). All corneal thickness values and % decrease values are available in APPENDIX 1 Table 17.

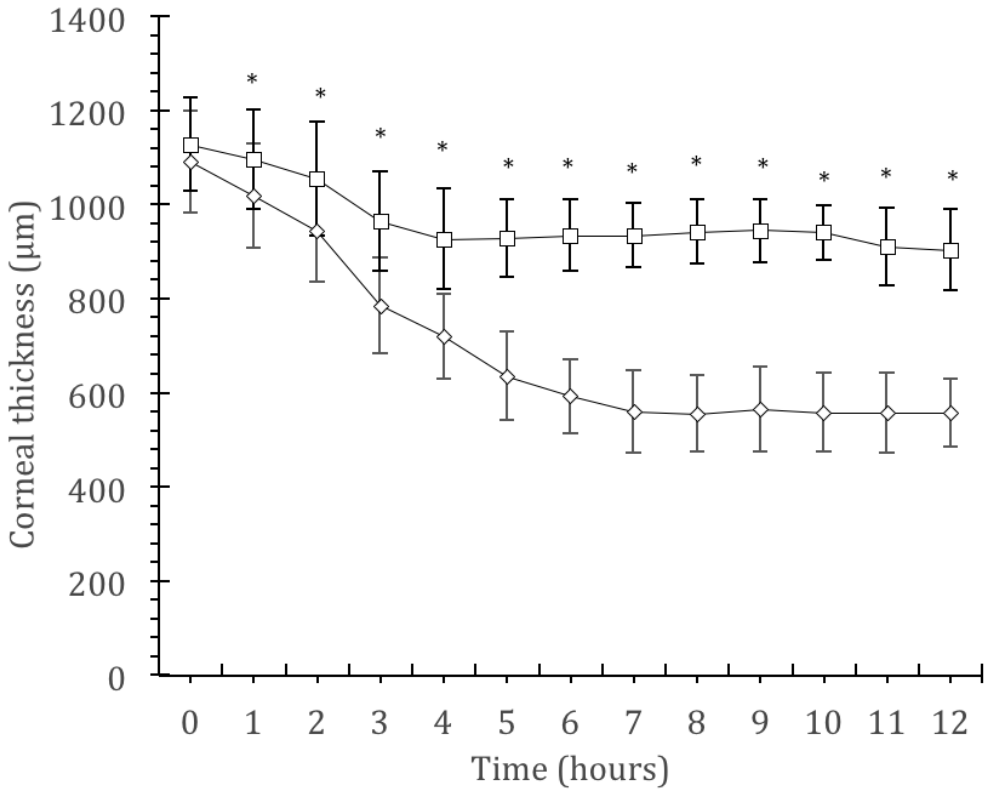


Figure 12 – Stromal de-swelling of *in vitro* organ cultured human corneas. Normal corneas (n=6) [◇] showed a more significant thickness decrease compared to decompensated corneas (n=5) [□] and were able to reach a near-physiological thickness of 557.51 ± 72.64 µm by the end of the 12-hour incubation period. The decompensated corneas maintained an edematous phenotype with thickness of 903.6 ± 86.51 µm. A single-factor ANOVA test showed that there was a statistically significant difference between the mean thicknesses of the normal and the decompensated corneas ($p<0.05$ was considered statistically significant). *Post hoc t test (Bonferroni correction) revealed statistically significant difference between all time points except point 0 hours.

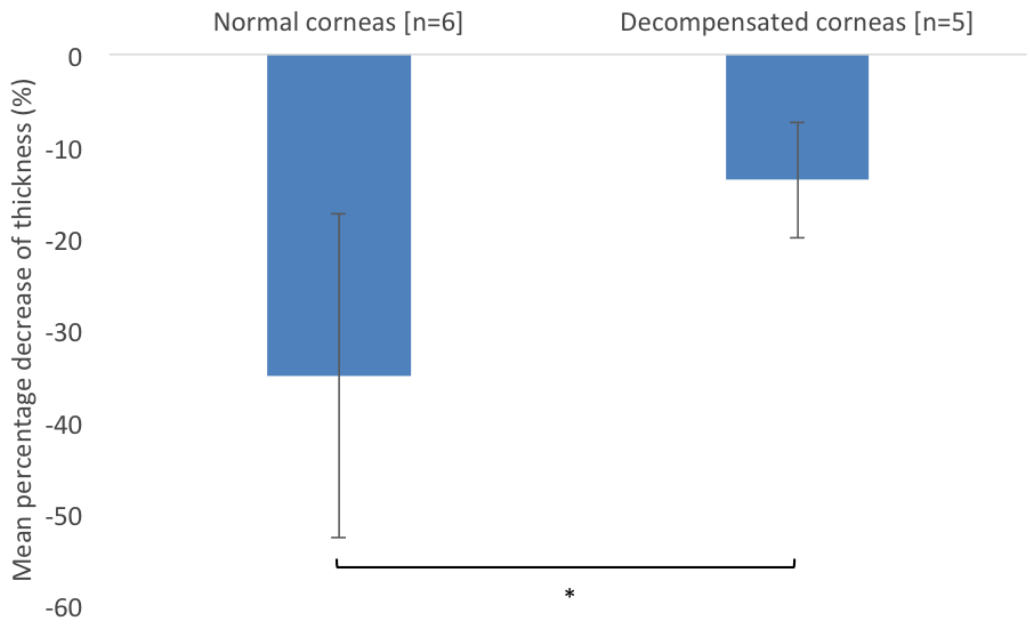


Figure 13 – Stromal de-swelling during a 12-hour incubation period. Normal corneas with endothelial cell density (ECD) 2002 ± 357.99 cells/mm^2 were used as a positive control for normal corneal endothelial cell function (the normal endothelial cell density for an elderly cornea is between 2200 and 2800 cells/mm^2). The percentage reduction of corneal thickness in the normal corneas was 48.27 ± 9.78 % after 12 hours of incubation and 19.27 ± 10.87 % for the decompensated corneas. A single-factor ANOVA test revealed that there was a statistically significant difference between the normal and the decompensated corneas. *p=0000004 (p<0.05 was considered statistically significant).

By the end of the experiment the normal corneas were significantly thinner ($p<0.05$) compared to the decompensated corneas and did not show any signs of posterior stromal folds. However, posterior stromal folds were observable in the decompensated corneas (See Figure 14).

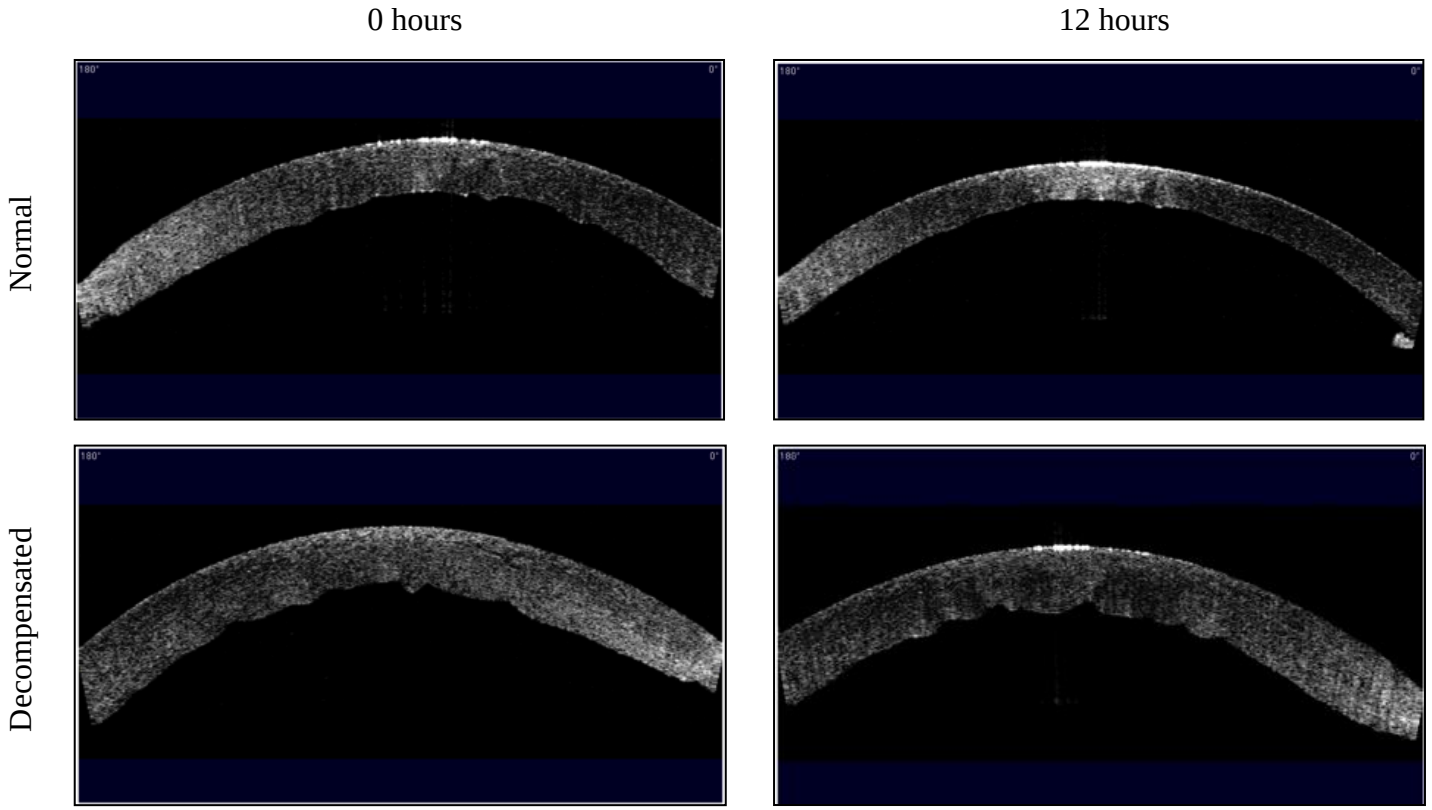


Figure 14 – Optical Coherence Tomography: high-resolution quadrant images of normal (n=6) and decompensated (n=5) corneas. The normal corneas have reached normal physiological thickness after 12 hours of air-interface organ culture, whereas the decompensated corneas remain oedematous.

2.4.3. Histology

Histological assessment with haematoxylin and eosin (H&E) staining of a decompensated cornea confirmed that the DM/Endothelium complex was completely removed and that a decompensation model was successfully created without disturbance to the posterior stromal collagen (See Figure 15).

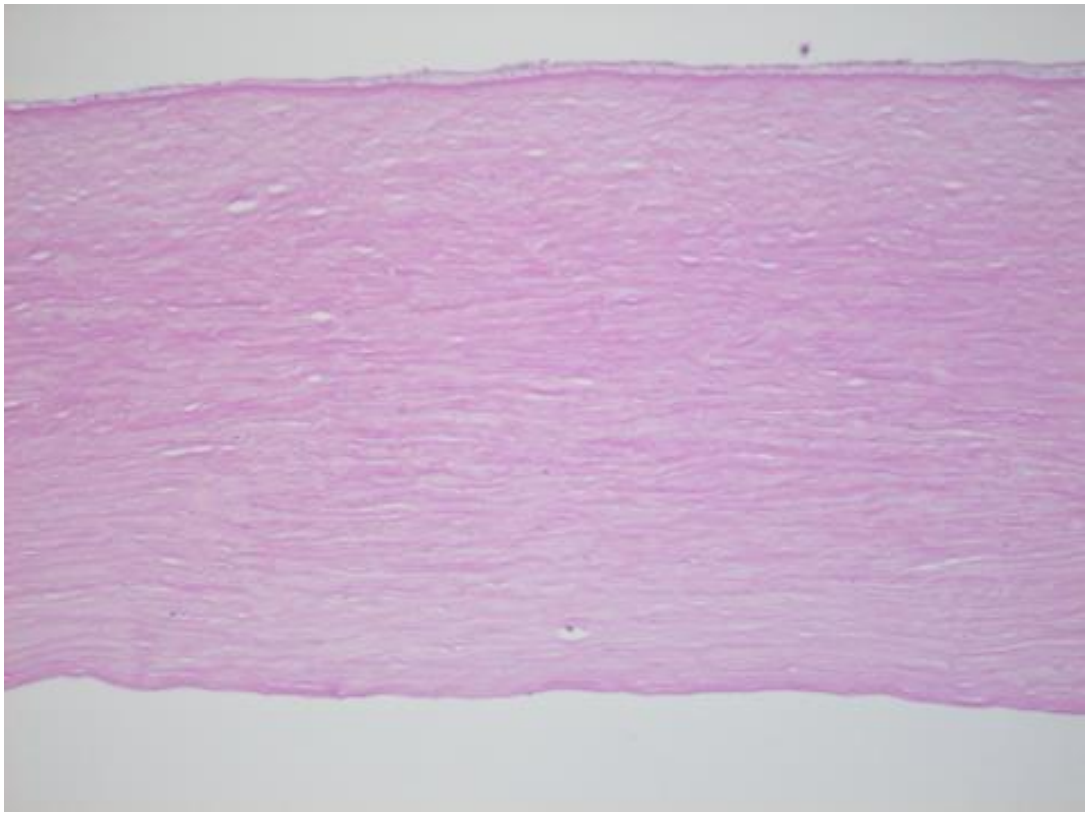


Figure 15 – Histological examination revealed the complete removal of the Descemet's membrane/Endothelium complex with no disturbance to the posterior stroma of cadaveric human donor corneas, confirming that an *in vitro* human corneal endothelial decompensation model was successfully induced [Haematoxylin and eosin (H&E) staining; x100].

2.5. Discussion

The human corneal endothelial cells form a monolayer at the posterior part of the cornea, which has a highly specialized function to maintain the corneal stroma in a controlled dehydrated state. This accounts for corneal transparency and is very important for normal vision. Damage or loss of the corneal endothelial monolayer leads to stromal oedema and loss of optical transparency of the cornea. Injury or damage to the corneal endothelium can be caused by dystrophies, such as Fuchs' dystrophy or trauma, which leads to corneal blindness. The human corneal endothelial cells are generally considered post-mitotic and have poor proliferative and self-renewal capacity *in vivo* and *in vitro* (Joyce, et al., 1996a and 1996b; Chen, et al., 1999). Human corneal endothelial disorders are currently treated using partial thickness transplantation techniques, such as DSAEK, DMEK and micro-thin DSAEK (Rajan, 2014). These treatments all rely on a partial-thickness graft harvested from a donor cornea, which is a major limiting factor due to lack of donor tissue availability in some parts of the world. Therefore, current research is focused on developing novel strategies for generating hCECs for therapeutic purposes. Cell-therapy would allow the use of a single donor cornea to produce a cell bank through isolation and *in vitro* culture of the corneal endothelial cells that could later be used for cell transplantation into patients. This treatment method has the potential to greatly reduce or even eliminate the need for donor material thus making this treatment available in countries where donor tissue is not readily available. In order to develop a cell-therapy treatment there is ongoing research for developing suitable *in vivo* or *in vitro* transplantation models and on optimizing primary hCEC isolation

and culture. In order to test this novel treatment method many groups have reported using a wide range of animal models including rabbit (Jumblatt, et al., 1978), rat (Mimura, et al, 2004a), cat (Proulx, et al, 2009) and primate (Koizumi, et al., 2007). However, certain limitations exist when using such models. For example, the rabbit and the rat have been documented as species whose corneal endothelium has the capacity to renew itself through proliferation (Van Horn, et al., 1977 and Tuft, et al., 1986). Since the human corneal endothelium lacks proliferative capacity using animal models whose endothelium can proliferate makes the generated data not directly applicable to humans. Therefore, it would be more beneficial to have a model that is more similar to humans, which will make any data generated with it more clinically relevant. The primate model has recently gained a lot of eminence for being more similar to the human corneal endothelium in its lack of proliferative response. However, even though this primate model is considered adequate for testing the feasibility of corneal endothelial cell transplantation the use of it in the lab remains very challenging and expensive because it requires special facilities and maintenance techniques. Also in many countries the use of animal models especially primates is very strictly controlled because of ethical issues. Ideally a human model would provide a testing platform that would generate data that is clinically relevant.

Therefore, in this study the aim was to establish an *in vitro* human corneal model of endothelial decompensation using cadaveric human corneas and to validate it for future use in testing the effectiveness of a cell-therapy treatment after transplanting cultured hCECs directly to the corneal stroma. Using an *in vitro* human model offers many advantages over animal models with the most important benefit being that it is

of human origin and also donor corneas are much easier and cost effective to maintain in the lab compared to animal models and allow for better control and reproducibility of the experiments.

The data from this chapter shows that when human donor corneas with intact DM/Endothelium complex and adequate cell density are cultured using air-interface organ culture their thickness will reach normal physiological levels. Whereas, the corneas whose DM/Endothelium is removed would maintain a severely oedematous phenotype when cultured in the same conditions. The data clearly demonstrates that this method of air-interface organ culture can adequately be utilized to determine and test the presence or absence of a functional corneal endothelial cell monolayer by using the corneal thickness as a surrogate measure of corneal endothelial cell function. This is very important for cell-therapy evaluation because it will enable testing whether normal corneal endothelial cell function has been restored to decompensated corneas (DM/Endothelium removed) by measuring the corneal thickness using Zeiss Visante OCT after corneal endothelial cell transplantation. This method is very effective in accurately measuring corneal thickness because it is able to acquire a cross section image, which reveals if posterior stromal folds are present (See Figure 16). It allows for thickness measurements to be taken at multiple locations of each cornea thus increasing the accuracy of the measurements as opposed to a single point measurement done by ultrasound pachymetry. In our experiments 28 measurements were taken at different locations for each cornea making the corneal thickness assessment very accurate (See Figure 8).

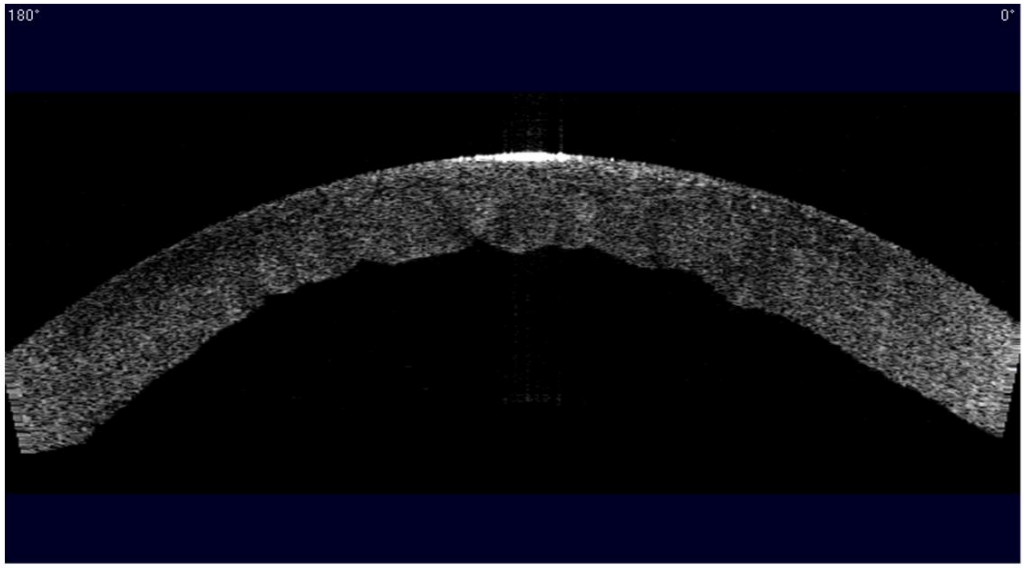


Figure 16 – Posterior stromal folds are visible on a corneal cross-section image acquired by optical coherence tomography.

The method of air-interface organ culture is very useful because it allows the corneas to be maintained in an environment that more closely simulates the normal physiological environment of the human cornea where the epithelial side has contact with the air. In Eye Banks normally human corneas are stored in immersion organ culture. But when stored in immersion even corneas with intact DM/Endothelium complex would swell. The mechanism by which the cornea maintains its thickness is interplay between the stromal swelling pressure driving the fluid inwards to the stroma and the active pump of ions driving the fluid outwards from the stroma. The stromal swelling pressure is the tendency of the stroma to swell as a result of its mechanical composition and is affected by the concentration of proteoglycans (Fischbarg and Maurice, 2004). However, since the corneal stroma is compressed between the epithelium and endothelium, a negative fluid pressure is created through an active ion transport that counteracts the stromal swelling pressure and causes the

corneal thickness to decrease (Dohlman, et al., 1962). When a donor cornea is stored in immersion, the storage environment does not allow such negative fluid pressure to be created, therefore causing even corneas with intact DM/Endothelium complex to become swollen. After transfer from immersion into air-interface organ culture, the cornea is no longer completely submerged in medium, with the exception of its endothelial side, and is able to de-swell (Aboalchamat, et al., 1999). Since the air-interface culture environment mimics more closely the natural conditions to which the cornea is normally subjected, these conditions allow the negative fluid pressure to be restored resulting in corneal de-swelling.

In our air-interface organ culture experiments, during both the 12-hour and the 14-day incubations, a slight thickness decrease in the decompensated corneas (DM/Endothelium removed) was observed, which was independent of CE pump function since these corneas were denuded of their native DM/Endothelium complex. This dehydration effect accounted for a total of 19.27 ± 4.86 % thickness decrease during the 12-hour incubation and a 9.83 ± 3.38 % decrease during the 14-day incubation experiments. Since the decompensated corneas had their DM/Endothelium completely removed prior to the experiments this minor decrease in thickness was attributed to the change of environment conditions after transfer from immersion to air-interface organ culture. The decompensated corneas maintained an oedematous phenotype with corneal thickness ≥ 780.83 ± 54.81 μm, whereas the normal corneas were able to reach and maintain physiological thickness of 555.86 ± 33.35 μm. When using this *in vitro* corneal model it was challenging to provide adequate hydration of the epithelial side of the cornea and to keep it

sufficiently moist throughout the entire incubation period. A plastic ring was attached to the artificial anterior chamber in order to hold culture medium and keep the corneal surface moist during incubation (See Figure 17).

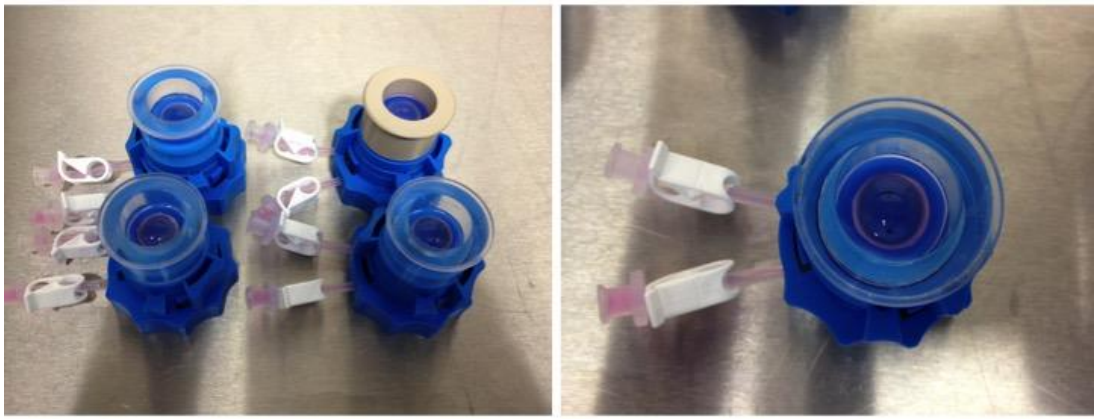


Figure 17 – A plastic ring attached to the artificial anterior mounting chamber was used to hold medium and moisturize the epithelial side of the corneas during the incubation period. However, its use was discontinued due to medium leaking out for some of the corneas causing inconsistencies in the experimental results.

However, that proved ineffective due to the medium leaking through the ring for some of the corneas, therefore the use of these plastic rings was discontinued. Additionally, due to the nature of the model it could only be filled with a limited amount of medium that was injected in the artificial anterior chamber. The medium was not constantly being perfused but was rather injected into the tubing of the model and was clamped in place. This resulted the corneas would start collapsing after some time in air-interface organ culture due to decreasing medium pressure inside the artificial anterior mounting chamber. The corneal surface was moisturized by pipetting 200 µl medium to the epithelial side every 24 hours and the culture

medium of the artificial anterior mounting chamber was exchanged every 24 hours, however, this was not sufficient to prevent the corneas from gradually collapsing when incubating overnight. This would cause an inconsistency to the experimental procedure. Therefore, the long-term incubation period of 14 days proved to be a limiting factor that caused inconsistency in the control of experimental conditions. When analyzing the results from the 14-day incubation experiment it was observed that by the end of the first 24 hours in air-interface organ culture the normal corneas (with intact DM/Endothelium complex) had already reached the maximum reduction in corneal thickness suggesting that when normal corneal endothelial cell function is present the corneal thickness is restored to normal within 24 hours for the air-interface organ culture conditions that were used in this study. It was also observed that after the 4^{th} day of incubation the corneal thickness of the normal corneas gradually started to increase until it almost reached the same level as the decompensated corneas by the end of the 14-day incubation period. This gradual thickness increase could be attributed to a reduction in corneal endothelial cell viability and inadequate hydration of the corneas that could not be consistently controlled throughout the entire incubation period. This makes the current model limited to about 7 days in terms of efficacy to maintain normal corneal endothelial cell pump function. In order to optimize the experiment and to increase the consistency and control of experimental conditions the incubation period of the corneas was reduced to 12 hours. This reduction in incubation period allowed the culture conditions of the corneas to be more easily controlled and maintained consistent for all corneas through the entire experiment. By moistening the epithelial

side of the corneas every hour and by maintenance of the artificial anterior chamber through irrigation every hour. Hence, future studies for optimizing this corneal organ culture model should include optimizing the hydration of the epithelial side of the cornea by using a spraying device that would enable 24-hour constant moisturizing of the cornea. In addition to that a perfusion system to allow constant medium exchange and maintenance of a constant pressure to keep the corneas from collapsing would enable long-term testing of the effects of cell therapy over periods longer than 12 hours. In the normal physiological state some rhythmical changes of the aqueous humour flow have been reported with rates of 3 µl/min in the morning, 2.4 µl/min in the afternoon and 1.5 µl/min at night (Brubaker, 1989). Rhythmical changes of the intraocular pressure have also been observed with values reaching around 14 mmHg in the morning and around 11 mmHg in the afternoon (Sajja and Vemapti, et al., 2013). In our *in vitro* corneal model the endothelial side of the cornea was not continuously perfused but the medium was kept in place by pinch clamps on the tubing of the artificial anterior mounting chamber. As a result of that after about an hour of incubation a slight capsizing of the corneas would be observed caused by a pressure decrease. This effect seemed to be more pronounced on the corneas with intact CE function suggesting that normal CE function can affect the intraocular pressure. Therefore, in the 12-hour incubation experiment the culture medium was exchanged every 1-hour in order to restore the pressure in the *in vitro* model and to keep the corneas from capsizing. Albeit, the pressure levels in the artificial anterior chamber were not measured during any of the experiments. Designing an *in vitro* human corneal model with a normal aqueous human outflow

and intraocular pressure would be very beneficial in keeping the CECs healthy for a longer period of time. Measuring the pressure levels continuously throughout the incubation would allow keeping the corneas from capsizing and that would possibly increase the CE vitality over a long-term incubation period. In addition, for optimizing the *in vitro* model it would be beneficial to mimic the culture conditions as closely as possible to the normal physiological conditions. For example, the ambient humidity is about 30%, however in our case the corneas were incubated in a humidified CO_2 incubator with 95% humidity levels. Interestingly, it has been demonstrated that the pre-corneal humidity does not have any substantial effect on the corneal thickness and de-swelling (Cohen, et al., 1990), therefore culturing the corneas at 95% humidity would not be expected to cause any negative effect on the stromal hydration. Nevertheless, relative humidity has been shown to have an effect on the aqueous tear loss with significant increase of tear loss at 20-25% humidity (McCulley, et al., 2006). Another aspect that could affect the long-term efficiency for maintaining the CE healthy in the *in vitro* model would be dependent on the type of media used for continuous perfusion of the posterior side of the cornea. It has been demonstrated that the aqueous humour plays a role in the CE proliferation control via TGF-β, which helps keep the hCECs in G1 stage (Chen, et al., 1999). Ideally a fluid with similar composition as the aqueous humour might prove to prolong the viability of the CE. In our *in vitro* model we used culture medium to moisturize the epithelial side of the corneas in order to simulate a tear film, however its composition is different from the physiological tear film and might not be as efficient in keeping the epithelium nourished and protected. One of the

decompensated corneas (DM/Endothelium complex removed) underwent histological examination in order to confirm the complete removal of the DM/Endothelium (See Figure 15). It shows a cross-section of the cornea and its epithelium, which is normally multi-layered and therefore appears much thicker than the CE monolayer, seems to be similar in thickness with the CE. This is perhaps indicative of epithelial cell loss during air-interface organ culture and suggests that the culture medium used for moisturizing the epithelium was not very effective in preserving it. For future optimization of the *in vitro* model it would conceivably be more efficient to use an artificial tear solution which would mimic the normal physiological conditions of the eye more closely thus improving the health of the corneal epithelium. This would be an important step in improving the *in vitro* model because even though the majority of the corneal hydration control depends on the CE, the epithelium has also been shown to play a minor role in the stromal hydration (Maurice and Giardini, 1951). The cadaveric human corneas that were used in our study as an *in vitro* corneal model were cultured at a temperature of 37^{o}C, which might not be optimal conditions since it has been shown that the temperature on the surface of the cornea is around 34^{o}C (Dixon and Blackwood, 1991). Hence, altering the culture temperature might also improve the longevity of this corneal model.

Donor cornea characteristics, such as initial corneal thickness, age, period in storage and ECD, were compared for sample-specific variation. In both the 12-hour and 14-day incubation experiments all corneas did not have any significant difference in age and period in storage prior to experimentation ($p>0.05$), therefore, these factors were not considered to have a significant contribution to sample variation.

The overall results in this study demonstrated that the corneal thickness of the normal corneas decreased more significantly compared to the decompensated corneas when cultured using air-interface organ culture. This means that using this i*n vitro* model the presence or absence of a functional corneal endothelial cell monolayer could successfully be demonstrated by measuring its effect on the corneal thickness of normal (intact DM/Endothelium complex) and decompensated (DM/Endothelium removed) corneas. Overall the results of this chapter show that this model can successfully be used for corneal endothelial cell transplantation testing.

2.6. Conclusion

Firstly, this study establishes the base line for stromal hydration behaviour of normal human corneas with adequate endothelial cell density in air-interface organ culture. Secondly, the oedematous phenotype of corneas denuded of their DM/Endothelium complex is demonstrated in comparison to normal control corneas with an intact DM/Endothelium complex. Thirdly, the *in vitro* system for human corneal organ culture used in this study can be successfully utilized for testing the efficiency of transplanted hCECs to decompensated human corneas, making it a valuable tool for cell-therapy evaluation in future studies. In addition, the above study identified that a 12-hour period would be the most appropriate time window to undertake CE functional assessment given the limitations of this *in vitro* set-up.

3. CHAPTER 3 – CELL THERAPY EVALUATION USING AN *IN VITRO* HUMAN CORNEAL MODEL OF ENDOTHELIAL DECOMPENSATION

3.1. Chapter overview

In this chapter the *in vitro* human CE decompensation model, which was established in chapter 2, was used to test human corneal endothelial cell (hCEC) transplantation as a potential treatment of CE disorders in humans. It was found that primary (passage 0) and (passage 2) hCECs have the capacity to restore normal functional CE monolayer after transplantation to the corneal stroma in the absence of a DM. Immortalized HCEC-12 cells were also used as a model for testing hCEC function after transplantation due to their high proliferative capacity that enables them to be readily expanded in culture and allows the generation of sufficient cell quantities that could be used for cell transplantation studies. The corneal endothelial cell function was tested up to 10 days after hCECs were transplanted to the corneal stroma. The corneal thickness was used as a surrogate measure of CE function and was measured using Zeiss Visante OCT over a 12-hour incubation period. The data from this chapter shows that after hCEC transplantation to the corneal stroma a functional CE monolayer is regenerated demonstrating the plausibility of using a cell-therapy approach for treating CE disorders in humans.

3.2. Brief introduction

Current treatments for corneal blindness rely on human cadaveric donor corneas for graft harvesting and transplantation. This limits treatment availability in countries where access to donor tissue is inadequate.

Hence, this chapter aims to evaluate the attachment, monolayer formation and functional potential of cultured human corneal endothelial cells after cell suspension transplantation directly to the posterior corneal stroma using an *in vitro* human corneal decompensation model. It was hypothesized that direct cell transfer to the posterior corneal stroma would result in endothelial cell attachment and will recover normal CE function in decompensated human corneas. The effect of hCEC transplantation on the corneal thickness was tested. The stromal thickness was used as a surrogate measure of hCEC function. Corneal thickness measurements were taken every hour over a 12-hour incubation. The outcome of hCEC transplantation to human donor corneas was evaluated using Zeiss Visante OCT for corneal thickness measurement and by histological assessment of the hCEC monolayer post-transplantation. Primary hCECs (passage 0) and hCECs (passage 2) were used for cell transplantation testing. Immortalized corneal endothelial cells (HCEC-12 cell line) were also used as a model for testing hCEC transplantation because they are readily available and due to their capacity to be easily expanded in cell culture.

3.3. Materials and Methods

3.3.1. List of materials used

Human corneas were obtained from Bristol and Manchester Eye Banks, UK; HCEC-12 – (ACC 646), Leibniz Institute DSMZ – German Collection of Microorganisms

and Cell Cultures; Human bone marrow-derived mesenchymal stem cells (BM-MSCs) - (PCS-500-012), The European Collection of Cell Cultures (ECACC); Mesenchymal Stem Cell Basal Medium – (PCS-500-030), ATCC Primary Cell Solutions; OptiMEM – (51985-026), Invitrogen by Life Technologies; Foetal bovine serum (FBS) – (F0804-500ML), Sigma Aldrich; Epidermal growth factor (EGF) – (E9644), Sigma Aldrich; Calcium chloride – (21115), Sigma Aldrich; Gentamicin – (G1397), Sigma Aldrich; Trypsin/EDTA – (T3924), Sigma Aldrich; Phosphate buffered saline (PBS) – (P3813), Sigma Aldrich; 24-well plates – (CLS3527), Sigma Aldrich; 6-well plates – (CLS3516), Sigma Aldrich; 12-well plates – (CLS3513), Sigma Aldrich; T25 flasks – (12-565-351), Thermo Scientific; T75 flasks – (12-565-350), Thermo Scientific; 15 ml centrifuge tubes – (14-955-160), Thermo Scientific; 50 ml centrifuge tubes – (14-955-161), Thermo Scientific; 0.06% VisionBlue™ (trypan blue); Barron artificial anterior chamber – Barron Precision Instruments; TrytonX-100 – (T8787), Sigma Aldrich; Hoechst 33258 – (94403), Sigma Aldrich; DAKO fluorescent mounting medium – (S3023), Dako; Anti-Na^+/K^+ ATPase primary antibody (mouse monoclonal) - (ab7671), Abcam, Cambridge, UK; Anti-ZO-1 primary antibody (rabbit polyclonal) - (ab59720), Abcam, Cambridge, UK.

Goat anti-rabbit (goat polyclonal) IgG secondary antibody (Alexa Fluor 488) – (ab150077), Abcam, Cambridge, UK; Goat anti-mouse (polyclonal) IgG secondary antibody (Alexa Fluor 488) – (ab150117), Abcam, Cambridge, UK; Multiwell Chambered Coverslips – (24779), Invitrogen; BD Plastipak 10 ml syringes – (SYR188), Medisave; Millex 0.22 um syringe filters – (F7648-50EA), Sigma Aldrich; Zeiss Visante OCT.

3.3.2. Primary hCEC culture

Cornea donor information is available in APPENDIX 2 - Table 20.

- Number of corneas – n=22.
- CE viability was assessed using 0.06% VisionBlue™ (trypan blue) staining prior to primary cell isolation.
- Donor age – 70 ± 13 years old (43-86 range).
- Days in storage – 49.3 ± 21 days (21-88 range).

3.3.2.1. Preparation of bone marrow-derived mesenchymal stem cell-conditioned medium

All primary hCEC cultures were done using mesenchymal stem cell-conditioned medium (MSC-CM), which was prepared according to published protocol by Nakahara, et al. (2013). Human bone marrow-derived mesenchymal stem cells (BM-MSCs) were seeded at 1.3 x 10^4 cells/cm^2 and were cultured for 1 day in DMEM, 10% FBS, 1% penicillin/streptomycin. The cells were then washed with PBS. The medium was then replaced with Opti-MEM-I, 8% FBS, 5ng/ml EGF, 20µg/ml ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate and 50 µg/ml gentamicin. The BM-MSCs were maintained for an additional 24 hours. The medium was then collected and centrifuged at 2000 rotations per minute (rpm) for 10 min and the supernatant was then filtered through a 0.22µm syringe filter. The filtered medium was then used as mesenchymal stem cell conditioned medium (MSC-CM). All primary cultures were cultured at 37°C, 5% CO_2, humidified incubator Heracell™ (relative humidity – 95%). BM-MSCs passages 2 – 6 were used for conditioning.

3.3.2.2.Isolation of primary human corneal endothelial cells

Primary human corneal endothelial cells were isolated from human donor corneas as follows:

1) The corneas were placed endothelial side-up in a trephine base.
2) The endothelium was stained with 0.06% VisionBlue™ (trypan blue) vital stain for 30 sec in order to minimize the cell toxicity effect of the trypan blue vital stain.
3) The Descemet's membrane was then washed and covered with unsupplemented culture medium to avoid drying of the endothelium.
4) Using a surgical knife the Descemet's membrane was scored at its periphery without disturbing the underlying stroma.
5) The DM/Endothelium complex was then peeled using forceps.
6) The cells were then dissociated from the membranes using 1 ml 0.05 % Trypsin/EDTA for 10-15 min at 37^{o}C.
7) After all the cells were dissociated from the Descemet's membrane MSC-CM supplemented with 8 % foetal bovine serum (FBS) was added to stop the trypsin reaction.
8) The cells were centrifuged and the supernatant was removed.
9) 1 ml fresh MSC-CM was then added to the cell pellet.
10) The cells were then transferred to a 24-well plate and were cultured in a humidified incubator at 37^{o}C, 5 CO_2 (relative humidity – 95%).
11) The cells were incubated until confluence was reached – between 7-21 days. The medium was replaced every 2 days.

3.3.3. HCEC-12 culture

The immortalized human corneal endothelial cell line HCEC-12 was used for culture and cell therapy testing due to its high proliferative capacity, which allowed it to be easily expanded in culture. These cells are a useful experimental tool because they seem to retain some characteristics of the normal CE, such as expression of CE-like morphology at confluence and CE pump function (Aboalchamat, et al., 1999). The HCEC-12 cell line, established in 1996 from normal corneal endothelial cells of a 91-year-old Caucasian woman transformed with the early region of the Simian virus 40 (SV40) genome including genes encoding large T-antigen and small t-antigen, was obtained from the Leibniz Institute DSMZ (German collection of Microorganisms and Cell Cultures). HCEC-12 cells were cultured using F99 (mixture of Ham's F12 and Medium 199 in 1:1 ratio), 10% FBS in a humidified incubator with 5% CO_2 at 37ºC (relative humidity – 95%). The medium was changed every 2 days. The cells were seeded out at 50 cells/mm^2. Confluent cultures were split 1:2 to 1:4 every 4-5 days using 0.05% trypsin/EDTA.

3.3.4. Immunocytochemistry

Primary antibodies against human Na^+/K^+ ATPase (ab7671), zona occludens 1 (ZO-1) (ab59720) and secondary anti-mouse and anti-rabbit antibodies were obtained from Abcam, Cambridge, UK. HCEC-12 and primary hCECs were cultured on a glass slide with 4 separate chambers and the cells within each chamber were stained with a different antibody. Cells were seeded at 1000 cells/mm^2. HCEC-12 were cultured in F99 (Ham's F12/Medium 199 1:1 mixture), 8% FBS. Each chamber contained 1 ml final volume culture medium. Primary hCECs were cultured using

OptiMEM-I, 8 % FBS, 5 ng/ml epidermal growth factor (EGF), 20 μg/ml ascorbic acid, 200 mg/L calcium chloride, 0.08 % chondroitin sulfate, 50 μg/ml gentamycin. Both HCEC-12 and primary hCECs were incubated overnight at 37ºC, 5 % CO_2, 95% humidity before staining with antibodies.

A commonly used procedure for antibody staining was done as follows: the culture medium was aspirated from the glass slide. The glass slide was then washed in phosphate-buffered saline (PBS) 3 times. 10 % Paraformaldehyde (PFA) solution was used to fix the cells for 10 minutes at room temperature. After the PFA was removed the cell were washed with PBS 3 times. Blocking solution (10% FBS, 0.1% TritonX-100 in PBS) was added and the cells were incubated for 1 hour at room temperature. The primary antibodies for Na^+/K^+ ATPase and ZO-1 were diluted in blocking solution in a 1:100 dilution as per the manufacturer's protocols. The solution with each antibody was then added to the respective chamber on the glass slide. The cells were incubated with the primary antibodies overnight at 4ºC. The next day the primary antibody solution was removed and the cells were washed 3x with PBS for 10 min. The secondary antibodies were diluted in blocking solution with Hoechst DNA stain. The slides were then wrapped in foil to protect the photosensitive fluorophores. The diluted secondary antibody/Hoechst mixture was then added and the cells were incubated for 1 hour at room temperature. After 1 hour the secondary antibody/Hoechst solution was removed and the cells were rinsed 3x with PBS for 10 minutes each. Before mounting the slides were quickly washed with H_2O and were then dried off. DAKO mounting medium was used to mount a coverslip to the glass slide, which was then allowed to dry at room temperature for 4

hours wrapped in foil. They were then viewed using a Leica Microsystems CMS confocal microscope.

3.3.5. Human donor corneas used for human corneal endothelial cell transplantation

Human donor corneas (n=17) for this study were supplied by Bristol Eye Bank, Bristol, UK and Manchester Eye Bank, Manchester, UK in three separate batches during separate time intervals (cornea donor information is available in APPENDIX 3 Table 21. The corneas were stored in Eagle's Minimum Essential Medium (MEM) supplemented with 2 % foetal bovine serum (FBS) at 37^{o}C. Transportation of the corneas was at 25^{o}C. No dextran was added to the medium at any point during storage and culture. Corneas age ranged between 46 - 81 years. Each of the three batches of corneas underwent removal of the DM/Endothelium complex in order to establish the CE decompensation model. Group 1 (n=6) was seeded with HCEC-12 cells, group 2 (n=5) was seeded with passage 0 primary hCECs and group 3 (n=6) was seeded with passage 2 cultured hCECs.

3.3.6. Corneal endothelial cell transplantation in an in vitro human corneal model of endothelial decompensation

All corneas had their DM/Endothelium complex removed as described in the methods section 2.3.4 and were divided into three groups. In group 1 was seeded with HCEC-12 cells via cell suspension in culture medium F99 (1:1 mixture of Ham's F12 and Medium 199), supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin. In groups 2 and 3 primary cultured hCECs (passage 0 and passage 2 respectively) were used for seeding via cell suspension in culture

medium OptiMEM-I, 8 % FBS, 5 ng/ml epidermal growth factor (EGF), 20 µg/ml ascorbic acid, 200 mg/L calcium chloride, 0.08 % chondroitin sulfate, 50 µg/ml gentamycin.

3.3.6.1.Group-1 corneas

Cell therapy group-1 (n=6) corneas were used test the capacity of immortalized HCEC-12 cells to attach and form a functional corneal endothelial cell monolayer after transplantation to the posterior corneal stroma. For that purpose HCEC-12 cells were cultured to confluence using F99 (mixture of Ham's F12 and Medium 199 in 1:1 ratio), 10% FBS. The medium was changed every 2 days. After reaching confluence the cells were harvested using 0.05% trypsin/EDTA and were centrifuged. A cell seeding density of 310,000 cells/200µl per cornea was used for transplantation.

3.3.6.2.Group-2 corneas

Cell therapy group-2 (n=5) corneas were used to test the capacity of normal primary hCECs to attach and form a functional corneal endothelial cell monolayer after transplantation to the posterior corneal stroma. For that purpose passage 0 primary human corneal endothelial cells were isolated from n=5 human donor corneas. These cells were then seeded to the posterior stroma of the same corneas at a cell seeding density of 260,000 cells/200µl per cornea.

3.3.6.3.Group-3 corneas

Cell therapy group-3 (n=6) corneas were used to test the potential of primary cultured hCECs to attach and form a functional corneal endothelial cell monolayer

after transplantation to the posterior corneal stroma. For that purpose, passage 2 cultured primary human corneal endothelial cells were harvested from a tissue culture flask using 0.05 % trypsin/EDTA, followed by centrifugation in order to pellet the cells. The cell pellet was then diluted with fresh medium to a desired cell concentration. A seeding density of 155,000 cells/200µl per cornea was used for transplantation. All three groups are summarized in Table 8. There was no statistically significant difference between the three groups when age was compared. There was no statistically significant difference between groups 1 and 2 and 1 and 3 when the period in storage was compared, however there was a statistically significant difference between groups 2 and 3 ($p=0.001$). There was statistically significant difference between the cell-seeding densities for the three groups; however, all three seeding densities were sufficient to restore normal corneal endothelial cell function. There was a statistically significant difference between the post-cell transplantation periods of incubation prior to functional evaluation but all three incubation periods (3, 7 and 10 days) were sufficient for the corneal endothelial cells to attach to the stroma and form a functional corneal endothelium. The primary cells used in groups 2 and 3 were allowed to incubate longer on the corneal stroma in order to ensure proper attachment and monolayer formation on the corneal stroma since the primary cells are more fragile compared to the immortalized cells. Cell seeding, allowing the cells to attach and form a monolayer and functional evaluation were done in three stages.

Table 8 – Summary of cell therapy cornea groups.

	Group 1 (n=6)	Group 2 (n=5)	Group 3 (n=6)
DM/Endothelium complex	Removed	Removed	Removed
Age (years)	60.5 ± 13.99 (range - 46-78)	66.4 ± 12.25 (range - 46-76)	74 ± 8.08 (range - 67-81)
Period in storage prior to experiment (days)	47.33 ± 13.6 (range - 30-60)	51.2 ± 7.01 (range - 44-60)	33.5 ± 0.57 (range - 33-34)
Cells used for transplantation	HCEC-12	Primary hCECs (passage 0)	Cultured hCECs (passage 2)
Cell seeding density per cornea	310,000 cells/200µl (2070 cells/mm^2)	260,000 cells/200µl (1740 cells/mm^2)	155,000 cells/200µl (1036 cells/mm^2)
CE functional evaluation (days after cell transplantation)	3	10	7
Time period during which CE function was assessed (hours)	12	12	12

Note: Descemet's membrane (DM). The corneas from each group were received in three separate batches. Each batch was processed individually and experiments were run separately for each batch.

3.3.6.4.Corneal endothelial cell transplantation – Stage 1 – Cell seeding

All human donor corneas in the three groups were held in inverted position with the endothelial side facing up (native DM/Endothelium removed) at the bottom of a 50ml centrifuge tube. Human corneal endothelial cells were transplanted by pipetting a cell suspension diluted in 200µl of culture medium to the posterior stroma. After cells were transferred to the posterior stroma of each cornea in 200µl culture medium they were allowed to settle onto the posterior stroma aided by gravity. The epithelial side of each cornea was maintained moist with 50µl culture medium. The corneas were then incubated at 37ºC, 5 % CO_2, 95% humidity. After 4 hours it was evident that both the immortalized (HCEC-12) and the primary hCECs were already staring to attach to the stroma but still appeared rounded, suggesting that attachment was still incomplete (See Figure 18).

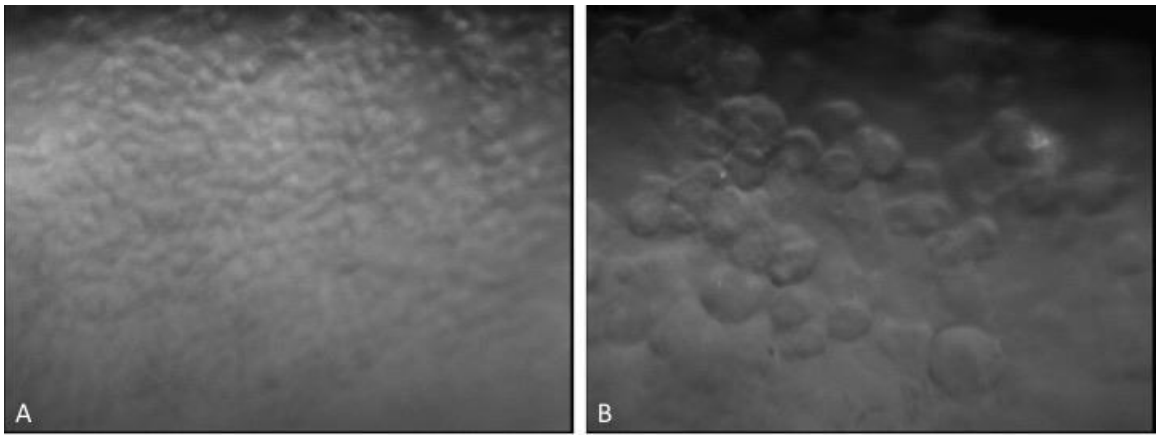


Figure 18 – Human corneal endothelial cell transplantation to *ex vivo* cultured human donor corneas. HCEC-12 (A) and primary human corneal endothelial cells (B) are attaching to the stroma 4 hours after transplantation but still appear rounded as a result of incomplete attachment evidencing an immature corneal endothelial cell monolayer [EB-2000xyz Eye Bank Specular Microscope: 450µm x 500µm field of view].

3.3.6.5.Corneal endothelial cell transplantation – Stage 2 – Cell attachment

After the initial 4 hours incubation the centrifuge tubes were then topped up with additional 10-20 ml of culture medium and the corneas were left to incubate in immersion organ culture in order to allow the monolayer to mature, tight junctions to be formed and the cells to adopt normal physiological morphology. Based on the proliferative capacity of the HCEC-12 cells and the primary hCECs the incubation time in immersion culture was set at 3 days for group-1 corneas, which were seeded with the HCEC-12 cells, and 7-10 days for groups 2 and 3, which were seeded with passage 0 and passage 2 primary hCECs. Previous studies have reported incubation periods of 7-14 days prior to morphological or functional evaluation (Aboalchamat, et al., 1999; Engelmann, et al., 1999 and Chen, et al., 2001). The culture medium was changed every second day. The cell seeding densities varied between the three groups based on possible cell yield in culture. Immortalized HCEC-12 cells, due to their proliferative capacity, were more readily available for use in cell transplantation studies. However, passage 0 and passage 2 primary hCECs were more scarce due to poor proliferation response in culture, hence, a lower cell seeding densities were used in groups 2 and 3. Albeit, all seeding densities were sufficient to produce a functional CE monolayer and were well above the critical CE density of 300-500 cells/mm^2 below which normal CE function gets compromised (Tuft and Coster, 1990).

3.3.6.6.Corneal endothelial cell transplantation – Stage 3 – Corneal endothelial functional assessment

Following these incubation periods, single corneas were removed from immersion culture and were placed in air interface organ culture using the *in vitro* air interface organ culture

model described in section 2.3.5. At this point stromal thickness measurements were done every hour for up to 12 hours to evaluate the physiological response. Previous studies have reported successful functional evaluation for 8-hour, 12-hour and 24-hour incubation periods (Aboalchamat, et al., 1999; Engelmann, et al., 1999 and Chen, et al., 2001).

3.3.6.7.Positive and negative controls

The behavior of the corneas with intact DM/Endothelium complex over a 12-hour incubation period was used as a positive control for the presence of normal corneal endothelial cell function, whereas corneas whose DM/Endothelium complex was removed served as a bullous keratopathy model (See 2.4.2)

3.3.7. Corneal endothelial cell density calculation

The area of a cornea was calculated using the formula for calculating the area of a dome: $A = 2 \pi r h$. Where 'r' is the radius of the cornea and 'h' is the anterior chamber depth or the distance between the corneal endothelium and the anterior surface of the intraocular lens. The average diameter of a human cornea was assumed to be about 11.7 ± 0.42 mm (Rüfer and Erb, 2005) and the average anterior chamber depth measured by Visante OCT (the same brand of instrument that was used in this study) was 2.98 ± 0.29 mm (Dinc, et al., 2010). Therefore, the approximate area of a human cornea was calculated to be equal to 149.51 mm^2. Corneal endothelial cell density was calculated by dividing the cell seeding density by the area of the cornea.

3.3.8. Optical Coherence Tomography assessment of corneal endothelial cell function after cell transplantation

Corneal thickness was measured as described in section 2.3.5.3.

3.3.9. Histology

Histology was used to assess the corneal endothelial cell monolayer after each experiment in order to confirm the post-transplantation effects, such as cell attachment, morphology and monolayer formation after transplantation directly to the stroma of the human corneas. After each experiment the human corneas were fixed in 10% formalin for 10 min at room temperature and were stored at 4^{o}C overnight. Histological processing, haematoxylin and eosin (H&E) staining and sample analysis was done in Addenbrooke's Hospital, Cambridge, UK.

3.3.10. Statistical analysis

Single factor ANOVA test was used for statistical analysis to determine if there was a statistically significant difference between the three corneal groups after a period of air-interface organ culture. Confidence levels were set at 95%. $P<0.05$ was considered statistically significant. Post hoc t tests with Bonferroni correction (p value divided by the number of tests) were used to compere the corneal thickness of the different groups of corneas at specific time points throughout the incubation period. The error bars in all graphs and bar charts represent the mean ± standard deviation (SD).

3.4. Results

3.4.1. HCEC-12 culture

After they became confluent the HCEC-12 cells had a cell density of 1800 ± 173 cells/mm^2, showed positive expression of Na^+/K^+ ATPase and ZO-1, which was also present throughout the cell membrane but had highest expression on the cell borders especially where the cells formed contact with each other (See Figure 19). These cells were used for transplantation into group-1 corneas.

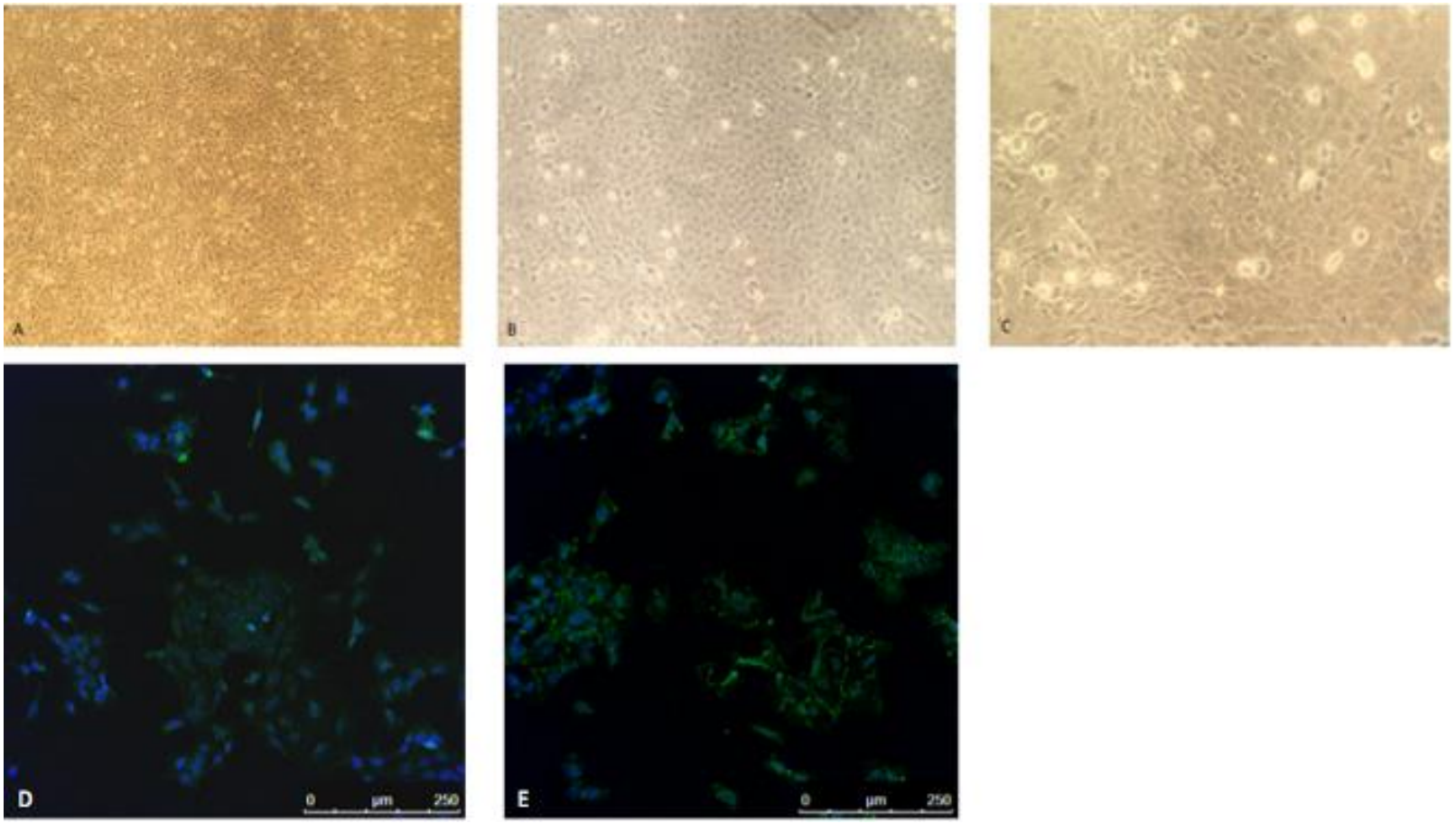


Figure 19 - HCEC-12 cells. Endothelial cell density – 1800 ± 173 cells/mm^2 [Phase-contrast; x40 (A), x100 (B), x400 (C)]. Na^+/K^+ ATPase staining (D), zona occludens-1 staining (E) [Confocal microscopy; x100].

3.4.2. Passage 0 primary human corneal endothelial cells

Passage 0 primary hCECs were isolated from a DM and were used for transplantation into group-2 corneas (See Figure 20).

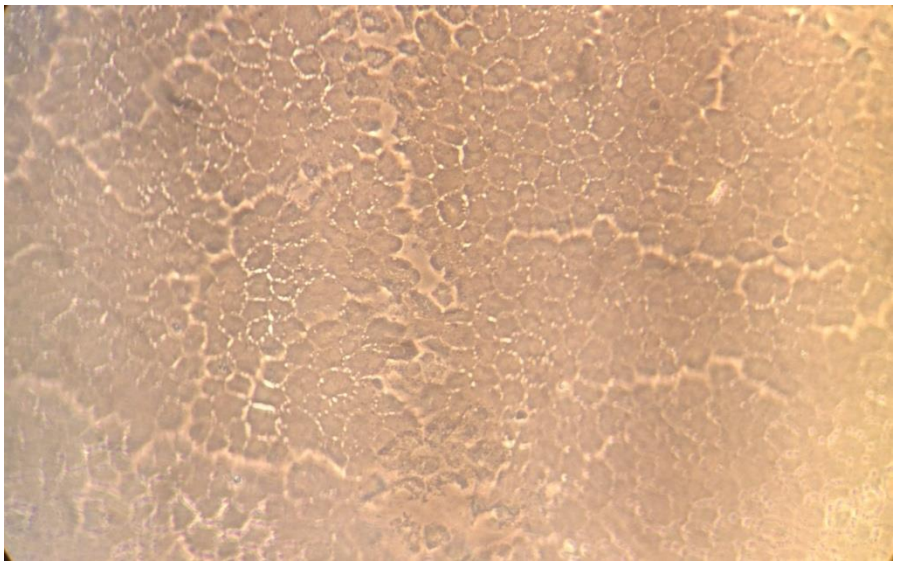


Figure 20 – Primary hCECs (passage 0) were dissociated from the Descemet's membranes of individual corneas and were used for transplantation into corresponding group-2 corneas. Endothelial cell density – 1900 cells/mm^2 [Phase-contrast; x400].

3.4.3. Primary human corneal endothelial cell culture

Out of the 23 corneas used for primary hCECs isolation and culture 7 cultures were successfully initiated and were able to reach confluence (See Table 9).

Table 9 - Primary cultures that successfully reached confluence.

Cornea number	Seeding density	Days to confluence	Cell density at confluence ± SD	Number of passages
1	192 cells/mm^2	15	-	1
2	314 cells/mm^2	10	366 ± 115 cells/mm^2	2
3	650 cells/mm^2	18	733 ± 378 cells/mm^2	0
4	500 cells/mm^2	26	400 ± 100 cells/mm^2	0
5	2200 cells/mm^2	3	1966 ± 378 cells/mm^2	1
6	1000 cells/mm^2	15	200 ± 100 cells/mm^2	1
7	1200 cells/mm^2	26	766 ± 588 cells/mm^2	2

Note: Cornea donor information sheets are available in APPENDIX 2 Table 20.

There was no statistically significant difference in age, period in eye bank storage and cell seeding density between those cultures that were successful and those that were not (See Table 10). Passage 2 primary cultures, which exhibited positive Na^+/K^+ ATPase and ZO-1 expression after sub-culture were used in transplantation into group-3 corneas (See Figure 21).

Table 10 – Comparison of the characteristics of donor cornea used for primary cell culture initiation.

Primary cultures	Age (years)	Period in eye bank storage (days)	Seeding density (cells/mm^2)
Successful (n=7)	69.5 ± 4.46	45.16 ± 11.58	977 ± 681
Unsuccessful (n=16)	70.4 ± 14.92	51.71 ± 22.78	498 ± 238
P value	Non-significant	Non-significant	Non-significant

Note: P<0.05 was considered statistically significant.

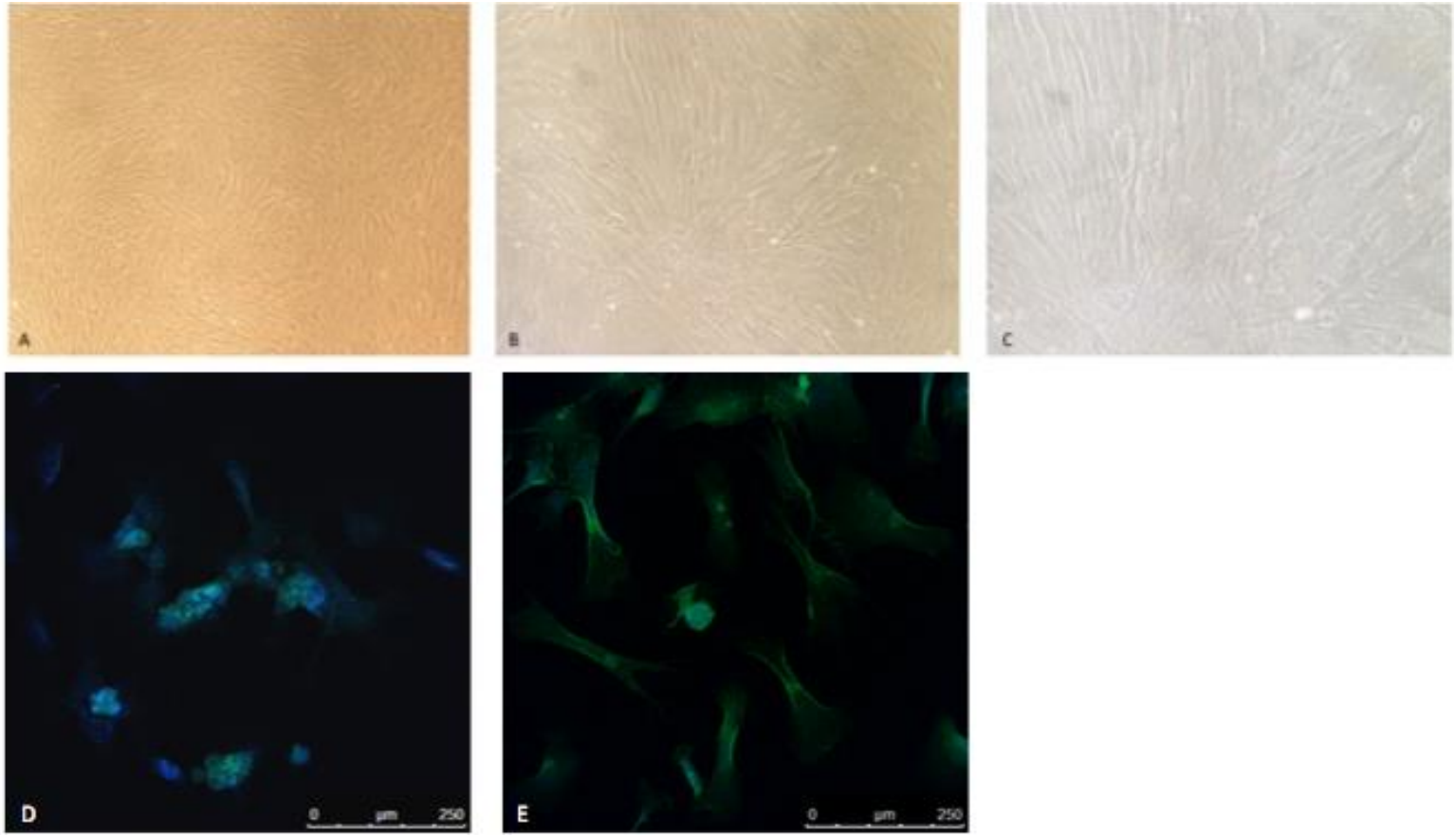


Figure 21 – Passage 2 primary human corneal endothelial cells sub-cultured at a cell density of 60 cells/mm^2 and reached confluence 9 days later. [Phase-contrast microscopy; x40 (A), x100 (B) and x200 (C)]. Na^+/K^+ ATPase staining (D), zona occludens-1 staining (E) [Confocal microscopy; x100].

3.4.4. Assessment of corneal endothelial cell function up to 10 days after cell transplantation

All cell therapy-treated corneas as well as the normal and decompensated corneas had an oedematous phenotype at the beginning of the experiment as a result of being stored in immersion organ culture conditions. The initial corneal thickness measurement at the start of the experiment for each group was: group 1 – 1218.02 ± 153.65 μm, group 2 – 1100.86 ± 86.15 μm and group 3 - 1143.56 ± 69.23 μm. After transfer to air-interface organ culture conditions a gradual thickness decrease was observed in all corneas. At the end of the 12-hour incubation group 1 had a corneal thickness of 458.91 ± 90.07 μm (a 63.15 ± 6.14 % decrease from its initial thickness), group 2 had an average thickness of 489.65 ± 94.62 μm (a 55.61 ± 7.44 % decrease from its initial thickness), and group 3 had a thickness of 613.7 ± 47.23 μm (a 46.19 ± 4.61 % decrease). All three cell therapy-treated groups reached a near normal physiological thickness between 5 and 7 hours of incubation and maintained it till the end of the incubation period. Their phenotype and thickness were similar to the normal corneas (See section 2.4.2) and were significantly thinner compared to the decompensated corneas, which maintained a severely oedematous phenotype throughout the entire period of incubation (See section 2.4.2). There was no statistically significant difference between the mean corneal thicknesses (μm) of the normal corneas and the cell therapy-treated groups 1, 2 and 3 during the 0-12 hours incubation ($p>0.05$). There was a statistically significant difference, however, between the mean thicknesses (μm) of the decompensated corneas and the cell-

therapy treated groups 1, 2 and 3 ($p<0.05$) [See Figure 22]. There was no statistically significant difference between the mean percentage corneal thickness decrease (%) of the normal corneas and the cell therapy-treated groups 1, 2 and 3 ($p>0.05$). There was, however, a statistically significant difference between the % thickness decrease of the decompensated corneas compared to the cell therapy-treated groups 1, 2 and 3 ($p<0.05$) [See Figure 23].

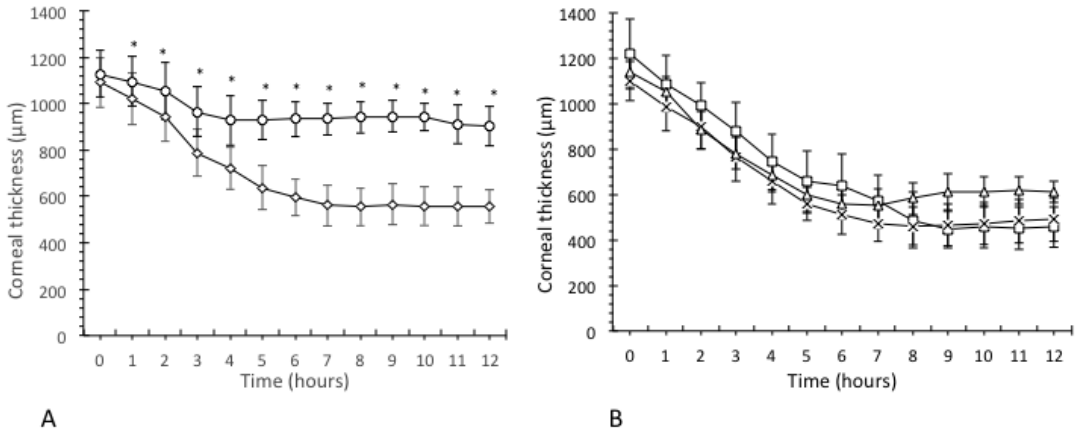


Figure 22 – Corneal endothelial cell function was assessed 3 to 10 days after human corneal endothelial cell (hCEC) transplantation by subjecting the corneas to a 12-hour experiment in air-interface organ culture incubation using the in vitro corneal model. Normal corneas (n=6) [◇] and decompensated corneas (n=5) [○] were used as controls for normal corneal endothelial cell function and bullous keratopathy respectively (A). Group 1 (n=6) [□], group 2 (n=5) [×], group 3 (n=6) [△] decompensated corneas underwent cell therapy treatment (B). A single-factor ANOVA test showed that there was no statistically significant difference between the mean thicknesses of the normal corneas and groups 1, 2 and 3 but there was a statistically significant difference between the mean thicknesses of the decompensated corneas and groups 1, 2 and 3. P<0.05 was considered statistically significant.

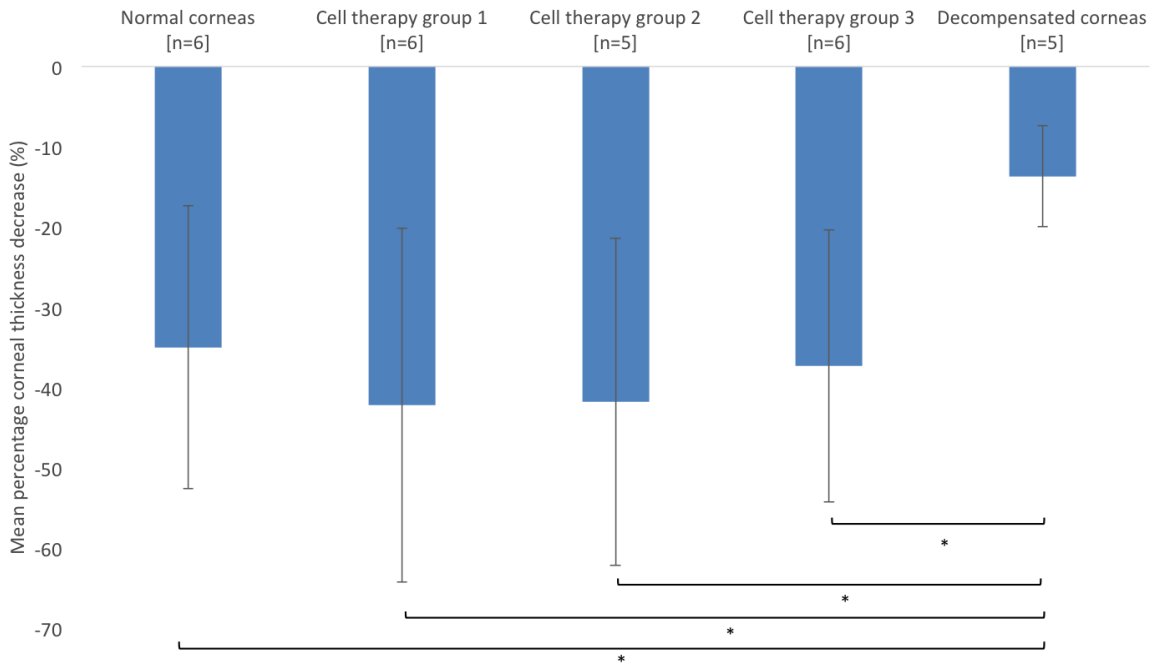


Figure 23 – The mean percentage (%) thickness decrease over 0-12 hours incubation was compared. The normal corneas showed an average 48.27 ± 9.78 % thickness decrease, group 1 - 63.15 ± 6.14 %, group 2 - 55.61 ± 7.44 %, group 3 - 46.19 ± 4.61 %, and the decompensated corneas - 19.27 ± 10.87 %. A single-factor ANOVA test showed that there was no statistically significant difference between the normal corneas and the cell therapy groups 1, 2 and 3 but there was a statistically significant difference between the decompensated corneas and cell therapy groups 1, 2 and 3. *P<0.05 was considered statistically significant.

At the end of the 12-hour experiment in air-interface organ culture incubation all three cell-therapy groups reached physiological thickness levels. Additionally, the posterior stromal folds that were visible at 0h were no longer present after the 12-hour period (See Figure 24).

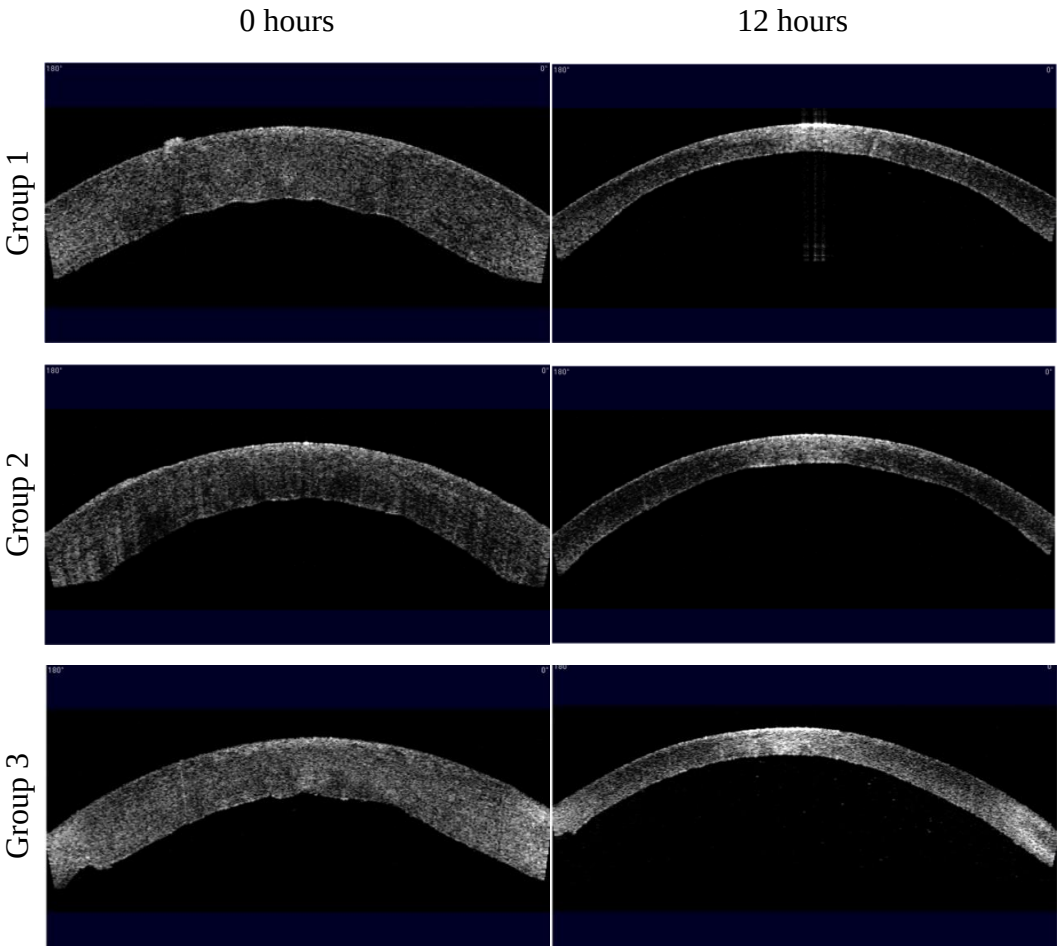


Figure 24 – Optical Coherence Tomography: high-resolution quadrant images of cell therapy treated corneas. All three cell-therapy groups are showing a significant reduction in thickness to normal physiological levels.

3.4.5. Endothelial cell attachment and formation of cell monolayer following cell transplantation

A corneal endothelial cell monolayer with a normal phenotype was observable in groups -1 and -2 attached to the corneal stroma 3 and 10 days after HCEC-12 and passage 0 primary hCECs transplantation respectively, however in group 3 the cells appeared multi-layered and spindle-shaped (See Figure 25).

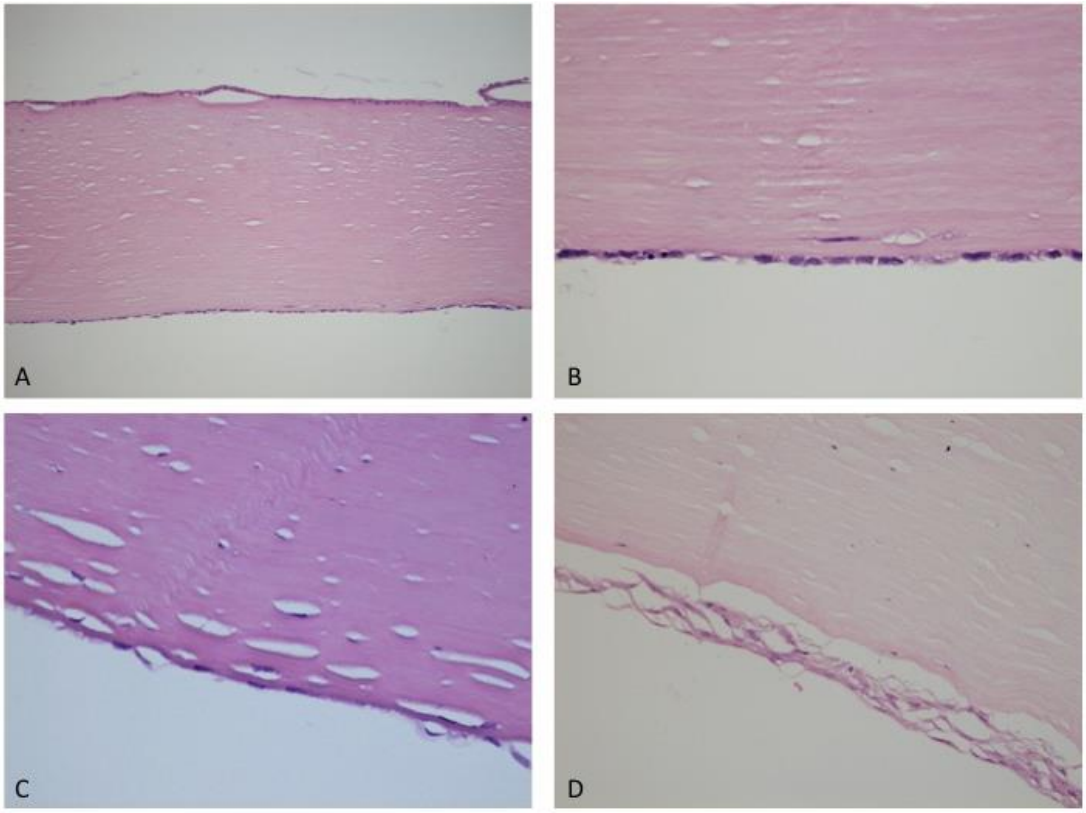


Figure 25 - Histological sections of cell therapy treated corneas. In Group 1 (A and B) and in group 2 (C) a monolayer of cells is present 3 and 10 days after transplantation to the corneal stroma respectively. In Group 3 corneas a multi-layer of cells was seen 7 days after transplantation possibly a result of using too high cell seeding density (D; Note: cell detachment from the stroma is an artifact of histological processing). Basement membrane secretion was not observed in any of the groups throughout the duration of the experiment [Haematoxylin and Eosin (H&E) staining; (A) x100; (B and C) x400; (D) x200].

3.5. Discussion

Studying the potential of human corneal endothelial cell transplantation as a treatment of corneal decompensation has currently generated significant interest.

The development of this novel approach can significantly reduce or perhaps even eliminate the need for donor tissue.

In this study hCECs were isolated and seeded at different seeding densities into 24-well plates and primary cultures were successfully initiated from 7 human corneas. Microscopic observations seemed to indicate that at high seeding densities, as in the case of cornea 5 (see Table 9), the primary culture was able to reach confluence in a shorter period of time and achieved higher endothelial cell density compared to primary cultures that were initiated at lower seeding densities. The comparison of the seeding density between the successful and unsuccessful cultures appeared to be statistically non-significant (See Table 10), however, this could be due to limited sample size. Nevertheless, microscopic observation seemed to point otherwise and seemed to suggest that the seeding density is an important factor for successful primary cell culture initiation and high seeding density enabled the cultures to reach confluence more quickly.

Many research studies have been published in recent years focused on developing a novel cell therapy approach for treating corneal blindness using a variety of animal transplantation models, however, there are a limited number of studies that have examined the plausibility of a cell-based therapy using human donor corneas as a transplantation model to restore a functional corneal endothelial cell monolayer (Engelmann, et al., 1993 and 1999; Böhnke, et al., 1999; Aboalchamat, et al., 1999; Chen, et al., 2001 and Joyce, et al., 2012). These studies that used human donor corneas with intact DMs for hCEC transplantation testing reported that at seeding densities of 50,000, 150,000 and 700,000 cells/cornea a density of 1800 – 2200

cells/mm^2 was achieved. Additionally, 20 days after transplantation the corneas showed significant thickness decrease and reached physiological thickness after perfusion culture over an incubation period between 8 – 24 hours (Engelmann, et al., 1993; Engelmann, et al., 1999; Aboalchamat, et al., 1999). In another study Chen, et al. (2001) tested hCEC transplantation using *in vitro* cultured human donor corneas denuded of their native endothelium, but still with intact DM. Their results corroborated previous studies by Engelmann, et al., (1993), Engelmann, et al., (1999) and Aboalchamat, et al., (1999) and reported that at seeding densities between 250,000 and 500,000 cells a post-transplantation average cell density of 1895 ± 178 cells/mm^2 was reached 7-14 days after transplantation. However, Chen et al., (2001) only assessed the cell attachment and morphology but not the corneal endothelial cell pump function, which still leaves the question whether seeding the hCECs directly to the corneal stroma would have a negative effect on their attachment and function. In all of these studies cell-therapy outcomes showed very encouraging results of significant corneal thickness decrease. A limitation of these human corneal models, however, is that in such an experimental set up, it might be very difficult to ascertain if there were any residual host endothelial cells that were left behind on the host DM as they might confound the observed results. Additionally, after the removal of the native corneal endothelium the Descemet's membrane was left intact and was allowed to serve as a natural substrate for the newly transplanted cells. Since the DM is the natural substrate for the corneal endothelial cells it would enable and facilitate corneal endothelial cell attachment and monolayer formation. It has been shown that the presence of an intact DM

promotes endothelial cell migration on human corneas (Soh, et al., 2016). It has also been shown that the DM stimulates CE regeneration in rabbits (Bhogal, et al., 2017; Chen, et al., 2017a). But in reality in some patients the DM has to be removed prior to transplantation due to fibrosis and the cell-therapy would have to be applied directly to the corneal stroma. Because the DM and the corneal stroma have different composition and structure this might affect corneal endothelial cell, attachment, morphology and function. This structural difference of the underlining substrate might affect cell attachment, morphology and function.

There is very limited evidence for the cell fate of hCECs after transplantation to bare posterior human corneal stroma. Patel, et al. (2009), on the other hand, showed that a method using superparamagnetic microspheres (SPMs) incorporation into hCECs and an external magnet were capable of inducing hCEC attachment to the corneal stroma. The technique employs a method for co-culture of hCECs with magnetic microspheres, which allows the microspheres to be absorbed by the cells, and thus allowing the cells to be attracted towards a magnet. This study gave an important insight that hCECs attachment to the corneal stroma can be facilitated using SPMs. However, it is still questionable whether the cells have the ability to attach to the stroma unaided. The use of such method and equipment are not readily available in most hospitals and therefore could severely limit its clinical application. Additionally, the long-term effect of the magnetic spheres on the viability, morphology and function of hCECs after transplantation is not known. Another limitation of the study of Patel, et al., (2009) is that hCEC function was not tested after transplantation to the stroma. Therefore, the effect of the structure of the

underlining substrate on the corneal endothelial cell function after transplantation remains unknown. Choi, et al., (2010) used 120-200 μm slices of decellularized human corneal stroma for hCEC transplantation and showed that the seeded cells still retained expression of Na^+/K^+ ATPase and ZO-1. This positive expression of CE functionality markers suggested that the transplanted hCECs will still be functional but this was not experimentally demonstrated. Moreover, since this study used decellularized stroma it still remains unknown how the absence of the stromal keratocytes would affect the hCECs biology and physiological function over time.

Therefore, in order to build on the limitations of previous published literature it was aimed in this study to test the plausibility of hCEC transplantation directly onto the corneal stroma, and to determine how this change of substrate affects their attachment, morphology and function combined with histological evaluation. Corneal de-swelling, measured by Zeiss Visante OCT, was used as a surrogate measure of corneal endothelial ion pump function. For that purpose, human donor corneas, denuded of their native DM/Endothelium complex were used to establish an *in vitro* human corneal model of endothelial decompensation suitable for hCEC transplantation testing. This model could be used to demonstrate the corneal de-swelling in normal and decompensated corneas *in vitro* and to compare them with cell-therapy treated corneas. The results of this chapter clearly demonstrate that human corneal endothelial cells were able to attach to the stroma under the action of gravity, which facilitated cell sedimentation when the corneas were incubated with the endothelial side facing upwards. After transplantation directly to the posterior stroma the hCECs formed a endothelial cell monolayer as evidenced by histology

and were able to reduce the corneal thickness of the recipient corneas to normal physiological levels measured by Zeiss Visante OCT. These results are very significant because they show that in cases, where the DM has had to be removed due to fibrosis and scarring, cell-therapy could still be successfully applied as an effective treatment and could restore normal corneal endothelial cell function. In these experiments the cell suspension was sedimented and cell attachment was facilitated under the influence of gravity. Such attachment was histologically shown to be stable and firm forming a monolayer of normal-shaped cells with high viability 3-10 days after transplantation. However, the minimum duration that is required for this gravity-induced attachment is not yet confirmed. In our study we demonstrated that after transplantation to the bare posterior corneal stroma the hCECs are able to function normally and to regulate stromal hydration and restore normal corneal thickness in decompensated human corneas.

In this study cell densities of 260,000 cells/cornea and 155,000 cells/cornea respectively were used for transplantation, which yielded on average a cell density of 2000 ± 212 cells/mm^2 after transplantation. The *in vitro* model for air-interface culture of human donor corneas was restricted to a 12-hour CE functionality testing experiment and CE function was tested between 3 to 10 days after transplantation.

There are a number of limitations that restrict the use of this model and these include donor availability with signed research consent. For this study mainly elderly corneas were used and the scarcity of young cadaveric corneal donors for research is another issue. In this project gravity-aided cell settlement was used to achieve cell attachment to posterior cornea in our *in vitro* model. This might pose some

challenges when it comes to translating these results to clinical application. For example, after cell transplantation the patient would have to be kept in a facedown position for up to 4 hours to aid cell attachment. Also the effect of aqueous humour circulation on cell attachment is unknown. It may cause washing off of the transplanted cell suspension, which in turn might cause blockage in the trabecular meshwork and a raise in intraocular pressure. If the intraocular pressure increases too much this can cause other complications, such as glaucoma. Therefore, the cell transplantation technique, that is used to deliver cell-therapy to patients, needs to be re-assessed and modified so that it can be optimized for clinical use. In this study the human *in vitro* corneal model could only be used between 12-hour incubation experiments and up to 14 days, which did not allow for long-term assessment of cell transplantation outcomes, such as long-term effect on cell morphology, attachment, proliferation, cell survival, scar formation and fibrosis. The nature of the *in vitro* model posed a limitation to infection control since the corneas had to be exposed to the open air when corneal thickness measurements were performed using the Zeiss Visante OCT. Additionally, the model although portable, is still constraining to testing corneal transparency. Another limiting factor of using this model is that Human Tissue Act (HTA) regulations restricting only certain labs suitable to test these models using human tissue and there is a long period of time required in order to put in place the necessary licenses for any work to commence. Other limitations of the model and strategies for improving its longevity were discussed in the Discussion section of CHAPTER 2 – ESTABLISHING AND VALIDATION OF AN *IN VITRO* HUMAN CORNEAL MODEL OF ENDOTHELIAL DECOMPENSATION.

In this chapter a cell suspension in F99 or OptiMEM-I culture media was seeded onto human donor corneas. The anterior chamber of the eye has aqueous humour circulating inside and bathing the CE which has a different composition compared to culture medium. Also the lack of circulation in the *in vitro* model that was used in this chapter facilitated cell attachment after seeding because the cells could remain undisturbed where otherwise in an *in vivo* model they would probably be washed off to some degree by the circulating aqueous. This could pose additional clinical complications in patients since the washed off cells might block the aqueous humour drainage system and possibly lead to increased intraocular pressure which in turn might lead to glaucoma. There is also a possibility that the washed off cells might reach other parts of the body via the circulation. This could pose serious consequences if the transplanted cells become spontaneously transformed and gain capacity to proliferate uncontrollably and form tumors and are able to spread to other parts of the body. Other research studies that have explore cell injection of a cell suspension in intraocular irrigating solution into an *in vivo* animal model found that this technique by itself is not effective in restoring a CE monolayer as compared to transplantation using a collagen sheet as a carrier (Koizumi, et al., 2007). Later studies found that the use of a Rho kinase inhibitor Y-27632 was able to improve cell attachment after injection of cells suspended in DMEM culture medium into the eye of primates and rendered this transplantation technique efficient in recovering a functional CE (Okumura, et al., 2016). Interestingly, in it appears that the use of culture medium for cell injection was an effective method for delivering viable cells to the eye of this primate model. Another study that used *in vivo* rabbit and primate

models also reported a successful outcome after injecting cells suspended in endothelial serum-free culture medium using Y-27632 inhibitor (Shen, et al., 2017).

However, additional very extensive work is required before the cell-therapy evaluation can be used for clinical trial studies. For example, by improving the culture conditions and the *in vitro* proliferation of hCECs a cell bank can be established, which would greatly increase the cell-therapy availability. Studying the cell fate in culture and after transplantation, finding ways to inhibit proliferation once the cells have been transplanted, testing different methods for administering cell therapy and the use of substrates, testing for any possible side effects, such as migration of the cells to the anterior chamber and any possible carcinogenic potential are all aspects that need to be studied very carefully before cell therapy can be approved for clinical trials. Additionally, the rejection mechanisms mediated by the host immune system against these cultured cells should also be investigated. Other important factors to consider include the cell source, i.e. from a single donor or multiple donors, gender-specific effects and the ability of the cells to maintain normal shape and functional potential and not cause any fibrosis over the long term after transplantation. But probably the single greatest limiting factor of cell-therapy development is the difficulty of culturing primary human corneal endothelial cells.

In this study the limited proliferative potential of the primary hCECs negatively affected the cell yield in culture, this resulted in obtaining limited number of cells, therefore a range of different seeding densities, 1036 cells/mm^2 for passage 2 and 1740 cells/mm^2 – for passage 0 had to be used for hCEC transplantation testing. Therefore, the immortalized HCEC-12 cell line was added to the experimental

model for testing corneal endothelial cell transplantation since it has a high proliferative potential and could generate a high cell yield when cultured allowing cell seeding densities of up to 2070 cells/mm^2 per cornea. However, since this is an immortalized cell line it is deemed unsuitable to be used for transplantation and treatment due to its potential carcinogenic and possible uncontrolled proliferative properties. The main challenges in human corneal endothelial cell culture, such as lack of proliferation, incapacity for long-term cultivation and *in vitro* expansion, and morphological transformation, hinder the establishment of a clinical-grade human corneal endothelial cell bank that could be used as a source of cell therapy for corneal endothelial disorders. With the existing culture protocols, hCECs can be cultured for several passages but eventually the cultures are lost to senescence. They might also undergo morphological transformation when cultured (Roy, et al., 2015). Typical *in vivo* characteristics of the hCECs with normal morphology include: polygonal-shaped cells that form a monolayer and are arrested in gap 1 (G1) phase of the cell cycle and expressing zona occludens-1 (ZO-1), Na^+/K^+ ATPase and N-cadherin. Spindle-shaped morphology in cultured human corneal endothelial cells (hCECs) can be triggered by a number of factors including transforming growth factor-β (TGF-β) (Chen, et al., 1999), interleukin-1β (IL-1β) and fibroblast growth factor-2 (FGF-2) (Kay, et al., 1994) through NF-kB (Lee and Kay., 2012) and connexin43 (Cx43) also play a role in hCEC spindle-shaped morphology (Nakano, et al., 2008).

The results of this study when passage 0 primary cells and HCEC-12 cells were used for cell therapy corroborated previously reported results by Engelmann, et al.,

(1993), Engelmann, et al., (1999), Aboalchamat, et al., (1999), Chen, et al., (2001) and Patel, et al., (2009) and demonstrated that after hCEC transplantation directly to the corneal stroma similar results were obtained in comparison with hCEC transplantation to the DM. Nevertheless, Engelmann, et al., (1993) stated that when morphologically altered hCECs to a spindle-shaped phenotype were used for transplantation on the DM of human corneas they formed multilayer of elongated cells which did not revert back to normal corneal endothelial phenotype for up to two weeks after transplantation and were unable to restore normal corneal thickness. In this chapter it was experimentally confirmed that when seeding passage 2 cultured hCECs (spindle-shaped) directly to the corneal stroma of human corneas after complete removal of the DM they did form a multilayer that did not revert back to normal CE morphology for up to 10 days of incubation, however, normal corneal thickness was restored in a similar manner as when passage 0 hCECs with normal CE morphology were used. These results are potentially very intriguing as they might be showing that spindle-shaped hCECs, which have a much higher proliferative potential compared to normal-shaped hCECs, might be a suitable source for cell therapy and treating corneal endothelial disorders. Yet further experimentation is required to confirm these results.

Cultivation of hCECs remains a challenge and in order to improve and enhance *in vitro* human corneal endothelial cell culture it is necessary to identify new cell markers for corneal endothelial progenitor cells, which can be used to identify, isolate and culture hCECs with high proliferative potential. Positive regulatory domain zinc finger proteins (PRDMs) have been implicated in the control of cell

differentiation and maintenance of stem cell potential in a variety of tissues but their expression and role in corneal endothelial cell biology has not been studied yet, except for PRDM5, which has been shown to play a role in Brittle Cornea Syndrome (Burkitt Wright, et al 2011). There are 17 PRDMs in total, all of which with slightly different functions. Therefore, the focus of the next chapter of this study is to test the expression of PRDM proteins and to construct a full expression profile of all PRDMs in the corneal endothelium and cultured corneal endothelial cells. This could give important clues about the role of these proteins in cell fate determination and regulation of proliferation in hCECs.

3.6. Conclusion

The DM is not necessary for hCEC transplantation. Seeding hCECs directly to the corneal stroma is sufficient to regenerate the CE monolayer and to restore normal corneal thickness. The use of cultured human corneal endothelial cells as a cell-therapy approach for treating corneal endothelial disorders was confirmed to be efficient using an *in vitro* human model.

4. CHAPTER 4 – POSITIVE REGULATORY DOMAIN ZINC FINGER PROTEIN EXPRESSION PROFILE IN HUMAN CORNEAS AND CULTURED HUMAN CORNEAL ENDOTHELIAL CELLS[1]

4.1. Chapter overview

This chapter explores the expression profile of positive regulatory domain zinc finger proteins (PRDMs) in the human corneal endothelium and cultured human corneal endothelial cells using reverse-transcriptase polymerase chain reaction (RT-PCR), immunocytochemistry and immunohistochemistry. PRDM proteins have been linked to the control of stemness and differentiation and their role in the CE has not been studied yet. Only PRDM5 has been shown to control extracellular matrix formation and mutations in PRDM5 lead to Brittle Cornea Syndrome. And more recently it was found that PRDM4 deletion causes a gender-specific abnormal corneal morphology in mice (Phenotyping Centre - MRC Harwell web reference), therefore studying their expression in the CE and cultured hCECs might give some clues to their role in hCEC proliferation control and can potentially be used for enhancing hCEC culture.

[1] Rolev, K., O'Donovan, D.G., Georgiou, C., Rajan, M.S. and Chittka, A., 2017. Identification of Prdm genes in human corneal endothelium. *Experimental eye research*, [e-journal] 10.1016/j.exer.2017.02.009. Available through: Primo.

4.2. Brief introduction

The corneal endothelial cells are found on the posterior side of the human cornea and form a monolayer of cells that serves as a barrier between the aqueous humour and the stroma. After damage to the endothelium following a disease process, as a result of previous eye surgery or as a result of mechanical damage the CE can loose its function, which results in corneal swelling (Parekh et al. 2016). After any such damage the human corneal endothelial cells are unable to regenerate themselves due to having a low proliferative potential and due to being arrested in the G1 phase of the cell cycle (Joyce, et al., 1996b). Recently researchers have found some evidence suggesting that human corneal endothelial progenitor cells exist in the periphery of the endothelium and a number of markers, such as nestin, telomerase, Oct-3/4, Wnt-1, SOX-2, PAX-6, p75 NTR and LGR5 have been used in order to identify these progenitors (Whikehart, et al., 2005; McGowan, et al., 2007; He, et al., 2012; Hara, et al., 2014 and Hirata-Tominaga, et al., 2013). It is of a great interest to understand the mechanisms that control hCEC proliferation and differentiation in order to successfully develop a cell-therapy approach for treating corneal endothelial disorders.

Positive-regulatory domain (PRDM) proteins are a family of 17 proteins that are transcriptional regulators and chromatin modifiers and play an important role in stem cells and a variety of developmental processes (Hohenauer and Moore, 2012) (See Table 11).

Table 11 - The PRDM proteins mediate their function via recruiting histone deacetylases and play crucial role in regulation of proliferation, cell differentiation and maintenance of stem cells.

	Function	Reference
PRDM1	Drives terminal B cell differentiation	Fog, et al., 2012
PRDM2	Tumour suppressor; Expressed in immature progenitor cells and mature monocytes and granulocytes	Fog, et al., 2012
PRDM3 (MDS-1)	Essential during the early stages of haematopoiesis	Fog, et al., 2012
PRDM3 (MECOM)	Essential during the early stages of haematopoiesis and in myeloid leukaemia stem cells. Essential for long-term haematopoietic stem cell function	Fog, et al., 2012 Zhang, et al., 2011 Eppert, at al., 2011
PRDM4	Inhibits proliferation during neuronal differentiation; interacts with p75NTR and controls neural development	Fog, et al., 2012 Chittka and Chao, 1999
PRDM5	Tumour suppressor through suppressing Wnt signaling; Regulates extracellular matrix development and maintenance in the cornea; Regulates haematopoiesis; Suppressor of Wnt/ß-catenin signaling; Regulates collagen type I gene transcription in developing bone	Fog, et al., 2012 Shu, et al., 2011 Burkitt Wright, et al., 2011 Duan, et al., 2007 Meani, et al., 2009 Galli, et al., 2012
PRDM6	Regulates aortic smooth muscle cell differentiation and vascular differentiation	Fog, et al., 2012 Davis, et al., 2006
PRDM8	Expressed in mouse embryo nervous system; Neuronal differentiation; Expressed in post-mitotic neurons	Fog, et al., 2012 Kinameri, et al., 2008 Komai, et al., 2009
PRDM10	Expressed in mouse embryo nervous system	Fog, et al., 2012 Park and Kim, 2010
PRDM12	Potential tumor suppressor for chronic myeloid leukaemia	Reid and Nacheva, 2003
PRDM14	Required for derivation of embryonic stem cells	Hohenauer and Moore,

		2012
PRDM16	Regulates brown adipocyte differentiation; Expressed in neural stem/progenitor cells; Essential during the early stages of haematopoiesis; Neuronal differentiation	Fog, et al., 2012 Kinameri, et al., 2008 Seale, et al., 2007 Kajimura, et al., 2009

Note: Research articles describing the roles of PRDMs 7, 9, 11, 13 and 15 in stem cells and cell proliferation control were not found in the published literature.

Of all the PRDMs only PRDM4 and 5 have been associated with the cornea. PRDM5 plays an important role in the extracellular matrix development and maintenance in the human cornea and mutations in the *PRDM5* gene lead to the development of Brittle Cornea Syndrome (Burkitt Wright, et al., 2011). PRDM4 also seems to be having a role in the corneal biology as *PRDM4* knockout mice show abnormal corneal phenotypes (Phenotyping Centre - MRC Harwell web reference). It has been demonstrated that PRDM4 interacts with p75NTR (Chittka, et al., 1999). Expression of p75NTR has been associated with high proliferative capacity in hCECs (Hara, et al., 2014). In addition, PRDM4 has been shown to control neural stem cell proliferation and differentiation (Chittka, et al., 2012) and since hCECs are derived from the neural crest it would be of interest to test the expression of PRDMs and PRDM4 in particular in hCECs. The expression and function of all other PRDM proteins and their role in corneal endothelial cell proliferation and progenitor cells have not been adequately explored and revealed yet. The purpose of this study was to demonstrate the PRDM protein expression profile in normal human corneas and cultured primary and immortalized human corneal endothelial cells, which will pave the way for further studying and understanding the role of PRDM proteins in the control of hCEC proliferation and differentiation. This will potentially enable the finding of new ways of optimizing hCEC culture for cell-therapy testing and future clinical use.

4.3. Materials and methods

4.3.1. List of materials used

Cell culture plastic dishes, flasks, well plates, centrifuge tubes, pipette tips,

eppendorf tubes and other plastics were purchased from ThermoFisher Scientific. Foetal bovine serum (FBS) – (F0804-500ML), was purchased from Sigma Aldrich. OptiMEM – (51985-026), was purchased from Invitrogen. Medium 199 – (31150022) was purchased from ThermoFisher Scientific. Ham's F12 – (11765054) was purchased from ThermoFisher Scientific. Trypsin/EDTA – (T3924), was purchased from Sigma Aldrich. Phosphate buffered saline (PBS) – (P3813) was purchased from Sigma Aldrich. Goat serum – (G9023) was purchased from Sigma Aldrich. Immortalized human corneal endothelial cell line HCEC-12 – (ACC 646), was purchased from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. CultureWell™ multiwell-chambered coverslips – (24779) were purchased from Invitrogen. Micro-prep RNA extraction Kit – (400805) obtained from Agilent Technologies. TRIzol® - (15596018) obtained from ThermoFisher Scientific. Chloroform – (C2432) was purchased from Sigma Aldrich. Isopropyl alcohol for molecular biology (I9516) was purchased from Sigma Aldrich. Ethanol for molecular biology (E7023) was purchased from Sigma Aldrich. SuperScript® II Reverse Transcriptase – (18064-014) obtained from Invitrogen. GoTaq® Flexi DNA polymerase – (M8305) obtained from Promega. Primary antibodies against human PRDM4 – (ab156867), PRDM5 – (ab79016), PRDM5 – (ab84572) were obtained from Abcam, Cambridge, UK. Goat anti-rabbit (goat polyclonal) IgG secondary antibody (Alexa Fluor 488) – (ab150077), was purchased from Abcam, Cambridge, UK; Goat anti-mouse (polyclonal) IgG secondary antibody (Alexa Fluor 488) – (ab150117) was purchased from Abcam, Cambridge, UK. PRDM1-16 forward and reverse primers were obtained from OriGene. DAKO

fluorescent mounting medium – (S3023), was purchased from Dako. Hoechst 33258 – (B2883) was purchased from Sigma Aldrich.

4.3.2. Human corneal tissue preparation

Human donor corneas were obtained from Bristol and Manchester Eye Banks, UK (See Table 12).

Table 12 – Human donor cornea information sheet.

Cornea	Age (years)	Gender	Cause of death	Time in storage	ECD (cells/mm^2)
1	69	Male	Ruptured aortic aneurysm	43 days	2300
2	64	Female	Pulmonary fibrosis	27 days	2800
3	87	Male	Unknown	69 days	-

Note: Endothelial cell density (ECD).

4.3.3. Cell culture

4.3.3.1.Primary human corneal endothelial cell isolation and culture

Primary hCEC culture from peripheral DM (2 mm) was initiated via primary explant culture. It was cut into small pieces with fine scissors and placed in an uncoated T25 (25 cm^2) cell culture flask. Culture was done using 1ml basal medium OptiMEM-I supplemented with 8 % FBS, 1% L-Glutamine, 1 % penicillin/streptomycin. Small amount of medium was used in order to allow the pieces of the peripheral DM to come in contact with the bottom surface of the flask and to promote cell outgrowth into the flask. The culture medium was not exchanged until primary cell outgrowth occurred. After that the culture medium was exchanged every 2^{nd} day until the culture reached confluence. After reaching confluence the primary culture was sub-cultured using 0.05 % trypsin/EDTA. Passage number 2 cells were used for transplantation studies. Cell culture was done in a 37^oC, 5% CO_2, humidified incubator Heracell™ (relative humidity – 95%).

4.3.3.2.HCEC-12 culture

HCEC-12 cells were cultured using F99 (mixture of Ham's F12 and Medium 199 in 1:1 ratio), 10% FBS. The medium was changed every 2 days. The cells were seeded out at 50 cells/mm^2. Confluent cultures were split 1:2 to 1:4 every 4-5 days using 0.05% trypsin/EDTA. Cell culture was done in a 37^oC, 5% CO_2, humidified incubator Heracell™ (relative humidity – 95%).

4.3.4. Ribonucleic acid extraction

Ribonucleic acid (RNA) was extracted from whole human donor corneas, Descemet's membrane/Endothelium complex, primary cultured human corneal endothelial cells (hCECs) and from cultured HCEC-12 cells (See Table 13).

Table 13 – Samples and methods used for RNA extraction.

Sample used for RT-PCR	Method of RNA extraction
Cornea (9.5 mm diameter)	Trizol®
Descemet's membrane/Endothelium complex (whole)	Micro-prep RNA extraction Kit
Primary cultured hCECs (passage 2)	Micro-prep RNA extraction Kit
HCEC-12	Micro-prep RNA extraction Kit

Note: Reverse transcriptase polymerase chain reaction (RT-PCR), human corneal endothelial cells (hCECs).

4.3.4.1.Trizol reagent

For RNA extraction from 9.5 mm corneas TRIzol reagent (Invitrogen) was used according to manufacturer's protocol.

4.3.4.2.Agilent Technologies Microprep Kit

RNA extractions were performed using the Micro-prep Kit (Cat No: 400805) obtained from Agilent Technologies. Total RNA from each sample was extracted according to the manufacturer's protocol.

4.3.4.2.1.Primary cultured human corneal endothelial cells

Primary human corneal endothelial cells (hCECs) were kept in culture for 7 days. The cells were harvested using trypsin/EDTA and total RNA was extracted using the Agilent technologies Micro-prep Kit.

4.3.4.2.2.HCEC-12

HCEC-12 cells were kept in culture for 7 days. The cells were harvested using trypsin/EDTA and total RNA was extracted using the Agilent technologies Micro-prep Kit. A cell number of 5 x 10^5 HCEC-12 cells, passage 6 were used for RNA extraction.

4.3.4.3.RNA quantification

The quality of the RNA was measured by NanoDrop spectrophotometer.

4.3.5. Complementary deoxyribonucleic acid synthesis

Complementary deoxyribonucleic acid (cDNA) was synthesized from 1µg total RNA using the SuperScript@ II Reverse Transcriptase kit (Invitrogen, Cat No: 18064-014) according to the manufacturer's protocol. Briefly, all reactions were assembled on ice in eppendorf tubes: 1µg of total RNA sample, 1µl random hexamers, 1µl dNTPs (deoxynucleotide triphosphates) mix (10mM), 7.7µl Diethyl pyrocarbonate (DEPC)-water, total sample volume=13µl. The mixture was heated to 65^{o}C for 5 min followed by a quick chill on ice. The eppendorf tubes were then briefly centrifuged. To each sample was then added: 5 x First-strand buffer - 4µl, 0.1 mole (M) dithiothreitol (DTT)-2µl. The samples were then mixed gently and were incubated at room temperature for 2 min. 1µl of reverse transcriptase was added in the +RT (plus reverse-transcriptase – the test sample) sample and 1µl of DEPC-water to the -RT (minus reverse-transcriptase – the negative control) sample to keep the volumes the same. All samples were then incubated at room temperature for 10 min followed by incubation at 42^{o}C for 50 min. Finally the reaction was inactivated at 70^{o}C for 15 min.

4.3.6. Primers for reverse transcriptase polymerase chain reaction (RT-PCR)

All primers that were used in this experiment are listed in Table 14.

Table 14 – Primer sequences.

Oligonucleotide	Sequence (5' → 3')	Product size (bp)
PRDM1 F	CAGTTCCTAAGAACGCCAACAGG (23)	122
PRDM1 R	GTGCTGGATTCACATAGCGCATC (23)	
PRDM2 F	TTGGGCTTGCTCAGGAGAAGAG (22)	140
PRDM2 R	GCTGCTATCTCAGGGTTGTCTTC (23)	
PRDM3 (MDS1) F	CCTTATGTGGGAGAGCAGAGGT (22)	131
PRDM3 (MDS1) R	GAAGGCTATTCCTACGTCTGAGC (23)	
PRDM3 (MECOM) F	CCTGCTTCAGATGGTTCCTTGC (22)	147
PRDM3 (MECOM) R	GGTGAAACAAGAATCCTGGAGAAG (24)	
PRDM4 F	CCAAAGCAGCTTGTTCTCCGTC (22)	95
PRDM4 R	TAGAGGTCCAAAGCAAGTCCGC (22)	
PRDM5 F	ACTCTGAGGAGAGACCGTTCCA (22)	144
PRDM5 R	AGCATCGCAGTGATGGCACTTG (22)	
PRDM6 F	GTGAGGAACACGCAGCATCTCT (22)	128
PRDM6 R	TTCTCCGCAGTGCCTTGCACAT (22)	
PRDM7 F	AGACGAAGAGGCAGCCAACAGT (22)	149
PRDM7 R	GCCACCAGGTTCTGCTCTTCAT (22)	

PRDM8 F	CTGTGTCCTGAGCCATACTTCC (22)	125
PRDM8 R	CCTTCTGAGGAACCATTTGCTGC (23)	
PRDM9 F	ACGAAGAGGCAGCCAACAATGG (22)	147
PRDM9 R	GCCACCAGGTTCTGCTCTTCAT (22)	
PRDM10 F	CCGCAAAGACTTCCTGTGTTCC (22)	120
PRDM10 R	GCTGATGCGGTCGGCTTTCTTG (22)	
PRDM11 F	CCAGGAAACCATTCACCGCAAC (22)	93
PRDM11 R	CCTCAGGTCTTCTGGGTTATCC (22)	
PRDM12 F	TGCACGTAACGAACAGGAGCAG (22)	118
PRDM12 R	GTGAGTTTCCGTACCACACCAG (22)	
PRDM13 F	CTAACTCCTTGGCTCAGTGGTTC (23)	90
PRDM13 R	CAGTACCAGCAGATGTAGCGCT (22)	
PRDM14 F	CCTTGTGTGGTATGGAGACTGC (22)	125
PRDM14 R	CTTTCACATCTGTAGCCTTCTGC (23)	
PRDM15 F	GGCACTTGTGAGAAGACCTTCC (22)	119
PRDM15 R	GAGGTTGCTGTTGGTGGAGAAG (22)	
PRDM16 F	CAGCCAATCTCACCAGACACCT (22)	145
PRDM16 R	GTGGCACTTGAAAGGCTTCTCC (22)	

4.3.7. Reverse transcriptase polymerase chain reaction

4.3.7.1.Sample preparation

Reagents for 1xPCR reaction – final volume 25µl: 5 x Flexi Buffer - 5µl, $MgCl_2$ (25mM) - 1.5µl, dNTP (10mM) - 1µl, forward primer - 1 µl, reverse primer - 1µl, Go Taq DNA polymerase - 0.25µl, cDNA - 1 µl, water - 15.25 µl.

4.3.7.2.PCR program

PCR program: 94ºC for 2 min, 40 cycles (94ºC for 30 sec, 59ºC for 30 sec, 72ºC for 30 sec), 72ºC for 5 min, final hold 4ºC.

4.3.8. Agarose gels electrophoresis

The gels were run for 1 hour and 30 minutes at 85 volts (V).

4.3.8.1.Gel preparation

2% agarose gels were prepared with 2 x 16 wells per gel (two rows of 16 wells per gel) and with 2 x 28 wells per gel (two rows of 28 wells per gel).

4.3.8.2.Loading the samples on the gel

5µl of each sample was mixed with 5µl loading dye to a 9µl final volume. The samples loaded on the gel by careful pipetting.

4.3.9. Immunofluorescence staining

Multiwell-chambered coverslips were pre-coated with poly-d-lysine (PDL). *In vitro* cultured HCEC-12 and primary hCECs were tested for PRDM protein expression. HCEC-12 and primary hCECs were seeded at 5000 cells/cm^2 on chambered

coverslips and were incubated for 24 hours at 37ºC and 5% CO_2. The cells were then fixed by 4% paraformaldehyde (PFA) for 10 minutes at room temperature. The cells were then washed with phosphate buffered saline (PBS) 3x for 15 minutes. Goat serum was used as a blocking solution for 1 hour at room temperature. Primary rabbit anti-PRDM4 antibody (ab156867), primary anti-PRDM5 antibody (ab79016) and primary anti-PRDM5 antibody (ab84572) were diluted to a 1:100 concentration and were added to the cells followed by overnight incubation at 4ºC. The cells were then washed with PBS 3x for 15 minutes and secondary goat anti-rabbit antibody (1:100 dilution) was added to the cells with Hoechst DNA stain (1µg/ml) followed by 1-hour incubation at room temperature. The cells were then washed with PBS 3x for 15 minutes. DAKO mounting medium with was used to mount glass coverslips onto the slides. The slides were then wrapped in foil and were left to dry out at room temperature in the dark for 2 hours. They were then viewed using the Leica Microsystems CMS confocal microscope.

4.3.10. Immunohistochemical labeling of human cornea

Immunohistochemistry was performed in Addenbrooke's hospital, Cambridge, UK. Cornea were fixed in formalin and embedded in paraffin for processing using LEICA BondTM automated system with LEICA Bond Polymer Refine Detection kit according to the manufacturer's instruction. Bond Polymer Refine Detection kit is a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system for the detection of tissue-bound mouse and rabbit IgG's. All sections were subjected to antigen heat retrieval at 100ºC for 20 minutes prior to staining procedure using either Epitope retrieval solution 1 (pH 6.0) for anti-PRDM4

antibodies or Epitope retrieval solution 2 (pH9.0) for anti-PRDM5 antibodies. Both antibodies were used at 1:500 dilutions. The staining program used was as follows: 1) peroxide block - 5min, 2) primary antibody was applied for 30 minutes, 3) polymer HRP was added for 8 minutes, 4) mixed 3,3'-diaminobenzidine (DAB) was added for 10 minutes. Sections were counterstained with haematoxylin for 5 minutes to visualize nuclei.

4.4. Results

4.4.1. Reverse transcriptase polymerase chain reaction detection of positive-regulatory domain proteins gene expression

Expression of PRDMs was detected: in the 9.5 mm human cornea - PRDMs 1, 2, 4, 5 and 10, in the corneal endothelium alone - PRDMs 2, 4, 5 and 10, in cultured hCECs - PRDMs 1, 2, 4, 5, 8 and 10 and in HCEC-12 cells - PRDMs 1, 2, 3 (MECOM), 4, 5, 6, 8, 10, 11 and 16 (See Figure 26 and Table 15). PRDMs 2, 4, 5 and 10 were consistently expressed by all samples. The HCEC-12 cells expressed the largest number of PRDMs (10 PRDMs in total).

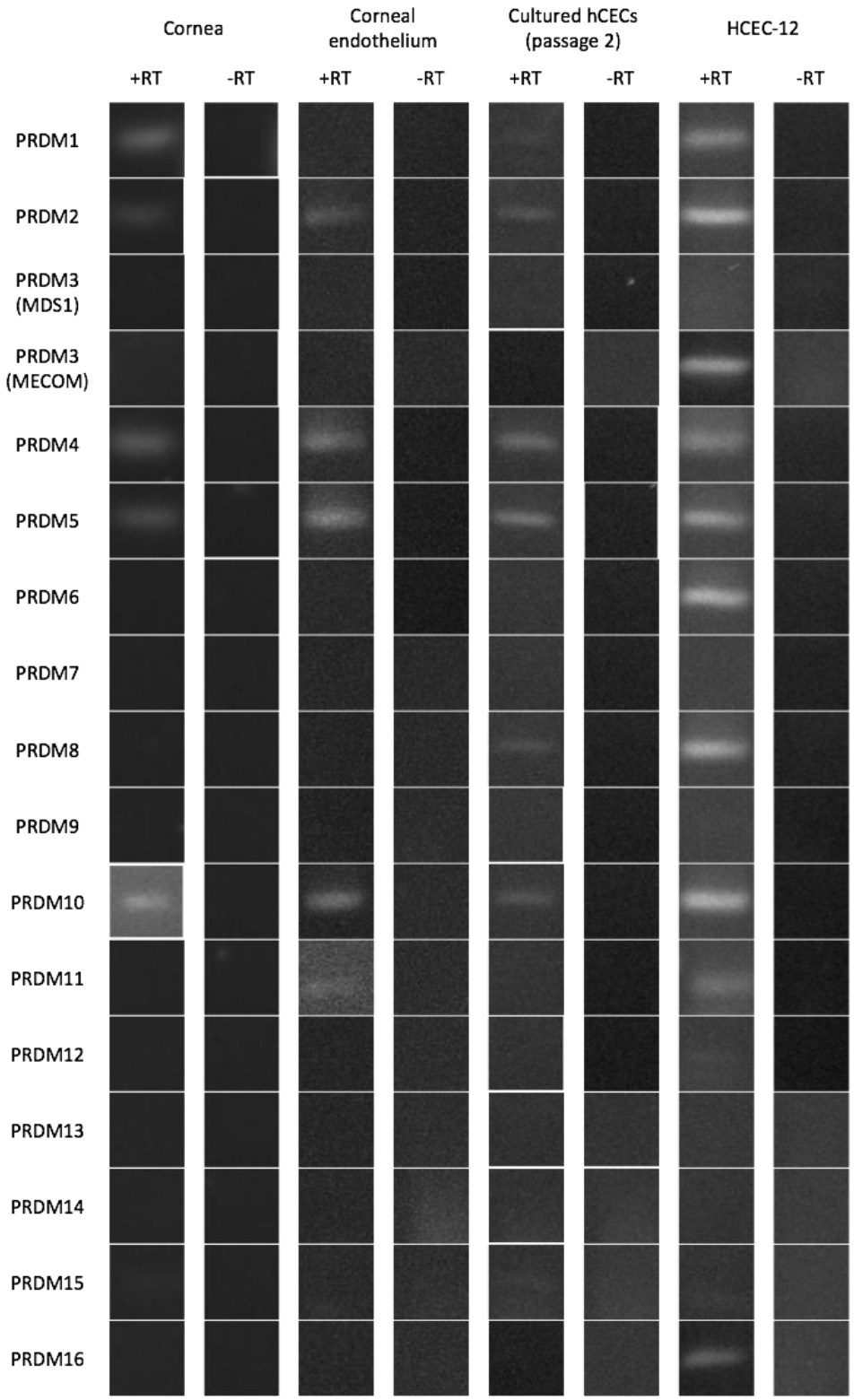


Figure 26 – Positive-regulatory domain (PRDM) protein gene expression profile in different samples.

Table 15 – PRDM gene expression. + denotes expression of mRNA.

	Normal Cornea	DM/CECs	Primary hCECs	Immortalised hCECs (HCEC-12)
PRDM1	+		+	+
PRDM2	+	+	+	+
PRDM 3 (MDS1)				
PRDM3 (MECOM)				+
PRDM4	+	+	+	+
PRDM5	+	+	+	+
PRDM6				+
PRDM7				
PRDM8			+	+
PRDM9				
PRDM10	+	+	+	+
PRDM11		+ (Weak)		+
PRDM12				
PRDM13				
PRDM14				
PRDM15				
PRDM16				+

Note: Descemet's membrane (DM); corneal endothelial cells (CECs); human corneal endothelial cells (hCECs).

4.4.2. Immunofluorescence detection of positive-regulatory domain proteins 4 and 5 expression

Protein expression of positive-regulatory domain (PRDM) proteins 4 and 5 was detected on HCEC-12 cells and passage 2 hCECs (See Figure 27 and Figure 28).

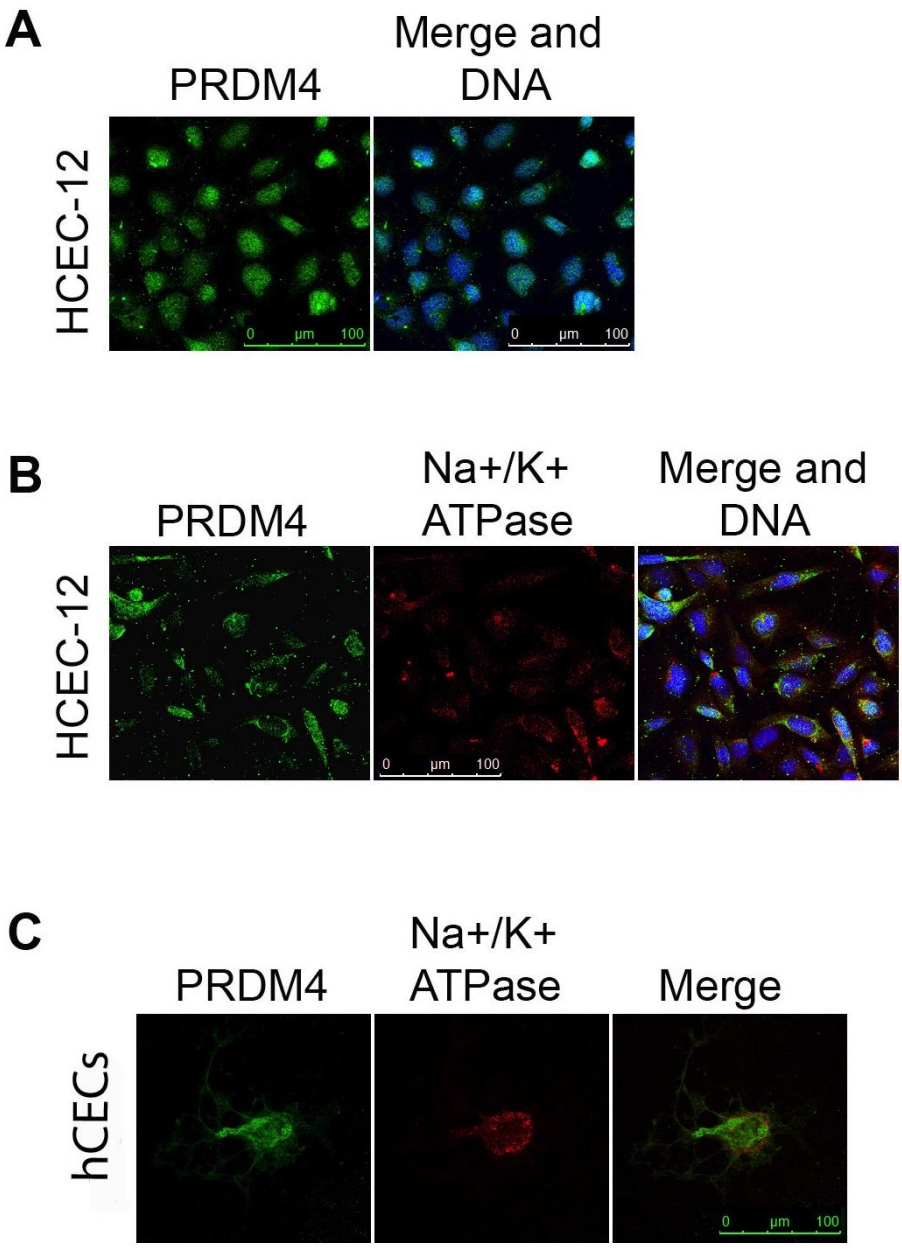


Figure 27 – Positive-regulatory domain zinc finger protein (PRDM) 4 and Na^+/K^+ ATPase expression by HCEC-12 cells (A and B) and by passage 2 cultured human corneal endothelial cells (hCECs) [C].

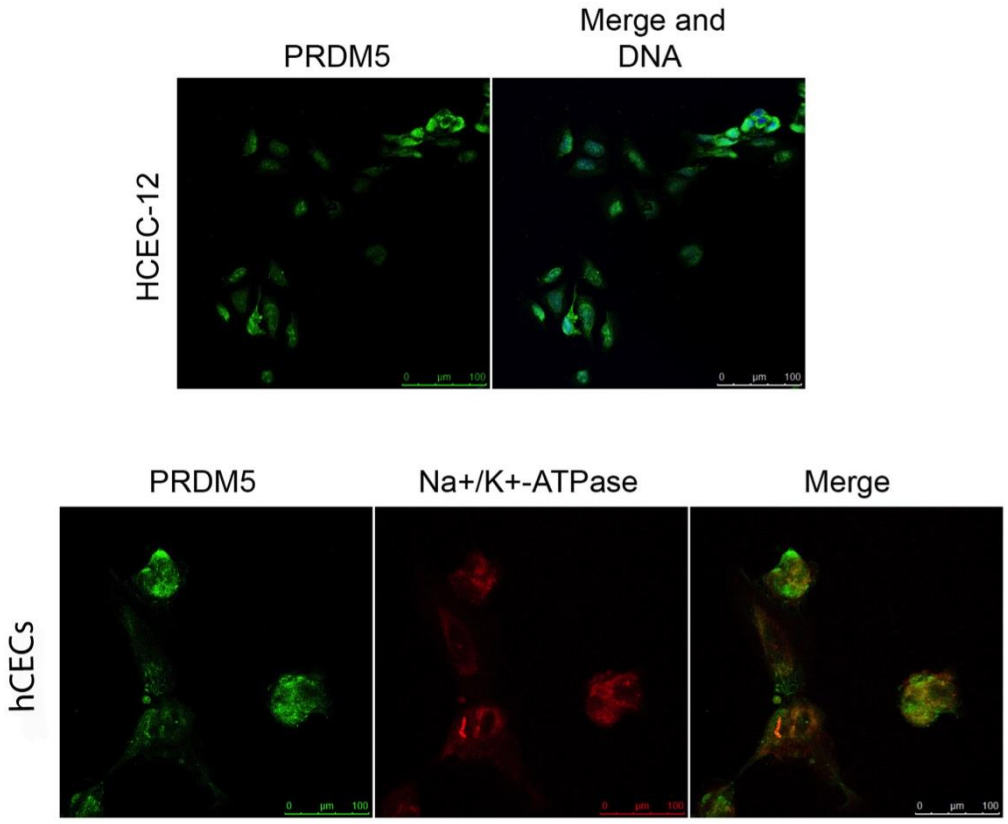


Figure 28 – Positive-regulatory domain zinc-finger protein 5 (PRDM5) expression by HCEC-12 cells (A) and PRDM5 and Na^+/K^+ ATPase expression by passage 2 cultured human corneal endothelial cells (hCECs).

4.4.3. Immunohistochemistry

Immunohistochemical analysis of human corneal cross-sections showed that there was no differential expression of PRDMs 4 and 5 within the center and the periphery of the cornea (See Figure 29).

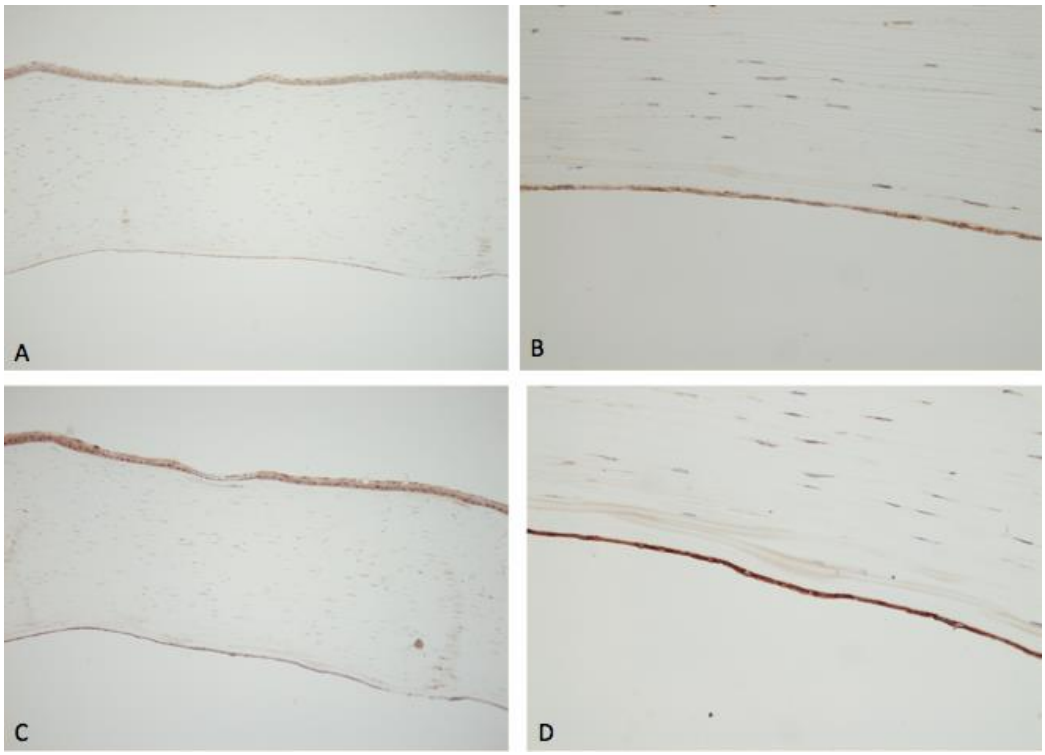


Figure 29 – Uniform expression of positive-regulatory domain protein 4 (PRDM4) throughout the entire corneal cross-section including central and peripheral corneal endothelium (x100) [A], positive-regulatory domain zinc-finger protein 4 (PRDM4) expression throughout the central corneal endothelium (x400) [B], uniform expression of positive-regulatory domain zinc-finger protein 5 (PRDM5) throughout the entire corneal cross-section including the central and the peripheral corneal endothelium. Positive expression is also visible throughout the corneal epithelium (x100) [C], PRDM5 expression is visible throughout the central corneal endothelium (x400) [D].

4.5. Discussion

Human corneal endothelial cells are generally notorious for their deficient proliferative ability. There is a significant lack of understanding of the mechanisms

that regulate proliferation both *in vivo* and *in vitro* of this cell monolayer that is so crucial for normal vision. Understanding the mechanisms that control proliferation of these cells would help in improving the *in vitro* corneal endothelial cell culture and will open up the possibility to use cell therapy as a novel treatment method for corneal endothelial disorders. Therefore, studying the expression of PRDM proteins, which are known to control differentiation and reprogramming of a wide variety of cells (Fog, et al., 2012), in the human corneal endothelium can open new doors in better understanding the mechanisms that control hCEC proliferation and can lead to the development of new strategies for enhancing hCEC culture.

So far the expression and role of PRDMs in the human cornea have not been studied. Only PRDM5 has been shown to play an important role in the development and maintenance of the extracellular matrix in the human cornea and mutations in PRDM5 lead to Brittle Cornea Syndrome (Burkitt Wright, et al., 2011). And a novel finding has shown that PRDM4 deletion causes a gender-specific abnormal corneal morphology (Phenotyping Centre - MRC Harwell web reference).

Therefore, this chapter endeavored to study the expression of PRDM proteins in the human corneal endothelium and to allow for future studies on the role of PRDM proteins in proliferation and cell fate control in hCECs. This expression profile has not been shown previously. In this chapter it was found that PRDMs 1, 2, 4, 5 and 10 were consistently expressed by the human corneal endothelium of whole corneas. In cultured passage 2 primary hCECs additionally expressed PRDM8. Intriguingly, cultured immortalized human corneal endothelial cell line HCEC-12 expressed PRDMs 3 (MECOM), 6, 8, 11 and 16 in addition to PRDMs 1, 2, 4, 5, 8 and 10.

These findings suggest that there might be interplay between different PRDM proteins in the control of human corneal endothelial cell proliferation since a larger number of PRDMs is expressed in HCEC-12 cells that have a very high proliferative capacity. Some PRDMs are involved in regulating TGF-β signaling, namely PRDM3 and PRDM16 can bind to and repress Smads, and in regulating Notch signaling (Fog, et al., 2012). TGF-β signaling and Notch signaling have been shown to be very important in the proliferation, senescence and morphological transformation in primary hCECs (Chen, et al., 1999; Joyce, et al., 2002; Okumura, et al., 2013; Li, et al., 2013).

PRDM1 drives terminal B cell differentiation (Fog, et al. 2012) and controls the germ cell lineage (Ohinata, et al., 2005). However, there is currently no information available on the role of PRDM1 in corneal development. PRDM2 has been shown to act as a tumor suppressor and to inactivate a number of tumors (Liu, et al., 2012; Tan, et al., 2014; Zhang, et al., 2015). Its role and function in the cornea has not yet been reported also. In this study PRDMs 4 and 5 were expressed by all samples that were tested. PRDM4 has been reported to interact with p75NTR and control neural development (Chittka and Chao, 1999). It acts as a transcriptional repressor (Chittka, et al., 2004), controls neural stem cell proliferation and differentiation (Chittka, et al., 2012) and its activity is regulated by epidermal growth factor (EGF) and nerve growth factor (NGF) (Chittka, 2013). It has been shown that there are human corneal endothelial cell populations, which express p75NTR, and which also possess very high proliferative capacity (Hara, et al., 2014). Therefore, knowing that progenitor hCECs express p75NTR it was of interest to explore the expression and role of

PRDM4 in the corneal endothelium and to see whether it is specifically expressed only in progenitor p75NTR expressing hCECs and whether it could be used as a progenitor cell marker of the corneal endothelial progenitor cells. Both PRDMs 4 and 5 were consistently expressed in all samples that were tested including whole cornea and primary and immortalized cultures, however, there was no differential expression of these proteins between the central and peripheral endothelium suggesting that there was no difference in the expression of these proteins between hCEC progenitors and mature hCECs.

HCEC-12 cells showed a very significant difference in their PRDM expression profile compared to the contact-inhibited CE of the normal cornea and compared to the primary cultured hCECs, which could be due to the SV40 immortalization. HCEC-12 expressed PRDM3 (MECOM isoform), which seems to control haematopoietic stem cell (HSC) quiescence has been associated with controlling the survival of the primary myeloid leukemia stem cells (Eppert, et al., 2011). Its expression in HCEC-12 cells could be due to their immortalization with the SV40 virus as part of the re-programming of these cells. Davis, et al. (2006) demonstrated in their study that PRDM6 promotes proliferation in smooth muscle cells while PRDMs 8 and 10 have been shown to play an important role in the mouse embryonic development (Kinameri, et al., 2008; Park and Kim, 2010). Moreover, PRDM8 has been implicated in specific aspects of eye development and differentiation of specific cell types within the visual system in mice (Komai, et al., 2009 and Jung, et al., 2015). However, its role in the corneal endothelium has not yet been elucidated and it is unclear whether it is expressed in cultured primary hCECs

as a result of re-programming occurring in the absence of contact inhibition during culture. PRDM16 plays an important role in controlling adipose, haematopoietic and cardiac tissue (Seale et al. 2007; Kajimura et al. 2009; Arndt et al. 2013; Chi and Cohen 2016) and regulates oxidative stress in haematopoietic and neural stem cells and is involved in haematological malignancies (Chi and Cohen, 2016). Hence, PRDM16 could be contributing to the re-programming of the HCEC-12 cells after their immortalization. The genes for PRDMs 1, 2, 3 and 16 each encode two different isoforms, whose biological roles in the control of proliferation are opposite (Zazzo, et al., 2013). Hence, it is of great importance to study the distribution of these different isoforms in the human corneal endothelium and in cultured human corneal endothelial cells in order to understand their role in the control of proliferation and differentiation.

In this study it was observed that some alterations occurred in the PRDM expression profile following *in vitro* culture and as a result of immortalization of the hCECs. This is very interesting because it was experimentally demonstrated that the contact-inhibited CE monolayer from a normal cornea expressed only PRDMs 1, 2, 4, 5 and 10, whereas, primary cultured and immortalized corneal endothelial cells expressed more PRDMs in addition to these expressed by the contact-inhibited monolayer. Since in the primary cultured hCECs contact inhibition has been disrupted by trypsin dissociation the cells have been sensitized to the mitogenic effect of growth factors present in the culture medium and started proliferating, as evidenced by reaching confluence being passaged over 2 times. In addition, the immortalization of the HCEC-12 cells via transfection with the small t-antigen and large T-antigen of the

SV40 virus is known to produce high cell proliferation. Therefore, these results might seem to indicate that the increased proliferation capacity in human corneal endothelial cells is accompanied by expression of additional PRDM proteins. Although, currently there is no available information on the role of PRDM proteins in the human corneal endothelium except for PRDM4 and PRDM5. Therefore, a further characterization of protein expression in the human cornea of PRDMs 4 and 5 was performed in this study in order to test their distribution within the corneal endothelial cell monolayer. And it was found that these two proteins were uniformly expressed throughout the central and peripheral corneal endothelium.

In summary, PRDMs are known to regulate proliferation, differentiation and senescence of haematopoietic stem cells, embryonic stem cells and induced pluripotent stem cells and have been implicated in some leukaemias. Therefore, it is of great interest to study the expression of PRDM proteins in the human corneal endothelium and to further elucidate their role in this particular cell type and their co-expression with p75NTR.

4.6. Conclusion

A wide range of PRDM expression was detected in a human cornea and cultured primary and immortalized human corneal endothelial cells. PRDMs 1, 2, 4, 5 and 10 were consistently detected in all samples tested in this study. However, primary cultured and immortalized human corneal endothelial cells expressed some additional PRDMs suggesting that *in vitro* culture, disruption of contact inhibition and increased proliferation might be accompanied by a difference in the PRDM expression profile, hence implying that PRDMs might have a role in the control of

hCEC proliferation and re-programming. No differential expression of PRDM 4 or 5 was found between the central and the peripheral endothelium of normal human corneas. Studying the expression of PRDM proteins in hCECs opens an interesting avenue for further exploration of their role in corneal endothelial cell development, proliferation and homeostasis and for helping the generation of hCECs for therapeutic purposes in future studies.

5. CONCLUDING REMARKS

Transplantation of corneal endothelial cells as a cell-therapy approach for treating corneal endothelial disorders has been the focus of many researchers in recent years. The human corneal endothelial cells are arrested in G1 phase of the cell cycle (Joyce, et al., 1996b; Chen, et al., 1999). This G1-phase arrest is mediated by TGF-β and contact inhibition (Joyce and Zeiske, 1997; Joyce, et al., 2002). Corneal endothelial cell culture plays a central role as a cell source for hCEC transplantation and therefore many studies have attempted to optimize the *in vitro* culture conditions so that sufficient numbers of cells can be generated and used for transplantation. Cells from both young and old donors seem to be able to proliferate in culture after stimulation with appropriate culture conditions in response to foetal bovine serum and FGF (Senoo and Joyce, 2000b; Zhu and Joyce, 2004). However, some age-related differences that negatively affect the cell cycle progression exist, such as increased expression of p16 and p21 in cells from older donors (Joyce and Harris, 2010; Enomoto, et al., 2006). Different methods of isolation, culture media, growth factors and substrates have been tested for optimal corneal endothelial cell culture (Engelmann, et al., 1988; Engelmann and Friedl, 1989; Rieck, et al., 1995; Peh, et

al., 2011; Okumura, et al., 2015; Peh, et al., 2015a and 2015b; Valtink, et al., 2016) including serum-free culture (Bednarz, et al., 2001; Møller-Pedersen, et al., 2001), which have yielded some degree of success but hCEC culture still remains a challenge.

It has been speculated for quite some time that the peripheral areas of the corneal endothelial monolayer harbour progenitor cells with high proliferative potential, which could be used for propagation in culture and later used for transplantation. Some studies have shown that hCECs, isolated from both the centre and the peripheral endothelium, retain proliferative potential (Konomi, et al., 2005). However, the cells from the peripheral region seem to possess higher proliferative potential regardless of donor age (Mimura and Joyce, 2006). Moreover, in another study by Hirata-Tominaga, et al., (2013) it was shown that leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is uniquely expressed in the peripheral region of the endothelium and that the LGR5 expressing cells have some stem cell properties. Later on Okumura, et al., (2014b) showed that roof plate-specific spondin 1 (R-spondin1), which is an LGR5 ligand significantly upregulated corneal endothelial cell proliferation. In addition, He, et al., (2012) showed that there are corneal endothelial cells that have high proliferative capacity and migrate towards the corneal center and as they migrate they fully differentiate and loose their proliferative capacity. A number of other studies have also identified the existence of human corneal endothelial cell progenitor cells (Whikehart, et al., 2005; McGowan, et al., 2007; Hara, et al., 2014; Walshe and Harkin, 2014). However, a

cell bank of clinically usable cells suitable for transplantation testing has not been established yet.

The development of cell-based therapy technologies has been a major focus in the search of new treatments for corneal blindness. These have been classified in a number of different sub-groups based on the cell source and various methods for applying them to treat corneal blindness, which include somatic cells, immortalized cells, *in vitro* and *in vivo* gene modification using viral vectors, genome editing, stem cells, iPSCs and cell differentiation and three-dimensional technologies (Mount, et al., 2015). Stem cells and iPSCs have been widely researched and reviewed and have shown some promise for clinical application but there are still many challenges remaining before these novel treatment methods can be translated to clinical use (Yuan and Fan, 2015). One of the biggest challenges to be overcome is the establishing of a suitable cell source for hCECs. In this study we used primary and immortalized hCECs in order to demonstrate the plausibility for hCEC transplantation to human corneas. However, both of these have certain limitations. In the case of primary cells the lack of proliferative capacity severely limits the amount of cells that can be generated for cell transplantation. Whereas, in the case of the immortalized cells they can be easily cultured and large numbers of functional cells can be generated but their carcinogenic properties and uncontrolled proliferation can cause complications including tumor formation and possible increased intraocular pressure due to cell flaking and obstructing the trabecular drainage system. Interestingly, research has shown that different methods of immortalization may provide a means for enabling *in vitro* expansion of the cells while abolishing their

proliferative capacity when transplanted *in vivo*. Stevanato, et al., (2009) showed that the c-Myc transcription factor/mutant estrogen receptor ($cMycER^{TAM}$) transgene immortalization is very suitable for clinical application because it is conditional and is based on the presence of 4-hydroxytamoxifen. Therefore, this enables the cell proliferation to be activated when the cells are cultured but after *in vivo* transplantation when 4-hydroxytamoxifen is no longer present this proliferative potential is lost. Further research into different cell immortalization techniques might hold a significant potential for the future development of a cell-therapy approach for treating corneal blindness but extensive testing both in *in vitro* and *in vivo* models is required before it can be translated to clinical application.

Even though there have been significant advances in the *in vitro* culture of hCECs and in the identification and isolation of human corneal endothelial progenitor cells, which seem to possess higher proliferative capacity than the fully differentiated hCECs, a cell bank for clinical use in transplantation and treatment of patients has not yet been established. This study has provided a human model for hCEC transplantation testing that can be successfully used for cell-therapy evaluation studies and producing clinically relevant data. Additionally, using this model it was demonstrated that after seeding to the posterior corneal stroma hCECs are able to regenerate a fully functional CE monolayer and to restore normal corneal thickness, which is a very significant finding for cases when the DM has to be removed prior transplantation due to fibrosis or other types of damage to the DM. However, further testing and evaluation of cell morphology, potential abnormal collagen deposition and fibrosis, cell migration to other parts of the cornea, corneal endothelial cell

culture optimization and corneal endothelial marker expression profiling are required before this treatment can be taken to clinical trials. Moreover, this study demonstrated the differential expression of PRDM proteins in normal CE and cultured hCECs with variable proliferative capacity due to disruption of the contact inhibition following trypsin dissociation. Further understanding of the role of the PRDM proteins in the proliferation and differentiation of hCECs could opens novel avenues for enhancing corneal endothelial cell culture and is another stepping stone towards the development of a cell-therapy approach for treating corneal endothelial disorders.

5.1. Final conclusions

1) The study established an *in vitro* human corneal endothelial decompensation model with a detailed description of stromal behaviour of human corneas after transfer from immersion to air-interface organ culture.
2) Demonstrated the potential of cultured hCECs to restore normal physiological stromal thickness after cell transplantation using an *in vitro* human model.
3) It was experimentally shown that the posterior human corneal stroma is a suitable substrate for direct transplantation of cultured hCECs following the complete removal of the DM/Endothelium complex, which allows successful cell attachment, monolayer formation and restoration of normal CE function.
4) The work has detailed a methodology for the assessment of the functional potential of cultured hCECs by using cadaveric human donor corneas an *in vitro* human corneal endothelial decompensation model.

5) The limitations related to the proliferative capacity of hCECs prompted the exploration of PRDM protein expression in hCECs. Positive expression of a number of PRDM proteins in the normal CE (contact inhibited) and in cultured hCECs (contact inhibition released via trypsin dissociation) was successfully demonstrated with an interesting observation of differential expression between the normal CE and cultured hCECs suggesting that PRDMs might play a role in the control of hCEC proliferation.

5.2. Future work and limitations

The two biggest limitations in the field of cell therapy research remain (1) the limited potential of hCECs for *in vitro* expansion due to poor proliferative capacity and (2) a suitable *in vitro* human model that will enable longer post-transplantation study periods of 1 month or more follow-ups. Improvements on the first limitation are necessary and will enable researchers to generate enough clinical-grade hCEC cultures for hCEC transplantation studies. Improvements on the second limitation will enable long-term studies of hCEC behavior post-transplantation such as morphological changes, abnormal collagen secretion, proliferation, spontaneous transformation, carcinogenic properties and immune response. In order to address these the following future work is proposed:

1) Optimize the *in vitro* human corneal model of endothelial decompensation possibly by using an irrigation system that allows constant perfusion of the corneas while maintaining constant pressure in addition to a spraying device that would allow moistening of the epithelial side of multiple corneas at the

same time. Employing this system will ensure the proper hydration of both the endothelial and the epithelial side of the cultured corneas and maintenance of constant intraocular pressure that will preserve the viability of the corneal endothelial cells for a longer incubation period longer than 14 days.

2) Establish *in vitro* models with a longer life span by using younger donor corneas.
3) Further test the use of hCECs that have obtained a spindle-shaped morphology after *in vitro* culture as a potential source for cell therapy and test their capacity to revert back to normal phenotype after transplantation.
4) Study cell fate of hCECs including morphological changes and proliferative potential following cell therapy.
5) Test the long-term physiological effects on stromal hydration and corneal transparency following cell therapy over a period of at least 1 month.
6) Safety studies on immune rejection and on the potential of uncontrolled proliferation and tumor formation following cell therapy.
7) Further studies on the interaction of PRDM4 and p75NTR in hCECs and their role in cell development and proliferation.
8) Develop cell culture strategies for enhancing hCEC culture for research and possible future use in cell therapy.
9) Explore the possibility of using whole human eyes as a transplantation model rather than just a corneoscleral rim.

LIST OF PUBLICATIONS DERIVED FROM THIS THESIS

Rolev, K., O'Donovan, D.G., Georgiou, C., Rajan, M.S. and Chittka, A., 2017. Identification of Prdm genes in human corneal endothelium. *Experimental eye research,* [e-journal] 10.1016/j.exer.2017.02.009. Available through: Primo.

Submitted to Cornea in November 2017: **Feasibility study of human corneal endothelial cell transplantation using an *in vitro* human corneal model.**

REFERENCE LIST

Aboalchamat, B., Engelmann, K., Böhnke, M., Eggli, P. and Bednarz, J., 1999. Morphological and Functional Analysis of Immortalized Human Corneal Endothelial Cells after Transplantation. *Experimental eye research,* [e-journal] 69 (5), pp. 547-553. Available through: Primo.

Ali, M., Raghunathan, V., Li, J.Y., Murphy, C.J. and Thomasy, S.M., 2016. Biomechanical relationships between the corneal endothelium and Descemet's membrane. Experimental eye research, [e-journal] 152, pp.57-70. 10.1016/j.exer.2016.09.004. Available through: Primo.

Anderson, J.M. and Van Itallie, C.M., 1995. Tight junctions and the molecular basis for regulation of paracellular permeability. *The American Journal of Physiology*, [e-journal] 269 (4), pp.G467. Available through: Primo.

Araki-Sasaki, K., Danjo, S., Kawaguchi, S., Hosohata, J. and Tano, Y., 1997. Human Hepatocyte Growth Factor (HGF) in the aqueous humor. Japanese journal of ophthalmology, [e-journal] 41 (6), pp.409-413. 10.1016/S0021-5155(97)00081-6. Available through: Primo.

Arndt, A., Schafer, S., Drenckhahn, J., Sabeh, M. ., Plovie, E., Caliebe, A., Klopocki, E., Musso, G., Werdich, A., Kalwa, H., Heinig, M., Padera, R., Wassilew, K., Bluhm, J., Harnack, C., Martitz, J., Barton, P., Greutmann, M., Berger, F., Hubner, N., Siebert, R., Kramer, H., Cook, S., Macrae, C. and Klaassen, S., 2013. Fine Mapping of the 1p36 Deletion Syndrome Identifies Mutation of PRDM16 as a Cause of Cardiomyopathy. *The American Journal of Human Genetics,* [e-journal] 93 (1), pp. 67-77. Available through: Primo.

Bahn, C.F., MacCallum, D.K., Lillie, J.H., Meyer, R.F. and Martonyi, C.L., 1982. Complications associated with bovine corneal endothelial cell-lined homografts in the cat. *Investigative ophthalmology & visual science,* [e-journal] 22 (1), pp. 73. Available through: Primo.

Bednarz, J., Rodokanaki-von Schrenck, A. and Engelmann, K., 1998. Different characteristics of endothelial cells from central and peripheral human cornea in primary culture and after subculture. *In Vitro Cellular & Developmental Biology-Animal,* [e-journal] 34 (2), pp. 149-153. Available through: google.

Bednarz, J., Doubilei, V., Wollnik, P.C.M. and Engelmann, K., 2001. Effect of three different media on serum free culture of donor corneas and isolated human corneal endothelial cells. *British Journal of Ophthalmology,* [e-journal] 85 (12), pp. 1416. Available through: Primo.

Bhogal M, Lwin CN, Seah XY, Peh G, Mehta JS., 2017. Allogeneic Descemet's Membrane Transplantation Enhances Corneal Endothelial Monolayer Formation and Restores Functional Integrity Following Descemet's Stripping. *Invest Ophthalmol Vis Sci*, 58 (10):4249-4260. doi: 10.1167/iovs.17-22106. PubMed PMID: 28850636.

Blake, D.A., Yu, H., Young, D.L. and Caldwell, D.R., 1997. Matrix stimulates the proliferation of human corneal endothelial cells in culture. *Investigative ophthalmology & visual science,* [e-journal] 38 (6), pp. 1119. Available through: Primo.

Böhnke, M., Eggli, P. and Engelmann, K., 1999. Transplantation of cultured adult human or porcine corneal endothelial cells onto human recipients in vitro. Part II: Evaluation in the scanning electron microscope. *Cornea,* [e-journal] 18 (2), pp. 207. Available through: Primo.

Bonanno, J.A., 2012. Molecular mechanisms underlying the corneal endothelial pump. *Experimental eye research,* [e-journal] 95 (1), pp. 2. Available through: Primo.

Bostan, C., Thériault, M., Forget, K.J., Doyon, C., Cameron, J.D., Proulx, S. and Brunette, I., 2016. In Vivo Functionality of a Corneal Endothelium Transplanted by Cell- Injection Therapy in a Feline Model. *Investigative ophthalmology & visual science,* [e-journal] 57 (4), pp. 1620. Available through: Primo.

Bourne, W.M., 2003. Biology of the corneal endothelium in health and disease. *Eye (London, England),* [e-journal] 17 (8), pp. 912. Available through: Primo.

Brubaker RF., 1989. Measurement of aqueous flow by fluorophotometry. *The Glaucomas*. pp. 337–44.

Burkitt Wright, E.M., Spencer, H., Daly, S., Manson, F.C., Zeef, L.H., Urquhart, J., Zoppi, N., Bonshek, R., Tosounidis, I., Mohan, M., Madden, C., Dodds, A., Chandler, K., Banka, S., Au, L., Clayton-Smith, J., Khan, N., Biesecker, L., Wilson, M., Rohrbach, M., Colombi, M., Giunta, C. and Black, G.M., 2011. Mutations in PRDM5 in Brittle Cornea Syndrome Identify a Pathway Regulating Extracellular

Matrix Development and Maintenance. *The American Journal of Human Genetics*, [e-journal] 88 (6), pp. 767-777. Available through: Primo.

Capella, J.A. and Kaufman, H.E., 1969. Human corneal endothelium. I. Normal morphology. *Documenta ophthalmologica.Advances in ophthalmology,* [e-journal] 26, pp.1. Available through: Primo.

Chen J, Li Z, Zhang L, Ou S, Wang Y, He X, Zou D, Jia C, Hu Q, Yang S, Li X, Li J, Wang J, Sun H, Chen Y, Zhu YT, Tseng SCG, Liu Z, Li W., 2017a. Descemet's Membrane Supports Corneal Endothelial Cell Regeneration in Rabbits. *Sci Rep*, 7(1):6983. doi: 10.1038/s41598-017-07557-2. PubMed PMID: 28765543; PubMed Central PMCID: PMC5539296.

Chen, K.H., Azar, D. and Joyce, N.C., 2001. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea,* [e-journal] 20 (7), pp. 731. Available through: Primo.

Chen, K.H., Harris, D.L. and Joyce, N.C., 1999. TGF- beta2 in aqueous humor suppresses S- phase entry in cultured corneal endothelial cells. *Investigative ophthalmology & visual science,* [e-journal] 40 (11), pp. 2513. Available through: Primo.

Chen, S., Liu, X., Wang, N., Wang, X., Xiong, Q., Bo, E., Yu, X., Chen, S. and Liu, L., 2017b. Visualizing Micro-anatomical Structures of the Posterior Cornea with Micro-optical Coherence Tomography. Scientific reports, [e-journal] 7 (1), pp.10752. 10.1038/s41598-017-11380-0. Available through: Primo.

Chi, J. and Cohen, P., 2016. The Multifaceted Roles of PRDM16: Adipose Biology and Beyond. *Trends in Endocrinology & Metabolism,* [e-journal] 27 (1), pp. 11-23. Available through: Primo.

Chittka, A., 2013. Differential regulation of SC1/ PRDM4 and PRMT5 mediated protein arginine methylation by the nerve growth factor and the epidermal growth factor in PC12 cells. *Neuroscience letters,* [e-journal] 550, pp. 87-92. Available through: Primo.

Chittka, A., Arevalo, J.C., Rodriguez-Guzman, M., Pérez, P., Chao, M.V. and Sendtner, M., 2004. The p75NTR- interacting protein SC1 inhibits cell cycle progression by transcriptional repression of cyclin E. *The Journal of cell biology,* [e-journal] 164 (7), pp. 985-996. Available through: Primo.

Chittka, A. and Chao, M.V., 1999. Identification of a Zinc Finger Protein Whose Subcellular Distribution Is Regulated by Serum and Nerve Growth Factor. *Proceedings of the National Academy of Sciences of the United States of America*, [e-journal] 96 (19), pp. 10705-10710. Available through: Primo.

Chittka, A., Nitarska, J., Grazini, U. and Richardson, W.D., 2012. Transcription factor positive regulatory domain 4 (PRDM4) recruits protein arginine methyltransferase 5 (PRMT5) to mediate histone arginine methylation and control neural stem cell proliferation and differentiation. *The Journal of biological chemistry,* [e-journal] 287 (51), pp. 42995. Available through: Primo.

Choi, J.S., Kim, E.Y., Kim, M.J., Khan, F.A., Giegengack, M., D'Agostino, R., Criswell, T., Khang, G. and Soker, S., 2014. Factors affecting successful isolation of human corneal endothelial cells for clinical use. *Cell transplantation,* [e-journal] 23 (7), pp. 845. Available through: Primo.

Choi, J.S., Williams, J.K., Greven, M., Walter, K.A., Laber, P.W., Khang, G. and Soker, S., 2010. Bioengineering endothelialized neo- corneas using donor- derived corneal endothelial cells and decellularized corneal stroma. *Biomaterials,* [e-journal] 31 (26), pp. 6738-6745. Available through: Primo.

Chou, M., Burnouf, T. and Wang, T., 2014. Ex vivo expansion of bovine corneal endothelial cells in xeno- free medium supplemented with platelet releasate. *PloS one,* [e-journal] 9 (6), pp.e 99145. Available through: Primo.

Cohen, S.R., Polse, K.A., Brand, R.J. and Mandell, R.B., 1990. Humidity effects on corneal hydration. *Investigative Ophthalmology and Visual Science,* [e-journal] 31 (7), pp.1282-1287. Available through: Primo.

Davis, C.A., Haberland, M., Arnold, M.A., Sutherland, L.B., McDonald, O.G., Richardson, J.A., Childs, G., Harris, S., Owens, G.K. and Olson, E.N., 2006. PRISM/ PRDM6, a Transcriptional Repressor That Promotes the Proliferative Gene Program in Smooth Muscle Cells. *Molecular and cellular biology,* [e-journal] 26 (7), pp. 2626. Available through: Primo.

Davson, H., 1955. The hydration of the cornea. The Biochemical journal, [e-journal] 59 (1), pp.24. 10.1042/bj0590024. Available through: Primo.

Dikstein, S. and Maurice, D.M., 1972. The metabolic basis to the fluid pump in the cornea. *The Journal of physiology,* [e-journal] 221 (1), pp.29. 10.1113/jphysiol.1972.sp009736. Available through: Primo.

Dinc, U.A., Gorgun, E., Oncel, B., Yenerel, M.N. and Alimgil, L., 2010. Assessment of Anterior Chamber Depth Using Visante Optical Coherence Tomography, Slitlamp Optical Coherence Tomography, IOL Master, Pentacam and Orbscan IIz. Ophthalmologica, [e-journal] 224 (6), pp.341-346. 10.1159/000313815. Available through: Primo.

Dixon, J.M. and Blackwood, L., 1991. Thermal variations of the human eye. Transactions of the American Ophthalmological Society, [e-journal] 89, pp.183. Available through: Primo.

Dohlman, C.H., Hedbys, B.O. and Mishima, S., 1962. The swelling pressure of the corneal stroma. *Investigative ophthalmology*, [e-journal] 1, pp. 158. Available through: Primo.

Duan, Z., Person, R.E., Lee, H., Huang, S., Donadieu, J., Badolato, R., Grimes, H.L., Papayannopoulou, T. and Horwitz, M.S., 2007. Epigenetic Regulation of Protein-Coding and MicroRNA Genes by the Gfi1-Interacting Tumor Suppressor PRDM5. *Molecular and cellular biology,* [e-journal] 27 (19), pp. 6889. Available through: Primo.

Elbaz U., Mireskandari K., Tehrani N., Shen C., Khan MS., Williams S., Ali A., 2017. Corneal Endothelial Cell Density in Children: Normative Data From Birth to 5 Years Old. *American Journal of Ophthalmology*. 173:134-138. doi: 10.1016/j.ajo.2016.09.036. Epub 2016 Oct 14. PubMed PMID: 27746297.

Engelmann, K., Böhnke, M. and Friedl, P., 1988. Isolation and long-term cultivation of human corneal endothelial cells. *Investigative ophthalmology & visual science,* [e-journal] 29 (11), pp. 1656. Available through: Primo.

Engelmann, K., Drexler, D. and Böhnke, M., 1999. Transplantation of adult human or porcine corneal endothelial cells onto human recipients in vitro. Part I: Cell culturing and transplantation procedure. *Cornea,* [e-journal] 18 (2), pp. 199. Available through: Primo.

Engelmann, K., Drexler, D., Draeger, J. and Böhnke, M., 1993. Endothelial cell transplantation in a model. *Der Ophthalmologe: Zeitschrift der Deutschen Ophthalmologischen Gesellschaft,* [e-journal] 90 (2), pp. 166. Available through: Primo.

Engelmann, K. and Friedl, P., 1995. Growth of human corneal endothelial cells in a serum-reduced medium. *Cornea,* [e-journal] 14 (1), pp. 62. Available through: Primo.

Engelmann, K. and Friedl, P., 1989. Optimization of culture conditions for human corneal endothelial cells. *In Vitro Cellular & Developmental Biology,* [e-journal] 25 (11), pp. 1065-1072. Available through: Primo.

Engler, C., Kelliher, C., Speck, C.L. and Jun, A.S., 2009. Assessment of attachment factors for primary cultured human corneal endothelial cells. *Cornea,* [e-journal] 28 (9), pp. 1050. Available through: Primo.

Enomoto, K., Mimura, T., Harris, D.L. and Joyce, N.C., 2006. Age differences in cyclin-dependent kinase inhibitor expression and rb hyperphosphorylation in human corneal endothelial cells. *Investigative ophthalmology & visual science,* [e-journal] 47 (10), pp. 4330. Available through: Primo.

Eppert, K., Takenaka, K., Eric, R.L., Waldron, L., Björn Nilsson, Peter, V.G., Klaus, H.M., Poeppl, A., Ling, V., Beyene, J., Angelo, J.C., Jayne, S.D., Stefan, K.B., Buske, C., Mark, D.M., Todd, R.G., Jurisica, I., Benjamin, L.E. and John, E.D., 2011. Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature medicine,* [e-journal] 17 (9), pp. 1086. Available through: Primo.

Fischbarg, J. and Maurice, D.M., 2004. An update on corneal hydration control. *Experimental eye research,* [e-journal] 78 (3), pp. 537-541. Available through: Primo.

Fog, C.K., Galli, G.G. and Lund, A.H., 2012. *PRDM proteins: Important players in differentiation and disease*: Bioessays. [e-journal]. Weinheim: 34 (1) pp. 50-60. Available through: Primo.

Galli, G.G., Honnens, d.L., Carrara, M., Hans, W., Wuelling, M., Mentz, B., Multhaupt, H.A., Fog, C.K., Jensen, K.T., Rappsilber, J., Vortkamp, A., Coulton, L., Fuchs, H., Gailus-Durner, V., Hrabě, d.A., Calogero, R.A., Couchman, J.R. and Lund, A.H., 2012. Prdm5 Regulates Collagen Gene Transcription by Association with RNA Polymerase II in Developing Bone (Prdm5 Promotes the Transcription of ECM Genes). *PLoS Genetics,* [e-journal] 8 (5), pp.e 1002711. Available through: Primo.

Geroski, D.H., Kies, J.C. and Edelhauser, H.F., 1984. The effects of ouabain on endothelial function in human and rabbit corneas. *Current eye research,* [e-journal] 3 (2), pp.331-338. 10.3109/02713688408997217. Available through: Primo.

Goodenough, D.A. and Paul, D.L., 2009. Gap junctions. *Cold Spring Harbor perspectives in biology,* [e-journal] 1 (1), pp.a 002576. Available through: Primo.

Gospodarowicz, D., Greenburg, G. and Birdwell, C.R., 1978. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer research,* [e-journal] 38 (11), pp. 4155. Available through: Primo.

Gospodarowicz, D., Greenburg, G. and Alvarado, J., 1979. Transplantation of Cultured Bovine Corneal Endothelial Cells to Rabbit Cornea: Clinical Implications for Human Studies. *Proceedings of the National Academy of Sciences of the United States of America,* [e-journal] 76 (1), pp. 464-468. Available through: Primo.

Gottsch, J.D., Sundin, O.H., Liu, S.H., Jun, A.S., Broman, K.W., Stark, W.J., Vito, E.C.L., Narang, A.K., Thompson, J.M. and Magovern, M., 2005a. Inheritance of a

novel COL8A2 mutation defines a distinct early-onset subtype of Fuchs corneal dystrophy. *Investigative ophthalmology & visual science,* [e-journal] 46 (6), pp. 1934. Available through: Primo.

Gottsch, J.D., Zhang, C., Sundin, O.H., Bell, W.R., Stark, W.J. and Green, W.R., 2005b. Fuchs corneal dystrophy: aberrant collagen distribution in an L450W mutant of the COL8A2 gene. *Investigative ophthalmology & visual science,* [e-journal] 46 (12), pp. 4504. Available through: Primo.

Graham, M.A., Rawe, I., Dartt, D.A. and Joyce, N.C., 2000. Protein kinase C regulation of corneal endothelial cell proliferation and cell cycle. *Investigative ophthalmology & visual science,* [e-journal] 41 (13), pp. 4124. Available through: Primo.

Gruschwitz, R., Friedrichs, J., Valtink, M., Franz, C.M., Müller, D.,J., Funk, R.H.W. and Engelmann, K., 2010. Alignment and cell-matrix interactions of human corneal endothelial cells on nanostructured collagen type I matrices. *Investigative ophthalmology & visual science,* [e-journal] 51 (12), pp. 6303. Available through: Primo.

Gu, X., Kay, E.P., Seong, G.J. and Lee, Y.G., 1996. Fibroblast growth factor 2 uses distinct signaling pathways for cell proliferation and cell shape changes in corneal endothelial cells. *Investigative Ophthalmology and Visual Science,* [e-journal] 37 (11), pp. 2326-2334. Available through: Primo.

Han, S.B., Ang, H., Balehosur, D., Peh, G., Chaurasia, S.S., Tan, D.T.H. and Mehta, J.S., 2013. A mouse model of corneal endothelial decompensation using cryoinjury. *Molecular vision,* [e-journal] 19, pp. 1222. Available through: Primo.

Hanson C., Arnarsson A., Hardarson T., Lindgård A., Daneshvarnaeini M., Ellerström C., Bruun A., Stenevi U., 2017. Transplanting embryonic stem cells onto damaged human corneal endothelium. *World Journal of Stem Cells*. 26 ;9(8):127-132. doi: 10.4252/wjsc.v9.i8.127. Available through: PubMed PMID: 28928909; PubMed Central PMCID: PMC5583531.

Hara, S., Hayashi, R., Soma, T., Kageyama, T., Duncan, T., Tsujikawa, M. and Nishida, K., 2014. Identification and potential application of human corneal endothelial progenitor cells. (Report). *Stem Cells and Development,* [e-journal] 23 (18), pp. 2190. Available through: Primo.

Harrison, T., He, Z., Boggs, K., Thuret, G., Liu, H. and Defoe, D., 2016. Corneal endothelial cells possess an elaborate multipolar shape to maximize the basolateral to apical membrane area. Molecular Vision, [e-journal] 22, pp.31-39. Available through: Primo.

Hassell, J.R. and Birk, D.E., 2010. The molecular basis of corneal transparency. *Experimental eye research,* [e-journal] 91 (3), pp. 326-335. Available through: Primo.

Hayashi, K., Sueishi, K., Tanaka, K. and Inomata, H., 1986. Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. Graefe's Archive for Clinical and Experimental Ophthalmology, [e-journal] 224 (5), pp.452-456. 10.1007/BF02173362. Available through: Primo.

He, Z., Campolmi, N., Gain, P., Ha Thi, B.M., Dumollard, J., Duband, S., Peoc' H, M., Piselli, S., Garraud, O. and Thuret, G., 2012. Revisited Microanatomy of the Corneal Endothelial Periphery: New Evidence for Continuous Centripetal Migration of Endothelial Cells in Humans. *Stem cells,* [e-journal] 30 (11), pp. 2523-2534. Available through: Primo.

Hirata- Tominaga, K., Nakamura, T., Okumura, N., Kawasaki, S., Kay, E.P., Barrandon, Y., Koizumi, N. and Kinoshita, S., 2013. Corneal endothelial cell fate is maintained by LGR5 through the regulation of hedgehog and Wnt pathway. *Stem cells,* [e-journal] 31 (7), pp. 1396-1407. Available through: google.

Hirsch, M., Renard, G., Faure, J. and Pouliquen, Y., 1977. Study of the ultrastructure of the rabbit corneal endothelium by the freeze-fracture technique: Apical and lateral junctions. Experimental eye research, [e-journal] 25 (3), pp.277-288. 10.1016/0014-4835(77)90094-X. Available through: Primo.

Hohenauer, T. and Moore, A.W., 2012. The Prdm family: expanding roles in stem cells and development. *Development (Cambridge, England),* [e-journal] 139 (13), pp. 2267. Available through: Primo.

Iliff, B., Riazuddin, S.A. and Gottsch, J., 2012. *The genetics of Fuchs'corneal dystrophy*: Expert Review of Ophthalmology.[e-journal]. London: Informa Healthcare.7 (4) pp. 363-375. Available through: Primo,.

Ishino, Y., Sano, Y., Nakamura, T., Connon, C.J., Rigby, H., Fullwood, N.J. and Kinoshita, S., 2004. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Investigative ophthalmology & visual science,* [e-journal] 45 (3), pp. 800. Available through: Primo.

Jakobiec, F.A. and Bhat, P., 2010. Retrocorneal Membranes: A Comparative Immunohistochemical Analysis of Keratocytic, Endothelial, and Epithelial Origins. *American Journal of Ophthalmology,* [e-journal] 150 (2), pp. 230-242.e2. Available through: Primo.

Jakus, M.A., 1956. Studies on the cornea. II. The fine structure of Descement's membrane. *The Journal of biophysical and biochemical cytology,* [e-journal] 2 (4), pp. 243. Available through: Primo.

Johnson, D.H. and Tschumper, R.C., 1987. Human trabecular meshwork organ culture. A new method. *Investigative ophthalmology & visual science,* [e-journal] 28 (6), pp. 945. Available through: Primo.

Joyce, N.C. and Zieske, J.D., 1997. Transforming growth factor-beta receptor expression in human cornea. *Investigative ophthalmology & visual science,* [e-journal] 38 (10), pp. 1922-1928. Available through: google.

Joyce, N.C., Meklir, B., Joyce, S.J. and Zieske, J.D., 1996b. Cell cycle protein expression and proliferative status in human corneal cells. *Investigative ophthalmology & visual science,* [e-journal] 37 (4), pp. 645. Available through: Primo.

Joyce, N.C., 2012. Proliferative capacity of corneal endothelial cells. *Experimental eye research,* [e-journal] 95 (1), pp. 16. Available through: Primo.

Joyce, N.C., 2003. Proliferative capacity of the corneal endothelium. *Progress in retinal and eye research,* [e-journal] 22 (3), pp. 359-389. Available through: Primo.

Joyce, N.C. and Harris, D.L., 2010. Decreasing expression of the G 1 - phase inhibitors, p21Cip1 and p16INK4a, promotes division of corneal endothelial cells from older donors. *Molecular Vision,* [e-journal] 16, pp. 897-906. Available through: Primo.

Joyce, N.C., Harris, D.L., Markov, V., Zhang, Z. and Saitta, B., 2012. Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. *Molecular Vision,* [e-journal] 18, pp. 547-564. Available through: Primo.

Joyce, N.C., Harris, D.L. and Mello, D.M., 2002. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. *Investigative ophthalmology & visual science,* [e-journal] 43 (7), pp. 2152. Available through: Primo.

Joyce, N.C., Navon, S.E., Roy, S. and Zieske, J.D., 1996a. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. *Investigative ophthalmology & visual science,* [e-journal] 37 (8), pp. 1566. Available through: Primo.

Joyce, N.C. and Zhu, C.C., 2004. Human corneal endothelial cell proliferation: potential for use in regenerative medicine. *Cornea,* [e-journal] 23 (8), pp. 8. Available through: Primo.

Ju, C., Gao, L., Wu, X. and Pang, K., 2012. A human corneal endothelium equivalent constructed with acellular porcine corneal matrix. *The Indian journal of medical research,* [e-journal] 135 (6), pp. 887-894. Available through: Primo.

Jumblatt, M.M., Maurice, D.M. and McCulley, J.P., 1978. Transplantation of tissue-cultured corneal endothelium. *Investigative ophthalmology & visual science,* [e-journal] 17 (12), pp. 1135. Available through: Primo.

Jumblatt, M., M., Maurice, M., D. and Schwartz, D., B., 1980. A GELATIN MEMBRANE SUBSTRATE FOR THE TRANSPLANTATION OF TISSUE CULTURED CELLS. Transplantation, [e-journal] 29 (6), pp.498-499. 10.1097/00007890-198006000-00013. Available through: Primo.

Kajimura, S., Seale, P., Kubota, K., Lunsford, E., Frangioni, J.V., Gygi, S.P. and Spiegelman, B.M., 2009. Initiation of myoblast to brown fat switch by a PRDM16–C/ EBP-β transcriptional complex. *Nature,* [e-journal] 460 (7259), pp. 1154. Available through: Primo.

Kay, E.P., Gu, X. and Smith, R.E., 1994. Corneal endothelial modulation: bFGF as direct mediator and corneal endothelium modulation factor as inducer. *Investigative ophthalmology & visual science,* [e-journal] 35 (5), pp. 2427. Available through: Primo.

Kay, E.D.P., Cheung, C.C., Jester, J.V., Nimni, M.E. and Smith, R.E., 1982. Type I collagen and fibronectin synthesis by retrocorneal fibrous membrane. *Investigative Ophthalmology and Visual Science,* [e-journal] 22 (2), pp. 200-212. Available through: Primo.

Kay, E.P., 1989. Expression of types I and IV collagen genes in normal and in modulated corneal endothelial cells. *Investigative Ophthalmology and Visual Science,* [e-journal] 30 (2), pp. 260-268. Available through: Primo.

Kaye, G.I., Mishima, S., Cole, J.D. and Kaye, N.W., 1968. Studies on the cornea. VII. Effects of perfusion with a Ca++-free medium on the corneal endothelium. Investigative ophthalmology, [e-journal] 7 (1), pp.53. Available through: Primo.

Kaye, G.I., Sibley, R.C. and Hoefle, F.B., 1973. Recent studies on the nature and function of the corneal endothelial barrier. Experimental eye research, [e-journal] 15 (5), pp.585-613. 10.1016/0014-4835(73)90070-5. Available through: Primo.

Keven Williams, K. and Watsky, M.A., 2002. Gap junctional communication in the human corneal endothelium and epithelium. *Current Eye Research, 2002,* [e-journal] 25; Vol. 25 (1; 1), pp. 29; 29-36; 36. Available through: Primo.

Kimoto, M., Shima, N., Yamaguchi, M., Hiraoka, Y., Amano, S. and Yamagami, S., 2014. Development of a bioengineered corneal endothelial cell sheet to fit the

corneal curvature. *Investigative ophthalmology & visual science,* [e-journal] 55 (4), pp. 2337. Available through: Primo.

Kinameri, E., Inoue, T., Aruga, J., Imayoshi, I., Kageyama, R., Shimogori, T. and Moore, A.W., 2008. Prdm Proto-Oncogene Transcription Factor Family Expression and Interaction with the Notch-Hes Pathway in Mouse Neurogenesis (Prdm Genes in Neurogenesis). *PLoS ONE,* [e-journal] 3 (12), pp. e3859. Available through: Primo.

Klintworth Gordon, K., 2009. Corneal dystrophies. *Orphanet Journal of Rare Diseases,* [e-journal] 4 (1), pp. 7. Available through: Primo.

Koizumi, N., Sakamoto, Y., Okumura, N., Okahara, N., Tsuchiya, H., Torii, R., Cooper, L.J., Ban, Y., Tanioka, H. and Kinoshita, S., 2007. Cultivated corneal endothelial cell sheet transplantation in a primate model. *Investigative ophthalmology & visual science,* [e-journal] 48 (10), pp.4519. Available through: Primo.

Komai, T., Iwanari, H., Mochizuki, Y., Hamakubo, T. and Shinkai, Y., 2009. Expression of the mouse PR domain protein Prdm8 in the developing central nervous system. *Gene Expression Patterns,* [e-journal] 9 (7), pp.503-514. Available through: Primo.

Konomi, K., Zhu, C., Harris, D. and Joyce, N.C., 2005. Comparison of the proliferative capacity of human corneal endothelial cells from the central and peripheral areas. *Investigative ophthalmology & visual science,* [e-journal] 46 (11), pp.4086. Available through: Primo.

Laganowski, H. C., Sherrard, E. S., Muir, M. G., & Buckley, R. J., 1991. Distinguishing features of the iridocorneal endothelial syndrome and posterior polymorphous dystrophy: value of endothelial specular microscopy. *The British Journal of Ophthalmology*, [e-journal] 75 (4), pp.212–216.

Lee, H.T. and Kay, E.P., 2003. Regulatory role of PI 3-kinase on expression of Cdk4 and p27, nuclear localization of Cdk4, and phosphorylation of p27 in corneal endothelial cells. *Investigative Ophthalmology and Visual Science,* [e-journal] 44 (4), pp.1521-1528. Available through: Primo.

Lee, J.G. and Kay, E.P., 2012. NF-κB is the transcription factor for FGF-2 that causes endothelial mesenchymal transformation in cornea. *Investigative ophthalmology & visual science,* [e-journal] 53 (3), pp.1530. Available through: Primo.

Leung, E.W., Rife, L., Smith, R.E. and Kay, E.P., 2000. Extracellular matrix components in retrocorneal fibrous membrane in comparison to corneal endothelium and Descemet's membrane. *Molecular Vision,* [e-journal] 6 (4), pp.15-23. Available through: Primo.

Levis, H.J., Peh, G.S.L., Toh, K., Poh, R., Shortt, A.J., Drake, R.A.L., Mehta, J.S. and Daniels, J.T., 2012. Plastic Compressed Collagen as a Novel Carrier for Expanded Human Corneal Endothelial Cells for Transplantation (PC Collagen for Endothelial Transplantation). [e-journal] 7 (11), pp.e50993. Available through: Primo.

Li, C., Dong, F., Jia, Y., Du, H., Dong, N., Xu, Y., Wang, S., Wu, H., Liu, Z. and Li, W., 2013. Notch Signal Regulates Corneal Endothelial-to-Mesenchymal Transition. *The American Journal of Pathology,* [e-journal] 183 (3), pp.786-795. Available through: Primo.

Li, W., Sabater, A.L., Chen, Y., Hayashida, Y., Chen, S., He, H. and Tseng, S.C.G., 2007. A novel method of isolation, preservation, and expansion of human corneal endothelial cells. *Investigative ophthalmology & visual science,* [e-journal] 48 (2), pp.614. Available through: Primo.

Liang, Y., Liu, W., Han, B., Yang, C., Ma, Q., Song, F. and Bi, Q., 2011. An in situ formed biodegradable hydrogel for reconstruction of the corneal endothelium. *Colloids and Surfaces B: Biointerfaces,* [e-journal] 82 (1), pp.1-7. Available through: Primo.

Liu Z, Y., Wang J, Y., Liu H, H., X, M.M., Wang C, L., X, Z.P., Tao Y, Q., Lu Y, C., Liao J, C. and Hu G, H., 2012. Retinoblastoma protein-interacting zinc-finger gene 1 (RIZ1) dysregulation in human malignant meningiomas. *Oncogene,* [e-journal] 32 (10), pp.1216. Available through: Primo.

Lu, X., Chen, D., Liu, Z., Li, C., Liu, Y., Zhou, J., Wan, P., Mou, Y. and Wang, Z., 2010. Enhanced survival in vitro of human corneal endothelial cells using mouse embryonic stem cell conditioned medium. *Molecular Vision,* [e-journal] 16, pp.611-622. Available through: Primo.

Lu, J., Lu, Z., Reinach, P., Zhang, J., Dai, W., Lu, L. and Xu, M., 2006. TGF-β2 inhibits AKT activation and FGF-2-induced corneal endothelial cell proliferation. Experimental cell research, [e-journal] 312 (18), pp.3631-3640. 10.1016/j.yexcr.2006.08.004. Available through: Primo.

Marshall, G.E., Konstas, A.G. and Lee, W.R., 1993. Collagens in ocular tissues. *The British journal of ophthalmology,* [e-journal] 77 (8), pp.515. Available through: Primo.

Martola, E. and Baum, J.L., 1968. Central and Peripheral Corneal Thickness: A Clinical Study. Archives of Ophthalmology, [e-journal] 79 (1), pp.28-30. 10.1001/archopht.1968.03850040030009. Available through: Primo.

Massoudi, D., Malecaze, F. and Galiacy, S., 2016. Collagens and proteoglycans of the cornea: importance in transparency and visual disorders. *Cell and tissue research,* [e-journal] 363 (2), pp.337-349. Available through: Primo.

Maurice, D.M. and Giardini, A.A., 1951. Swelling of the Cornea in vivo after the Destruction of its Limiting Layers *. British Journal of Ophthalmology, [e-journal] 35 (12), pp.791. 10.1136/bjo.35.12.791. Available through: Primo.

Maurice, D.M., 1972. The location of the fluid pump in the cornea. The Journal of physiology, [e-journal] 221 (1), pp.43. 10.1113/jphysiol.1972.sp009737. Available through: Primo.

Maurice, D.M., Mcculley, J.P. and Schwartz, B.D., 1981. The use of cultured endothelium in keratoplasty. Vision research, [e-journal] 21 (1), pp.173-174. 10.1016/0042-6989(81)90156-5. Available through: Primo.

Mcculley, J.P., Uchiyama, E., Aronowicz, J.D. and Butovich, I.A., 2006. Impact of evaporation on aqueous tear loss. *Transactions of the American Ophthalmological Society,* [e-journal] 104, pp.121. Available through: Primo.

McGowan, S.L., Edelhauser, H.F., Pfister, R.R. and Whikehart, D.R., 2007. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. *Molecular vision,* [e-journal] 13, pp.1984. Available through: Primo.

Meani, N., Pezzimenti, F., Deflorian, G., Mione, M. and Alcalay, M., 2009. The Tumor Suppressor PRDM5 Regulates Wnt Signaling at Early Stages of Zebrafish Development (PRDM5 Modulates Wnt Signaling). *PLoS ONE,* [e-journal] 4 (1), pp.e4273. Available through: Primo.

Melles, G.R.J., Lander, F. and Rietveld, F.J.R., 2002. Transplantation of Descemet's membrane carrying viable endothelium through a small scleral incision. *Cornea,* [e-journal] 21 (4), pp.415. Available through: Primo.

Mergler, S. and Pleyer, U., 2007. The human corneal endothelium: New insights into electrophysiology and ion channels. Progress in retinal and eye research, [e-journal] 26 (4), pp.359-378. 10.1016/j.preteyeres.2007.02.001. Available through: Primo.

Mimura, T., Amano, S., Usui, T., Araie, M., Ono, K., Akihiro, H., Yokoo, S. and Yamagami, S., 2004a. Transplantation of corneas reconstructed with cultured adult human corneal endothelial cells in nude rats. *Experimental eye research,* [e-journal] 79 (2), pp.231-237. Available through: Primo.

Mimura, T. and Joyce, N.C., 2006. Replication competence and senescence in central and peripheral human corneal endothelium. *Investigative ophthalmology & visual science,* [e-journal] 47 (4), pp.1387. Available through: Primo.

Mimura, T., Yamagami, S., Yokoo, S., Araie, M. and Amano, S., 2005. Comparison of rabbit corneal endothelial cell precursors in the central and peripheral cornea. *Investigative ophthalmology & visual science,* [e-journal] 46 (10), pp.3645. Available through: Primo.

Mimura, T., Yamagami, S., Yokoo, S., Usui, T., Tanaka, K., Hattori, S., Irie, S., Miyata, K., Araie, M. and Amano, S., 2004b. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. *Investigative ophthalmology & visual science,* [e-journal] 45 (9), pp.2992. Available through: Primo.

MISHIMA, S. and KUDO, T., 1967. In vitro incubation of rabbit cornea. Investigative ophthalmology & visual science, 6 (4), pp.329-339.

Mount, N.M., Ward, S.J., Kefalas, P. and Hyllner, J., 2015. *Cell- based therapy technology classifications and translational challenges*: Philosophical Transactions of the Royal Society of London.Biological Sciences.[e-journal]. 370 (1680) pp.20150017. 10.1098/rstb.2015.0017. Available through: Primo,.

Møller-Pedersen, T., Hartmann, U., Møller, ,H.J., Ehlers, N. and Engelmann, K., 2001. Evaluation of potential organ culture media for eye banking using human donor corneas. *British Journal of Ophthalmology,* [e-journal] 85 (9), pp.1075. Available through: Primo.

Murphy, C., Alvarado, J. and Juster, R., 1984b. Prenatal and postnatal growth of the human Descemet's membrane. *Investigative ophthalmology & visual science,* [e-journal] 25 (12), pp.1402. Available through: Primo.

Murphy, C., Alvarado, J., Juster, R. and Maglio, M., 1984a. Prenatal and postnatal cellularity of the human corneal endothelium. A quantitative histologic study. *Investigative ophthalmology & visual science,* [e-journal] 25 (3), pp.312. Available through: Primo.

Nakahara, M., Okumura, N., Kay, E.P., Hagiya, M., Imagawa, K., Hosoda, Y., Kinoshita, S. and Koizumi, N., 2013. Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. *PloS one,* [e-journal] 8 (7), pp.e69009. Available through: Primo.

Nakano, Y., Oyamada, M., Dai, P., Nakagami, T., Kinoshita, S. and Takamatsu, T., 2008. Connexin43 knockdown accelerates wound healing but inhibits mesenchymal transition after corneal endothelial injury in vivo. *Investigative ophthalmology & visual science,* [e-journal] 49 (1), pp.93. Available through: Primo.

Nayak, S.K. and Binder, P.S., 1984. The growth of endothelium from human corneal rims in tissue culture. *Investigative ophthalmology & visual science,* [e-journal] 25 (10), pp.1213-1216. Available through: google.

Numata, R., Okumura, N., Nakahara, M., Ueno, M., Kinoshita, S., Kanematsu, D., Kanemura, Y., Sasai, Y. and Koizumi, N., 2014. Cultivation of corneal endothelial cells on a pericellular matrix prepared from human decidua- derived mesenchymal cells. *PloS one,* [e-journal] 9 (2), pp.e88169. Available through: Primo.

Ohinata, Y., Payer, B., Dónal O'Carroll, Ancelin, K., Ono, Y., Sano, M., Barton, S.C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M. and Azim Surani, M., 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature,* [e-journal] 436 (7048), pp.207. Available through: Primo.

Okumura, N., Kakutani, K., Numata, R., Nakahara, M., Schlötzer-Schrehardt, U., Kruse, F., Kinoshita, S. and Koizumi, N., 2015. Laminin-511 and -521 enable efficient in vitro expansion of human corneal endothelial cells. *Investigative ophthalmology & visual science,* [e-journal] 56 (5), pp.2933. Available through: Primo.

Okumura, N., Kay, E.P., Nakahara, M., Hamuro, J., Kinoshita, S. and Koizumi, N., 2013. Inhibition of TGF-β signaling enables human corneal endothelial cell expansion in vitro for use in regenerative medicine. *PloS one,* [e-journal] 8 (2), pp.e58000. Available through: Primo.

Okumura, N., Kinoshita, S. and Koizumi, N., 2017. Application of Rho Kinase Inhibitors for the Treatment of Corneal Endothelial Diseases. Journal of Ophthalmology, [e-journal] 2017 10.1155/2017/2646904. Available through: Primo.

Okumura, N., Koizumi, N., Ueno, M., Sakamoto, Y., Takahashi, H., Tsuchiya, H., Hamuro, J. and Kinoshita, S., 2012. ROCK Inhibitor Converts Corneal Endothelial Cells into a Phenotype Capable of Regenerating In Vivo Endothelial Tissue. *The American Journal of Pathology,* [e-journal] 181 (1), pp.268-277. Available through: Primo.

Okumura, N., Nakano, S., Kay, E.P., Numata, R., Ota, A., Sowa, Y., Sakai, T., Ueno, M., Kinoshita, S. and Koizumi, N., 2014a. Involvement of Cyclin D and p27 in Cell Proliferation Mediated by ROCK Inhibitors Y-27632 and Y-39983 During Corneal Endothelium Wound Healing. *Investigative ophthalmology & visual science,* [e-journal] 55 (1), pp.318. Available through: Primo.

Okumura, N., Sakamoto, Y., Fujii, K., Kitano, J., Nakano, S., Tsujimoto, Y., Nakamura, S., Ueno, M., Hagiya, M., Hamuro, J., Matsuyama, A., Suzuki, S., Shiina, T., Kinoshita, S. and Koizumi, N., 2016. Rho kinase inhibitor enables cell-based therapy for corneal endothelial dysfunction. *Scientific reports,* [e-journal] 6, pp.26113. Available through: Primo.

Okumura, N., Nakamura, T., Kay, E.P., Nakahara, M., Kinoshita, S. and Koizumi, N., 2014b. R-spondin1 regulates cell proliferation of corneal endothelial cells via the Wnt3a/β-catenin pathway. *Investigative ophthalmology & visual science,* [e-journal] 55 (10), pp.6861. Available through: Primo.

Okumura, N., Ueno, M., Koizumi, N., Sakamoto, Y., Hirata, K., Hamuro, J. and Kinoshita, S., 2009. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. *Investigative ophthalmology & visual science,* [e-journal] 50 (8), pp.3680. Available through: Primo.

Parekh, M., Ferrari, S., Sheridan, C., Kaye, S. and Ahmad, S., 2016. Concise Review: An Update on the Culture of Human Corneal Endothelial Cells for Transplantation. *STEM CELLS Translational Medicine,* [e-journal] 5 (2), pp.258-264. Available through: Primo.

Park, J. and Kim, K.C., 2010. Expression patterns of PRDM10 during mouse embryonic development. *BMB reports,* [e-journal] 43 (1), pp.29. Available through: Primo.

Park Kay, E.D., Smith, R.E. and Nimni, M.E., 1985. Type I collagen synthesis by corneal endothelial cells modulated by polymorphonuclear leukocytes. *Journal of Biological Chemistry,* [e-journal] 260 (8), pp.5139-5146. Available through: Primo.

Patel, S.V., Bachman, L.A., Hann, C.R., Bahler, C.K. and Fautsch, M.P., 2009. Human corneal endothelial cell transplantation in a human ex vivo model. *Investigative ophthalmology & visual science,* [e-journal] 50 (5), pp.2123. Available through: Primo.

Peh, G.S.L., Adnan, K., George, B.L., Ang, H., Seah, X., Tan, D.T. and Mehta, J.S., 2015b. The effects of Rho-associated kinase inhibitor Y-27632 on primary human corneal endothelial cells propagated using a dual media approach. *Scientific reports,* [e-journal] 5, pp.9167. Available through: Primo.

Peh, G.S.L., Toh, K., Ang, H., Seah, X., George, B.L. and Mehta, J.S., 2013. Optimization of human corneal endothelial cell culture: density dependency of successful cultures in vitro. *BMC research notes,* [e-journal] 6, pp.176. Available through: Primo.

Peh, G.S.L., Chng, Z., Ang, H., Cheng, T.Y.D., Adnan, K., Seah, X., George, B.L., Toh, K., Tan, D.T., Yam, G.H.F., Colman, A. and Mehta, J.S., 2015a. Propagation of human corneal endothelial cells: a novel dual media approach. *Cell transplantation,* [e-journal] 24 (2), pp.287. Available through: Primo.

Peh, G.S.L., Toh, K., Wu, F., Tan, D.T. and Mehta, J.S., 2011. Cultivation of Human Corneal Endothelial Cells Isolated from Paired Donor Corneas (Culture of

Human Corneal Endothelial Cells). *PLoS ONE,* [e-journal] 6 (12), pp.e28310. Available through: Primo.

Pipparelli, A., Arsenijevic, Y., Thuret, G., Gain, P., Nicolas, M. and Majo, F., 2013. ROCK inhibitor enhances adhesion and wound healing of human corneal endothelial cells. *PloS one,* [e-journal] 8 (4), pp.e62095. Available through: google.

Proulx, S., Bensaoula, T., Nada, O., Audet, C., d'Arc Uwamaliya, J., Devaux, A., Allaire, G., Germain, L. and Brunette, I., 2009. Transplantation of a tissue-engineered corneal endothelium reconstructed on a devitalized carrier in the feline model. *Investigative ophthalmology & visual science,* [e-journal] 50 (6), pp.2686. Available through: Primo.

Rajan M, S., 2014. Surgical strategies to improve visual outcomes in corneal transplantation. *Eye,* [e-journal] 28 (2), pp.196. Available through: Primo.

Rajan, M.S., Watters, W., Patmore, A. and Marshall, J., 2005. In vitro human corneal model to investigate stromal epithelial interactions following refractive surgery. *Journal of Cataract & Refractive Surgery,* [e-journal] 31 (9), pp.1789-1801. Available through: Primo.

Reid A, G. and Nacheva E, P., 2003. A potential role for PRDM12 in the pathogenesis of chronic myeloid leukaemia with derivative chromosome 9 deletion. Leukemia, [e-journal] 18 (1), pp.178. 10.1038/sj.leu.2403162. Available through: Primo.

Rieck, P., Oliver, L., Engelmann, K., Fuhrmann, G., Hartmann, C. and Courtois, Y., 1995. The role of exogenous/endogenous basic fibroblast growth factor (FGF2) and transforming growth factor beta (TGF beta-1) on human corneal endothelial cells proliferation in vitro. *Experimental cell research,* [e-journal] 220 (1), pp.36. Available through: Primo.

Rolev, K., O'Donovan, D.G., Georgiou, C., Rajan, M.S. and Chittka, A., 2017. Identification of Prdm genes in human corneal endothelium. *Experimental eye research,* [e-journal] 10.1016/j.exer.2017.02.009. Available through: Primo.

Roy, O., Leclerc, V., Bourget, J., Thériault, M. and Proulx, S., 2015. Understanding the process of corneal endothelial morphological change in vitro. *Investigative ophthalmology & visual science,* [e-journal] 56 (2), pp.1228. Available through: Primo.

Rüfer, F., Schröder, A. and Erb, C., 2005. White-to-white corneal diameter: normal values in healthy humans obtained with the Orbscan II topography system. *Cornea,* [e-journal] 24 (3), pp.259. Available through: Primo.

Sajja, S. and Vemapti, P., 2013. Diurnal Variation of Intra Ocular Pressure (IOP) in Healthy Individuals: A Pilot Study. *Scholars Journal of Applied Medical Sciences,* 1 (5), pp.488-492.

San Choi, J., Kim, E.Y., Kim, M.J., Giegengack, M., Khan, F.A., Khang, G. and Soker, S., 2013. In vitro evaluation of the interactions between human corneal endothelial cells and extracellular matrix proteins. *Biomedical materials,* [e-journal] 8 (1), pp.014108. Available through: google.

Schmedt, T., Silva, M.M., Ziaei, A. and Jurkunas, U., 2012. Molecular bases of corneal endothelial dystrophies. *Experimental eye research,* [e-journal] 95 (1), pp.24. Available through: Primo.

Schwartz, M., 2004. Rho signalling at a glance. *Journal of cell science; J.Cell Sci.*, [e-journal] 117 (23), pp.5457-5458. Available through: Primo.

Seale, P., Kajimura, S., Yang, W., Chin, S., Rohas, L.M., Uldry, M., Tavernier, G., Langin, D. and Spiegelman, B.M., 2007. Transcriptional Control of Brown Fat Determination by PRDM16. *Cell Metabolism,* [e-journal] 6 (1), pp.38-54. Available through: Primo.

Senoo, T., Obara, Y. and Joyce, N.C., 2000a. EDTA: a promoter of proliferation in human corneal endothelium. *Investigative ophthalmology & visual science,* [e-journal] 41 (10), pp.2930. Available through: Primo.

Senoo, T. and Joyce, N.C., 2000b. Cell cycle kinetics in corneal endothelium from old and young donors. *Investigative ophthalmology & visual science,* [e-journal] 41 (3), pp.660. Available through: Primo.

Sheerin, A.N., Smith, S.K., Jennert- burston, K., Brook, A.J., Allen, M.C., Ibrahim, B., Jones, D., Wallis, C., Engelmann, K., Rhys- williams, W., Faragher, R.G.A. and Kipling, D., 2012. Characterization of cellular senescence mechanisms in human corneal endothelial cells. *Aging Cell,* [e-journal] 11 (2), pp.234-240. Available through: Primo.

Shen, L., Sun, P., Zhang, C., Yang, L., Du, L. and Wu, X., 2017. Therapy of corneal endothelial dysfunction with corneal endothelial cell-like cells derived from skin-derived precursors. Scientific reports, [e-journal] 7 (1), pp.13400. 10.1038/s41598-017-13787-1. Available through: Primo.

Sherrard, E.S., Novakovic, P. and Speedwell, L., 1987. Age-related changes of the corneal endothelium and stroma as seen in vivo by specular microscopy. Eye (London, England), [e-journal] 1 (Pt 2), pp.197. Available through: Primo.

Sherrard, E.S. and Rycroft, P.V., 1967a. Retrocorneal membranes. I. Their origin and structure. British Journal of Ophthalmology, [e-journal] 51 (6), pp.379. 10.1136/bjo.51.6.379. Available through: Primo.

Sherrard, E.S. and Rycroft, P.V., 1967b. Retrocorneal membranes. II. Factors influencing their growth. British Journal of Ophthalmology, [e-journal] 51 (6), pp.387. 10.1136/bjo.51.6.387. Available through: Primo.

Shima, N., Kimoto, M., Yamaguchi, M. and Yamagami, S., 2011. Increased proliferation and replicative lifespan of isolated human corneal endothelial cells with L-ascorbic acid 2-phosphate. *Investigative ophthalmology & visual science,* [e-journal] 52 (12), pp.8711. Available through: Primo.

Shu, X., Geng, H., Li, L., Ying, J., Ma, C., Wang, Y., Poon, F., Wang, X., Ying, Y., Yeo, W., Yu, J., Sung, J., Huang, S., Chan, A., Tao, Q., Srivastava, G. and Tsao, S., 2011. The epigenetic modifier PRDM5 functions as a tumor suppressor through modulating WNT/β-catenin signaling and is frequently silenced in multiple tumors. [e-journal] 6 (11) Available through: Primo.

Srinivas, P., S., 2010. Dynamic Regulation of Barrier Integrity of the Corneal Endothelium. *Optometry and Vision Science,* [e-journal] 87 (4), pp.E239-E254. Available through: Primo.

Stevanato, L., Corteling, R.L., Stroemer, P., Hope, A., Heward, J., Miljan, E.A. and Sinden, J.D., 2009. c-MycERTAM transgene silencing in a genetically modified human neural stem cell line implanted into MCAo rodent brain. *BMC neuroscience,* [e-journal] 10, pp.86. 10.1186/1471-2202-10-86. Available through: Primo.

Stocker, F.W., 1953. The endothelium of the cornea and its clinical implications. *Transactions of the American Ophthalmological Society,* [e-journal] 51, pp.669-786. Available through: Primo.

Stocker, F.W. and Reichle, K., 1974. Theodor Leber and the endothelium of the cornea. American Journal of Ophthalmology, [e-journal] 78 (6), pp.893. Available through: Primo.

Sumide, T., Nishida, K., Yamato, M., Ide, T., Hayashida, Y., Watanabe, K., Yang, J., Kohno, C., Kikuchi, A., Maeda, N., Watanabe, H., Okano, T. and Tano, Y., 2006. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology,* [e-journal] 20 (2), pp.392-394. Available through: Primo.

Tan, S., Hu, R., Liu, J., Tan, Y. and Liu, W., 2014. Methylation of PRDM2, PRDM5 and PRDM16 genes in lung cancer cells. *International journal of clinical and experimental pathology,* [e-journal] 7 (5), pp.2305. Available through: Primo.

Thomas, P.B.M., Mukherjee, A.N., O'Donovan, D. and Rajan, M.S., 2013. Preconditioned donor corneal thickness for microthin endothelial keratoplasty. *Cornea,* [e-journal] 32 (7), pp.e173. Available through: Primo.

Tuft, S.J. and Coster, D.J., 1990. The corneal endothelium. *Eye (London, England),* [e-journal] 4 (Pt 3), pp.389. Available through: Primo.

Tuft, S.J., Williams, K.A. and Coster, D.J., 1986. Endothelial repair in the rat cornea. *Investigative ophthalmology & visual science,* [e-journal] 27 (8), pp.1199. Available through: Primo.

Valtink, M., Donath, P., Engelmann, K. and Knels, L., 2016. Effect of different culture media and deswelling agents on survival of human corneal endothelial and epithelial cells in vitro. *Graefe's Archive for Clinical and Experimental Ophthalmology; Incorporating German Journal of Ophthalmology,* [e-journal] 254 (2), pp.285-295. Available through: Primo.

Van, D.B., Dhubhghaill, S.N.í., Koppen, C., Tassignon, M. and Zakaria, N., 2017. A review of the evidence for in vivo corneal endothelial regeneration. Survey of ophthalmology, [e-journal] 10.1016/j.survophthal.2017.07.004. Available through: Primo.

Van Horn, D.L. and Hyndiuk, R.A., 1975. Endothelial wound repair in primate cornea. *Experimental eye research,* [e-journal] 21 (2), pp.113-124. Available through: Primo.

Van Horn, ,D.L., Sendele, D.D., Seideman, S. and Buco, P.J., 1977. Regenerative capacity of the corneal endothelium in rabbit and cat. *Investigative ophthalmology & visual science,* [e-journal] 16 (7), pp.597. Available through: Primo.

Walshe, J. and Harkin, D.G., 2014. Serial explant culture provides novel insights into the potential location and phenotype of corneal endothelial progenitor cells. *Experimental eye research,* [e-journal] 127, pp.9-13. Available through: Primo.

Whikehart, D.R., Parikh, C.H., Vaughn, A.V., Mishler, K. and Edelhauser, H.F., 2005. Evidence suggesting the existence of stem cells for the human corneal endothelium. *Molecular vision,* [e-journal] 11, pp.816. Available through: Primo.

Williams, K.A., Keane, M., Galettis, R., Jones, V., Mills, R.A. and Coster, D.J., 2015. The Australian Corneal Graft Registry 2015 Report. *Ophthalmology, Eye and Vision Research Collected Works*.

Wulle, K.G., 1972. Electron microscopy of the fetal development of the corneal endothelium and Descemet's membrane of the human eye. *Investigative ophthalmology,* [e-journal] 11 (11), pp.897. Available through: Primo.

Yamaguchi, M., Shima, N., Kimoto, M., Ebihara, N., Murakami, A. and Yamagami, S., 2016. Optimization of Cultured Human Corneal Endothelial Cell Sheet Transplantation and Post-Operative Sheet Evaluation in a Rabbit Model. *Current eye research,* [e-journal], pp.1-7. Available through: Primo.

Yamaguchi, M., Ebihara, N., Shima, N., Kimoto, M., Funaki, T., Yokoo, S., Murakami, A. and Yamagami, S., 2011. Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. *Investigative ophthalmology & visual science,* [e-journal] 52 (2), pp.679. Available through: Primo.

Yamamoto, M., Quantock, A.J., Young, R.D., Okumura, N., Ueno, M., Sakamoto, Y., Kinoshita, S. and Koizumi, N., 2012. A selective inhibitor of the Rho kinase pathway, Y- 27632, and its influence on wound healing in the corneal stroma. *Molecular vision,* [e-journal] 18, pp.1727. Available through: Primo.

Yokoo, S., Yamagami, S., Yanagi, Y., Uchida, S., Mimura, T., Usui, T. and Amano, S., 2005. Human corneal endothelial cell precursors isolated by sphere-forming assay. *Investigative ophthalmology & visual science,* [e-journal] 46 (5), pp.1626. Available through: Primo.

Yuan, S. and Fan, G., 2015. Stem cell-based therapy of corneal epithelial and endothelial diseases. (Report). [e-journal] 10 (4), pp.495. 10.2217/rme.15.3. Available through: Primo.

Yue, B., Sugar, J., Gilboy, J. and Elvart, J., 1989. Growth of human corneal endothelial cells in culture. *Investigative ophthalmology & visual science,* [e-journal] 30 (2), pp.248-253. Available through: google.

Zaniolo, K., Bostan, C., Rochette Drouin, O., Deschambeault, A., Perron, M., Brunette, I. and Proulx, S., 2012. Culture of human corneal endothelial cells isolated from corneas with Fuchs endothelial corneal dystrophy. *Experimental eye research,* [e-journal] 94 (1), pp.22. Available through: Primo.

Zavala, J., G, R.L., C, A.R. and J, V., 2013. Corneal endothelium: developmental strategies for regeneration. *Eye,* [e-journal] 27 (5), pp.579. Available through: Primo.

Zazzo, E., Rosa, C., Abbondanza, C. and Moncharmont, B., 2013. *PRDM Proteins: Molecular Mechanisms in Signal Transduction and Transcriptional Regulation*: Biology.[e-journal]. Basel: MDPI AG.2 (1) pp.107-141. Available through: Primo.

Zhang, C., Zhu, Q., He, H., Jiang, L., Hu, L., Hu, G., Jiang, Y., Ding, X. and Lu, Y., 2015. RIZ1: a potential tumor suppressor in glioma. *BMC Cancer*, [e-journal] 15 Available through: Primo.

Zhang, K., Pang, K. and Wu, X., 2014. Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. *Stem cells and development*, [e-journal] 23 (12), pp.1340. Available through: Primo.

Zhang L., Anderson MC. and Liu CY., 2017. The role of corneal stroma: A potential nutritional source for the cornea. *Journal of Nature and Science*. 3 (8). pp: e428. Available through: PubMed PMID: 28936480; PubMed Central PMCID: PMC5605150.

Zhang, Y., Stehling-Sun, S., Lezon-Geyda, K., Juneja, S.C., Coillard, L., Chatterjee, G., Wuertzer, C.A., Camargo, F. and Perkins, A.S., 2011. PR-domain-containing Mds1-Evi1 is critical for long-term hematopoietic stem cell function. *Blood*, [e-journal] 118 (14), pp.3853. Available through: Primo.

Zhu, C. and Joyce, N.C., 2004. Proliferative response of corneal endothelial cells from young and older donors. *Investigative ophthalmology & visual science*, [e-journal] 45 (6), pp.1743. Available through: Primo.

Zhu, M., Yao, Q., Chen, J., Shao, C., Yan, C., Ni, N., Fan, X., Gu, P. and Fu, Y., 2016. Effects of corneal stromal cell- and bone marrow-derived endothelial progenitor cell-conditioned media on the proliferation of corneal endothelial cells. *International Journal Of Ophthalmology*, [e-journal] 9 (3), pp.332-339. 10.18240/ijo.2016.03.02. Available through: Primo.

Zhu, Y.-., Chen, H.-., Chen, S.-. and Tseng, S.C.G., 2012. Nuclear p120 catenin unlocks mitotic block of contact-inhibited human corneal endothelial monolayers without disrupting adherent junctions. *Journal of cell science*, [e-journal] 125 (15), pp.3636-3648. Available through: Primo.

Zhu, Y., Hayashida, Y., Kheirkhah, A., He, H., Chen, S. and Tseng, S.C.G., 2008. Characterization and comparison of intercellular adherent junctions expressed by human corneal endothelial cells in vivo and in vitro. *Investigative ophthalmology & visual science*, [e-journal] 49 (9), pp.3879. Available through: Primo.

Web Reference

Phenotyping Center - MRC Harwell; https://www.mousephenotype.org/data/charts?accession=MGI:1920093&allele_accession_id=MGI:5548625&zygosity=homozygote&parameter_stable_id=IMPC_EYE_007_001&pipeline_stable_id=HRWL_001 (accessed Jan 8/2017).

APPENDIX 1

Table 16 - Donor cornea information sheet: 12-hour air-interface organ culture experiment.

Cornea number	Age (years)	Gender	Cause of death	Time of enucleation	Period in Eye Bank storage	Endothelial cell density (ECD)
1	89	Female	Not reported	28.02.2014	31 days	1670 cells/mm^2
2	76	Female	Intracranial – 1a Acute Spontaneous CVA2 Hypertension	11.10.2014	29 days	1840 cells/mm^2
3*	65	Female	1a Bowel infarction & Advanced polycystic Kidney and Liver Disease	30.10.2014	29 days	Not available
4**	29	Female	Hypoxic brain damage – all causes	06.11.2014	35 days	Not available
5	74	Male	Not reported	18.05.2015	57 days	2200 cells/mm^2
6	74	Male	Not reported	18.05.2015	57 days	2000 cells/mm^2
7	65	Female	1a Bowel infarction & Advanced polycystic Kidney and Liver Disease	30.10.2014	29 days	Not available
8	29	Female	Hypoxic brain damage – all causes	06.11.2014	35 days	Not available
9	76	Female	Intracranial – 1a Acute Spontaneous CVA2 Hypertension	11.10.2014	29 days	Not available

Note: Corneas 3 and 4 were first used in the normal group (intact Descemet's membrane/Endothelium complex). After the OCT measurements were taken the Descemet's membrane/Endothelium complex from both corneas was removed and the corneas were included in the decompensated group.

Table 17 - 12-hour air-interface organ culture experiment results: *in vitro* corneal model validation.

***In vitro* air-interface organ culture**	**Normal corneas – ECD - 2002 ± 357.99 cells/mm^2 (n=6)**		**Decompensated corneas (n=5)**		**Post hoc t test (Bonferroni corrected)**	
Time (hours)	Corneal thickness (µm) ± SD	Percentage Corneal thickness decrease ± SD	Corneal thickness (µm) ± SD	Percentage Corneal thickness decrease ± SD	Corneal thickness comparison (P value)	Percentage thickness decrease (%) comparison (P value)
0	1092.74 ± 108.03	-	1128. 51 ± 98.91	-	Non-significant	-
1	1020.16 ± 111.46	6.47 ± 6.67 %	1096.29 ± 105.93	2.78 ± 5.7 %	*p<0.0001	*p<0.0001
2	943.67 ± 106.99	13.4 ± 7.42 %	1054.54 ± 121.46	6.61 ± 6.53 %	*p<0.0001	*p<0.0001
3	786.39 ± 101.63	27.6 ± 9.48 %	965.15 ± 105.59	14.21 ± 8.45 %	*p<0.0001	*p<0.0001
4	721.13 ± 89.91	33.44 ± 9.73 %	927.54 ± 106.6	17.62 ± 7.9 %	*p<0.0001	*p<0.0001
5	636.44 ± 94.11	41.24 ± 9.75 %	929.42 ± 83.5	17.34 ± 6.86 %	*p<0.0001	*p<0.0001
6	594.31 ± 78.7	44.96 ± 9.7 %	934.7 ± 76.37	16.82 ± 6.93 %	*p<0.0001	*p<0.0001
7	560.35 ± 87.02	47.99 ± 10.75 %	934.3 ± 68.74	16.78 ± 7.24 %	*p<0.0001	*p<0.0001
8	555.86 ± 81.7	48.37 ± 10.63 %	942.62 ± 68.7	16 ± 7.77 %	*p<0.0001	*p<0.0001
9	566.33 ± 90.68	47.42 ± 11.14 %	945.85 ± 67	15.7 ± 7.84 %	*p<0.0001	*p<0.0001
10	559.13 ± 84.03	48.12 ± 10.48 %	941.04 ± 58.61	16.06 ± 8.04 %	*p<0.0001	*p<0.0001
11	558.68 ± 85.1	48.15 ± 10.57 %	911.07 ± 83.21	18.67 ± 10.02 %	*p<0.0001	*p<0.0001
12	557.51 ± 72.64	48.27 ± 9.78 %	903.6 ± 86.51	19.27 ± 10.87 %	*p<0.0001	*p<0.0001

Note: *p<0.0003 was considered statistically significant; endothelial cell density (ECD); standard deviation (SD).

Table 18 – Donor cornea information sheet: 14-day air-interface organ culture experiment.

Cornea number	Age (years)	Gender	Cause of death as specified on donor information sheet	Time of enucleation (dd/mm/yyyy)	Period in Eye Bank storage	Endothelial cell density (ECD)
1	68	Male	1a Acute MI 1B Coronary Artary Disease 2 Renal Failure & Diabetes	30.10.2013	25 days	1600 cells/mm^2
2	72	Female	Intracranial haemorrhage	19.10.2013	36 days	1500 cells/mm^2
3	64	Male	Myocardial infarction	17.10.2013	38 days	1600 cells/mm^2
4	69	Female	Lung cancer	16.12.2013	39 days	2172 cells/mm^2
5	75	Female	Peritonitis	02.10.2013	21 days	1900 cells/mm^2
6	75	Male	Prostate Cancer	02.10.2013	21 days	1975 cells/mm^2
7	64	Male	Myocardial infarction	17.10.2013	38 days	1200 cells/mm^2
8	69	Female	Lung cancer	16.12.2013	39 days	2207 cells/mm^2

Table 19 –14-day air-interface organ culture experiment results: *in vitro* corneal model validation.

***In vitro* air-interface organ culture**	**Normal corneas – ECD – 1718 ± 306.31 cells/mm^2 (n=4)**		**Decompensated corneas (n=4)**		**Post hoc t test (Bonferroni corrected)**	
Time (days)	Corneal thickness (µm) ± SD	Percentage corneal thickness (%) decrease ± SD	Corneal thickness (µm) ± SD	Percentage corneal thickness (%) decrease ± SD	Corneal thickness comparison (P value)	% thickness decrease comparison (P value)
0	876.41 ± 88.62	-	918.68 ± 98.27	-	*p<0.0001	-
1	582.59 ± 70.31	33.32 ± 6.89	879.73 ± 109.58	4.21 ± 6.62	*p<0.0001	*p<0.0001
2	571.33 ± 113.51	33.98 ± 16.01	844.32 ± 99.93	7.89 ± 6.53	*p<0.0001	*p<0.0001
3	587.77 ± 114.76	31.98 ± 16.79	837.72 ± 118.48	8.89 ± 7.58	*p<0.0001	*p<0.0001
4	583.29 ± 110.75	32.90 ± 14.55	823.74 ± 95.56	10.14 ± 7.32	*p<0.0001	*p<0.0001
5	610.04 ± 91.18	29.69 ± 12.93	817.02 ± 73.37	10.74 ± 5.86	*p<0.0001	*p<0.0001
6	627.87 ± 100.48	27.44 ± 14.82	848.79 ± 87	7.30 ± 7.29	*p<0.0001	*p<0.0001
7	657.12 ± 86.17	24.07 ± 13.3	780.83 ± 109.63	14.88 ± 9	*p<0.0001	*p<0.0001
8	667.02 ± 105.57	22.66 ± 16.57	831.761 ± 102.31	9.43 ± 5.7	*p<0.0001	*p<0.0001
9	633.35 ± 107.93	26.50 ± 16.67	821.11 ± 108.69	10.50 ± 8.53	*p<0.0001	*p<0.0001
10	640.30 ± 104.62	25.77 ± 16.06	809.48 ± 89.43	11.54 ± 8.42	*p<0.0001	*p<0.0001
11	729.63 ± 68.08	15.80 ± 12.2	828.89 ± 95.91	9.64 ± 6.25	*p<0.0001	*p<0.0001
12	681.97 ± 91.76	21.22 ± 14.57	798.11 ± 90.92	12.72 ± 9.27	*p<0.0001	*p<0.0001
13	758.68 ± 64.38	12.67 ± 10.75	820.20 ± 81.8	10.41 ± 6.51	*p<0.0001	*p<0.0001
14	746.29 ± 104.34	14.39 ± 12.13	826.81 ± 96.29	9.83 ± 6.77	*p<0.0001	*p<0.0001

Note: *p<0.002 was considered statistically significant; endothelial cell density (ECD); standard deviation (SD).

APPENDIX 2

Table 20 - Donor information sheet for all corneas that were used for primary cultures.

Cornea number	Age (years)	Gender	Cause of death	Time of enucleation	Period in storage
1	73	Female	Pulmonary Fibrosis	09.02.2014	59 days
2	73	Female	Pulmonary Fibrosis	09.02.2014	59 days
3	63	Female	Infections – 1a Urinary Tract Infection	14.03.2014	39 days
4	82	Female	Cerebral Infarction	12.03.2014	34 days
5	67	Male	Cardiovascular	03.09.2014	34 days
6	67	Male	Cardiovascular	03.09.2014	34 days
7	81	Female	Renal failure	04.09.2014	33 days
8	75	Male	-	30.01.2014	69 days
9	75	Male	-	30.01.2014	69 days
10	43	Male	CVA	20.03.2014	21 days
11	43	Male	CVA	20.03.2014	21 days
12	74	Female	Metastatic Esophageal Cancer	28.02.2014	46 days
13	80	Male	Intracranial haemorrage	15.03.2014	31 days
14	86	Female	Mettastatic Carcinoma of the Breast	11.03.2014	35 days
15	63	Male	Intracranial haemorrage	29.03.2014	43 days
16	-	-	-	-	-
17	-	-	-	-	-
18	81	Female	Renal failure	04.09.2014	33 days
19	45	Female	Not specified	05.12.2014	88 days
20	73	Female	Not specified	19.12.2014	74 days
21	73	Female	Not specified	19.12.2014	74 days
22	83	Female	Pneumonia	20.10.2014	73 days

APPENDIX 3

Table 21 – Donor cornea information sheet. All corneas had their DM/Endothelium complex removed and were used for hCEC transplantation testing.

Cornea number	Age (years)	Gender	Cause of death	Time of enucleation (dd/mm/yyyy)	Period in Eye Bank storage
1	46	Female	Intracranial haemorrhage	06.06.2015	60 days
2	57	Male	Unknown	10.06.2015	56 days
3	52	Female	Cancer (other than brain tumour)	12.06.2015	54 days
4	52	Female	Cancer (other than brain tumour)	12.06.2015	54 days
5	78	Male	Cancer (other than brain tumour)	26.06.2015	30 days
6	78	Male	Cancer (other than brain tumour)	26.06.2015	30 days
7	46	Female	Intracranial haemorrhage	06.06.2015	60 days
8	67	Female	Hypoxic brain damage - all causes	12.06.2015	54 days
9	67	Female	Hypoxic brain damage - all causes	12.06.2015	54 days
10	76	Female	Septicaemia	22.06.2015	44 days
11	76	Female	Septicaemia	22.06.2015	44 days
12	73	Male	Unknown	21.08.2014	21 days
13	73	Male	Unknown	21.08.2014	21 days
14	67	Male	Cardiovascular	03.09.2014	34 days
15	67	Male	Cardiovascular	03.09.2014	34 days
16	81	Female	Renal failure	04.09.2014	33 days
17	81	Female	Renal failure	04.09.2014	33 days

Table 22 - hCEC transplantation experiment results: cell therapy evaluation during a 12-hour air-interface organ culture.

***In vitro* air-interface organ culture**	**Group 1 (n=6) - seeded with 2070 cells/mm^2 [HCEC-12]**		**Group 2 (n=5) - seeded with 1740 cells/mm^2 [passage 0 primary hCECs]**		**Group 3 (n=6) - seeded with 1036 cells/mm^2 [passage 2 primary hCECs]**	
Time (hours)	Corneal thickness (µm) ± SD	Percentage corneal thickness decrease (%) ± SD	Corneal thickness (µm) ± SD	Percentage corneal thickness decrease (%) ± SD	Corneal thickness (µm) ± SD	Percentage corneal thickness decrease (%) ± SD
0	1218.02 ± 153.65	-	1100.86 ± 86.15	-	1143.56 ± 69.23	-
1	1089.67 ± 122.48	10.06 ± 7.71 %	987.87 ± 106.25	10.32 ± 5.65 %	1051.45 ± 72.37	7.95 ± 5.26 %
2	992.68 ± 101.48	17.72 ± 9.58 %	900.24 ± 98.98	18.33 ± 4.83 %	886.27 ± 83.81	22.5 ± 5.34 %
3	878.41 ± 129.58	26.62 ± 14.65 %	767.1 ± 109.63	30.38 ± 7.71 %	781.06 ± 69.66	31.69 ± 4.5 %
4	744.08 ± 121.94	38.06 ± 12.13 %	656.59 ± 97.96	40.47 ± 6.64 %	686.74 ± 76.12	39.89 ± 6.05 %
5	656.55 ± 137.55	45.14 ± 13.67 %	560.77 ± 73.63	49.02 ± 5.81 %	598.67 ± 74.87	47.52 ± 6.75 %
6	637.69 ± 138.96	46.79 ± 13.45 %	510.41 ± 86.31	53.67 ± 6.78 %	557.19 ± 66.51	51.21 ± 5.45 %
7	569.64 ± 118.89	52.86 ± 10.01 %	474.24 ± 79.39	56.98 ± 5.99 %	553.47 ± 74.54	51.4 ± 7.35 %
8	487.19 ± 122.93	59.75 ± 10.12%	461.39 ± 82.56	58.16 ± 6.36 %	583.11 ± 72.82	48.91 ± 6.44 %
9	448.33 ± 85.45	62.95 ± 6.78 %	467.68 ± 92.64	57.63 ± 7.19 %	611.38 ± 84.27	46.42 ± 7.5 %
10	457.91 ± 91.94	62.2 ± 7.48 %	474.9 ± 93.84	56.98 ± 7.24 %	612.2 ± 65.98	46.31 ± 6.3 %
11	451.4 ± 91.86	63.76 ± 6.38 %	483.50 ± 95.77	56.14 ± 7.81 %	619.63 ± 60.97	45.67 ± 5.82 %
12	458.91 ± 90	63.15 ± 6.14 %	489.65 ± 94.62	55.61 ± 7.44 %	613.7 ± 47.23	46.19 ± 4.61 %